FORMIC ACID OXIDATION IN *ASPERGILLUS NIGER*

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Nature has developed a number of ways for enzymic oxidation of formic acid. These may be summarized in the following reactions:

$$HCOO^- + DPN^+ \longrightarrow CO_2 + DPNH \quad (a)$$

$$HCOO^- + \text{cyt.} \longrightarrow CO_2 + \text{red. cyt.} \quad (b)$$

$$HCOO^- + H^+ + H_2O_2 \longrightarrow CO_2 + 2H_2O \quad (c)$$

The DPN-dependent system is found in peas[1]. An oxidation independent of DPN and TPN that can be linked to cytochromes and methylene blue is found in *Escherichia coli*[2]. In animal tissues formate has been found to be oxidized to CO_2 by a catalase–peroxide complex, the peroxide being supplied from a nonenzymic oxidation of certain sulfhydryl compounds[3,4]. A fourth mode of oxidation has been postulated for *Aspergillus niger*, in which the oxidation is partial, leading to oxalic acid[5,6]:

$$2HCOO^- + \tfrac{1}{2}O_2 \longrightarrow C_2O_4^{--} + H_2O \quad (d)$$

This postulate was based on the large yields of oxalic acid obtainable when *A.niger* mycelium mats were incubated on a formate medium.

In the present communication, a formate oxidizing system will be described that does not correspond to any of these schemes. In addition, evidence will be presented that makes the operation of reaction (d) in the organism unlikely.

MATERIALS AND METHODS

Production of mycelium for enzyme extraction

Five strains of *A.niger* were available for these studies. A strain designated B.L. was used in most of the experiments because of its good growth characteristics in submerged culture.

The fungus was grown with heavy aeration in an enriched Czapek-Dox medium[7], containing in one liter, 50 g glucose, 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, and 25 ml 10% malt extract. The pH was adjusted to 6.8. The inoculum was grown on a sporulation medium containing per liter, 90 g glucose, 0.45 g NH_4NO_3, 0.07 g KH_2PO_4, 0.06 g $MgSO_4 \cdot 7H_2O$, and 60 ml beer wort[7]. For a fungus production employing 8 l of medium, two 300 ml Erlenmeyer flasks each containing 50 ml of sporulation medium would be inoculated and incubated at 30°C. After 4 to 6 days the mycelial mats were removed and broken into small pieces in a sterile Waring blendor. This suspension was then transferred to a cylindrical 10 l flask containing 8 l of sterile medium and a bit of silicone grease as antifoaming agent. Air from a compressor was filtered and dispersed in the medium by means of a sintered glass sparger. A heavy growth of an evenly distributed mycelium mass resulted after 40–50 hours incubation at 30°. The mycelium was conveniently harvested by filtering the suspension through gauze, and wringing it into a moist cake by hand. Yields at this stage were between 100 and 200 g. The mycelium was then resuspended in water, and again pressed dry. There was always a weight-loss of about 20% accompanying this washing process.

Initially, cells produced in this way were incubated for a further 24 hours in a medium containing sodium formate instead of glucose. Later these two processes were combined in one, with

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50 g glucose and 12.5 g sodium formate per liter as carbon source, and the other components as before. Incubation time and yield were as with glucose alone. The mycelial cakes were stored at −10°.

Replacement cultures for oxalate production

The procedure described by BERNHAUER was used[5]. Mycelial pads were grown at 35° in 50 ml of a glucose-salt medium contained in 300 ml Erlenmeyer flasks. After 2 to 3 days the medium was removed and the pads washed on the underside with sterile water until the wash water no longer reduced Fehling's solution. The pads were then made to float on a medium of 50 ml water, phosphate buffer, or formate, or a combination of these, and again placed in the incubator at 35°. Samples were drawn at intervals for the determination of oxalate.

In the experiments with ^{14}C-labelled formate, the Erlenmeyer flask was equipped so that a CO_2-free airstream could be drawn through it. The air was subsequently passed through a gas washing flask containing 100 ml 2% KOH, where the CO_2 evolved was absorbed.

Preparation of cell-free extracts

Initially cell-free extracts were made by grinding fresh mycelium with water and alumina in a chilled mortar. An extract of considerably higher specific activity for formate oxidation was obtained when mycelium that had been stored at −10° was ground without abrasive to a fine powder, and then thawed and extracted with water. Routinely 8 g of mycelium was ground, and extracted with 20 ml water for 20 min at 0–4°. The debris was then centrifuged down at 10,000 *g* in 10 min, and reextracted with 10 ml water. The combined supernatants contained 1.5–3 mg protein per ml.

Assay methods

Protein was measured with the biuret method[8]. The oxidation of formate was followed in the Warburg apparatus at 30° with air as the gas phase. Enzyme activities were determined at pH 6.0 based on the O_2-uptake in a 10 min period, starting either 5 or 10 min after the tipping in of the substrate from the side-arm. 2,6-Dichlorophenol indophenol was used as described previously[9], and phenazine methosulfate added in a concentration of 40 mg/l when used.

Catalase activity was demonstrated in the same system as used for formate oxidase assay, adding 10 μl 3% H_2O_2 as substrate from the sidearm and following the O_2-production. Oxalate was precipated with $CaCl_2$ after having adjusted the pH in the sample to 3.5 with glacial acetic acid. The precipitate when destined for permanganate titration was washed with dilute calcium acetate and dissolved in sulfuric acid.

For plating of radioactive samples, the calcium oxalate was washed 3 times in dilute $CaCl_2$ and dissolved in *N* HCl. The samples were plated directly in an amount giving negligible self-absorption, and counted in a gas flow counter to 3,000 counts per sample.

The ^{14}C-labelled sodium formate was obtained from the Radiochemical Centre, Amersham, England.

RESULTS

Conversion of formate to oxalate

A survey of the oxalate-producing ability was made with five strains of *A.niger* by placing preformed mycelial pads on 1.9% sodium formate solution and assaying for oxalate. "Yields" varying from 15 to 90% of the formate added were recorded after 41 hours. Further studies with the strain that gave the highest yield revealed that very little oxalate appeared if the pad was placed on water alone. pH fell during the incubation from neutrality to 2. A medium of 2% K_2HPO_4 was then used so as to prevent the pH from falling too rapidly. When 1.9% sodium formate was added originally, the content of sodium oxalate was assayed to 1.7% after 24 hours, and when no formate was added there was 0.7% oxalate after the same period. This confirms the findings of BUTKEWITSCH[10], and shows that a substantial part of the oxalate formed during formate incubation may be derived from the mycelial mat. The question was still open, however, whether some of the oxalate formed could possibly have arisen in a direct way from formate. Experiments with ^{14}C-labelled formate were carried out in order to throw further light on the problem.

The results of an experiment with 1% formate in water is given in Table I. A

TABLE I

INCORPORATION OF THE FORMATE CARBON INTO OXALATE

	μmoles	*c.p.m.* × 10^{-6}	*c.p.m./μmole*
Formate added	7360	31.1	4220
Medium at 48 hours		2.2	
Oxalate	3880	2.1	540
Carbonate (in KOH)		18.9	
Mycelium		10.1*	

The experiment was carried out as described above, with 1 % sodium formate in 50 ml water over a period of 48 hours.

* By difference, $5 \cdot 10^6$ c.p.m. was recovered in the extract resulting from 3 weeks treatment of the cells with cold *N* HCl.

"yield" of oxalate of 105% based on formate added was obtained. The labelling shows, however, that only 6.4% of the oxalate could have been formed by a direct condensation of formate molecules; 93.6% of the oxalate thus originated from sources other than formate.

The possibility remained that even this 6.4% did not represent the C_1–C_1 condensation envisaged by BERNHAUER, but rather formate that had been converted to CO_2 and then reincorporated into oxalacetate before its cleavage into oxalate and acetate[11,12]. A second experiment was therefore performed with unlabelled formate in the presence of labelled carbonate. The source of labelled carbonate used was part of the KOH solution mentioned under MATERIALS AND METHODS after neutralization to pH 8. (Adjustment of pH to 5 in an aliquot of this immediately released 97% of the radioactivity into the air, justifying the assumption that the labelled material really was carbonate.)

Furthermore this time radioactivity was incorporated into the oxalate molecule, resulting in a specific activity of 147 c.p.m./μmole. This is 27% of the specific activity obtained above. The specific activity of the starting solution (c.p.m. per μmole carbonate + formate) was, however, also only ¼ of the activity recorded in Table I. It thus appears unnecessary to postulate that formate is dehydrogenated directly to form oxalate. Its role in the oxalate synthesis can be ascribed partly to its proton consumption when metabolized, and partly to its contribution of CO_2 molecules, some of which susbsequently become incorporated into oxalate. In the following the system that carries out this oxidative decarboxylation of formate will be dealt with.

The adaptive nature of the enzyme

When whole cells grown on the glucose medium were tested for their ability to oxidize formate, only negligible activity was detectable. The possibility suggested itself that formate-oxidizing ability might be induced if the fungus were grown in the presence of formate. It was not possible simply to replace glucose with formate, as this led to an impractically low yield of cells. The fungus was consequently first produced in good yield on the glucose medium, and then transferred to a medium containing formate instead of glucose. Fig. 1 shows how this final incubation on formate induces in the cells an ability to oxidize formate that is as prominent as the glucose-oxidizing ability.

This extra incubation with formate could be omitted when some formate was included in the glucose medium. The amount of formate that could be added was found

to be critical, since concentrations as low as 2% sodium formate in the medium were found to suppress the growth markedly.

Experiments were also carried out comparing the oxidation of glucose and formate by cell-free extracts produced respectively from cells grown on glucose and a mixture of glucose and formate. A slight oxidation of formate even by the glucose-grown cells was observed, the incubation with formate however leading to a 7–10 fold higher rate.

Purification of formic acid oxidase

The cell-free extract prepared according to the present method is deficient in aerobic oxidative activities other than formate and glucose oxidation. There is thus no oxidation of citrate, succinate, fumarate, malate, acetate, glycolate, oxalate, alcohol, or mannose, although the organism is known to contain the tricarboxylic acid cycle enzymes[13] as well as those carrying out glycolysis[14]. 30% of the protein content of the extract is sedimented during 30 min centrifugation at 100,000 *g*. The formate oxidase activity remains with the glucose oxidase in the supernatant.

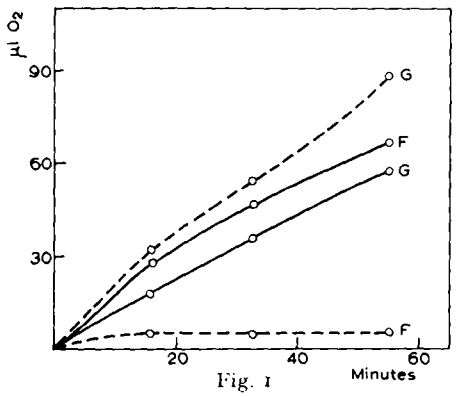

Fig. 1

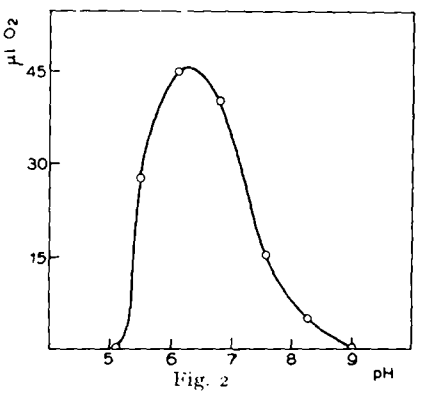

Fig. 2

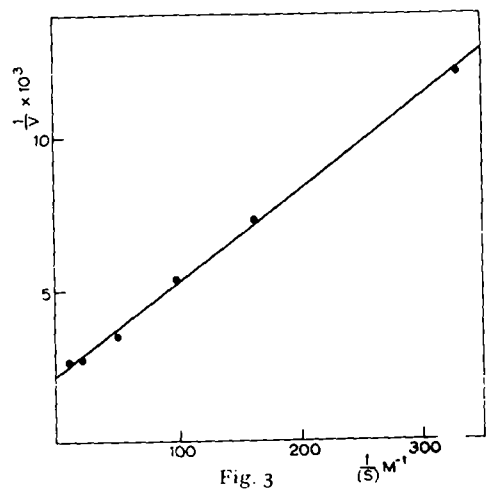

Fig. 3

Fig. 1. The adaptive nature of formate oxidation. The manometer vessels contained 250 µmoles phosphate buffer of pH 6.0, 30 µmoles $MgCl_2$, 100 µmoles substrate, 200 mg moist mycelium, 0.2 ml 20% KOH in the center well, and water to 3.2 ml. G represents oxidation of glucose, F of formate. Broken lines represent cells not preincubated with formate, whole lines preincubated cells. The endogenous respiration has been subtracted. It amounted to 20% of the total O_2-uptake with glucose.

Fig. 2. The influence of pH. The vessels contained 150 µmoles succinate, phosphate, or tris-(hydroxymethyl)aminomethane buffer, 100 µmoles formate, 0.3 ml enzyme concentrate, 0.2 ml 20% KOH, and water to 1.2 ml. Plotted is the O_2-uptake between 10 and 20 min after tipping in of the substrate.

Fig. 3. Michaelis constant. The vessels contained 120 µmoles phosphate buffer, 0, 3, 6, 10, 20, 50 and 100 µmoles formate, 0.6 ml enzyme concentrate for the three lowest formate concentrations, 0.3 ml for the two next concentrations, and 0.2 ml for the highest. The center well contained 0.2 ml 20% KOH, water was added to 1 ml, and pH was 6.0. (*S*) is molar concentration of formate, *V* is µl O_2 per mg protein per hour.

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When the extract proteins are precipitated with ammonium sulfate to saturation and redissolved in phosphate buffer, practically all the formate oxidase activity is recovered. During a fractionation of an unbuffered extract with solid ammonium sulfate, formic oxidase is precipitated between 25 and 40% saturation in a yield of 80%. Only 30% of the extract protein arrives in this fraction. A large fraction of this protein does not dissolve again, creating a sediment and turbidity in the solution. The turbidity remains even after long centrifugation at 10,000 g, but is easily sedimented after 2 hours dialysis against water or 0.02 M phosphate buffer followed by overnight freezing. Routinely the degree of purification achieved in these steps was 10-fold. The specific activities in the 25–40 fraction were in the range 400–1200 μl O_2/hour/mg protein, depending on the activity in the extract and a variable loss in enzymic activity during the purification. The characteristic properties with regard to the oxidative process have been studied at this purity level of the enzyme, and are described below.

Some properties of the enzyme

It was natural to compare the formate oxidase activity to the glucose oxidase activity present in the same extract. The two activities are similar in that they are both carried out by soluble enzymes that use oxygen as the electron acceptor. That these two enzymic activities are not properties of the same protein was, however, strongly indicated already by the inducible nature of the formate oxidation. This was confirmed by the ammonium sulfate fractionation in which the glucose oxidase was precipitated between 65 and 80% saturation, whereas, as noted above, formate oxidase appears between 25 and 40%.

The aerobic oxidation takes place with maximal rate at a pH of 6.2, the activity declining to essentially zero at pH 5 and 9, as shown in Fig. 2. When in separate experiments the buffers employed were compared at pH values within their common buffer regions, no effect was observed to result from replacing one by the other.

A comparatively high concentration of formate is needed to give half maximal rate. From a Lineweaver-Burk plot (Fig. 3) with seven formate concentrations in the range 0.003 to 0.100 M a K_s of 0.013 M was obtained.

Mechanism of the reaction

When CO_2 production was measured simultaneously with the O_2-uptake using Warburg's indirect method, a respiratory quotient (R.Q.) of 1.7–2.0 was obtained, both with crude extracts and with the enzyme concentrate. When the enzyme was incubated anaerobically with formate, no evolution of CO_2 (or H_2) occurred. An oxidative decarboxylation to CO_2 and H_2O thus appears to take place.

No dialyzable cofactors participate in the reaction. Most of the enzyme activity is intact after ammonium sulfate fractionation and dialysis, and the activity that is lost is not recovered by addition of boiled extract, magnesium ions, or a wide variety of known coenzymes, including cocarboxylase. Although the majority of the experiments were performed in the presence of phosphate buffer, the experiments cited above show that succinate or tris(hydroxymethyl)aminomethane equally well permit the reaction to proceed.

DPN does not stimulate the oxidation, and is not reduced by formate in the presence of the enzyme. In fact, the only really effective electron acceptor that has

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been found so far is oxygen itself. No reaction takes place with methylene blue. With 2,6-dichlorophenol indophenol oxidation of formate appears to occur, but only at a rate that is 0.05% of the rate with oxygen. Addition of phenazine methosulfate increases this rate twofold.

An enzymic reaction of the nature disclosed above would be expected to lead to the production of hydrogen peroxide as the immediate product, together with CO_2. This should have given an R.Q. of 1, not 2, as observed. Tests for catalase activity, however, showed this to be abundantly present both in the extract and in the oxidase concentrate. Thus, even though the primary product may have been H_2O_2, the result observed would be complete combustion to CO_2 and H_2O.

Evidence concerning the postulated role of H_2O_2 as an intermediate was sought by inhibiting catalase with HCN. Addition of HCN to a concentration of 0.01 *M* produced a reduction in the R.Q. by an average of 30% in three separate experiments for the period between 5 and 15 minutes after tipping in the substrate. Higher concentrations were not more effective. In some experiments the addition of HCN failed to reduce the R.Q., and with the cell-free extract, which has higher catalase activity than the oxidase fraction, no effect was observed. The partial inhibition of catalase did not result in a more rapid O_2 uptake, the decrease in R.Q. appearing as a consequence of a reduced rate of CO_2 production. It thus appears that HCN also inhibits the oxidase to some extent.

Attempts to demonstrate the formation of H_2O_2 by a secondary peroxidatic oxidation of an otherwise nonoxidazable substrate catalyzed by the catalase present failed. Alcohol was used as the secondary substrate, since it is attacked readily by the catalase-H_2O_2 complex[15]. In the oxidation of glucose by the glucose oxidase fraction of the extract, addition of alcohol or formate significantly increased the total O_2-uptake.

It was possible, however, to show oxidation of iodide ion that was added to the regular assay system in a concentration of 0.01 *M*. Within 5 to 10 minutes, the liquid was colored distinctly brown, and turned dark blue upon addition of starch indicator. With formate or enzyme alone no color appeared. This test for H_2O_2 is a qualitative one, the observable iodine merely being part of a complex steady state system which nonenzymically leads to breakdown of H_2O_2[16]. Titration with thiosulfate thus revealed the presence of only a fraction of a micromole of iodine in the reaction vessel.

DISCUSSION

There does not appear to be any basis for assuming the presence in the organism of a system that carries out a C_1–C_1 condensation of formate to oxalate. Studies of metabolism by means of the replacement culture technique is clearly beset with many pittalls. The large amounts of oxalate produced originate from substances in the mycelium rather than the formate of the medium. The formate is however effectively absorbed and metabolized by the cells, leaving practically none in the medium after 48 hours (Table I). A large part is seen to be converted into CO_2.

In oxidizing formate, the organism does not follow any of the mechanisms (a–c) outlined above that are found in plant, bacterial, or animal tissues. In being soluble, the present oxidase resembles the DPN-linked plant enzyme. In not being dependent on DPN it corresponds more to the *E.coli* enzyme, which however is of particulate

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nature, and together with hydrogenase plays a role in the so-called hydrogenlyase system, which catalyzes the formation of CO_2 and H_2 from formate[2].

Although reaction (c) cannot account for the present findings, this reaction is likely to play a role in the organism *in vivo*, and in the enzyme preparations as long as catalase is present. The evidence presented above strongly indicates that H_2O_2 is the immediate reaction product together with CO_2 with the present enzyme. Two ways are then open for the breakdown of H_2O_2 by catalase, the catalase-H_2O_2 complex reacting respectively with H_2O_2 and formate. The increase in total O_2-uptake in glucose oxidation caused by addition of formate showed that this peroxidatic oxidation of formate does occur in the present fungus system. Addition of alcohol did not raise the O_2-consumption in formate oxidation, and this might then mean that a competition with formate is established that is unfavorable for the peroxidatic oxidation of alcohol. The reaction mechanism may then be written as:

$$HCOO^- + H^+ + O_2 \longrightarrow CO_2 + H_2O_2 \qquad (e)$$

followed in the presence of catalase by

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \qquad (f)$$

$$HCOO^- + H^+ + H_2O_2 \longrightarrow 2H_2O + CO_2 \qquad (g)$$

The enzyme was found to be adaptive in nature. A small but significant formic oxidase activity could be detected also in glucose-grown cells. This could, however, have been induced by small amounts of formate that are likely to be formed endogenously during the metabolism of glucose.

The sluggishness with which it reacts with common artificial electron acceptors is striking. Only further purification will make it possible to find the reason for this behavior. It is possible that we are here dealing with another metalloprotein, or with a flavo-protein like glucose oxidase, only with a more strongly shielded prosthetic group, or, less likely, with an enzyme of a nature quite different from these.

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SUMMARY

Preformed mycelial pads of *Aspergillus niger* discharge large amounts of oxalate when incubated with phosphate buffer or sodium formate. Radioactive formate becomes incorporated to a small but significant extent into oxalate, probably by way of carbonate. The latter is readily formed from formate by the organism or extracts thereof, provided the organism has been exposed to formate during growth. This inducible and soluble enzyme has been partly purified and some of its properties studied. It carries out the oxidative decarboxylation of formate with oxygen as the only effective electron acceptor and H_2O_2 as the reaction product together with CO_2. No dialyzable cofactors participate in the reaction. The enzyme has a pH optimum of 6.2 and a Michaelis constant of 0.013 *M*.

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INTERMEDIATES IN THE ENZYMIC OXIDATION OF CATECHOL

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INTRODUCTION

There is general agreement that the first step in the oxidation of catechol to "catechol-melanin" in the presence of polyphenol oxidase is to *o*-benzoquinone. This has been confirmed by chemical[1], spectrographic[2] and polarographic[3] studies. There has been considerable controversy about the subsequent reactions[2,4,5,6]. Much of the controversy has revolved around the quantitative oxygen uptake at different ratios of enzyme to substrate and different substrate concentrations. This has been re-investigated using paper chromatographic methods together with the manometric. Under some conditions phenolic spots other than catechol are detected on the paper. Some of these substances are also produced by inorganic oxidants and have been isolated by chromatography on cellulose powder columns.

MATERIALS

The catechol was sublimed immediately before use to obtain chromatographically pure material, m.p. 105° C. Mushroom polyphenol oxidase (purchased from the Treemond Company, New York) was used. As supplied, the material was in aqueous solution assaying 3,300 catecholase units per ml or 3,000 catecholase units per mg dry matter by MILLER AND DAWSON'S[7] chronometric method.

RESULTS AND DISCUSSION

A. Manometry and paper chromatography

The curves obtained for oxygen uptake at different ratios of polyphenol oxidase to catechol were very similar to those obtained by WRIGHT AND MASON[8], and fully con-

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