Oxidase–Peroxidase Enzymes of *Datura innoxia*

OXIDATION OF REDUCED NICOTINAMIDE–ADENINE DINUCLEOTIDE IN THE PRESENCE OF FORMYLPHENYLACETIC ACID ETHYL ESTER

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The oxidase–peroxidase from *Datura innoxia* which catalyses the oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester and formic acid was also found to catalyse the oxidation of NADH in the presence of Mn^{2+} and formylphenylacetic acid ethyl ester. NADH was not oxidized in the absence of formylphenylacetic acid ethyl ester, although formylphenylacetoneitrile or phenylacetaldehyde could replace it in the reaction. The reaction appeared to be complex and for every mol of NADH oxidized 3–4 g-atoms of oxygen were utilized, with a concomitant formation of approx. 0.8 mol of H_2O_2 , the latter being identified by the starch–iodide test and decomposition by catalase. Benzoylformic acid ethyl ester was also formed in the reaction, but in a non-linear fashion, indicating a lag phase. In the absence of Mn^{2+} , NADH oxidation was not only very low, but itself inhibited the formation of benzoylformic acid ethyl ester from formylphenylacetic acid ethyl ester. A reaction mechanism for the oxidation of NADH in the presence of formylphenylacetic acid ethyl ester is proposed.

The oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester and formic acid and the oxidase–peroxidase nature of the iso-enzymes from *Datura* roots has been reported (Kalyanaraman *et al.*, 1975). Initial experiments indicated that the root extracts also catalysed the oxidation of NADH, for which there was an obligatory requirement for formylphenylacetic acid ethyl ester. Oxidase-peroxidases from plants are known to oxidize NADH in the presence of certain phenols such as 2,4-dichlorophenol and resorcinol in the obligatory presence of Mn^{2+} ions (Akazawa & Conn, 1958). Under these conditions NADH is also oxidized and the phenols are believed to be unaffected. The oxygen stoichiometry of the reaction, which is 0.5 mol of oxygen utilized/mol of NADH oxidized, lends support to the observation that the phenols are not oxidized. Moreover NAD^+ alone was identified as the product of the reaction. Several naturally occurring phenolic compounds having physiological activity, such as oestradiol and thyroxine, have also been shown to catalyse the oxidation of NADH (Williams-Ashman *et al.*, 1959; Klebanoff, 1959a,b). A uterine enzyme having similar activity differed from horseradish peroxidase in that there was a lag period in the initiation of the reaction, which was

abolished by the addition of H_2O_2 (Hollander & Stephens, 1959).

In the present paper we give evidence to show that both formylphenylacetic acid ethyl ester oxidation and NADH oxidation in the presence of formylphenylacetic acid ethyl ester are catalysed by the same enzyme. The NADH oxidase activity of the enzymes has also been studied in detail and a possible reaction mechanism to explain the complex nature of the reaction, namely, the concomitant oxidation of both NADH and formylphenylacetic acid ethyl ester, is given.

Materials and Methods

Formylphenylacetic acid ethyl ester and formylphenylacetoneitrile were prepared by methods described in the preceding paper (Kalyanaraman *et al.*, 1975). All other chemicals were commercial samples of highest purity. Catalase was obtained from Worthington Biochemicals Corp., Freehold, N.J., U.S.A., and yeast alcohol dehydrogenase was obtained from Medimplex, Budapest, Hungary, as a freeze-dried powder.

Formylphenylacetic acid ethyl ester oxidase (iso-enzymes I and II) were purified and assayed by the methods described in the previous paper (Kalyanaraman *et al.*, 1975). The oxidation of NADH was measured by the decrease in E_{340} . In 1 ml of standard reaction mixture were 0.2M-sodium phosphate buffer, pH 7.0, 1.25 μ mol of formylphenylacetic acid

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ethyl ester, 0.15 μmol of NADH, 0.25 mM- Mn^{2+} and 10–20 ng of DEAE-cellulose-purified enzyme (isoenzyme I). The progress of the reaction, at 25°C, was followed by the change in E_{340} at intervals of 30 s in a Beckman DU spectrophotometer. The control contained all components except NADH.

H_2O_2 was measured by the method of Yamazaki *et al.* (1956). For this 5 ml of the above reaction mixture was acidified with 1 ml of 1.5 M- H_2SO_4 , and 1 ml of 1% KI solution and 1 drop of saturated ammonium molybdate solution were then added. The liberated iodine was titrated against 0.1 mM- $\text{Na}_2\text{S}_2\text{O}_3$, with soluble starch as the indicator. From the titre value the amount of H_2O_2 present in the reaction mixture was calculated. Protein was determined by the procedure of Lowry *et al.* (1951), with dry bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as the standard.

Results

Co-purification of formylphenylacetic acid ethyl ester oxidase and formylphenylacetic acid ethyl ester-dependent NADH oxidase activities

Table 1 compares the specific activities of formylphenylacetic acid ethyl ester oxidation and formylphenylacetic acid ethyl ester-dependent NADH oxidation during the various stages of purification of formylphenylacetic acid ethyl ester oxidase from *Datura innoxia* root extracts. Both these activities were enriched in a parallel manner during all stages of purification of the enzyme, indicating that these activities are catalysed by one enzyme, a view consistent with the oxidase-peroxidase nature of the enzyme (Kalyanaraman *et al.*, 1975).

Role of metal ions

The NADH oxidase activity was very low in the absence of any added metal ions (Table 2). Mn^{2+} at 0.1–0.2 mM concentrations enhanced NADH oxida-

tion by about 12-fold, whereas Co^{2+} at 0.25 mM activated the reaction only twofold. Mn^{2+} was therefore included in the reaction mixture in all further experiments at a final concentration of 0.25 mM. Cu^{2+} and Ni^{2+} at 0.025 mM inhibited the Mn^{2+} -activated NADH oxidation by 88 and 92% respectively.

Time-course of NADH oxidation

Fig. 1 shows the time-course of NADH oxidation. There was a lag of about 2 min before NADH oxidation proceeded at a steady rate. Oxidation of NADH did not occur when either formylphenylacetic acid ethyl ester or enzyme was omitted from the reaction mixture, indicating the dependence of the enzymic reaction on formylphenylacetic acid ethyl ester.

Effect of formylphenylacetic acid ethyl ester concentration

The effect of increasing formylphenylacetic acid ethyl ester concentration on the rate of NADH oxidation is shown in Fig. 2. An affinity constant of 4 mM was obtained for formylphenylacetic acid ethyl ester when the reciprocal of the velocity of NADH oxidation was plotted against the reciprocal of formylphenylacetic acid ethyl ester concentration, which is about the same as the K_m for formylphenylacetic acid ethyl ester in its direct oxidation (Kalyanaraman *et al.*, 1975). Thus the enzyme shows the same affinity for formylphenylacetic acid ethyl ester for either of the two reactions.

Effect of other compounds in promoting NADH oxidation by the enzyme

Compounds other than formylphenylacetic acid ethyl ester were tested for their ability to promote NADH oxidation by the enzyme, and the results are presented in Table 3. Some of these compounds,

Table 1. Comparison of formylphenylacetic acid ethyl ester oxidase and formylphenylacetic acid ethyl ester-dependent NADH oxidase activities during purification of the enzyme from *D. innoxia*

For details of the purification see the preceding paper (Kalyanaraman *et al.*, 1975).

Purification step	Formylphenylacetic acid ethyl ester oxidase (μmol of O_2 utilized/min per mg of protein)	NADH oxidase (μmol of NADH oxidized/min per mg of protein)	Ratio <i>a/b</i>
	(a)	(b)	
Crude high-speed supernatant	170	62	2.74
C ₇ I enzyme	310	102	3.02
(NH_4) ₂ SO ₄ (40–80%-satd. fraction)	561	217	2.68
C ₇ II enzyme	1360	450	3.00
DEAE-cellulose chromatography (isoenzyme I)	5950	2370	2.51

Table 2. Effect of metal ions on the oxidation of NADH by formylphenylacetic acid ethyl ester oxidase

The enzyme was preincubated in the standard reaction mixture with NADH (0.15 μ mol) and the metal ions for 2 min before initiation of the reaction by the addition of formylphenylacetic acid ethyl ester (1.25 μ mol). The activity was determined as described in the text.

Metal ion	Concn. (mM)	NADH oxidized (nmol/min)
None	—	1.65
Mn^{2+}	0.025	11.20
	0.1	19.70
	0.25	19.40
Co^{2+}	0.25	3.22
Mg^{2+}	0.25	1.65
Zn^{2+}	0.25	1.65
Cd^{2+}	0.25	1.65
Mn^{2+}	0.25	2.27
+ Cu^{2+}	0.025	
Mn^{2+}	0.25	1.55
+ Ni^{2+}	0.025

Fig. 1. Time-course of NADH oxidation by *Datura* oxidase (isoenzyme I)

The activity of the enzymic oxidation of NADH was measured under standard assay conditions (+ Mn^{2+}), and in the absence of Mn^{2+} (— Mn^{2+}). Non-enzymic control (—) and the reaction in the absence of formylphenylacetic acid ethyl ester (---) are also shown.

themselves oxidized by the enzyme (Kalyanaraman *et al.*, 1975), could promote NADH oxidation to a limited extent. A comparison of the ratios of the rates of the direct oxidation and of NADH oxidation promoted by the corresponding substrates shows (Table 3) that whereas formylphenylacetic acid ethyl ester and phenylacetaldehyde yielded similar ratios for the two reactions, formylphenylacetonitrile was vastly superior in promoting NADH oxidation compared with its own oxidation rate.

Fig. 2. Effect of formylphenylacetic acid ethyl ester concentration on NADH oxidation by *Datura* oxidase (isoenzyme I)

Double-reciprocal plot of NADH oxidation ($1/v$) versus concentration of activator, formylphenylacetic acid ethyl ester ($1/[A]$). Inset: plot of activator, formylphenylacetic acid ethyl ester (FPAE) concentration in mM versus rate of NADH oxidation (nmol of NADH oxidized/min). Reaction mixture (1 ml) in 0.2M-phosphate buffer, pH7, contained enzyme, 150nmol of NADH, 0.25mM- $MnSO_4$ and formylphenylacetic acid ethyl ester at the appropriate concentrations.

Oxygen uptake during NADH oxidation

Enzymic oxidation of NADH was followed at two different concentrations of formylphenylacetic acid ethyl ester in two ways under identical conditions, (i) by following the disappearance of NADH with time by the fall in E_{340} and (ii) by following the O_2 uptake during the reaction. A similar experiment was carried out by using formylphenylacetonitrile instead of formylphenylacetic acid ethyl ester (Table 4). For every mol of NADH oxidized during the steady-state condition, 1.7 and 1.0mol of O_2 were utilized in the presence of formylphenylacetic acid ethyl ester and formylphenylacetonitrile respectively. The O_2 -consumption values are higher than that reported for NADH oxidation by horseradish peroxidase in the presence of resorcinol, wherein only 0.5mol of O_2 is utilized/mol of NADH oxidized during the reaction, which is equivalent to the amount of O_2 needed for the conversion of NADH into NAD^+ . The greater utilization of O_2 in the formylphenylacetic acid ester-dependent NADH oxidation indicated a complex reaction, which was expected, since unlike resorcinol, formylphenylacetic acid ether ester was oxidatively cleaved by the *Datura* enzyme.

Table 3. *Oxidation of NADH in the presence of formylphenylacetic acid ethyl ester and other compounds*

The reaction was followed under standard assay conditions by using the specific substrate instead of formylphenylacetic acid ethyl ester, but with appropriate concentrations of the enzyme. The rates for the direct oxidation of these compounds in the presence of Mn^{2+} and enzyme are taken from Table 5 in the preceding paper (Kalyanaraman *et al.*, 1975).

Compound	Activity (μ mol of NADH oxidized/min per mg of protein) (a)	Direct oxidation (μ mol/min per mg of protein) (b)	Ratio of the rate of direct oxidation of the compound and rate of NADH oxidation b/a
Formylphenylacetic acid ethyl ester	2370	4500	1.90
Formylphenylacetoneitrile	340	1.5	0.0044
Phenylacetaldehyde	12	16	1.33
Phenylpropionaldehyde	0	0	0
Atropic acid*	0	0	0
Resorcinol	107	0	0

* $C_6H_5-C(=CH_2)CO_2H$.

Table 4. *Oxygen uptake during oxidation of NADH in the presence of formylphenylacetic acid ethyl ester or formylphenylacetoneitrile*

Conditions of the reaction were the same as in the standard assay mixture except that the specified concentrations of formylphenylacetic acid ethyl ester or formylphenylacetoneitrile were used. In duplicate reaction mixtures, rates of NADH oxidation and of rate of O_2 uptake were measured.

Substrate	Concn. (mM)	Rate of NADH oxidation (nmol of NADH oxidized/min) (a)	Rate of O_2 consumption (nmol utilized/min) (b)	Ratio b/a
Formylphenylacetic acid ethyl ester	1.25	9.8	16.3	1.68
	2.50	18.6	30.5	1.64
Formylphenylacetoneitrile	0.05	11.7	11.3	0.97
	0.1	21.3	23.0	1.07

Identification of the reaction products

To resolve the question of extra O_2 uptake an effort was made to identify the products formed during NADH oxidation.

(i) *Formation of NAD^+* . NAD^+ was shown to be the final oxidation product of NADH oxidation as follows. After oxidation had proceeded under standard conditions of assay for 15 min when more than 90% of NADH had been utilized, as monitored by the fall in E_{340} , the reaction mixture was diluted 1:1 with 0.1 M-sodium pyrophosphate buffer, pH 9.0, containing 50 μ l of ethanol and 25 μ g of purified yeast alcohol dehydrogenase. Within 2 min of the addition of the dehydrogenase the E_{340} increased to maximal value which accounted for over 95% of the NADH originally present in the reaction mixture. This indicated that NAD^+ formed by the oxidation of NADH had not been oxidatively degraded any further.

(ii) *Concomitant formation of benzoylformic acid ethyl ester from formylphenylacetic acid ethyl ester during NADH oxidation*. Fig. 3 represents the formation of benzoylformic acid ethyl ester during

NADH oxidation in the presence of formylphenylacetic acid ethyl ester and Mn^{2+} , and also shows benzoylformic acid ethyl ester formation in the control reaction mixture where no NADH was present. Benzoylformic acid ethyl ester formation in the experimental reaction mixture showed a lag phase during NADH oxidation, but its concentration increased during later stages of the reaction. In the control (no NADH) the rate of formation of benzoylformic acid ethyl ester was linear with time even from the beginning of the reaction. However, the amount of benzoylformic acid ethyl ester formed when about 75% of added NADH was oxidized contributed to only 60% of O_2 utilized.

(iii) *Formation of H_2O_2* . Since the formation of NAD^+ and benzoylformic acid ethyl ester could not account for all the O_2 utilized during the enzymic oxidation of NADH, other oxygenated products were expected to be formed in the reaction. One possible candidate was H_2O_2 , since its formation in non-stoichiometric amounts has been reported during the oxidation of NADH by a uterine peroxidase (Beard & Hollander, 1962). The reaction

mixture was therefore analysed for H_2O_2 during the active phase of NADH oxidation. After 75% of the NADH was oxidized under standard assay conditions, the reaction mixture was acidified with H_2SO_4 and treated with the KI-starch reagent. An intense blue coloration was observed, indicating the oxidation of KI to iodine. The formation of H_2O_2 or a labile organic peroxide was thus suspected. Omission of NADH from the reaction mixture gave no colour with starch-iodide, and omission of any of the other components, namely formylphenylacetic acid ethyl ester, Mn^{2+} or enzyme, gave only a very faint blue colour, which was probably due to the H_2O_2 formed by the autoxidation of NADH (Yamazaki & Piette,

1963). That H_2O_2 may be the compound formed was strengthened by the observation that adding catalase to the reaction mixture, after NADH oxidation but before its acidification, gave no blue colour at all with the starch-iodide reagent.

Results of the determination of H_2O_2 formation during the enzymic oxidation of NADH are given in Table 5. For every mol of NADH oxidized about 0.8 mol of H_2O_2 was formed.

Effect of H_2O_2

Akazawa & Conn (1958) reported that the addition of H_2O_2 stimulated the oxidation of NADH by horseradish peroxidase in the presence of resorcinol and also abolished the requirement for Mn^{2+} in the reaction mixture. Hence the effect of H_2O_2 on formylphenylacetic acid ethyl ester-dependent oxidation of NADH by the *Datura* enzyme was tested in the presence and absence of Mn^{2+} . The results are summarized in Table 6. In the presence of Mn^{2+} , increasing the concentration of H_2O_2 correspondingly

Fig. 3. Time-course of oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester by *Datura* oxidase (isoenzyme I) in the presence and absence of NADH

At various time-intervals, 1 ml samples were withdrawn from each of two 10 ml reaction mixtures, with and without NADH, and assayed for the formation of benzoylformic acid ethyl ester. Standard reaction mixture (10 ml) in 0.2M-phosphate buffer, pH7, contained enzyme, 12.5 μmol of formylphenylacetic acid ethyl ester, 0.25 mM- Mn^{2+} and either 0 or 1.5 μmol of NADH.

Table 5. H_2O_2 formation during the formylphenylacetic acid ethyl ester-dependent oxidation of NADH by formylphenylacetic acid ethyl ester oxidase

The reaction was carried out under standard assay conditions. Samples (5 ml) of the reaction mixture were withdrawn at various time-intervals and assayed for H_2O_2 as described in the Materials and Methods section. Oxidation of NADH was followed spectrophotometrically under the same conditions.

Time (min)	NADH oxidized (nmol) (a)	H_2O_2 formed (nmol) (b)	Ratio <i>b/a</i>
4	16	19.6	1.23
8	44	39.6	0.9
12	71	55	0.77
16	96	75	0.79
20	110	92	0.84

Table 6. Effect of H_2O_2 on NADH oxidation by formylphenylacetic acid ethyl ester oxidase in the presence and absence of Mn^{2+}

The enzyme was preincubated with NADH and the specified concentration of H_2O_2 with or without Mn^{2+} before initiation of the reaction by the addition of the substrate. The reaction was carried out under standard assay conditions. No oxidation of NADH occurred when the enzyme was omitted from the reaction mixture.

Concn. of H_2O_2 (mM)	Activity (+ Mn^{2+}) (nmol of NADH oxidized/min)	Inhibition (%)	Activity (- Mn^{2+}) (nmol of NADH oxidized/min)	Activation (%)
0 (control)	12.9	—	1.34	—
0.005	10.7	17.5	1.9	38
0.01	8.4	35.0	1.9	35
0.1	6.4	50.0	4.2	200

enhanced the inhibition of NADH oxidation. H_2O_2 had no effect on the initial lag phase in the reaction. In the absence of Mn^{2+} , however, H_2O_2 stimulated NADH oxidation by about 200%. The initial lag phase was not pronounced in this case, but the maximal rate of oxidation never reached the value observed in the presence of Mn^{2+} alone. No oxidation of NADH occurred in the absence of the enzyme.

Effect of catalase

Catalase is known to be a potent inhibitor of peroxidase-catalysed oxidations. Its effect on the rate of oxidation of NADH is shown in Fig. 4. The addition of $2.5 \mu g$ of catalase per ml inhibited the reaction by 90%. Boiled catalase had no effect on the reaction.

Effect of NADH on the oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester in the absence of Mn^{2+}

Reference has been made above to the observation that in the absence of added Mn^{2+} , the oxidation of NADH occurred at a very low rate (Table 2). On the

Table 7. Effect of NADH on the oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester in the absence of Mn^{2+}

The enzyme was preincubated with NADH for 2 min and the reaction was started by the addition of substrate (1.25 mM). The reaction was followed by measurement of O_2 uptake and by measuring the amount of benzoylformic acid ethyl ester formed in the reaction. No oxidation of NADH occurred under these conditions.

Concn. of NADH (mM)	Inhibition of O_2 uptake (%)	Inhibition of benzoylformic acid ethyl ester formation (%)
0.1	100	100
0.05	85	85
0.025	68	77
0.005	37	43
0.002	0	0

other hand, during the phase of rapid oxidation of NADH in the presence of Mn^{2+} there was a lag in the formation of benzoylformic acid ethyl ester (Fig. 3). Hence the effect of NADH on benzoylformic acid ethyl ester formation and O_2 uptake was studied in the absence of added Mn^{2+} . Table 7 summarizes the effect of NADH on benzoylformic acid ethyl ester formation and O_2 uptake. NADH inhibited formylphenylacetic acid ethyl ester oxidation completely at concentrations of about 0.1 mM. At lower concentrations, partial inhibition was observed and at 0.005 mM NADH inhibition was 43%. O_2 consumed under these conditions appeared to be almost totally utilized for the formation of benzoylformic acid ethyl ester from formylphenylacetic acid ethyl ester. Inhibition to the

Fig. 4. Effect of catalase of NADH oxidation by *Datura* oxidase (isoenzyme I)

The enzyme was preincubated with NADH, Mn^{2+} and various amounts of catalase for 2 min before initiation of the reaction by the addition of formylphenylacetic acid ethyl ester. The reaction was carried out under standard assay conditions. Concentration of catalase ($\mu g/ml$ of reaction mixture) and percentage inhibition (in parentheses) were as follows: ●, no catalase (control); ▲, 0.5 μg (37%); △, 1.25 μg (75%); ○, 2.5 μg (88%).

Table 8. Oxidation of NADH by *D. innoxia* isoenzymes I and II and by horseradish peroxidase

The respective enzymes were incubated with 0.15 μmol of NADH, 0.25 mM- Mn^{2+} and with either resorcinol or formylphenylacetic acid ethyl ester at 1.25 mM in 1.0 ml of standard reaction mixture. The fall in E_{340} was followed with time.

Compound	Activity (μmol of NADH oxidized/min per mg of protein)		
	Isoenzyme I	Isoenzyme II	Horseradish peroxidase
Formylphenylacetic acid ethyl ester	2370	1080	571
Resorcinol	107	202	3500

same extent was observed with NADPH instead of NADH but not with NAD^+ or NADP^+ .

Comparison of NADH-oxidation activities of isoenzymes I and II of formylphenylacetic acid ethyl ester oxidase-peroxidase from D. innoxia and horseradish peroxidase

The physiological role of peroxidase isoenzymes has been attributed to the difference in their catalytic abilities. Hence an attempt was made to compare isoenzymes I and II of formylphenylacetic acid ethyl ester oxidase (Kalyanaraman *et al.*, 1975) between themselves and with commercial horseradish peroxidase. The results are given in Table 8. The rate of NADH oxidation dependent on formylphenylacetic acid ethyl ester was about 2½ times as high with isoenzyme I as with isoenzyme II, whereas isoenzyme II exhibited greater ability to catalyse NADH oxidation in the presence of resorcinol. However, NADH oxidation in the presence of resorcinol was markedly lower than in the presence of formylphenylacetic acid ethyl ester with both the *Datura* isoenzymes. Although NADH oxidation in the presence of resorcinol was about 35-fold greater with horseradish peroxidase than with the *Datura* isoenzyme I, the oxidation of NADH in the presence of formylphenylacetic acid ethyl ester was only 25% that of isoenzyme I.

Discussion

Plant peroxidases are known to catalyse a number of unusual reactions (Paul, 1963) involving molecular O_2 as the oxidant. A single primary reaction mechanism for the wide variety of reactions catalysed by horse-radish peroxidase has not been proposed. One of the most fancied reaction mechanisms involves the primary formation by a peroxidative oxidation from the substrate of free radicals, which then undergo further decomposition non-enzymically to yield the observed products (Yamazaki *et al.*, 1965). Inhibition by catalase of a number of oxidative reactions catalysed by peroxidases has been cited as evidence for the above mechanism (Yamazaki, 1966), although the presence of H_2O_2 , even in traces, has been demonstrated only in the oxidation of triose reductone by turnip peroxidase (Yamazaki *et al.*, 1956). The monophenols are believed to undergo primary oxidation to give the corresponding phenoxyl radicals, which then react with the substrate to give the free radical of the substrate. Such a reaction mechanism in lesser detail was proposed by Akazawa & Conn (1958) for the oxidation of NADH by horseradish peroxidase. A similar mechanism was also proposed for the oxidation of NADH by uterine peroxidase (Beard & Hollander, 1962). The oxygen in all these instances was used only for the oxidation of NADH.

The formylphenylacetic acid ethyl ester-dependent oxidation of NADH is more complicated, since the oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester takes place during the reaction, particularly when NADH concentration falls. Moreover, a peroxy compound, most probably H_2O_2 , is formed simultaneously in near-stoichiometric amounts, which is an unusual feature of the reaction. The mechanism for the direct oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester has been described previously (Kalyanaraman *et al.*, 1975), wherein a positively charged free radical of formylphenylacetic acid ethyl ester is initially formed, which reacts with molecular O_2 to give the substrate peroxide free radical. The substrate peroxide free radical then dismutates with another molecule of the substrate to yield the substrate peroxide anion and a further substrate free radical. The substrate peroxide anion then cyclizes and cleaves, by analogy with the tryptophan pyrrolase action (Ishimura *et al.*, 1968), to yield benzoylformic acid ethyl ester and formic acid. The same free radical of formylphenylacetic acid ethyl ester could be involved in the oxidation of NADH as well, as depicted in the following series of reactions:

The substrate free radical ($\text{R}\cdot$) formation is initiated by a peroxidase type of reaction (step 1). $\text{R}\cdot$ reacts with NADH to yield $\text{NAD}\cdot$ (step 2), which reacts with O_2 to yield NAD^+ and $\text{O}_2^{\cdot -}$ radical (step 3). $\text{O}_2^{\cdot -}$ could either dismutate (step 4) or react with substrate, R , to generate H_2O_2 and $\text{R}\cdot$ (step 5). Step 6 depicts the normal oxidation of the substrate free radical, $\text{R}\cdot$, to yield the regular oxidation products as described in the preceding paper. Mn^{2+} could activate any of the steps (2)–(5) but not (1)–(6) since only NADH oxidation is activated by Mn^{2+} .

The overall stoichiometry of the reaction will, however, depend on the competition of O_2 and NADH for $\text{R}\cdot$ (steps 2 and 6). A balance sheet of O_2 utilized and NADH oxidized with the concomitant formation of benzoylformic acid ethyl ester, formic acid and H_2O_2 is shown in Table 9, which is compiled from the data presented in Table 5 and Fig. 3. The

Table 9. *Stoichiometry of the enzymic oxidation of NADH by formylphenylacetic acid ethyl ester oxidase*

The data are compiled from Fig. 3 and Table 5. The theoretical O₂ uptake was calculated from the following equation:

$$\text{O}_2 \text{ uptake (nmol)} = \frac{\text{NADH utilized (nmol)}}{2} + \frac{\text{H}_2\text{O}_2 \text{ formed (nmol)}}{2} + \text{benzoylformic acid ethyl ester formed (nmol)}$$

Time (min)	NADH oxidized (nmol)	H ₂ O ₂ formed (nmol)	Benzoylformic acid ethyl ester formed (nmol)	O ₂ uptake (nmol)	Theoretical O ₂ uptake (nmol)
4	16	19.6	9	27	27
8	44	40.0	38	69	80
12	71	55.0	41	119	104
16	96	75.0	75	161	160

O₂-uptake values appear to agree reasonably well with the theoretical O₂-uptake values calculated from the rest of the data. Formylphenylacetic acid ethyl ester is thus a rare example of a substrate for oxidase-peroxidases, which can itself be oxidized as well as promote NADH oxidation, the latter property being unique for peroxidase substrates which are themselves oxidized.

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