A Comparison of Horseradish Peroxidase and Manganese Ions as Catalysts for the Oxidation of Dihydroxyfumaric Acid

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With horseradish peroxidase as catalyst the main product was dihydroxytartrate, but small amounts of glycolaldehyde, mesoxalic semialdehyde, mesoxalate and possibly glyoxal were also formed. Mn^{2+} catalysis gave rise only to mesoxalate and oxalate. When oxygen uptake was followed by a manometric method the rate of the peroxidase-catalysed reaction was proportional to oxygen concentration and marked inhibition by cyanide was obtained only at low buffer concentration. The catalytic effects of peroxidase and Mn^{2+} were almost always additive. Chelating agents inhibited the Mn^{2+} -catalysed reaction, but had either no effect or a slight accelerating effect on the peroxidase-catalysed reaction. It is concluded that Mn^{2+} does not function as cofactor in the peroxidase-catalysed oxidation.

Banga & Philipott (1939) reported that manganese and iron salts catalyse the oxidation of DFA,† and Theorell & Swedin (1939) found that manganese salts would restore DFA oxidase activity to plant extracts that had been inactivated by dialysis. Later, Swedin & Theorell (1940) showed that purified preparations of DFA oxidase exhibited the spectrum of peroxidase and that Mn^{2+} was not required for full activity. Using rapid-recording techniques, Chance (1952) found that the peroxidase-DFA reaction was biphasic at 4°. The first phase, reaction of oxygen with DFA to yield hydrogen peroxide, apparently required both Mn^{2+} and peroxidase, whereas the second phase, reaction of hydrogen peroxide with a second molecule of DFA, required only peroxidase. Authors of later publications appear to have tacitly assumed that no cofactors are required for full activity of the peroxidase-catalysed oxidation, yet no adequate refutation of the earlier conclusions has been made (Keilin & Hartree, 1951).

In an attempt to assess any possible role for Mn^{2+} I am describing the chemistry of the oxidations of DFA that are catalysed by Mn^{2+} and by peroxidase, and also comparing the rates of oxidation of DFA by these two catalysts added singly or together. From comparisons between different samples of DFA I have concluded that

some of the discrepancies between earlier papers were caused by impurities, especially by iron present in DFA. In the present experiments I endeavoured to avoid heavy-metal contamination as far as possible.

Some of these results have been briefly reported elsewhere (Hartree, 1967).

MATERIALS AND METHODS

Peroxidase

This was obtained from horseradish roots (Keilin & Hartree, 1951). The purpurogallin number (Keilin & Hartree, 1951) of samples exceeded 1000 and haematin contents were between 1.57 and 1.61%. Stock 50 μM solutions were stored at -20°.

DFA

This was prepared by the method of Hartree (1953b), dehydrated over P_2O_5 at 0.01 mm. Hg and stored over P_2O_5 .

Purification of reagents and cleaning of glassware

All glassware was cleaned in $\text{CrO}_3\text{-H}_2\text{SO}_4$, rinsed in deionized water, left in 6N-HCl for at least 1 hr. and rinsed again in deionized water and finally in glass-distilled water. Acetic acid was redistilled and sodium acetate of acceptable purity was selected from reagent-grade samples. The criterion of acceptability was that the rate of O_2 uptake under standard conditions (see below) did not exceed 1 $\mu\text{l.}$ of O_2 /min. Analytical-grade KH_2PO_4 (1M) was shaken repeatedly with 0.01 vol. of 1% (w/v) 8-hydroxyquinoline in chloroform until the extracts had the same colour as the

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† Abbreviations: DFA, dihydroxyfumaric acid (referred to in early papers as dihydroxymaleic acid; see Hartree, 1953a; Gupta, 1955); R.Q., respiratory quotient.

8-hydroxyquinoline solution. Excess of chelating agent was extracted with chloroform and KH_2PO_4 was crystallized from the evaporated solution. KOH, as a 1N solution, was passed through Zeo-Karb 225 (K^+ form; 200 mesh) (The Permutit Co. Ltd., London, W. 4) that had been pretreated by alternate washings (six in all) with hot 2N-HCl and warm N-KOH. Ammonium pyrrolidinedithiocarbamate (obtained from British Drug Houses Ltd., Poole, Dorset, as 'ammonium tetramethylene dithiocarbamate') was used as supplied.

Manometric methods

Uptake of O_2 was measured with Barcroft differential manometers containing reaction volumes of 3.0 ml. Centre wells contained 2N-NaOH (except in determinations of R.Q.). Unless otherwise stated the temperature was 20° and DFA was added to manometers in the dry form, being weighed directly into dangling cups. The platinum hooks were without influence on the measured reactions of DFA. Buffers were sodium acetate-acetic acid or KOH- KH_2PO_4 . 'Standard conditions' are defined as 50 μ moles of solid DFA added to 3 ml. of 0.16M-sodium acetate buffer, pH 4.8, in a manometer.

Enzyme activity is expressed as μ g.atoms of O utilized/mg. of peroxidase/min. Since the DFA/O stoichiometry of the enzymic oxidation is close to 2 this unit approximates to that recommended by the I.U.B. Commission on Enzymes.

Reference compounds (metabolites of DFA)

Dihydroxytartaric acid was from British Drug Houses Ltd. and disodium mesoxalate was kindly provided by Dr R. Hill, F.R.S. Glyoxylic acid was prepared by the method of Mohrschutz (1926), glyoxal was prepared by the method of Harries & Temme (1907) and a solution of glycolaldehyde was obtained by heating 0.4 g. of DFA with 5 ml. of water for 20 min. at 100°. Mesoxalic semialdehyde was isolated by a method based on that of Fenton & Ryffel (1902). A solution of 20 m-moles of FeSO_4 in 50 ml. of water was treated with 5 ml. of 30% (w/v) H_2O_2 and slowly heated to 100°. The suspension of $\text{Fe}_2(\text{SO}_4)_3$ was added slowly to a stirred suspension of 10 m-moles of DFA in 200 ml. of water at 40°. The resulting green solution (free from Fe^{3+}) was brought to pH 7.0 by gradual addition of powdered $\text{Ba}(\text{OH})_2$ and centrifuged. The clear solution was treated with a little Celite Analytical Filter Aid (Johns Manville Co., London, S.E. 1), adjusted to pH 8 with KOH, stirred for 1 hr. and filtered. This procedure precipitated most of the residual iron. The remainder was removed by passing the filtrate through Zeo-Karb 225 (H^+ form). The solution was concentrated to 40 ml. *in vacuo* and finally freeze-dried. The resulting yellow gum had the properties described by Fenton & Ryffel (1902). It yielded the expected derivative with DNP-hydrazine (see below) and after treatment with a solution of AgNO_3 in aq. NH_3 it yielded mesoxalic acid (identified as the DNP-hydrazone).

Preparation of DNP-hydrazones

Brady reagent was prepared by heating 25 m-moles of DNP-hydrazine with 750 ml. of 2.5N-HCl to 100° and filtering. In method α the hot reagent was mixed with a solution of 2.5 m-moles of reference compound, kept at 60°

for 1 hr. and then overnight at 20°. In method β the reaction time was the same but the temperature was 20° throughout. Glycolaldehyde DNP-hydrazone was prepared at 5° (see below). In all cases the precipitates were washed with N-HCl followed by water, recrystallized and dried over P_2O_5 .

Dihydroxytartaric acid bis-DNP-hydrazone (method α). This was crystallized by mixing hot saturated solutions in ethanol (3 vol.) and butan-1-ol (1 vol.). On slow heating the yellow product turns red at 100°, begins to sinter at 198° and melts at 255–258°; after rapid heating to 100° it melts at 215° (Found: C, 40.7; H, 3.78; N, 19.0. Calc. for $\text{C}_{16}\text{H}_{10}\text{N}_8\text{O}_{12} \cdot 2\text{C}_2\text{H}_5\text{OH}$: C, 40.3; H, 3.71; N, 18.7%. Loss in weight after 24 hr. at 110°, 14.7. Calc. for $2\text{C}_2\text{H}_5\text{OH}$, 15.4%). Addition of 2 vol. of CCl_4 to a hot saturated solution in dioxan yielded clusters of yellow needles containing dioxan of crystallization. During 1 week in high vacuum over silica gel and P_2O_5 the product became red and reached constant weight. It had m.p. 259–260° after sintering at 250° (Found: C, 37.8; H, 2.16; N, 21.9. Calc. for $\text{C}_{16}\text{H}_{10}\text{N}_8\text{O}_{12}$: C, 37.9; H, 1.99; N, 22.1%).

Mesoxalic semialdehyde bis-DNP-hydrazone (method α). The orange-red product was dissolved in hot acetic acid (in which it is sparingly soluble) and crystallized by adding 0.75 vol. of water and cooling. It had m.p. 268–270° after rapid heating to 250°; otherwise there was no defined m.p. (Found: C, 39.1; H, 2.22; N, 24.3. Calc. for $\text{C}_{15}\text{H}_{10}\text{N}_8\text{O}_{10}$: C, 39.0; H, 2.18; N, 24.2%).

Mesoxalic acid DNP-hydrazone (method α). This was crystallized by treating a hot ethanolic solution with 2 vol. of hot 2N-HCl. It had m.p. (rapid heating) 208–210°; Clift & Cook (1932) give 202° (Found: C, 36.0; H, 2.16; N, 18.5. Calc. for $\text{C}_9\text{H}_6\text{N}_4\text{O}_8$: C, 36.2; H, 2.03; N, 18.8%).

Glyoxylic acid DNP-hydrazone (method α). The yellow derivative was recrystallized three times from ethanol-water (7:3, v/v). It had m.p. 193° after rapid heating to 180°; Brady (1931) gives m.p. 190° (Found: C, 37.8; H, 2.56; N, 21.9. Calc. for $\text{C}_8\text{H}_6\text{N}_4\text{O}_6$: C, 37.8; H, 2.56; N, 22.1%).

Glycolaldehyde DNP-osazone (glyoxal bis-DNP-hydrazone). By method α glyoxal and glycolaldehyde yielded the same product, which was very sparingly soluble in common organic solvents. When twice recrystallized from large volumes of acetic acid and dried *in vacuo* over KOH it was obtained as red-brown crystals, m.p. 325°; Strain (1935) gives m.p. 326–328° (Found: C, 40.3; H, 2.49; N, 26.7. Calc. for $\text{C}_{14}\text{H}_{10}\text{N}_8\text{O}_8$: C, 40.2; H, 2.41; N, 26.8%).

Glycolaldehyde mono-DNP-hydrazone. Though treatment of glycolaldehyde with Brady reagent at 60° yielded only the osazone, method β yielded the osazone together with a product having the reactions of a mono-DNP-hydrazone (Table 1). To prepare this mono-DNP-hydrazone, glycolaldehyde (from 0.15 g. of DFA; 2 mol.prop.) in 10 ml. of water was mixed at 0° with 30 ml. of Brady reagent (1 mol.prop.). After 10 min. at 0° the yellow precipitate was collected, dissolved in 10 ml. of hot ethanol and cooled. After removal of a small red-brown precipitate the clear solution was treated with 10 ml. of hot water and cooled slowly to 0°. The resulting orange-yellow needles were recrystallized twice in the same way. It had m.p. 155°; Collatz & Neuberg (1932) give m.p. 155–156° and Glasstone & Hickling (1936) give m.p. 158°, in both cases after many recrystallizations (Found: C, 39.9; H, 3.59; N, 23.7. Calc. for $\text{C}_8\text{H}_8\text{N}_4\text{O}_5$: C, 40.0; H, 3.36; N, 23.3%).

Paper chromatography of DNP-hydrazones

Solvent mixtures that have proved useful in the separation of DNP-hydrazones of monocarboxylic keto acids (Block, Durum & Zweig, 1958) all gave rise to serious streaking with most of the derivatives described above. This was overcome adequately by use of a phosphate-citrate buffer and of mixtures containing this buffer (solvents I, II and III; Table 1).

Two-dimensional separations (descending technique) were made on sheets of Whatman no. 1 paper (32 cm. $\times$ 45 cm.). Mixed DNP-hydrazones (in pyridine) were spotted at a point (X) 10 cm. from the top (i.e. from a 32 cm. edge) and 10 cm. from the left side. The first run was made in solvent I. Under normal conditions the solvent front becomes irregular. Therefore after the spot had been applied the lower 75% of the paper was dipped into solvent, the paper was hung in the tank with its lower edge dipping into solvent and more solvent was added to the trough. The two advancing fronts met at the level of the spot. The paper was developed for 20 hr. and dried at room temperature. When separated components were to be eluted for quantitative assays, colour reactions were carried out either on a duplicate paper or on products separated simultaneously from a spot applied at Y, a point 2 cm. from the right edge of the paper. After development a narrow strip carrying the chromatogram of material at Y was cut off and treated appropriately (Table 1).

After the first run the paper was cut horizontally just above the major (slower) spot of dihydroxytartrate DNP-hydrazone, which moves about 6 cm. under the defined conditions. The upper portion carried the DNP-hydrazones of glyoxal and mesoxalic semialdehyde together with any DNP-hydrazine, all fused and near the origin. This paper was run in the second dimension in solvent III. The lower portion of the chromatogram carried the DNP-hydrazones of dihydroxytartrate (two spots), glycolaldehyde, mesoxalic acid and glyoxylic acid. The spot of glycolaldehyde (mono-)DNP-hydrazone trailed; therefore this component was separated for analysis by a one-dimensional run in solvent II.

Detection and assay of DNP-hydrazones separated by paper chromatography

All derivatives were visible as yellow to brown spots on untreated chromatograms. Such spots were more prominent when viewed by transmitted light from a mercury-vapour lamp fitted with Woods-glass filter. Identification of components was aided by colour changes caused by NH_3 and NaOH (Table 1). DNP-hydrazine and mesoxalic semialdehyde DNP-hydrazone move at similar speeds, but clear separation was obtained in solvent III. Similarly the DNP-hydrazones of glycolaldehyde and mesoxalic acid became adequately separated by solvent II when the front had advanced 25 cm.

All DNP-hydrazones except that of glycolaldehyde were analysed as follows. Spots were cut from unsprayed chromatograms and extracted at 20° in 1 ml. of *N*-NaOH in aq. 50% (v/v) methanol. Blanks cut from the same chromatograms were similarly treated. The coloured extracts were centrifuged and their extinctions measured in 10 mm. micro-cuvettes. The durations of extraction and the

Table 1. Paper chromatography of possible products from the reaction of DNP-hydrazine with oxidized DFA

The solvents used were: I, phosphate-citrate buffer, pH 8.2 (98 mm. Na_2HPO_4 -14 mm. citric acid); II, 2-methylbutan-2-ol-ethanol-phosphate-citrate buffer-water (16:6:5:5, by vol.); III, α -picoline-phosphate-citrate buffer-propan-1-ol (1:2:3, by vol.). The spray reagent was: 18 *N*-NaOH-methanol (1:9, v/v). R_F values in solvent I are approximate since they increase slightly as the loading increases and since the most reproducible migrations are obtained under conditions that do not allow measurements of R_F values (see the text). $\square$, Spot trails; $\circ$, evanescent colour. Where double spots are recorded the R_F value of the minor spot is *italicized*.

	R_F values			Colours of spots		
	Solvent I	Solvent II	Solvent III	Untreated	After NH_3 vapour	Sprayed
Glyoxal bis-DNP-hydrazone	0	0	0	Brown	Brown	Blue
Glycolaldehyde DNP-hydrazone	[0.56]	0.92	0.90	Yellow	Yellow-brown	Red-brown
Glyoxylic acid DNP-hydrazone	0.46	{ 0.59 0.71 }		Yellow	Yellow-brown	Pink-brown
Mesoxalic acid DNP-hydrazone*	0.68	{ 0.48 0.86 }		Yellow	Yellow	Yellow-brown
Mesoxalic semialdehyde bis-DNP-hydrazone	0.01	0.62	0.95	Yellow-brown	Purple-brown	Blue
Dihydroxytartaric acid bis-DNP-hydrazone†	{ 0.15 0.26 }	{ 0.54 0.62 }		Orange-yellow	Deep yellow	Violet
DNP-hydrazine	0	0.93	1.00	Yellow	Yellow	Violet-brown (Grey-brown)

* The spots obtained with solvent II each moved as two spots when rechromatographed in the same solvent.

† When rechromatographed in solvents I and II the faster spot moved as two spots and the slower spot as one spot.

wavelengths for *E* measurements were: glyoxal bis-DNP-hydrazone, 4 hr. and 577 m μ ; mesoxalic semialdehyde bis-DNP-hydrazone, 4 hr. and 560 m μ ; dihydroxytartaric acid bis-DNP-hydrazone, 2 hr. and 560 m μ ; mesoxalic acid DNP-hydrazone, 1 hr. and 448 m μ . Double spots were combined and extracted with the one portion of methanolic NaOH. Standard quantities of reference DNP-hydrazone were put through the same chromatographic procedure to obtain data for calibration curves. By this procedure 0.002 μ mole of a DNP-hydrazone is determined with an error of 5–10%.

Glycolaldehyde in a mixture of metabolic products was estimated by chromatography, in solvent II, of DNP-hydrazone prepared by method β . A series of standards was run on the same paper: these were prepared (method β) from known quantities of glycolaldehyde. The weight of glycolaldehyde represented by the 'unknown' spot was obtained by making use of the logarithmic relationship between area of spot and quantity applied (see Levine & Chargaff, 1951).

Insoluble product of the reaction of DFA with oxygen and peroxidase

Under standard conditions and in the presence of peroxidase a copious crystalline precipitate appeared in the manometer flask. This product does not decolorize iodine solution and its solution lacks the characteristic u.v.-absorption band of DFA (Hartree, 1953a). It was isolated on a larger scale as follows. A solution of 2.00 g. of DFA in 600 ml. of 0.25 M-acetate buffer, pH 5.0, was mixed with 0.2 μ mole of peroxidase and stirred vigorously for 1 hr. at 20°. The mixture was left standing at 5° and next day the crystals were collected, washed with cold water and dried in air (1.52 g.). The following analyses established that the product was disodium dihydroxytartrate (Found: Na, 16.50. Calc. for $C_4H_4Na_2O_8 \cdot 3H_2O$, 16.42%); a sample was dried *in vacuo* (0.1 mm. Hg) over P_2O_5 (Found: Na, 17.08; calc. for $C_4H_4Na_2O_8 \cdot 2.5H_2O$, 16.97%); further drying over P_2O_5 at 100° yielded the anhydrous salt (Found: Na, 20.55. Calc. for $C_4H_4Na_2O_8$, 20.36%). Fenton (1898) established that the disodium salt yields the two hydrates under these conditions. Treatment of the trihydrate with phenylhydrazine acetate yielded a yellow product, which was recrystallized from acetic acid. It had m.p. 227° (decomp.); Ziegler & Locher (1887) give m.p. > 200° (Found: C, 59.3; H, 4.29; N, 17.8. Calc. for bisphenylhydrazone of dihydroxytartaric acid; C, 58.9; H, 4.27; N, 17.2%).

Other analytical methods

Oxalate. The solution (4 ml.) was mixed in a centrifuge tube with 1 ml. of acetic acid and treated at 100° with an excess of M-calcium acetate. The precipitate was centrifuged down, washed with 0.2 M-calcium acetate, dissolved in N- H_2SO_4 and titrated at 80° with 0.1 N-permanganate.

Sodium. The sample (salt of DFA) was moistened with 6 N-HCl and heated slowly to 600°. The residue was again treated with HCl, reheated at 600° and weighed as NaCl.

RESULTS

Choice of experimental conditions

The pH optimum of the peroxidase-catalysed oxidation of DFA in acetate buffer is 4.6–5.0 and in

this region the completed oxygen uptake, which is higher than at other pH values, is close to 0.5 mole/mole of DFA. Lowering of the buffer concentration leads to increases in both the peroxidase-catalysed oxidation and the autoxidation of DFA. Under standard conditions the rate of autoxidation is low but the final oxygen uptake reaches the maximum value (0.5 mol.prop.). From experiments (in air) under standard conditions with and without 10 nm-peroxidase a specific activity of about 2000 was obtained. Rates of oxygen uptake in the system are approximately proportional to oxygen concentration. However, in pure oxygen rates of autoxidation and of destruction of enzyme are high.

Catalysis by Mn^{2+} shows a broad pH optimum at 7–10. The rate of oxidation is not appreciably affected by the composition or concentration of the buffer.

Products of aerobic oxidation of DFA

Oxidation products were recovered from manometer flasks after DFA had been shaken with either peroxidase or Mn^{2+} until oxygen uptake had ceased. For each experiment 50 μ moles of DFA were added as a fresh solution in 2 ml. of 45 mM-potassium hydroxide. The buffers were 0.16 M-sodium acetate (final pH 4.8) or 0.05 M-potassium phosphate (final pH 7.3); the catalysts were 27 nm-peroxidase or 67 μ M-manganese chloride. EDTA was added at 1 mM concentration. Eleven manometers were used: the components in the flasks and the duration of experiments are given in Table 2. When oxygen uptakes were complete the products from reactions A1, A2, B1, B2, C1, C2, D1 and D2 were converted into DNP-hydrazone and analysed chromatographically (see below). Comparisons of manometer readings for reactions A1 and B1 (oxygen uptake) with readings for reactions A3 and B3 (oxygen uptake minus carbon dioxide production) permitted calculations of R.Q. Reaction C3 was carried out in a manometer flask with side bulb from which acid was delivered after oxygen uptake had ceased. The resulting manometer readings, together with the final reading for reaction C1, provided the R.Q. of the Mn^{2+} -catalysed reaction.

Treatment of products from reactions A1, B1 and D1. In each case the mixture was cooled in ice and centrifuged, two 1 ml. lots of buffer being used to rinse out the flask and wash the crystalline precipitate. These precipitates were dehydrated over phosphorus pentoxide and weighed as disodium dihydroxytartrate ($C_4H_4Na_2O_8 \cdot 2.5H_2O$). Chromatography of the derived DNP-hydrazone showed that only dihydroxytartrate was present. The supernatant solutions from reactions A1 and B1 were each treated with 20 ml. of Brady reagent under the conditions of method α (heating at 60°). The

Table 2. *Design of manometric experiments leading to assays of oxidation products of DFA*

Details of the components are given in the text.

Reaction	Components		Duration of experiment (min.)
	In main compartment (3.0 ml.)	In centre well (0.3 ml.)	
A1, A2*	DFA, acetate pH 4.8, peroxidase	2N-NaOH	60
A3	DFA, acetate pH 4.8, peroxidase	Acetate	60
B1, B2*	DFA, acetate pH 4.8, peroxidase, EDTA	2N-NaOH	60
B3	DFA, acetate pH 4.8, peroxidase, EDTA	Acetate	60
C1, C2*	DFA, phosphate pH 7.3, MnSO ₄	2N-NaOH	90
C3	DFA, phosphate pH 7.3, MnSO ₄	Phosphate†	90
D1, D2*	As reaction A1, but without peroxidase	2N-NaOH	60

* Duplicate experiments.

† For this experiment the manometer flask had a side bulb from which 0.3 ml. of *m*-citric acid was added after 90 min.

precipitated DNP-hydrazones (fraction *Z*) were collected by centrifuging and washed twice with *n*-hydrochloric acid. The DNP-hydrazones remaining in solution were extracted with ethyl acetate (6 ml., 4 ml.) and the extract was evaporated *in vacuo*. The residue was dissolved in 4 ml. of hot butan-1-ol and kept overnight at 28°. DNP-hydrazine was filtered off and the clear fluid was evaporated. This residue, and fraction *Z*, were freed from hydrochloric acid in a desiccator over potassium hydroxide and combined as a solution in 2 ml. of pyridine.

The precipitate formed in reaction D1 was identified microscopically, and by iodine addition, as disodium dihydroxyfumarate. The supernatant fluid from this precipitate was treated as described in the preceding paragraph to give a pyridine solution of DNP-hydrazones.

Treatment of products from reactions A2, B2 and D2. These were worked up as described for reactions A1, B1 and D1 respectively except that DNP-hydrazones were prepared by method β (no heating at 60°).

Treatment of products from reactions C1 and C2. No precipitates formed in these reaction mixtures. DNP-hydrazones were prepared from the products of reaction C1 by method α and from the products of reaction C2 by method β .

Tests carried out on the products from reactions A3, B3 and C3. The precipitates that formed in reactions A3 and B3 were converted into DNP-hydrazones by method α . Paper chromatography revealed only the bis-DNP-hydrazone of dihydroxytartaric acid. The clear solutions from the three experiments were analysed for oxalate.

Comparison of reactions catalysed by peroxidase and Mn²⁺. The information in Table 3 was obtained as follows. Products from reactions A1, B1 and C1 provided analytical values for dihydroxytartaric acid, mesoxalic acid and mesoxalic semialdehyde in

columns A, B and C. In addition, values for glyoxal plus glycolaldehyde were obtained since both compounds yielded the same bis-DNP-hydrazone under the experimental conditions. Products from reactions A2 and B2 provided values for glycolaldehyde. Determinations of oxalate and of R.Q. are described above: calculated values of R.Q. are based on Scheme 1. Results of control experiments in the absence of catalyst (column D) are expressed qualitatively. In no experiment was glyoxylic acid DNP-hydrazone detected. Estimates of glyoxal plus glycolaldehyde are more accurate than their separate estimations owing to the greater error (approx. 10%) in determining the latter. It therefore remains uncertain whether glyoxal should be considered as an oxidation product of DFA.

Effects of metal ion-binding reagents on the catalysed oxidations of DFA

Under standard conditions, with peroxidase as catalyst, oxidation is not inhibited by 1 mM concentrations of EDTA, 2,2'-bipyridyl, 8-hydroxyquinoline or ammonium pyrrolidinedithiocarbamate. In fact, EDTA usually caused slight accelerations in the rate of oxygen uptake. Cyanide was also ineffective as inhibitor unless the acetate concentration was very low (less than 1 mM), when buffering capacity was maintained by adding DFA as a solution 80% neutralized with potassium hydroxide. Reproducibility was not good in the experiments with cyanide and this was due, at least in part, to the impossibility of controlling cyanide concentrations at low pH even when potassium cyanide was present in the centre wells.

With Mn²⁺ as catalyst chelating agents, even 2,2'-bipyridyl, were inhibitory at pH values between 4 and 7. Cyanide, however, never showed more than a slight inhibitory action at all buffer concentrations.

Table 3. *Products arising during the oxidation of 50 μ moles of DFA*

These results are based on the experiments A-D listed in Table 2.

	Solvent system(s)		Amounts found (μ moles)			D* (DFA)		
			A (DFA + peroxidase)	B (DFA + peroxidase + EDTA)	C (DFA + Mn ²⁺)			
	First dimension	Second dimension						
Dihydroxytartrate								
i, as cryst. disodium salt	I		27.1	33.5	{ 34.7 2.1 }	36.8	0	+ (b)
ii, in solution			6.4					
Glyoxal	I	III	0.6		0.5		0	$\pm$
Glycolaldehyde	II		5.3		6.9		0	$\pm$
Mesoxalate	I		2.1		1.5		12.1	+
Mesoxalic semialdehyde	I	III	4.2		5.1		0	+ (a)
Oxalate			0		0		37.0	—
Total products			45.7		50.8		49.1	
R.Q.								
Found			0.74		0.71		0.73	
Calculated			0.83		0.76		0.78	

* By method α , a predominates: by method β , b predominates. Scheme 1. Probable steps in the oxidation of DFA catalysed by Mn^{2+} and by peroxidase. Broken lines denote reactions that may occur in part during treatment with DNP-hydrazine.*Combined action of peroxidase and Mn^{2+} on DFA*

Under standard conditions with 1–30 nm-peroxidase and 10–100 μ M-manganese sulphate, the rate and extent of oxidation of DFA in the presence of both catalysts did not exceed the sum of the rates and extents observed when the catalysts were tested separately. At 0°, however, and with low concentrations of peroxidase, a slight synergistic effect was detectable, though after the first 10 min. rates became additive as at 20°.

DISCUSSION

Dihydroxytartrate is the main product of the peroxidase-catalysed oxidation of DFA (Scheme 1). This confirms the conclusion reached by Mason & Anan (1959) on the basis of the i.r. spectrum of the sodium salt that crystallized out during the

reaction. The breakdown of DFA to mesoxalic semialdehyde can be considered as a spontaneous reaction: thus manometric experiments either in the absence or in the presence of peroxidase showed that dihydroxytartrate is decarboxylated at an appreciable rate whereas its oxidation proceeds only very slowly. Corresponding experiments with mesoxalic semialdehyde gave parallel results except that decarboxylation was slower. Mesoxalate may perhaps arise through oxidation of the semialdehyde by hydrogen peroxide, or other oxidizing entity, formed during oxidation of DFA to dihydroxytartrate. Glycolaldehyde is formed slowly in aqueous solutions of DFA and could be detected among the products from control experiments (Table 3, column D). However, the quantity formed in the peroxidase-catalysed reaction is far greater and it must be assumed that most of the glycol-

aldehyde is formed in the enzymic reaction. The effect of EDTA on the yield of dihydroxytartrate is consistent with its influence on the course of oxygen uptake and suggests that some side reactions may be catalysed by trace-metal impurities. In manometric experiments the yield of dihydroxytartrate also increases as the peroxidase concentration is raised. In such cases the reaction is completed more rapidly and presumably there is less time for side reactions to occur.

The results of Mn^{2+} catalysis can be interpreted unambiguously. Only two products were found and mesoxalate is undoubtedly an intermediate in the formation of oxalate since its oxidation to oxalate is catalysed by Mn^{2+} . Because not more than 50 μ moles of oxalate plus mesoxalate were formed from 50 μ moles of substrate it follows that oxalate is not formed by oxidative cleavage at the double bond of DFA.

Differences between peroxidase and Mn^{2+} as catalysts can be summarized as follows: (i) the two catalyses are chemically distinct; (ii) chelating agents inhibit only the Mn^{2+} -catalysed reaction; (iii) at very low buffer concentrations only the peroxidase-catalysed reaction is sensitive to cyanide. These results, together with the finding that in all experiments but one the catalytic effects of Mn^{2+} and peroxidase were additive, point clearly to the conclusion that the peroxidase-DFA reaction has no requirement for Mn^{2+} . Similarly, I have been unable to demonstrate a requirement for a phenolic carrier, though earlier workers in this field recorded considerable activation of the oxidase reaction by quinol and some other phenols (Robežnieks, 1938; Banga & Philipott, 1939; Theorell, 1940, 1943).

I must stress, however, that in the experiments of this paper the substrate, DFA, was added to peroxidase either in the solid form or as a freshly prepared partially neutralized solution. If such solutions are aged for several hours, during which time their DFA contents decline by only about 10%, they become much less susceptible to the action of peroxidase. The rate of peroxidase-catalysed oxidation of such aged solutions can be restored to a considerable extent by addition of quinol or of Mn^{2+} . The progressive decline in the

ability of peroxidase to catalyse the oxidation of an aging DFA solution is due to spontaneous conversion of a small proportion of the DFA into inhibitory products (E. F. Hartree, unpublished work). This decline is not due to *trans-cis* isomerization as suggested by Banga & Philipott (1939). It appears that these inhibitory products were responsible for the earlier claims that Mn^{2+} and a phenolic carrier were essential cofactors in the peroxidase-DFA reaction.

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