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The Dihydroxyfumaric Acid Oxidase-Peroxidase Reaction

I. The Inhibition by Citrate

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Dihydroxyfumaric acid rapidly decomposes in water, and fresh solutions must be used. Ethanol accelerates the enzymic reaction in citrate.

Citrate inhibits the system dihydroxyfumaric acid-peroxidase-oxygen markedly at pH 4.8 but less at pH 3.75. The inhibition is non-competitive to the donor. Dihydroxyfumaric acid-peroxidase-oxygen forms no compound III in citrate. Citrate does nor inhibit the peroxidatic step.

Both rate and stoichiometry depend upon the concentration of the reactants. The ratio 1.5:1 in citrate between the acid and the peroxide indicates the involvment of molecular oxygen, and it is tentatively suggested that the peroxide might modify the enzyme to make it act as an oxidase even in citrate. The ratios of donor to oxygen = 1 at pH 5 and > 4 at pH 3.75 are obtained under certain conditions.

Some effects of buffer ions on oxidase-peroxidase reactions have been noticed.[1] Morita and Kameda[2] found the rate of oxidation of DHF* to be three times higher in acetate than in phosphate with both JRP a and c. Waygood et *al.*[3] observed that citrate inhibited the oxidase step with indoleacetic acid. On the other hand Yamazaki[4] found no difference between citrate and acetate at pH 3.7 in the peroxidase catalyzed reaction between methylene blue and triose reductone.

When repeating some of the above experiments, we confirmed that the addition of solid DHF, or of a fresh, aqueous solution of DHF, to HRP in acetate under aerobic conditions produced the red colour and absorption spectrum of HRP compound III. However, when the acetate buffer was replaced by citrate no spectral change occurred. When a DHF-solution, prepared anaerobically and stored under argon for a couple of hours, was added to HRP in acetate only a slight and transitory change in colour could be noticed. This led us to reinvestigate the reactions between DHF and HRP.

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* Abbreviations: DHF dihydroxyfumaric acid, HRP and JRP horse radish and Japanese radish peroxidase.

MATERIAL AND METHODS.

HRP was prepared as previously described.[5] CMC quantitatively retained the basic peroxidase, and the peroxidase in the present study therefore was only of type II. It was crystallized twice from ammonium sulphate before used. In the chromatogram on CMC a blue zone always migrated behind the peroxidase. It was caused by a copper-containing protein, the spectrum of which showed a maximum at 605 mμ. This protein was also quantitatively removed from HRP II by CMC.

DHF was prepared according to Hartree [6] and crystallized twice from aqueous acetone. No iron could be detected by the sulphosalicylic acid method [7] after the wet ashing of a 20-mg batch with the final volume 5 ml. DHF crystallized with two molecules of water and was converted to and stored in the anhydrous form over P_2O_5. $\varepsilon_{292} = 9300$ M^{-1} cm^{-1} in water.

Chemicals of analytical grade (Hopkins & Williams Ltd.) and quartz-distilled water were used throughout. For anaerobic work suitably designed cuvettes with side-bulbs and wide-bore stopcoks were used. Argon (≤ 2 p.p.m. O_2. AGA, Stockholm) was bubbled through the contents for 2 h prior to the addition of DHF, HRP, etc. Care was taken to wash the oxygen from the glass walls above the solution during the argon bubbling.

A Beckman DK 2 A spectrophotometer (pen movement 10.6 mm = 10 mμ/min) and a Braun manometer set SL 85 (gas volume about 12 ml) were used.

The initial lag phase in the DHF oxidation in spectrophotometer and manometer experiments is influenced upon by pH, the concentration of the enzyme, the age of the DHF solution, and by traces of H_2O_2. It prevents the general use of an exact kinetic expression for the rate of oxidation of DHF, and even $1/t$, where t is the time for 10 %—50 % decrease in the initial A_{292}, may become seriously misleading. In the present study the overall activity is usually expressed as $-\Delta A_{292}/\Delta t$, where $\Delta A = 0.2$ after the termination of the lag phase. The effects under study are of such a magnitude that this expression is sufficiently accurate.

RESULTS

Solvent effect. A fresh aqueous solution of DHF shows a light absorption maximum at 292 mμ. When the solution was made up anaerobically and stored under argon its maximum absorption shifted towards shorter wavelengths, broadened and decreased. The light absorption of the stock solution, the iodine-reducing capacity, and the intensity of the grey-brown colour given by ferric chloride decreased roughly in parallel. Plots of log absorbancy of the stock solution against time (Fig. 1) gave a slope usually non-linear and differing from one part of the UV spectrum to another with the highest value in the range 270—330 mμ, where it was essentially constant. Thus several substances

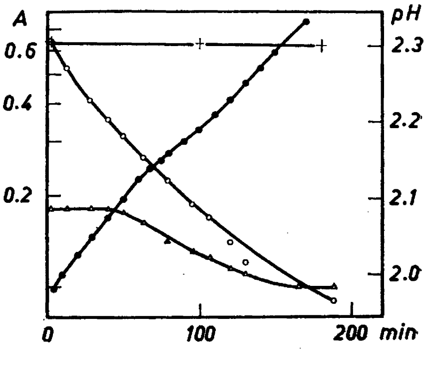

Fig. 1. Changes in aqueous (○, △) or ethanolic (+) 17 mM DHF, made up and stored under argon at 25°. Aliquots of 10 μl were diluted with 2.50 ml of 10 mM acetate of pH 4.80 and read at 292 (○, +) and 250 (△) mμ. pH (●) was followed directly in an anaerobic, aqueous, 17 mM solution.

were present in the aging DHF solution. In 17 mM aqueous DHF, which is a convenient stock solution, the light absorption at 250 mμ remained unchanged for about 50 min, *i.e.* DHF and its immediate derivative(s) have an isosbestic point at this wavelength. Simultaneously pH increased with a change in rate at about 70 min.

In an alcoholic solution of DHF very little change in A_{292} occurred in several hours under the same conditions. This circumstance, together with the somewhat higher solubility of DHF in ethanol, makes it tempting to use stock solutions of DHF in ethanol. Fig. 2 shows, however, that the addition of a minute quantity of ethanol to HRP-DHF in citrate will markedly influence the reaction. The effect is quantitatively similar to the one caused by a small amount of H_2O_2 on the autoxidation of acetylated ferrocytochrome c.[7] Hence fresh, aqueous solutions of DHF should be used. In the sequel an aliquot was transferred from the DHF-solution to the cuvette exactly 2 min after the dissolution of DHF unless otherwise mentioned; every experiment required a number of weighings.

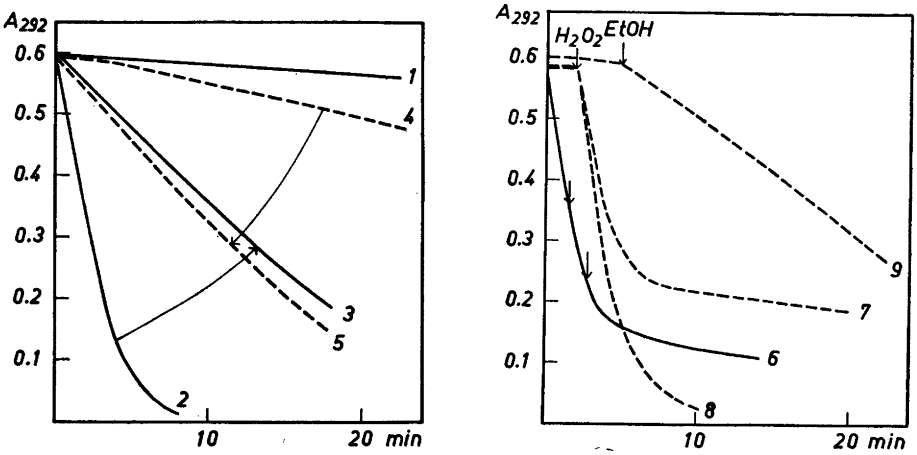

Fig. 2. a and b. Oxidation of DHF in acetate and citrate. 10 μl or more (see text) from an anaerobic 17 mM stock solution were added to 2.50 ml of buffer. HRP (30 μl of a 16 μM solution) was added at time O (2 min after DHF). Air, pH 4.80, 25°. All curves are corrected for dilution. 1. Blank curve, no HRP, 10 mM acetate. 2. Oxidation by HRP, in 10 mM acetate. 3. Same as 2 but DHF stock solution stored anaerobically for 80 min. 4. Same as 2 but 10 mM citrate instead of acetate. 5. Same as 4 but DHF stock solution stored for 80 min. 6. Same as 2 but 0.1 ml 10 mM citrate added at the arrows. 7. Same as 4 but 20 μl 3.7 mM H_2O_2 added at 2 min. 8. Same as 7 but 0.1 ml 3.7 mM H_2O_2. 9. Same as 4 but 10 μl ethanol at 5 min.

Citrate effect. The rate of disappearance of DHF in terms of A_{292} in the presence of peroxidase and O_2 was much higher in acetate than in citrate (Fig. 2 a). The addition of citrate to the complete system in acetate during the course of the reaction within a few minutes reduced the rate to that in citrate (Fig. 2 b). The citrate inhibition was non-competitive to DHF. (Fig.

3). When the citrate buffer was equilibrated with pure oxygen instead of air prior to the addition of DHF and HRP the lag phase was possibly somewhat prolonged but the change was moderate at the most.

Some inhibition of the DHF oxidase activity under the same conditions as in Fig. 2 b was also achieved with 10 mM solutions of maleate (40 % of the activity in acetate), fumarate (30 %), malonate (20 %), and glutarate(10 %), but no inhibition was given by oxalate and succinate.

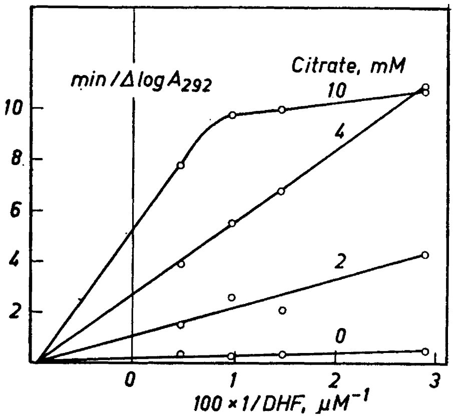

Fig. 3. Inhibition of DHF oxidation in acetate by citrate. The figures give the citrate concentrations in 30 mM acetate. HRP 0.19 μM, pH 4.80, air, 25°. Ordinate: $1/k$, expressed as $t \times (\Delta \log A_{292})^{-1}$ with ΔA as defined.

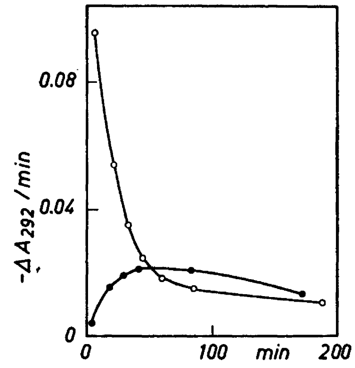

Fig. 4. The effect of storage of the DHF stock solution on the rate of the peroxidase-catalyzed decrease in A_{292}. ○ 10 mM acetate, ● 10 mM citrate. HRP 0.19 μM, air, pH 4.8, 25°.

When the DHF stock solution was stored anaerobically the rate of the enzymic reaction gradually increased in citrate and decreased in acetate (Fig. 2 a and 4). About one hour after the preparation of the stock solution the rates in the two buffers were equal, and then they decreased roughly in parallel. As A_{292} of the stock solution decreased correspondingly larger volumes were used for the enzyme experiments to make the initial A_{292} consistent. When the DHF stock solution was made up in 0.1 M HCl the decrease in rate of reaction in acetate and increase in citrate was about twice as fast.

Citrate had much less effect on the peroxidase than upon the oxidase reaction. The addition of peroxide immediately started the reaction, and when the peroxide was exhausted the initial, slow rate in citrate reappeared (Fig. 2 b). When 0.09 μmole of H_2O_2 were added to the cuvette, containing 0.17 μmole of DHF and 5×10^{-4} μmole of HRP and aerated, the initial rate $-\Delta A_{292}/\Delta t$ was unchanged in 1-25 mM citrate and only little less in 50 mM.

Effect of pH. pH had a strikingly similar effect upon $-\Delta A_{292}/\Delta t$ for a fresh DHF-solution in citrate and for an aged solution in acetate or citrate (Fig. 5). In all cases the rate of the reaction was slow at pH 4.5—

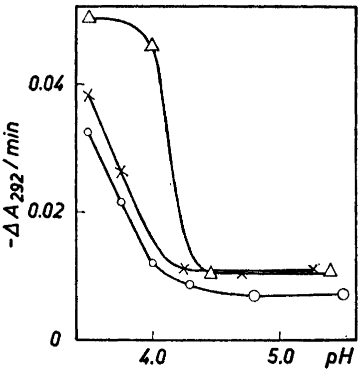

Fig. 5. Reaction velocity in 10 mM citrate (○) and glutarate (△) for a fresh DHF solution and in acetate (×) for a 3 h-solution. The aged solution reacted in the same way in citrate as in acetate. HRP 0.19 µM, DHF 68 µM. Air, 25°.

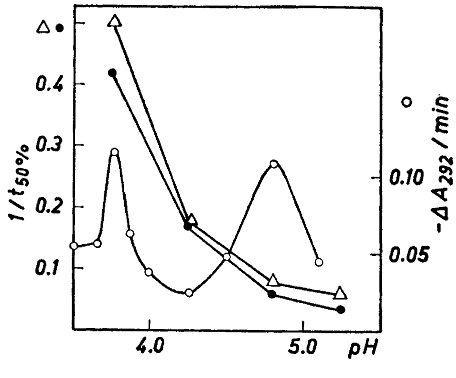

Fig. 6. The effect of pH upon the rate of oxidation of fresh DHF in 30 mM acetate (○). HRP 0.19 µM, DHF 68 µM, air, 25°. In ● as before but the solution was made 30 µM in H_2O_2. In △ as above but 0.9 mM DHF and 2.1 µM HRP, no peroxide added. ○, ● 292 mµ △ 337 mµ. Left scale △ and ●, right scale ○.

5.5 but increasing towards lower pH. With a fresh solution the shape of the activity/ pH-curve in acetate varied much with the concentration of HRP and DHF, but at suitable concentrations two maxima could be discerned (Fig. 6). A lag phase appeared in the experiments with the low concentrations of the reactants but not at the higher concentrations or when peroxide was added at the beginning. The addition of peroxide in quantities less than equivalent with DHF abolished the lag phase and increased the rate about 10-fold at pH 3.75 but little at pH 4.8. Very much the same velocities as with

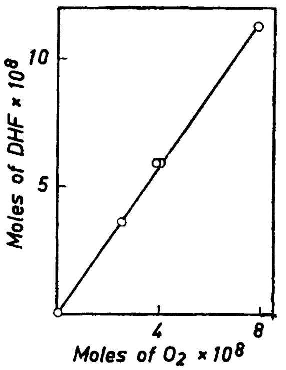

Fig. 7 a. Titration of DHF with acetate buffer, equilibrated with O_2 (1.2 mM O_2) DHF 68 µM, HRP 0.19 µM, 30 mM acetate, pH 4.8, argon, 25°.

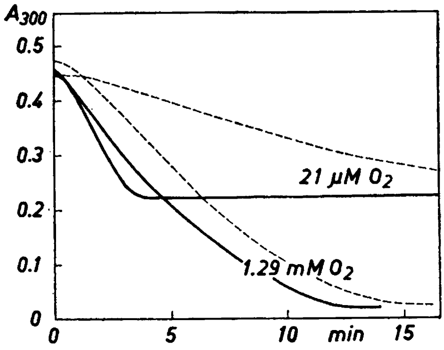

Fig. 7 b. Rate of decrease in A_{300} (glass-cuvettes) in 30 mM acetate at pH 4.80 (——) and 3.75 (– – –) at two oxygen concentrations. Concentrations as in *a.*

the peroxide were obtained (Fig. 6) with the concentrations in the manometer experiments to be described.

Stoichiometry. Under aerobic conditions in citrate buffer at pH 4.8 the ratio DHF: H_2O_2 was 1.5 (Fig. 2 b), which should be compared with the value 1.0 from anaerobic titrations with H_2O_2.[8]

The spectrophotometric titration of DHF with oxygenated water in O_2-free acetate at pH 4.8 in the presence of HRP gave the overall ratio DHF:O_2 1.45 (Fig. 7 a). At pH 3.75 the reaction was slow and well defined final levels were not attained (Fig. 7 b). To elucidate the figure 1.45 the decrease in light absorption, the oxygen uptake, and the change in the state of HRP were compared (Fig. 8). Since manometer experiments require a high concentration of DHF the spectrophotometer record was taken at 337 mμ. Changes in HRP were followed by repeated scanning of the region 380—430 mμ with interpolation of the times. The existence or a rapid and a slow fase in the oxygen uptake was confirmed,[9,8,2,10] and at pH 3.75 and 4.25 the uptake was hampered for some minutes after the termination of the rapid phase. A similar observation was made by Anan and Mason.[10] The oxidation of DHF, in terms of A_{337}, was completed during the end of the rapid phase.[10] During the 75 % disappearance of DHF the ratio DHF:O_2 was about 1 at pH 4.8—5.2 but at pH 3.75 higher than any value previously reported ($\approx$ 9). DHF:O_2 was 0.9 at the end of the rapid phase at pH 4.8, and 1.3 when the oxygen uptake came to a temporary halt after 4 min at pH 3.75 and 4.25. At all pH values the adjacent slow reaction brought the ratio to 0.65—0.85. In the experiments in Fig. 8 (as well as at pH 3.50 and 5.25) the rapid phase lasted for about 4 min. The total uptake during this time was only 45—60 μl O_2, and the gas and liquid phases should have been in equilibrium.

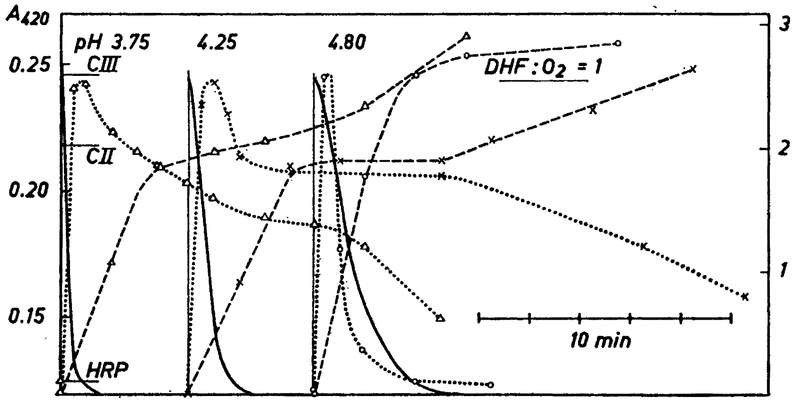

Fig. 8. The oxidation of DHF in terms of A_{337} (——) and oxygen uptake (— — —), and the variations in the Soret band, 420 mμ (.....). HRP 2.1 μM, DHF 0.9 mM, acetate buffer 30 mM, total volume 2.82 ml in all experiments. 25°, air. HRP, C II, C III denote relative levels of the free peroxidase and its complexes II and III. DHF:O_2 = 1 indicates O_2-uptake equimolar to the DHF present. Right scale denotes μmoles O_2 or DHF.

The changes at 420 mμ give the state of HRP. The enzyme existed as compound III during the oxidation of DHF at pH 3.75 and 4.25, whereas at pH 4.80 some DHF remained after the disappearance of compound III. During the cessation of the oxygen uptake at pH 3.75 and 4.25 HRP was present as compound II, and simultaneuosly to the resumption of the oxygen uptake the enzyme returned to its original, free form. The compound II-period at pH 4.25 in Fig. 8 gives an apparent value of $k_4 \leq 500$ M^{-1} sec^{-1} for the reaction between compound II and the DHF derivative(s) emerging from the rapid phase, thus some 40 times less than for the reaction with DHF.[8]

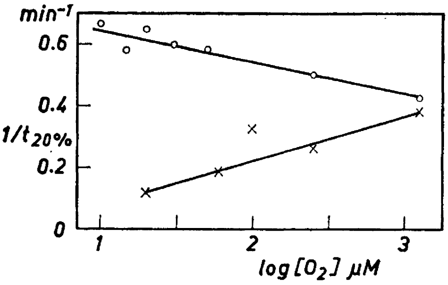

Fig. 9. Rate of reaction at pH 3.75 (×) and 4.80 (O) at various oxygen concentrations. DHF 68 μM, HRP 0.19 μM, 30 mM acetate, 25°.

The variations in the rate of oxidation of DHF with the oxygen concentration are given in Fig. 9. In these experiments $1/t_{20\ \%}$ had to be used for the velocity since rates at less than equivalent concentrations of oxygen were included. At pH 4.80 the velocity somewhat decreased at higher oxygen tensions and the highest values were actually observed at the lowest oxygen tension, with the added O_2 less than equimolar to the DHF in the cuvette. At pH 3.75 the rate increased with increasing oxygen tension. Chance[8] found K_m to be 80 μM at pH 4.0 and 4°, and Fig. 9 suggest a value of this order of magnitude. The straight lines were drawn to fit the experimental values.

DISCUSSION

The general shape of the curves in Fig. 4 suggests two consecutive reactions to occur in the stock solution with DHF as the substance initially present. Weaker acids were formed, A_{max} shifted hypsochromically and decreased at rates that varied with the wavelength, and the rate of the enzymic reaction decreased faster for a stock solution made up in hydrochloric acid than in water. This fits with the stepwise decarboxylation of HDF *via* hydroxymalonic seminaldehyde or hydroxypyruvate to glycolaldehyde demonstrated by means of ^{14}C labelled compounds.[11]

The lag phase in the oxidation of DHF by HRP and O_2 in acetate is abolished by a trace of H_2O_2, which initiates the reaction and makes it proceed with the consumption of oxygen.[9] Heavy metals are catalysts for the autoxidation of enediols, and in model experiments with cytochrome c as DHF oxidase H_2O_2 and Mn^{2+} apparently replaced each other.[9] An assumption that citrate inhibits the DHF—HRP—O_2 reaction by forming a catalytically inactive chelate with metal ions accidentally present does not account for all observations. The ratio of oxidized DHF to added H_2O_2 was 1.5 in citrate under aerobic conditions (Fig. 2 b) *i.e.* the reaction soon stopped when the peroxide was exhausted. The addition of citrate to DHF—HRP—O_2 in acetate brought the reaction to a halt except for what could be attributed to remaining peroxide (Fig. 2 b). Finally citrate inhibited non-competitively to DHF (Fig. 3) and a DHF-metal chelate could hence hardly be operating.The results in Fig. 2 become comprehensible if peroxide exerts a twofold action: As an oxidant of DHF (1:1[8]) and as a modifier of the enzyme to make it fit to catalyze the oxidase reaction even in the presence of citrate. The effect of ethanol (Fig. 2) lends some support to this notion. The stoichiometry of H_2O_2:DHF$=2:1$ has been rejected.[8]

DHF seems to react in two ways: At pH 4.5—5.5 by an oxidase-peroxidase mechanism in acetate but not in citrate, and at a more acid reaction, pH $<$ 4, by some other mechanism which operates equally well in citrate and acetate. The reactivity in citrate of the enediol DHF parallels that of the 3-carbon compounds, both of which can acquire an enediol form, and it is a reasonable assumption that DHF in citrate behaves as any other enediol. The oxidation of DHF at pH 4.8 in acetate is inhibited by other redogenic substanses in Yamazaki's classification [12] (as we have confirmed for ascorbic acid). This fact, together with the lack of a marked difference between maleate and fumarate as inhibitors, makes it probable that DHF is linked to HRP *via* one or both of its hydroxyl groups. Several substances react with an oxidase-peroxidase mechanism, but DHF seems to be unique in its ability to form a compound of type III and to hydroxylate. The question then arises about the role of the carboxyl groups, adjacent to the enediol arrangement, for the formation of compound III.

The noncompetition of DHF and citrate implies different sites of action. A reaction between DHF and some group(s) on the protein is one possibility to consider. George and Irvine [13,14] found anionic oxidants to react slowly with tyrosine, tryptophan, and cysteine but to cause rapidly spectral changes in HRP. Fergusson [15] added histidine to the group of reactive amino acids, and histidine is a target in the photooxidation of myoglobin.[16,17] It seems plausible that DHF could use the same site(s) on HRP as the anionic oxidants but for the opposite purpose, to add electrons to the protein and further to the ultimate acceptor, O_2 or H_2O_2. Another possible site for the binding of DHF would obviously be the haem itself, in a manner non-competitive to the citrate.*

Banga, Phillipot, and Szent-Györgyi [18] stated that DHF oxidase has a sharp maximum at pH 4, and Chance [8] studied the reaction at pH 4.0. Morita and Kameda [2] found the optimum at pH 4.8. Yamazaki [4] found two maxima in the methylene blue-reductone-peroxidase reaction. Under certain conditions two discrete maxima for the overall DHF oxidase activity are found (Fig. 6). DHF reacts differently at these acidities in the following respects:

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* As a matter of fact citrate does not give a spectroscopically operable compound with HRP (to be published).

	pH 3.75	pH 4.80
End-levels in O_2 titrations	ill-defined	sharp
Effect of H_2O_2 on DHF-oxidation in aerobic acetate	10-fold increase	little
Effect of increasing pO_2 on rate of DHF oxidation	increasing	decreasing
Rate of oxidase reaction in citrate	high	low

Fig. 8 confirms [10] that the oxidation of DHF, in terms of light absorption, is completed during the rapid phase, and shows that at the actual concentrations the reaction is faster at pH 3.75 than at pH 4.80. The adjacent slow O_2-consumption does not concern DHF as such but only its oxidation products, *i.e.* diketosuccinate [19–21,2] and the products thereof. Interestingly enough these products do not consume O_2 by autoxidation but by the mediation of HRP. As long as HRP is bound in the form of compound II no O_2 is taken up. At pH 4.80 (Fig. 8) HRP returns rapidly from compound III to free enzyme and before all DHF is gone, whereas at pH 3.75 and 4.25 DHF and compound III disappear about simultaneously. There is a lag period at pH 4.80, and the data permit no conclusions about the effect of DHF on the stability of compound III. The data do, however, suggest an interaction between O_2 and H_2O_2, and the occurrence of the two maxima, at pH 3.75 and 4.80, may depend upon the initial balancing of the concentrations of DHF, HRP, and O_2.

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