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The Effect of Ions on the Catalytic Activity of Enzymes: The Old Yellow Enzyme

WILLIAM J. RUTTER* and BRITA ROLANDER

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The present communication is concerned with a study of the effect of ions on the activity of the old yellow enzyme system. In addition to a general inhibitory effect by anions, certain specific effects were observed. Fe^{3+} ions can, for example, act as the electron acceptor in the reaction, and in fact, with chelated iron compounds as the electron acceptor, the reaction rate increases markedly. The old yellow enzyme, as isolated, is apparently not a metallo flavoprotein.

The implications of the date with regard to the physiological activity of the old yellow enzyme are discussed.

The old yellow enzyme (OYE) ** facilitates the oxidation of TPNH (and to a lesser extent DPNH) according to equations (1) and (2).

$$\text{TPNH} + \text{H}^+ + \text{yellow enzyme} \rightleftharpoons \text{TPN}^+ + \text{leuco enzyme} \qquad (1)$$

$$\text{leuco enzyme} + \text{oxygen or dye} \rightarrow \text{H}_2\text{O}_2 \text{ or leuco dye} + \text{yellow enzyme} \qquad (2)$$

This enzyme was discovered by Warburg and Christian [1], and later purified extensively and studied by Theorell [2]. It has recently been crystallized by Theorell and Åkesson [3].

OYE preparations are characterized by a low turnover number in oxygen (approximately 35 moles O_2/min/mole enzyme at 30°C for the crystalline enzyme [3]), and also a very slow reaction rate with cytochrome c. Under aerobic conditions [4] reaction with oxygen takes precedence over reaction with cytochrome c. On this basis, OYE could be considered the TPN counterpart to the diaphorases.

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* U. S. Public Health Service Fellow, 1954—55. Present address, Division of Biochemistry, University of Illinois, Urbana, Illinois, USA.

** Abbreviations used in this paper are OYE, old yellow enzyme; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; Zwischenferment, yeast glucose-6-phosphate dehydrogenase; EDTA, ethylene diamine tetraacetic acid.

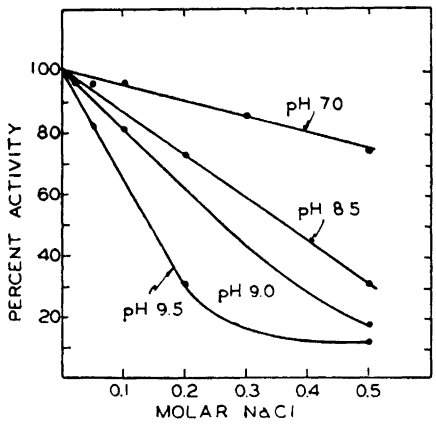

Fig. 1. Inhibition of activity of the Zwischenferment OYE system by sodium chloride. The measurements were made in conventional Warburg apparatus, each flask contained 15 μM glucose-6-phosphate; 0.5 μM TPN; 2 mg *Zwischenferment* (activity under optimal conditions, 1 μM TPNH formed/min/mg); 334 μg OYE; 29 mμM catalase; 100 μM buffer (glycine, pH 9.0, 9.5; glycylglycine, pH 8.5, 7.0); salts were added to the final concentration indicated; total volume 3.0 ml. Under these conditions 4.2 μM O_2 disappeared in 10 min, in control flasks at pH 8.5.

Theorell and Nygaard [5-7] have studied in a thorough manner the reaction of the old yellow protein, and the prosthetic group, FMN:

$$FMN + \text{old yellow enzyme apoprotein} \rightleftharpoons \text{old yellow enzyme} \qquad (3)$$

It was found that the ionic environment, especially the anionic species, influenced the reaction markedly.

METHODS

The old yellow enzyme was prepared by a modification of methods used for some time in the laboratory of Professor Theorell. A preparation having a β 280/β 465 ratio of 12.8 corresponding to 80 % purity was used in these experiments. Glucose-6-phosphate dehydrogenase (*Zwischenferment*) was prepared by the method of Warburg and Christian [8] and a modification of the procedure of Kornberg [9].

TPN, 90 % pure, was procured from Sigma Company. Oxygen consumption by the glucose-6-phosphate dehydrogenase — old yellow enzyme system was measured in the conventional Warburg apparatus. TPNH oxidation was followed spectrophotometrically or by the decrease in the intensity of fluorescence [10].

RESULTS

Effects of anions on the Zwischenferment— old yellow enzyme system. The influence of anions on the coupled Zwischenferment-old yellow enzyme system was studied using O_2 as the electron acceptor. Because of the experience already gained with Zwischenferment reaction [11], it was possible to keep this enzyme activity in considerable excess. The TPN concentration was

Table 1. Inhibition of the activity of the Zwischenferment—OYE system by ions.

Salt	pH	Molar concentration for 50 % inhibition
Fluoride	9.5	0.25*
	9.0	**
Chloride	9.5	0.14
	9.0	0.25
	8.5	0.37
Bromide	9.5	0.13
	8.5	0.24
Iodide	9.5	0.05
	8.5	0.08
Phosphate	9.5	0.04
Nitrate	9.0	0.14
Sulfate	9.5	0.07
Carbonate	9.5	0.03
Formate	9.5	0.14
Citrate	9.5	0.05
EDTA	9.5	0.01
Cholate	9.5	0.005

* 0.2 M NaF produces 40 % inhibition, higher concentrations produce no greater inhibtion
** only 10 % inhibition at 0.5 M NaF

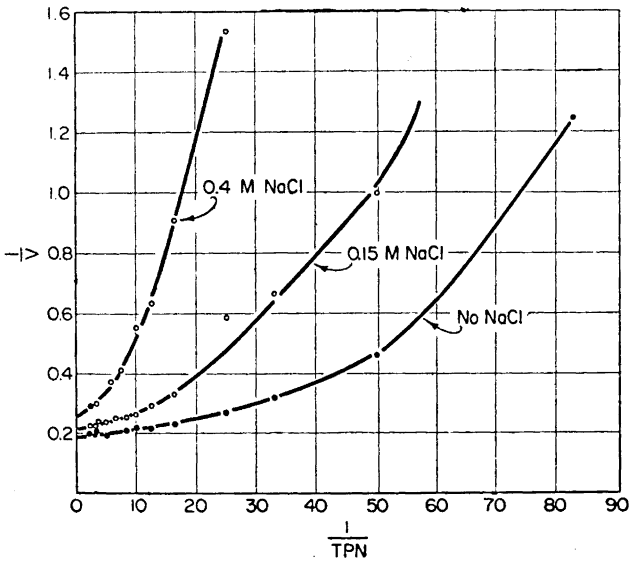

Fig. 2. Effect of TPN concentration on the inhibition of activity of the Zwischenferment OYE system by salts. Experimental conditions are essentially those described under Fig. 1; salt concentration, and TPN concentration varied as indicated.

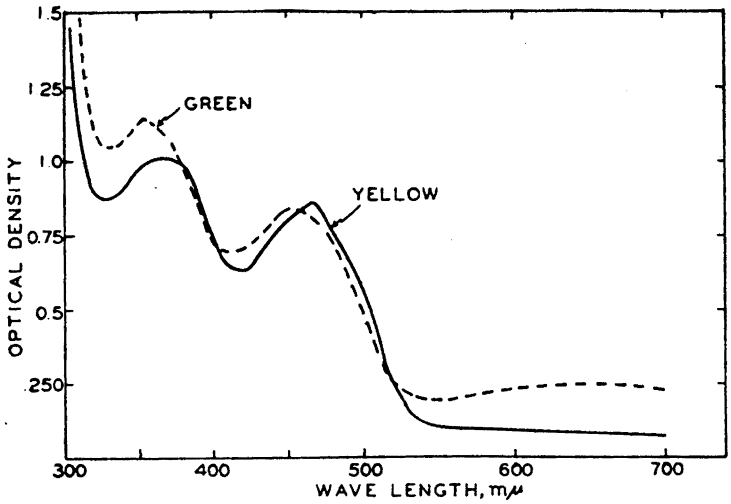

Fig. 3. Absorption spectra of OYE in acidic and basic ammonium sulfate solutions. OYE used in these experiments was approximately 80 % pure, spectra were taken in approximately 0.50 saturated $(NH_4)_2SO_4$, pH 10.2 (by addition of concentrated ammonium hydroxide, and in the same solution after bubbling with N_2.)

kept at a level of several times required to saturate the system under control conditions. Under these conditions the pH of optimal activity was 8.3 It was found that various salts inhibited oxygen uptake by the system, especially on the alkaline side of the pH activity curve. The inhibitory effect of sodium chloride, for example, as a function of pH, is presented in Fig. 1. Similar data were obtained with other salts. The degree of inhibition was in large measure a reflection of the anionic species present. Table 1 summarizes the data obtained with various salts. The inorganic salts may be placed in the order of decreasing effectiveness PO_4, CO_3, $I > SO_4$, $NO_3 > Br > Cl > F$.

The possibility that the inhibitory effects were simply related to a decreased affinity of the system for TPN was tested. The inhibition could be partly reversed by the addition of additional TPN to the system. As shown in Fig. 2, the saturation of the system with TPN was a function of the ions present in the assay system. A substantial inhibition remained, however, even after extrapolation to infinite TPN concentration. It was also confirmed that additional Zwischenferment and glucose-6-phosphate did not affect the inhibition. These observations suggested that some of the inhibitory effects might be on the catalytic process associated with the old yellow enzyme. This result was interesting in view of the effects of anions on the dissociation of the old yellow enzyme observed by Theorell and Nygaard. Addition of large amounts of FMN did not, however, increase the reaction rate in the presence of salts, and calculations from their data show that dissociation as measured by appearance of fluorescence is still much too small to account for inhibition by salts in the presence of excess TPN.

The solubility of oxygen in the medium is not significantly altered by salts at the concentrations used in these experiments, but the reaction of the old yellow enzyme with oxygen may be markedly affected.

Of particular interest was the strong inhibition observed with divalent anions and especially with EDTA.

The transformation of the old yellow enzyme into a green-colored protein. It was observed that addition of saturated ammonium sulfate solutions, adjusted to pH 10—10.5 with ammonium hydroxide, to solutions of the old yellow enzyme produced greenish solution. This did not reflect a complete dissociation of FMN from the protein, since OYE could be precipitated in this green form, redissolved in buffer (becoming yellow instantly) and reprecipitated in the green form without leaving absorption at 465 mμ. It was found that if the ammonia were removed, by bubbling a gas through the solution, for example, the yellow color would also reappear. Fig. 3 presents the absorption spectra of the green compound, and the spectra of the same solution after bubbling with oxygen. There was less than 4 % difference in the fluorescence of the solutions of the yellow and green proteins. It would not, however, be wise to conclude that the green form of OYE is nonfluorescent as is the yellow form, since the high concentrations of NH_4^+, $SO_4^=$, and NH_3 might effectively quench fluorescence, if present.

Several cycles of conversion from the green to the yellow form did not influence the catalytic activity of the enzyme.

Metal ion content of old yellow enzyme preparations. The strong inhibition of activity of old yellow enzyme preparations by EDTA (see Table 1) prompted an examination of the relationship of various metal ions to this catalysis. The effect of prolonged dialysis against chelating agents and subsequent addition of metal ions was studied, and in addition, the metal content of OYE preparations was assayed by emission spectroscopic and chemical methods.

Table 2. Effect of metal ions on oxygen consumption by Zwischenferment—OYE system. Conditions essentially the same as those described in the legend of Figure 1. Glycylglycyl buffer, pH 7.8, 200 μgm OYE (dialyzed against 0.001 M versene), metal ions were added at the concentration listed, as their chloride or sulfate salts.

		μM O_2/10 min	% Activity
1.	Control	2.01	100
2.	» + 10^{-3} M Mg^{2+}	2.02	100
3.	» + 10^{-2} M Mg^{2+}	1.08	54
4.	» + 10^{-3} M Cu^{2+}	1.93	95
5.	» + 10^{-2} M Cu^{2+}	0.81	40
6.	» + 10^{-5} M Mn^{2+}	2.11	105
7.	» + 10^{-4} M Mn^{2+}	1.94	97
8.	» + 10^{-3} M Mn^{2+}	0.48*	24
9.	» + 10^{-5} M Fe^{3+}	2.03	100
10.	» + 10^{-4} M Fe^{3+}	2.01	100
11.	» + 10^{-3} M Fe^{3+}	0.47	23
12.	» + 10^{-2} M Fe^{3+}	0.06	3

* Visible precipitate formed

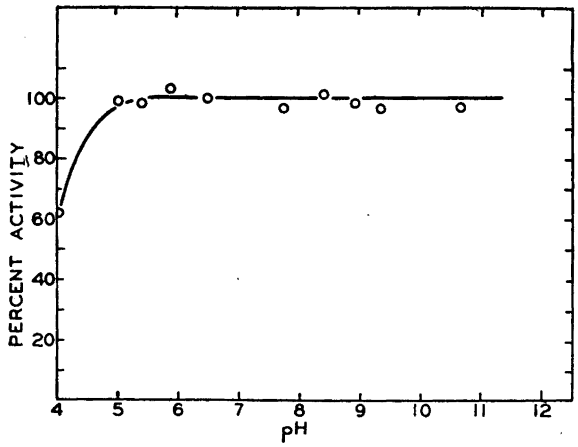

Fig. 4. Oxidation of TPNH by OYE as a function of pH. Each reaction mixture contained 50 μg OYE β 280/β 464 = 12.8 (~80 % pure), 0.15 μM TPNH, 100 μM buffer (acetate pH 4.0, 5.0; histidine, pH 5.4, 5.8; phosphate, pH 6.4, 6.7, 7.2; glycylglycine, pH 7.4, 7.8, 8.3; glycine pH 8.7, 9.1, 10.2, 10.7), total volume 3 ml. 100 % activity under these conditions represented an optical density change at 340 mμ of 0.045.

Prolonged dialysis against EDTA at pH 9.4, 7.5, and 5.0 did not decrease the specific activity; in fact, when most of the EDTA was removed by dialysis against triple-distilled water, the specific activity consistently increased 10—15 %.

The metal content of OYE preparations dialyzed against dilute buffer, and against EDTA solutions was measured in cooperation with Kandidat Molt, of Sveriges Geologiska Undersökning, Geokemiska avdelningen. Emission spectroscopic assays were made for the presence of Zn, Fe, Mn, Cu, Mg and Ca. Fairly large and variable quantities of Mg and Ca and insignificant amounts of Fe, Mn, Cu, Zn were found. The zinc and iron content of OYE preparations was also assayed by chemical methods with a similar result. In addition, a preparation of OYE of approximately 80 % purity which had

Table 3. Fe^{3+} and oxidation of TPNH by OYE. The control reaction mixture contained 0.1 μM TPNH, 50 μg OYE, 100 μM glycylglycine, pH 8.2, total volume, 3.0 ml. The experimental cuvettes contained in addition $FeCl_3$ in the amount indicated, and 1 μM *a,a'*-dipyridyl when indicated. Measurements were made spectrophotometrically at 340 mμ with appropriate correction for absorption of other materials.

		TPNH Oxidized μM/min
1.	Control	0.0212
2.	» + 10^{-3} M Fe^{3+}	0.0249
3.	» + 10^{-4} M Fe^{3+}	0.024
4.	» + *a,a'*-dipyridyl	0.023
5.	» + 10^{-4} M Fe^{3+} + *a,a'*-dipyridyl	0.030

been dialyzed against dilute ammonia buffer, was kindly assayed by Professor B. L. Vallee of Harvard University. Only traces of Zn, Fe, Cu, and Mn were found, in all cases less than 0.1 mole metal/mole protein.

It is concluded that the old yellow enzyme, as isolated at least, is not a metallo flavoprotein involving Fe, Zn, Cu, or Mn. Mg and Ca are commonly present in many enzyme preparations, and it is presumed, though not proved, that these cations are contaminants not involved in the catalytic function of OYE.

Effect of metal ions on the catalytic activity of old yellow enzyme. The effect of metal ions on the activity of the Zwischenferment-OYE system assayed by measurement of oxygen uptake was studied. As shown in Table 2, it was found that no significant stimulation of activity occurred under the conditions employed, but that concentrations of Mn^{2+} or Fe^{3+} higher than 10^{-4} M caused marked inhibition. Since a visible precipitate accompanied Mn^{2+} inhibition, but none occurred with Fe^{3+} inhibition, further studies of this inhibitory phenomena were made primarily with Fe^{3+} ions. At 10^{-3} M, Fe^{3+} did not markedly influence the activity of isolated Zwischenferment, nor did increased TPN concentration influence the inhibition of oxygen uptake of the coupled system. It was, therefore, likely that the inhibition of oxygen uptake by Fe^{3+} concerned the activity of OYE itself. Experiments on the activity of OYE were carried out by measuring the oxidation of added TPNH spectrophotometrically. Fig. 4 presents the remarkable relationship between pH and activity for this reaction; essentially full activity is measured over the range from pH 5.0 to pH 10.

The Fe^{3+} ions did not inhibit TPNH oxidation by OYE in the concentration ranges 10^{-5} to 10^{-1} M, but instead acted as an electron acceptor. The formation of Fe^{2+} during the reaction was detected by spectrophotometric measurement of its complex with α,α'-dipyridyl. Table 3 presents the results of these experiments. The apparent inhibition of the Zwischenferment system by Fe^{3+} is likely a result of its competition with oxygen for electrons. Since the solubility of oxygen is 10^{-3} M, these data suggest Fe^{3+} to be a more efficient electron acceptor than oxygen for OYE.

Table 4. Effect of various electron acceptors on TPNH oxidation by OYE. The control reaction mixture contained 0.5 μM TPNH, 50 μg OYE, 100 μM glycylglycine, pH 8.2, total volume 3.0 ml. The experimental cuvettes contained, in addition when indicated: DCPIP (2,6-dichlorophenolindophenol); ferricyanide at a final concentration of 4×10^{-4} M; H_2O_2, 0.05 ml 30 % solution; catalase, 50 μg crystalline beef liver preparation. Measurements were made spectrophotometrically at 340 mμ; corrections for absorption of other materials were made when necessary.

		TPNH oxidized μM/min
1.	Control	0.022
2.	» + DCPIP	0.032*
3.	» + H_2O_2 + catalase	0.12*
4.	» + 4×10^{-4} M Fe $(CN)^{3-}$	0.18*
5.	» + 10^{-4} M Fe^{3+} + H_2O_2 + catalase	0.13

* Corrected for endogenous TPNH oxidation which occurred at a slow rate in the absence of substrate.

Table 4 presents a typical experiment in which different electron acceptors were used. Coupling to the dye dichlorophenolindophenol did not alter the rate markedly, but the presence of a chelated iron compound $Fe(CN)_6$ stimulated the catalytic rate 8 to 10-fold over the rate observed under ordinary conditions. The addition of H_2O_2 and catalase also caused a substantial increase in rate. This might be the reflection of an increased oxygen tension, although rates could not be obtained by bubbling oxygen through the solutions. It is possible that a more specific reaction is involved.

Attempts to find in bottom yeast a system which would stimulate oxidation of TPNH by OYE preparation have thus far been unsuccessful.

DISCUSSION

Although the old yellow enzyme is present in high concentration in yeast (1 mg OYE/g dry weight) and although the enzyme has been studied extensively, its physiological role is yet unclear (like the majority of flavoprotein enzymes). Its turnover number with oxygen or cytochrome c as electron acceptor is very low. Several experimental observations are consistent with the postulate that the old yellow enzyme has been separated from its appropriate acceptor during the course of purification. Haas [12] has shown that the reduction of OYE by TPNH according to equation (1) occurs at 60 times the rate of the oxidation of the leuco enzyme by oxygen. The present experiments indicate that appropriate electron acceptors indeed allow a much faster rate. It is not surprising that Fe^{3+} acts as an electron acceptor. Kaplan *et al.*[13] have recently shown that this is perhaps a general phenomenon with flavoprotein enzymes. It is pertinent, however, that Fe^{3+} is a more efficient electron acceptor than oxygen, and that with ferricyanide very considerable increases in catalytic rates are obtained. It would be unwise to conclude from this that the biological electron acceptor system for OYE is mediated through Fe^{3+} or another suitable metal ion, but the attractiveness of this possibility is manifest. The possible role of metals in the activity of flavoprotein enzymes has been recently discussed by Mahler [14].

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