XANTHINE DEHYDROGENASE OF CHICKEN LIVER

by

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In a previous publication it was demonstrated[1] that xanthine dehydrogenase in tissue slices and preparations from chicken liver is linked with lactic dehydrogenase via cozymase (DPN). Earlier it had been shown by BERNHEIM AND BERNHEIM[2] that pyruvate could increase the activity of xanthine dehydrogenase in rat liver slices. RICHERT AND WESTERFELD[3] first demonstrated that in chicken liver homogenates xanthine dehydrogenase is a dehydrogenase, not an oxidase, when they found that it reacted only very slowly with oxygen compared to methylene blue. MORELL[1] has shown that this reaction with oxygen is even slower in partly purified enzyme preparations than in homogenates since the presence of low concentrations of pyruvate and DPN in the latter preparations increases the oxidation of xanthine as measured by the increase in uric acid production.

It was suggested[1] that the component of the electron-transferring chain between xanthine and lactic dehydrogenase was identified with, or occurred near, DPN-cytochrome *c* reductase. Further study has not revealed any evidence for a component mediating between xanthine dehydrogenase and cozymase as was thought possible at that time. The dehydrogenase must therefore be considered to react directly with DPN. MACKLER, MAHLER AND GREEN[4] have recently claimed that milk xanthine oxidase also reacts with DPN.

The present paper describes an investigation into the reduction of DPN by chicken liver xanthine dehydrogenase and other reactions of this enzyme.

EXPERIMENTAL

Methods and reagents

Xanthine dehydrogenase activity was measured either by the colorimetric determination of uric acid produced per unit time as described by MORELL[1] or spectrophotometrically using a Hilger "Uvispec" spectrophotometer to measure the rate of reduction of DPN with xanthine as substrate. This measurement was made at pH 9.2 in pyrophosphate buffer by following the increase in optical density at 340 mμ. DPN, buffer and enzyme preparation were added to a 1 cm cell and xanthine added only after the optical density at 340 mμ was stable. Dialysed crude preparations usually gave some reduction of DPN before xanthine was added but partly purified preparations were virtually free of endogenous substrates. The reaction rates were calculated from the readings of optical density made up to two minutes after adding xanthine. These readings were approximately linear with time, as shown in Fig. 1, when the enzyme was saturated with either substrate or acceptor.

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Xanthine (mono-sodium salt) was purchased from Schwarz Laboratories and hypoxanthine (Nutritional Biochemicals Corp.) was supplied by Dr. R. K. MORTON. Cytochrome *c* (in solution in 0.9% NaCl) was donated by The Anglo-Swiss Drug Co., Sydney, and was dialysed against distilled water or the appropriate buffer before use. DPN (Sigma "90") and TPN "80" were purchased from the Sigma Chemical Co. The designated purity for the former (85%) was checked by complete reduction by xanthine and chicken liver xanthine dehydrogenase.

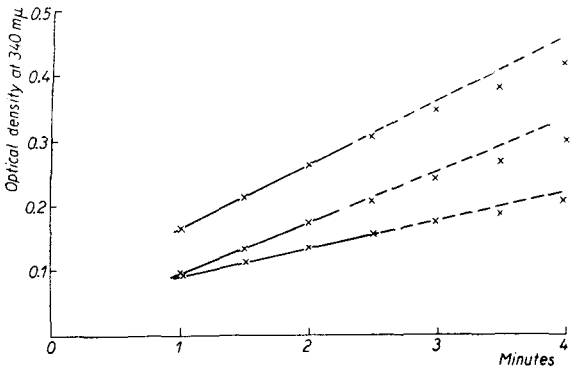

Fig. 1. Time course of reduction of $1 \cdot 10^{-4}$, $3 \cdot 10^{-4}$ and $10 \cdot 10^{-4}$ *M* DPN by chicken liver xanthine dehydrogenase and xanthine ($1.8 \cdot 10^{-3}$ *M*) in pyrophosphate buffer, pH 9.2, 0.016 *M*. Readings from these curves are used in Fig. 2.

Preparations of chicken liver xanthine dehydrogenase used in this study were either crude or partly purified. Those designated "crude" were prepared by homogenizing chicken liver in two volumes of water using a glass Teflon Elvehjem-Potter type homogenizer, centrifuging the homogenate at 8,000 r.p.m. for 15 minutes after adjustment of the pH to 5.5 and subsequently dialysing the centrifugate (pH adjusted to 7.4) against the appropriate buffer or distilled water. "Partly purified" preparations were made by a procedure which will be described in detail in a later paper. The preparations were light orange in colour and showed only faint oxyhaemoglobin bands using the hand spectroscope. The extent of purification was only by a factor of 5–10 times relative to the original homogenate. The colour of these solutions was due neither to flavins nor haemoglobin.

The preparation of milk microsomes used was kindly supplied by Dr. R. K. MORTON who has described their preparation[5]. This preparation had a high concentration of xanthine oxidase as determined spectrophotometrically with 2,6-dichlorphenol indophenol as acceptor. The percentage xanthine oxidase was calculated to be 3–5% using the kinetic values for the activity of the nearly pure enzyme (μmole substrate oxidized/minute/mg enzyme) published by MACKLER *et al.*[4].

RESULTS

The reduction of DPN

Chicken liver xanthine dehydrogenase. The linkage of the xanthine and lactic dehydrogenases found in tissue slices and preparations from chicken liver[1] and the finding by MACKLER *et al.*[4] that milk xanthine oxidase can react with DPN suggested the direct reaction of the chicken liver enzyme with DPN as hydrogen acceptor. It has been found that chicken liver xanthine dehydrogenase can reduce DPN almost as rapidly as it can reduce methylene blue. This is in contrast to milk xanthine oxidase which reacts with DPN only very slowly (see later).

If a component exists which mediates between xanthine dehydrogenase and DPN this should be revealed during purification of the enzyme. By removing this component, purification would prevent the reaction of the enzyme with DPN without affecting its reaction with methylene blue. A tenfold purification did not affect the rate of reaction of the enzyme with DPN as compared with methylene blue. In this experiment the ratio of activity with DPN ($1.2 \cdot 10^{-3}$ *M*) to activity with methylene blue ($1 \cdot 10^{-3}$ *M*) was 1/1.23 for the "crude" enzyme preparation and 1/1.17 after tenfold purification. Thus, the chicken liver xanthine dehydrogenase appears to react directly with DPN. Complete purification of the enzyme will be needed to establish this point beyond doubt.

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The Michaelis constant for the enzyme with DPN as acceptor (K_m-DPN) was determined using both crude and partly purified enzyme preparations and with different ranges of DPN and xanthine concentration. When the enzyme was saturated with xanthine values for K_m-DPN of $1.32 \cdot 10^{-4}$ M (see Fig. 2) and $1.07 \cdot 10^{-4}$ M were obtained. Because higher levels of xanthine were found to be inhibitory (see later), concentrations of xanthine less than that giving the theoretical maximum activity (V_{max}) were also used and the rate of reduction of DPN was calculated from the first two readings (20 and 45 seconds) because the readings were not linear with time. The values for K_m-DPN calculated from several such experiments are considered unsatisfactory because of the considerable scatter in the Lineweaver-Burk graphs and are not given.

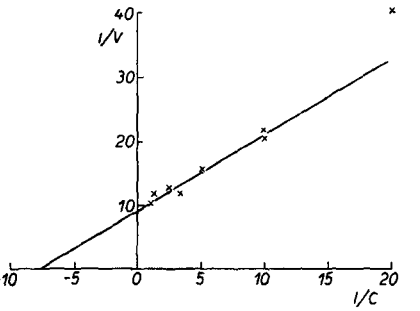

Fig. 2. Graphic evaluation of Michaelis constant for chicken liver xanthine dehydrogenase (reaction with DPN as electron acceptor) using a "crude" enzyme preparation (see *Methods*) and $1.8 \cdot 10^{-3}$ M xanthine as substrate, c = millimolar concentration of DPN and v = velocity in activity test. $K_m = 1.32 \cdot 10^{-4}$ M; for duplicate determination see text.

Comparison with milk xanthine oxidase. It was stated earlier that chicken liver xanthine dehydrogenase reduces DPN nearly as fast as it reduces methylene blue (ratio = 1/1.23) and that this reaction with DPN is fast relative to the reduction of DPN by milk xanthine oxidase. MACKLER *et al.*[4] have claimed that DPN can serve as electron acceptor in the milk xanthine oxidase system (hypoxanthine as substrate). However, calculation of the rate of this reaction from Fig. 1A of their paper gives a rate of $7 \cdot 10^{-3}$ μmole DPN reduced/minute/mg enzyme. From Table II of the same paper the rate of reduction of indophenol dye is given as 1.11 μmole hypoxanthine oxidized/minute/mg enzyme. Thus, in the presence of hypoxanthine milk xanthine oxidase reduces indophenol dye approximately 150 times as fast as DPN. This finding has been confirmed in the present study using xanthine as substrate and several different preparations of milk xanthine oxidase. Two of these preparations were made according to the method of HORECKER AND HEPPEL[6] and were purified only as far as the first optically clear solution of enzyme. These solutions were prepared in the crude state to prevent or minimize any alteration in the properties of the xanthine oxidase. Under anaerobic conditions, in the presence of $9 \cdot 10^{-4}$ M xanthine and with phosphate buffer, pH 7.4, 0.1 M, these preparations reduced DPN at less than 1/80th the rate of reaction with oxygen.

The third milk xanthine oxidase preparation used consisted of a suspension of milk microsomes (see *Methods*). Microsomal xanthine oxidase was tested for its ability to reduce DPN because it was thought that the enzyme attached to particles may have properties different from those of the solubilized enzyme. The reaction mixture contained DPN ($7.5 \cdot 10^{-4}$ M), hypoxanthine ($5 \cdot 10^{-4}$ M), pyrophosphate buffer, pH 8.1, 0.05 M and microsome suspension. The amount of microsome suspension reduced, in a separate determination, 0.06 μmole indophenol dye/minute/mg microsome protein. The determination of DPN reduction was made anaerobically in Thunberg tubes which were incubated at room temperature for 30 minutes. On opening the tubes an aliquot of the contents was de-proteinized with perchloric acid,

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neutralized, diluted appropriately and the optical density read at 340 mμ in the spectrophotometer. Control tubes were set up in the same manner except for the absence of hypoxanthine. In this experiment no $DPNH^+$ was detected. In order to show that DPN was not destroyed during the experiment alcohol and alcohol dehydrogenase were added after the measurement of optical density at 340 mμ. A rapid increase of absorption at 340 mμ ensued. The sensitivity of the test was such that less than $7 \cdot 10^{-4}$ μmole DPN was reduced/min by the same amount of microsome preparation as used in the reaction with indophenol. Thus, the microsomal xanthine oxidase preparation reduced DPN at a rate less than 1/85th the rate of reduction of indophenol.

Other properties of chicken liver xanthine dehydrogenase

Effect of varying xanthine concentration. High concentrations of xanthine were found to inhibit the activity of xanthine dehydrogenase. This is demonstrated in Fig. 3 where the effect of varying the xanthine concentration on enzyme activity (enzyme saturated with DPN) is plotted according to the method of LINEWEAVER AND BURK[7]. At concentrations of xanthine considerably above K_m-xanthine high values for the reciprocal of reaction velocity were obtained. However, for concentrations of xanthine below K_m-xanthine a linear curve was found from which the value $2.9 \cdot 10^{-4}$ M was obtained for K_m-xanthine (see Fig. 3). Two other determinations of K_m-xanthine using low concentrations of xanthine gave values of $1.7 \cdot 10^{-4}$ M and $3.1 \cdot 10^{-4}$ M, the average for the three determinations being $2.6 \cdot 10^{-4}$ M.

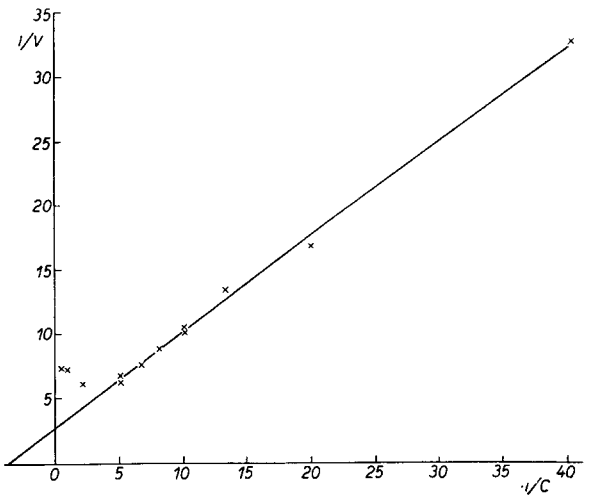

Fig. 3. Graphic evaluation of Michaelis constant for chicken liver xanthine dehydrogenase (reaction with xanthine as substrate) using a "partly purified" enzyme preparation (see *Methods*) and $1.2 \cdot 10^{-3}$ M DPN as electron acceptor. $K_m = 2.9 \cdot 10^{-4}$ M; for replicate determinations see text.

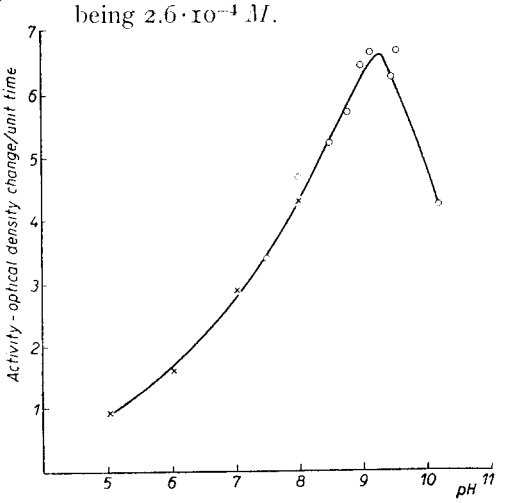

Fig. 4. The effect of pH on the activity of chicken liver xanthine dehydrogenase. ×—×, citratephosphate buffer, O- - -O, pyrophosphateglycine-NaOH buffer. The substrate was xanthine and the acceptor methylene blue. For methods of enzyme activity measurement see text. "Partly purified" enzyme preparation (see *Methods*) was used.

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pH optimum. The effect of pH on xanthine dehydrogenase activity is demonstrated in Fig. 4. A sharp optimum at pH 9.2–9.6 was found for both methylene blue and DPN as electron acceptors using either "crude" or "partly purified" (see *Methods*) enzyme preparations.

Lack of reaction with cytochrome c *or TPN.* A number of chicken liver preparations were tested for the ability to reduce cytochrome *c* in the presence of xanthine. The reduction of cytochrome *c* was measured spectrophotometrically at 550 mμ. Even well dialysed, "partly purified" preparations of enzyme gave some reduction of cytochrome *c* in the absence of added substrate. Xanthine was added to such reaction mixtures after the rate of reduction of cytochrome *c* by endogenous substrate had reached a minimum. A slight increase in the rate of reduction then took place but rapidly slowed down (see Fig. 5). The amount of enzyme preparation used in this experiment was found to reduce $2.2 \cdot 10^{-2}$ μmole DPN/minute. From Fig. 5 it has been calculated that this concentration of enzyme has reduced less than $9 \cdot 10^{-4}$ μmole cytochrome *c*/minute; *i.e.* the reaction with DPN is more than 25 times as fast.

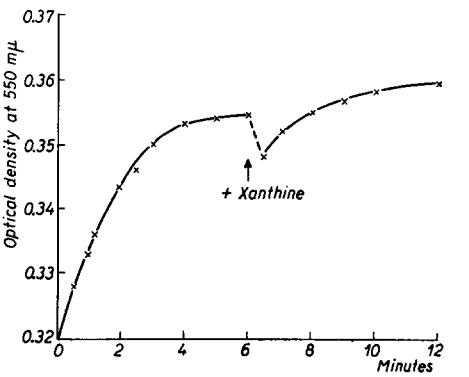

Fig. 5. Reduction of cytochrome *c* by "partly purified" chicken liver xanthine dehydrogenase preparation (see *Methods*). The reaction mixture contained 0.1 ml enzyme preparation, cytochrome *c*, $1.8 \cdot 10^{-5}$ *M* and phosphate buffer, pH 7.5, 0.01 *M*. Xanthine, $1 \cdot 10^{-3}$ *M*, final concentration, was added as indicated. Full reduction of cytochrome *c* would have given an optical density change of 0.355.

Effect of cyanide. DIXON AND KEILIN[8] have demonstrated the slow, irreversible inhibition of milk xanthine oxidase by cyanide. The following experiment shows that chicken liver xanthine dehydrogenase is also irreversibly inhibited by cyanide. A "partly purified" enzyme preparation was incubated at 37° C with 0.01 *M* sodium cyanide at pH 9.2 in glycine buffer. Determinations at 0, 10, 30 and 60 minutes after mixing with cyanide showed 0, 43, 71 and 93% decrease respectively in enzyme activity compared to a control without cyanide. After dialysis of the mixtures against cold, distilled water for two hours with stirring the enzyme activity of the cyanide-treated preparation was less than 5% of the untreated preparation.

Acetaldehyde as substrate. All chicken liver preparations examined reduced DPN in the presence of acetaldehyde. However, no evidence was obtained excluding the presence of a separate specific aldehyde dehydrogenase.

DISCUSSION

The identity of the *in vivo* electron acceptor for chicken liver xanthine dehydrogenase seems relatively more easily determined than that for the milk xanthine dehydrogenase which has been reported to react with oxygen, cytochrome *c* and DPN as well as with many organic dyes and with nitrate. It has been shown in the present paper that chicken liver xanthine dehydrogenase reduces DPN almost as fast as it reduces methylene blue. Together with earlier evidence[1] that xanthine can

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reduce pyruvate rapidly in chicken liver slices and that, in homogenates, this reduction is dependant upon DPN, this strongly indicates that DPN is an electron acceptor of xanthine dehydrogenase *in vivo*. As yet there is no evidence of any other acceptor for this enzyme in chicken liver.

The fact that chicken liver xanthine dehydrogenase reacts with methylene blue suggests that this enzyme also has a flavin or other reducible prosthetic group. The participation of the dehydrogenase in the reduction of DPN suggests that, like milk xanthine oxidase, it has an oxidation-reduction potential considerably lower than most flavin enzymes.

The slow rate of reduction of DPN by milk xanthine oxidase mentioned earlier in this paper is interesting in this connection. According to MACKLER *et al.*[4] the E'_0 for this enzyme system is well below that of the DPN-DPNH$^+$ system. In fact these authors claim an E'_0 for the enzyme system of —0.45 volt. The present author has calculated an E'_0 value of —0.44 volt from their experimental findings that 1.1 and 44 μM hypoxanthine (E'_0 for hypoxanthine-xanthine system = —0.37 volt) reduced the enzyme flavin ($1.7 \cdot 10^{-2}$ μM) to the extent of 40 and 80% respectively. A similar calculation using their finding that 100 μM acetaldehyde (E'_0 for acetaldehyde-acetate < -0.45 volt) produced 57% reduction gave an E'_0 value of < -0.57! It is suggested that this absurd value may be due to inactivation of the enzyme by acetaldehyde under the initially aerobic conditions used in MACKLER *et al.*'s experiment. In the present author's hands highly purified preparations of milk xanthine oxidase are very quickly inactivated by acetaldehyde under aerobic conditions.

Since milk xanthine oxidase appears to have an oxidation-reduction potential much lower than that of the DPN-DPNH$^+$ system the slow rate of reduction of DPN by this enzyme is presumably due to the specificity of the enzyme with regard to its electron acceptors. While DPN is almost certainly the *in vivo* acceptor for chicken liver xanthine dehydrogenase the evidence at the moment suggests that cytochrome *c* is more likely to play this role for the milk enzyme. Since MACKLER *et al.*[4] have shown that molybdenum is required by the milk enzyme for the reduction of cytochrome *c* or of other one electron acceptors it will be of interest, when a suitable method of purifying chicken liver xanthine dehydrogenase is available, to determine whether this enzyme also contains molybdenum. Of interest also in this connection is that MACKLER *et al.*[4] have suggested that the low potential of the milk enzyme may be due, in part, to the presence of molybdenum in the enzyme.

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SUMMARY

Xanthine dehydrogenase of chicken liver is shown to reduce DPN nearly as fast as it reduces methylene blue. Strong evidence is produced showing that DPN is the *in vivo* acceptor for this enzyme and comparisons with milk xanthine oxidase are made. Other properties of the enzyme are described.

RÉSUMÉ

La xanthine déshydrogénase du foie de poulet réduit presque aussi vite le DPN qu'elle réduit le bleu de méthylène. Les résultats obtenus suggèrent fortement que le DPN est, *in vivo*, l'accepteur de cet enzyme et des comparaisons avec la xanthine oxydase lactique ont été faites. D'autres propriétés de l'enzyme sont décrites.

ZUSAMMENFASSUNG

Es wird gezeigt, dass DPN von der Xanthin-Dehydrogenase der Hühnerleber fast so schnell reduziert wird als Methylenblau. Die Ergebnisse machen es sehr wahrscheinlich, dass in der lebenden Zelle DPN der Akzeptor für dieses Enzym ist, welches mit Milch-Xanthinoxidase verglichen wird. Andere Eigenschaften des Enzyms werden beschrieben.

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