THE OXIDATION OF REDUCED XANTHINE DEHYDROGENASE IN CHICKEN LIVER

by

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The mechanisms of oxidation of reduced flavin enzymes are known with any degree of exactness in only a few cases. There is good evidence that cytochrome *c* reductases (or diaphorases) are linked to the cytochrome system in particles structurally organised with respect to the electron transferring chain. These flavin enzymes, in the crude or purified state, do not react at a significant rate with oxygen. Other flavin enzymes, however, such as D-amino acid oxidase, liver aldehyde oxidase (not requiring cozymase) and xanthine dehydrogenase (from milk and rat liver) react with oxygen at a significant rate. Of these enzymes only milk xanthine dehydrogenase has been shown[1] to be associated with cell particles. The others appear to be readily soluble and have been considered widely to react *in vivo* directly with oxygen. With xanthine dehydrogenase in particular, however, there has been increasing evidence that oxygen is not the direct hydrogen acceptor *in vivo*.

It is well established[2,3,4] that cytochrome *c* is reduced in the presence of xanthine and milk xanthine dehydrogenase preparations. MORELL[4] has shown that cytochrome *c* and oxygen are approximately equally efficient as electron receptors in the milk xanthine dehydrogenase system. Recently RICHERT AND WESTERFELD[5] have shown that xanthine dehydrogenase of chicken liver homogenates reacts only very slowly with oxygen compared with methylene blue. IRZYKIEWICZ (private communication) has also found that the xanthine dehydrogenases in homogenates of certain insect larvae (*Tenebrio molitor*, *Tineola bisselliella*) react with methylene blue some twenty to fifty times faster than with oxygen.

BERNHEIM AND BERNHEIM[6] have shown that xanthine dehydrogenase in rat liver slices can also utilize pyruvate as acceptor. Although xanthine dehydrogenase in chicken liver is a readily soluble enzyme (see later) it seems clear from the findings mentioned above that oxygen is not the immediate electron acceptor for this enzyme *in vivo*. The purpose of the present work, therefore, has been to study the mechanism of re-oxidation of the reduced xanthine dehydrogenase in chicken liver slices and preparations.

EXPERIMENTAL

Method of measuring xanthine dehydrogenase activity

The standard method for estimation of enzyme activity was by uric acid production in the presence of methylene blue as hydrogen acceptor. The oxidation of xanthine by liver slices or prepa-

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rations in the presence of other hydrogen acceptors and hydrogen acceptor systems has been compared using this method for standard reference. Uric acid was measured by the slightly modified method of FOLIN[7]. Fortunately, methylene blue did not interfere in the colorimetric method of determining uric acid concentration because it was almost completely removed by adsorption during the preliminary tungstate deproteinization of aliquots. The specificity of the uric acid determination was checked in several experiments using uricase to destroy uric acid. Colour development due to compounds other than uric acid was found to be negligible.

Determinations of xanthine dehydrogenase activity were made in 25 ml flat bottomed conical flasks held in a rack attached to, and shaken by, two manometer frames of a conventional Warburg apparatus. With this arrangement aliquots (0.1–0.2 ml) could be removed for uric acid analysis at any time during the experiment. For anaerobic experiments similar flasks were adapted so that nitrogen could be passed through them to remove oxygen. In the rubber stoppers of these flasks a glass tube, topped with rubber tubing which was clamped during most of the experiment, was used for the addition of the enzyme fraction or for the removal of aliquots. A pressure of nitrogen was maintained slightly greater than atmospheric during anaerobic experiments. In all experiments the incubation temperature was approximately 38° C, the volume of reaction medium 3.0–3.2 ml and the final concentration of reagents as follows: xanthine (Schwartz), $3 \cdot 10^{-3}$ *M*; DPN (cozymase), $5 \cdot 10^{-4}$ *M*; methylene blue, $6.7 \cdot 10^{-4}$ *M*; pyruvate, $2 \cdot 10^{-2}$ *M*; α-ketoglutarate, fumarate and succinate, $3 \cdot 10^{-2}$ *M*. Pyruvic, α-ketoglutaric, fumaric and succinic acids were neutralized with dilute ammonia. Approximately 35 mg (dry weight) of slices and 80 mg (dry weight) of homogenates were used. Aliquots of the reaction media for uric acid determination were taken after 10 minutes and 40 minutes where slices were used and at 0 minutes and 30 minutes for homogenates and supernatants. In experiments with methylene blue incubation periods were only 15 minutes.

Chicken livers were obtained from a commercial slaughterhouse. Slices were cut by hand to a thickness of 0.40–0.45 mm the suspending medium being isotonic phosphate buffer, pH 7.4. All homogenates were prepared in a glass "Telfon" homogenizer using approximately 1 g liver per 2 ml isotonic phosphate buffer, pH 7.4. Liver supernatants were prepared from homogenates by centrifuging at 33,000 *g* for 45 minutes to remove all whole cells, nuclei, mitochondria and most microsomes. The partly (three-fold) purified preparation referred to in Table I was made from supernatant (No. 2, Table I) by ammonium sulphate fractionation. Where applied, dialysis was performed overnight at 4° C against 0.9% sodium chloride or isotonic phosphate buffer, pH 7.4.

RESULTS

Rates of production of uric acid from xanthine for chicken liver slices and preparations, under various conditions, are given in Tables I and II. In Table I the results are expressed as a percentage of the activity of the same amount of enzyme in the presence of $6.7 \cdot 10^{-4}$ *M* methylene blue. These results confirm the finding by RICHERT AND WESTERFELD[5] that methylene blue is much more efficient as hydrogen acceptor, compared to oxygen, in the reaction with chicken liver xanthine dehydrogenase and xanthine, than when rat liver or milk xanthine dehydrogenases are the catalysts. With these mammalian enzymes, methylene blue (about $6 \cdot 10^{-4}$ *M*) increases xanthine oxidation by a factor of only 1.5–3.0, whereas from data in Table I this ratio is of the order of 15 to 18 for undialysed chicken liver homogenates and supernatants. For the partly purified chicken liver preparation this ratio rose to 83. Thus, not only does xanthine dehydrogenase of chicken liver react slowly with oxygen compared to the similar xanthine dehydrogenases of rat liver and milk, but slight purification greatly reduces this rate.

The low autoxidizability of the chicken liver xanthine dehydrogenase and the anaerobic production of uric acid in slices (see Table II) and homogenates (Table I) suggested that oxidation of xanthine in chicken liver might be linked with the reduction of some other metabolite. Following the work of BERNHEIM AND BERNHEIM[6] in which pyruvate was found to stimulate xanthine oxidation in rat liver slices it was thought that in intact chicken liver cells, the oxidation of xanthine might be coupled with the reduction of such a metabolite. In fact, pyruvate, α-ketoglutarate and fumarate were found to cause increased production of uric acid from xanthine in aerobic and anaerobic

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TABLE I

OXIDATION OF XANTHINE BY PREPARATIONS FROM CHICKEN LIVER

Preparation	*Conditions**	*Percent activity***	*Ratio****
Homogenate 1	undialysed; + —	2.2	
	undialysed; + xanthine	6.4	
	undialysed; + — (anaerobic)	1.7	
	undialysed; + xanthine (anaerobic)	2.3	
Homogenate 2	undialysed; + xanthine	8.4	
	undialysed; + xanthine + pyruvate	10.0	1.19
	dialysed; + xanthine	3.6	
	dialysed; + xanthine + pyruvate	5.4	1.50
Homogenate 3	dialysed; + xanthine	4.1	
	dialysed; + xanthine + pyruvate	5.3	1.29
	dialysed; + xanthine + pyruvate + DPN	8.6	2.10
	dialysed; + xanthine + fumarate	5.3	1.29
	dialysed; + xanthine + DPN	7.2	
Supernatant 1	undialysed; + xanthine	5.6	
	undialysed; + xanthine + pyruvate	5.6	1.00
	dialysed; + xanthine	3.2	
	dialysed; + xanthine + pyruvate	3.6	1.12
Supernatant 2	undialysed; + xanthine	5.7	
	undialysed; + xanthine + pyruvate	5.7	1.00
	dialysed; + xanthine	3.0	
	dialysed; + xanthine + pyruvate	3.0	1.00
	dialysed; + xanthine + pyruvate + DPN	16.0	5.33
P.P. Preparation	undialysed; + xanthine	1.2	
	undialysed; + xanthine + pyruvate + DPN	6.8	5.67
Slices homogenate	— + xanthine	11.8	
	— + xanthine + pyruvate	19.5	1.65
	undialysed; + xanthine	7.6	
	undialysed; + xanthine + pyruvate	10.0	1.32

* All experiments were aerobic unless otherwise designated. See text under EXPERIMENTAL for details of preparations and reactants. Brackets indicate where identical preparations were used in the first six experiments or that preparations in the last experiment were from the same liver.

** As a percentage of activity of an equal amount of enzyme in the presence of $6.7 \cdot 10^{-4}$ *M* methylene blue.

*** Ratio, activity in presence of xanthine plus pyruvate *etc.*/activity with xanthine only.

experiments using chicken liver slices, homogenates and other preparations from chicken liver (see Tables I and II). Lactate (0.02 *M*), succinate (0.02 *M*) or cyanide (10^{-3} *M*) did not affect the production of uric acid in the presence of xanthine and chicken liver slices. With rat liver BERNHEIM AND BERNHEIM[6] did not find any activation of xanthine dehydrogenase activity with added fumarate.

Results given in Table I show that pyruvate and α-ketoglutarate stimulate the production of uric acid from xanthine in dialysed homogenates more than in undialysed preparations. It is also demonstrated that DPN is required for full activity. Dialysed supernatants catalyse very little increased uric acid production when pyruvate is added owing to the absence of DPN. In dialysed homogenates, however, DPN may be released from the form bound in the mitochondria, thus allowing some increase in uric acid production in the presence of pyruvate. Results for supernatants in Table I indicate that dialysis probably has not removed all xanthine-reducible substrates.

In the last experiment recorded in Table I the rates of production of uric acid from xanthine for chicken liver slices and homogenate are compared. Slices and homogenate

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were prepared from the same liver and the values given are based on dry weight determinations for both slices (taken at end of experiment) and homogenate. Similar results have been obtained in a duplicate experiment and indicate that, for a given weight of tissue, xanthine dehydrogenase in slices is approximately twice as active as in homogenates.

TABLE II

EFFECT OF PYRUVATE, α-KETOGLUTARATE, FUMARATE AND SUCCINATE ON XANTHINE OXIDATION BY CHICKEN LIVER SLICES

Liver	*Conditions**	*μM uric acid/100 mg dry wt/hour*	*Ratio***
1	Aerobic + xanthine	17.6	
	Aerobic + xanthine + pyruvate	37.2	2.11
2	Aerobic + xanthine —	7.6	
	Aerobic + xanthine + pyruvate	10.5	1.38
3	Aerobic + xanthine —	10.6	1.71
	Aerobic + xanthine + pyruvate	18.1	
4	Aerobic + xanthine —	11.3	
	Anaerobic + xanthine —	6.9	
	Anaerobic + xanthine + α-ketoglutarate	16.3	1.52
	Anaerobic + xanthine + pyruvate	21.1	1.87
5	Aerobic + xanthine —	12.6	
	Aerobic + xanthine + pyruvate	26.8	2.13
	Anaerobic + xanthine —	10.2	
	Anaerobic + xanthine + pyruvate	27.2	2.66
6	Aerobic + xanthine —	6.1	
	Aerobic + xanthine + α-ketoglutarate	11.1	1.82
7	Aerobic + xanthine —	8.9	
	Aerobic + xanthine + α-ketoglutarate	11.3	1.27
8	Aerobic + xanthine —	7.4	
	Aerobic + xanthine + α-ketoglutarate	10.5	1.42
	Aerobic + xanthine + succinate	6.4	
	Aerobic + xanthine + fumarate	10.6	1.43

* For details of reactants see text under EXPERIMENTAL.
** See legend Table I.

DISCUSSION

The increase in uric acid production by adding DPN, pyruvate, α-ketoglutarate and fumarate to chicken liver slices or preparations strongly suggests that xanthine dehydrogenase is linked in some way to the DPNH oxidase system. BERNHEIM AND BERNHEIM[6] have previously recorded the stimulation of xanthine oxidation by added pyruvate in rat liver slices. They claimed, however, that a coupled oxidation-reduction reaction between the xanthine dehydrogenase and lactic dehydrogenase systems could not exist because they found that lactate did not reverse the effect of pyruvate in their experiments. However, the oxidation-reduction potentials of these systems are such that in the reaction

$$\text{xanthine} + \text{pyruvate} \rightleftharpoons \text{uric acid and lactate}$$

$$\text{the equilibrium constant, } K = \frac{[\text{uric acid}] \times [\text{lactate}]}{[\text{xanthine}] \times [\text{pyruvate}]} = ca.\ 10^6$$

for equimolar reactants. Hence, lactate would not be expected to reverse significantly the effect of pyruvate in such a linked dehydrogenase system.

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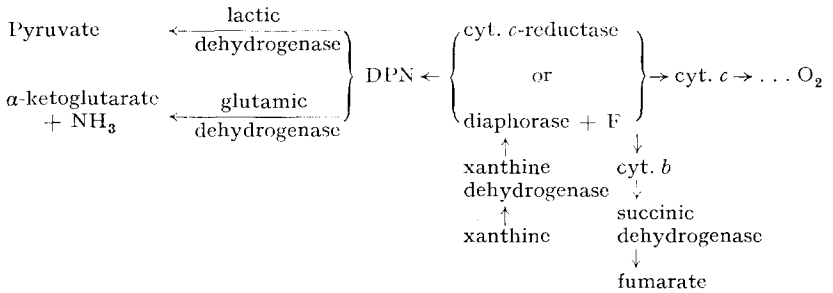
In Table I DPN and pyruvate have been shown to increase xanthine oxidation not only in chicken liver homogenates and supernatants but also in a supernatant purified three-fold by ammonium sulphate fractionation. It seems, therefore, that the linked dehydrogenase system is not easily inactivated by treatment with solutions of high osmotic strength. Comparison with the supernatant (No. 2, Table I) from which this preparation was derived does, however, indicate about 55–60% loss of activity. This may, of course, be due to separation of components by fractionation. The rather large variations shown for *Ratio* in Table II in experiments with dialysed preparations also suggest that, in processing, the link between dehydrogenases in these preparations has been partly destroyed or inactivated. The higher activity of xanthine dehydrogenase in liver slices compared to homogenates also bears out this hypothesis.

Details of the link between xanthine and lactic dehydrogenases in chicken liver (probably present in rat liver and possibly also in cow mammary tissue) are not yet known. Milk xanthine dehydrogenase preparations are known to reduce cytochrome *c* *in vitro*[2,3,4] but kinetic considerations rule out cytochrome *c* as a component in the direct link in this system because of its comparatively very high oxidation-reduction potential. Moreover, these experiments suggest the possibility that xanthine dehydrogenase may not directly reduce cytochrome *c* at a significant rate because the reduction of cytochrome *c* has only been accomplished with preparations known to contain, or probably containing, diaphorase (possibly DPN-cytochrome *c* reductase[4,3]). It is also relevant to consideration of the mechanism of this linked dehydrogenase system that pyruvate increases the oxidation of xanthine in liver slices even under aerobic conditions (see Table II). It is suggested that the following scheme best represents the link between the xanthine and lactic dehydrogenases using knowledge now available



In this scheme the direction of the arrows shows the possible paths of electrons from xanthine to pyruvate, α-ketoglutarate, fumarate or to oxygen via cytochrome *c*. "F" represents SLATER's factor. Cytochrome *b* is sited in this scheme according to SLATER[8] although this position is disputed by CHANCE[9].

As demonstrated in Table I a significant proportion of xanthine dehydrogenase linked to lactic dehydrogenase can be obtained in the supernatant which does not contain any mitochondria. It was found that virtually all (> 85%) of the xanthine dehydrogenase of the tissue appears in the supernatant. It seem likely, however, from consideration of the scheme postulated above that xanthine dehydrogenase is structurally organized in the liver cell so that it can react with the cytochrome-bearing particulate fraction. This is supported by the histological experiments of BOURNE[10] showing the reducing action of xanthine dehydrogenase in rat liver to be sited at the periphery of certain cell particles.

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Oxidation of xanthine according to the scheme postulated above suggests a considerable gain in efficiency to the liver compared to the wasteful production of hydrogen peroxide by direct reaction of the dehydrogenase with oxygen.

Oxidation of xanthine via the cytochromes would enable energy expended in building up ammonia to hypoxanthine to be recaptured to a large extent in oxidative phosphorylation reactions. This work also indicates that the function of catalase in the liver is not the destruction of hydrogen peroxide formed by xanthine dehydrogenase.

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SUMMARY

It has been shown that xanthine dehydrogenase in chicken liver slices or various preparations from chicken liver is linked to a lactic dehydrogenase system and that DPN is essential to this link. Evidence also indicates a link between xanthine dehydrogenase, glutamic dehydrogenase and succinic dehydrogenase. It is suggested on the evidence presented that xanthine dehydrogenase is linked with the DPNH oxidase system in chicken liver at, or near, DPN-cytochrome *c* reductase.

RÉSUMÉ

Dans des coupes et dans diverses préparations de foie du poulet, la xanthine déshydrogénase est liée au système de la lactique déshydrogénase par l'intermédiaire du DPN. Il y a également une liaison entre la xanthine déshydrogénase, la glutamique déshydrogénase et la succinique déshydrogénase. Les résultats obtenus permettent de suggérer que la xanthine déshydrogénase est liée, dans le foie du poulet, au système de la DPNH-oxydase au niveau, ou près, de la DPN-cytochrome *c*-réductase.

ZUSAMMENFASSUNG

Es wird gezeigt, dass die Xanthin-Dehydrogenase in Hühnerleber-Schnitten oder -Homogenaten mit einem Milchsäuredehydrogenase-System verbunden ist und dass DPN für diese Bindung notwendig ist. Ein Zusammenhang zwischen Xanthin-Dehydrogenase, Glutaminsäure-Dehydrogenase und Bernsteinsäure-Dehydrogenase scheint ebenfalls zu bestehen. Aus den Ergebnissen wird geschlossen, dass die Xanthin-Dehydrogenase mit dem DPNH-Oxydase-System durch die DPN-Cytochrom-*c*-Reduktase oder in ihrer Nähe verbunden ist.

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