CCCI. LIVER XANTHINE OXIDASE.

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In the past the Schardinger enzyme-xanthine oxidase system has received considerable attention, but investigations have been mainly confined to the preparation and properties of the enzyme as found in milk. Liver of most Mammalia is a potent source of this enzyme, but apart from the pioneer work of Battelli & Stern [1910], and Parnas [1910], the only detailed investigation of this source is that of Wieland & Frage [1929].

Milk is of course a much better starting material for the preparation of the enzyme, as not only is it almost free from accompanying substrates, but it is not a rich storehouse of enzymes like liver. Despite this it seemed of interest to study the properties of liver enzyme preparations, if only to compare them with those from milk.

The earlier work of Wieland and Frage, of which this communication forms an extension, was carried out with horse liver. The difficulty of obtaining supplies soon led to its abandonment in favour of ox liver. A further point in favour of ox liver is that, unlike horse liver, it is free from uricase [Rô, 1931], a fact later confirmed in this investigation. This makes the interpretation of figures obtained for the oxidation of purine bases to uric acid much easier as there is no further oxidative breakdown to allantoin to consider.

I. RESPIRATION OF MINCED LIVER AND ITS AQUEOUS EXTRACTS.

In studying the respiration of minced ox liver it became evident that owing to factors unknown at the time, certain of the results of Wieland & Frage could no longer be considered correct. These authors stated that the rate of oxygen uptake of minced liver suspended in phosphate buffer slowed down considerably after half an hour, whilst the corresponding development of CO₂ came to a standstill at the same time. In the experiments from which these conclusions were drawn a 40% solution of KOH was used to absorb CO₂ developed. This method of CO₂ absorption has been shown by Dixon & Elliott [1930] to be very inefficient, and when a small roll of filter paper soaked in 7% KOH is used instead, quite different results are obtained. As is shown in Table I both oxygen uptake and CO₂ output continue quite regularly, the rates fall off slowly with time, but there is no sudden decrease or cessation.

The CO₂ output is calculated by difference from the manometric readings obtained when no KOH is used to absorb the CO₂ produced.

This does not give a strictly accurate measure of the CO₂ output as both the phosphate buffer and the protein present will retain a certain amount of CO₂. The R.Q. 0.37 as calculated from the above data is therefore low. Figures are not yet available for the R.Q. of ox liver slices, but Gemmill & Holmes [1935] record 0.79 for rat liver slices. It therefore seems likely that mincing leads to a

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Table I.

Time in min.	O ₂ uptake $\mu\text{l./g.}$	CO ₂ output $\mu\text{l./g.}$
15	92	44
30	178	66
45	260	92
60	322	116
90	434	160
120	536	206

reduction in the R.Q. of ox liver, a conclusion which it is hoped to confirm at a later date. It is a well-known fact that mincing leads to inactivation of several enzymes in tissues. As xanthine oxidase is unaffected by such treatment, mincing frees the ultimate preparation from some of the enzymes likely to be present.

Acceleration of respiration by xanthine.

An aqueous extract of minced liver (1 ml. per g.), like the 'brei' itself, has quite a considerable self-respiration. If xanthine be added to a solution containing 1 ml. of such an extract buffered at pH 7.6, the respiration may or may not undergo an initial acceleration. It may take an hour or more for xanthine to exert an accelerating effect, but ultimately the extra oxygen uptake will amount to that required for the complete oxidation of xanthine to uric acid.

Similar experiments by Morgan *et al.* [1922] showed that with rat liver the oxygen uptakes were in accord with the complete conversion of xanthine into allantoin. As rat liver is rich in uricase and ox liver contains none the discrepancy is only apparent. The aqueous extracts are kept sterile by toluene; otherwise, as shown by Truszkowski [1930], bacterial contamination may lead to uricolytic action. Toluene does not seem to affect xanthine oxidase in any way.

Table II shows the varying effects of the addition of $M/100$ or $M/80$ xanthine on the rate of respiration of an aqueous extract of liver.

Table II.

Q_{O_2}	$Q_{O_2}^x$	Q_{O_2}	$Q_{O_2}^x$
104	129	70	152
100	149	82	154
62	172	164	164
127	131		

Q_{O_2} represents the amount of oxygen taken up by 1 ml. of an aqueous liver extract in 1 hour, whilst $Q_{O_2}^x$ represents the amount taken up when xanthine is also present. Uptakes are recorded in $\mu\text{l.}$

While $Q_{O_2}^x$ only varies some 15 % on either side of a mean, Q_{O_2} is subject to wide variations. $Q_{O_2}^x$ may be equal to Q_{O_2} , or it may be three times as great. The approximate constancy of $Q_{O_2}^x$ indicates that the activity of xanthine oxidase does not vary much from one liver to another, but variations in Q_{O_2} indicate a variable purine content.

However suboptimum concentrations of purine bases are so low that for a complete explanation of the respiration rate obtained a continuous formation of purine base by nucleosidase action must be assumed. The purine base is oxidized as soon as it is set free so that its rate of oxidation is governed by its rate of formation.

Precisely the same process takes place in the milk enzyme. According to Dixon & Lemberg [1934] the slow hydrolysis of inosine by xanthine oxidase preparations can be followed manometrically, as the hypoxanthine is oxidized to uric acid as soon as it is formed.

The above results are not inconsistent with the view that the bulk of the respiration of minced liver is due to the oxidation of xanthine. Although positive proof of this cannot be advanced there is evidence which fits in with this view. If the bulk of the respiration is due to xanthine oxidase it should be but little affected by cyanide for, as will be shown later, the enzyme is only partially inhibited at high cyanide concentrations. In agreement with earlier results of Dixon & Elliott [1929] it has been found that this is indeed the case, inhibition amounting to less than 20% with $M/1000$ HCN. The extent of cyanide inhibition is thus in harmony with, although it does not prove, the theory advanced.

If we consider the non-cyanide-sensitive part of the respiration to be due to purine base oxidation it should be possible to calculate the purine base content of the liver. In one case, typical of several, the self-respiration of 1 ml. of an aqueous liver extract in the presence of $M/1000$ HCN amounted to 173 μ l. after 6 hours' shaking, by which time the reaction had come almost to a standstill. If the whole of this oxygen uptake were due to xanthine oxidation the purine base content of the extract would be 2.35 mg./ml. while if due to hypoxanthine oxidation it would be 1.05 mg./ml. Thus the soluble purine base content of ox liver if the above views are correct should be between 105 and 235 mg. per 100 ml., which is consistent with the value 201 mg. per 100 ml. found by Fellenberg [1918].

Mincing the tissue does not necessarily destroy enzymic systems, but those systems requiring a coenzyme will have their activities largely reduced by the dilution effect of the dispersion of the coenzyme. Other enzymes such as the succinic dehydrogenase may make but little contribution to the respiration rate owing to the low substrate concentration associated with the enzyme. That this applies in the case of succinic dehydrogenase is shown by the very great acceleration in the rate of oxygen uptake when succinate is added to minced liver.

Effect of acetaldehyde on the respiration rate.

According to Wieland & Frage the addition of acetaldehyde has an accelerating influence on both the oxygen uptake and the CO_2 output of respiring liver extracts. This acceleration in oxygen uptake is however only apparent, as the increase is exactly equal to the rate of autoxidation of acetaldehyde when alkali is present to absorb CO_2 .

Thus after 1 hour's shaking the oxygen uptake of 218 μ l. shown by 0.25 g. of minced liver is increased to 317 by $M/25$ acetaldehyde. This concentration of acetaldehyde under the same conditions produces an oxygen uptake of 102 μ l. without any enzyme being present.

According to Andersson [1935] the dehydrogenation of acetaldehyde by the Schardinger enzyme is strongly inhibited by several purine derivatives such as adenosine, muscle adenylic acid and adenylypyrophosphate as well as by xanthine itself. The presence in liver of these substances accounts for the failure of the enzyme to bring about the oxidation of its substrate. This conclusion implies that the oxidation of acetaldehyde by the liver enzyme has no physiological significance. In milk, which is largely free from purine derivatives, added aldehydes are oxidized quite normally.

When no steps are taken to absorb the CO_2 produced one would expect that the addition of acetaldehyde would have no effect on the manometric readings, but actually the readings were distinctly higher. As oxygen uptake is unaffected, CO_2 output must therefore be inhibited, probably through an inhibition by the aldehyde of enzymic processes leading to CO_2 formation. In micro-respiration studies of the oxidation of aldehyde, CO_2 must therefore be absorbed even although it is not a product of oxidation of the substrate, unless one can prove quite definitely that no CO_2 is produced in the system at all.

II. PREPARATION OF XANTHINE OXIDASE FROM LIVER.

In the earlier work of Wieland & Frage [1929] an enzyme preparation is described obtained by the precipitation with acetone of a toluene-water extract of finely minced liver. If this precipitate is centrifuged down, exhaustively washed with acetone and given one final washing with ether (dried and distilled over sodium) an enzyme preparation is obtained in the form of a very finely divided powder (Prep. A).

It is insoluble in water, but its suspension functions as quite an active catalyst. It still possesses an appreciable self-respiration but the accelerations in the rate of oxygen uptake on addition of xanthine and aldehyde are quite marked.

Its insolubility makes it impossible to concentrate the enzyme by dissolving and reprecipitating. Fractional precipitation with acetone led to no definite concentration of the enzyme in any one particular fraction. Further, attempts to deplete the liver mince of its substrates, either by dialysis or impoverishment (shaking with oxygen for several hours) were not sufficiently successful to make such preliminary treatment before acetone precipitation of any great advantage.

In manometric experiments 0.2 or 0.25 g. proved a convenient amount to handle. With the Warburg technique this amount was suspended in 5 ml. $M/15$ phosphate buffer, pH 7.6, and the volume adjusted with distilled water so that after addition of substrate the total volume was 8 ml. In the experiments with the Barcroft differential manometer the total volume was 3 ml.

Of many substrates tried only purine bases and aldehydes accelerated the rate of oxygen uptake. There was no acceleration on the addition of succinic acid or alcohol despite the high activity in liver of their corresponding dehydrogenases.

In an endeavour to make the enzyme more water-soluble the minced liver was subjected to digestion with takadiastase and with trypsin, but both methods brought about a complete destruction of the xanthine oxidase.

The dehydration of minced liver with acetone has led, in the hands of Bernheim [1928, 2] and Harrison [1931, 1, 2], to a method by which several dehydrogenases could be extracted from the acetone-liver. An aqueous extract of acetone-liver may itself be used as an enzyme source but a solid preparation may be obtained by adding slowly, with stirring, an equal volume of a saturated solution of ammonium sulphate. A high-speed centrifuge is required to separate the precipitate formed. It is roughly dried on a porous plate to remove as much ammonium sulphate solution as possible, after which drying is completed *in vacuo* over sulphuric acid (Prep. B).

This preparation is completely soluble in water or phosphate buffer, and 1 ml. of a 10% solution in $M/15$ phosphate pH 7.6 is a convenient amount for manometric experiments.

Acetone-liver, like aqueous liver extracts, has a somewhat variable self-respiration but its oxygen uptake is always considerably accelerated by the addition of xanthine. Its phosphate extracts have a somewhat lower self-respiration and the final preparation is sometimes obtained completely free from self-respiration.

Oxidation of aldehydes.

These experiments were all carried out with Prep. A. If in manometric experiments on the oxidation of volatile aldehydes KOH is used to absorb any CO_2 that may be formed, there will be a rapid oxygen uptake due to a condensation reaction of the aldehyde at the surface of the KOH. This, unless eliminated or corrected for, will lead to very inaccurate results, and failure to realize this fact invalidates much of the previous work on the enzymic oxidation of these aldehydes.

In this particular work CO_2 must be absorbed, so the effect was corrected for by subtracting values for the non-enzymic autoxidation from the values recorded. This procedure seems justifiable as the two reactions leading to an oxygen uptake occur in different phases and should not interfere with one another.

Table III.

$\mu\text{l. O}_2$			
Acetaldehyde.			
Time min.	$M/10$	$M/25$	$M/50$
10	60	26	13
20	105	45	20
30	—	64	28
40	176	73	34
80	289	123	—
Propaldehyde.			
Time min.	$M/10$	$M/20$	
5	90	40	
10	142	66	
15	188	91	
20	226	111	
30	298	151	
40	364	191	
50	420	227	
60	472	261	

The figures quoted above are average values from often as many as ten determinations; individually the uptakes vary considerably. There is an approximate dependence of the rate of autoxidation on the aldehyde concentration.

In Figs. 1 and 2 are plotted the results obtained for the enzymic oxidations of acetaldehyde and propaldehyde, corrected in this way for autoxidation.

The rate of oxidation of acetaldehyde is independent of substrate concentration so long as this concentration is greater than $M/50$. In each of several such experiments the values for $M/100$ acetaldehyde were consistently lower, and for $M/250$ acetaldehyde lower still. The figures obtained were not precise enough for an exact evaluation of the Michaelis constant, but it is probably of the order $0.005 M$.

Propaldehyde undergoes autoxidation much more rapidly so that a larger proportion of the recorded uptake is due to this factor. Despite the greater error introduced there seems little reason to doubt that the initial rates of oxidation of propaldehyde are the same while the concentration ranges

from $M/100$ to $M/10$. In the later stages of the reaction this agreement is not maintained, and after an hour's shaking the uptake with $M/100$ aldehyde is double that with $M/10$. In the latter case reaction ceases after 30 min.

Fig. 1. $\circ-\circ$ $M/10$ acetaldehyde. $\triangle-\triangle$ $M/25$ acetaldehyde.
 $\square-\square$ $M/50$ acetaldehyde. $\times-\times$ $M/100$ acetaldehyde.

Fig. 2. $\circ-\circ$ $M/10$ propaldehyde. $\triangle-\triangle$ $M/25$ propaldehyde.
 $\square-\square$ $M/50$ propaldehyde. $\times-\times$ $M/100$ propaldehyde.

With acetaldehyde the uptake curve is not linear, and when we consider that the enzyme is protected against H_2O_2 inhibition by catalase and is unaffected by acetate and by mechanical shaking, we must conclude that acetaldehyde

inhibits its own dehydrogenase. Propaldehyde appears to have an even higher degree of toxicity and here the slowing up of the reaction rate is sufficiently marked for the greater effect of higher substrate concentrations to be evident.

If these conclusions are to be accepted incubation of xanthine oxidase with $M/10$ propaldehyde should cause complete destruction of the enzyme. To test this advantage was taken of the insolubility of Prep. A. After incubation at 37° for 1 hour the enzyme was centrifuged down, washed to remove products of reaction and resuspended in phosphate buffer. After this treatment it was found to be completely inactive. The centrifugate, on the other hand, could still function as a substrate for fresh enzyme so that it can be concluded that no toxic products are formed as a result of the reaction and that it is the substrate itself that is the inhibitor.

It is interesting to recall that Battelli & Stern [1913] showed that propionaldehyde exerted a greater toxic effect than acetaldehyde on succinic dehydrogenase, and that Bernheim [1928, 1] showed that the aldehyde dehydrogenase of potato was completely destroyed by incubation with acetaldehyde.

Oxidation of xanthine.

Using Prep. B, one completely free from self-respiration, the theoretical curve for the complete oxidation of xanthine to uric acid was realized, as shown in Fig. 3.

Fig. 3.

The rate was linear until oxidation was nearly complete and, after the calculated quantity of oxygen had been taken up (2 ml. $M/100$ xanthine requires $224 \mu\text{l. O}_2$), the reaction came to a standstill. The curve is identical with that obtained by Dixon [1925] when milk xanthine oxidase was mixed with a sufficiency of catalase. As is well known, liver is rich in catalase. $0.02 M \text{H}_2\text{O}_2$ is instantaneously decomposed by a phosphate extract of acetone-liver and the rate is only measurable after considerable dilution.

With this high catalase concentration the enzyme is completely protected against the toxic effect of H_2O_2 . The uptake curves are therefore linear and as the H_2O_2 is completely decomposed the uptakes for complete oxidation to uric acid are realized exactly.

The enzymic oxidation of xanthine by liver xanthine oxidase is perfectly normal. In Figs. 4 and 5 are plotted the results obtained by varying enzyme and substrate concentration.

The initial rate of oxidation is proportional to the enzyme concentration. A five-fold increase in the substrate concentration has no effect on the rate and the two curves only separate when the oxidation of $M/400$ xanthine approaches

Fig. 4.

Fig. 5.

Fig. 4. $\circ-\circ$ $M/80$ xanthine. $\times-\times$ $M/400$ xanthine. $\triangle-\triangle$ Self-respiration.

Fig. 5. $\circ-\circ$ 0.50 g. Prep. A. $\triangle-\triangle$ 0.25 g. Prep. A. $M/200$ xanthine.

completion. Beyond this point the curve obtained with $M/80$ xanthine still follows the same straight line. The persistence of the straight line up to the point where oxidation is almost complete indicates that the rate is independent of substrate concentration down to very high dilutions of the order of $M/4000$. This indicates a low K_m of the same order as that found by Dixon & Thurlow [1924] and by Booth [1935]. Apparently the purine bases do not inhibit their own oxidation when oxygen, and not methylene blue, is the hydrogen acceptor.

The straight line uptake curves do not pass through the origin but cut the time axis at a point corresponding to a time interval of 5 to 10 min. Dixon [1925] noticed a similar effect, for when strong catalase solutions were added to milk preparations, there was always an initial induction period.

Cyanide inhibition.

With both Prep. A and B cyanide inhibition of the oxidation of xanthine is quite marked although it does not approach the order of inhibition observed with the succinic dehydrogenase. The effect of varying concentrations of cyanide on the rate of oxidation of xanthine is shown in Fig. 6.

30 % of the activity, as measured by initial rate of reaction, is retained in the presence of $M/100$ HCN. Such a concentration would cause complete inhibition of O_2 uptake of succinic oxidase, while according to Dixon & Thurlow [1925] milk xanthine oxidase is unaffected by cyanide at this concentration. The essential difference between Dixon's milk preparations and liver xanthine

oxidase is in the high catalase content of the latter. A closer inspection of the inhibition by high cyanide concentrations shows that the oxidation comes almost to a standstill with less than a half of the xanthine oxidized. This is so reminiscent of the oxidation of xanthine by catalase-free xanthine oxidase which Dixon [1925] showed to be brought to a standstill by the accumulation of H_2O_2 , that it was concluded that similar conditions operated here through the complete inhibition of the catalase by HCN.

Fig. 6. I, Oxidation $M/400$ xanthine. II, Oxidation + $M/2500$ HCN. III, Oxidation + $M/500$ HCN. IV, Oxidation + $M/200$ HCN. V, Oxidation + $M/100$ HCN.

However, it has recently been shown by Dixon & Keilin [1936] that incubation of xanthine oxidase with HCN in the absence of substrates caused a progressive inactivation. As, in the above experiments, HCN was mixed with the enzyme and the substrate added after the attainment of temperature equilibrium, the above results might be due to this same type of inhibition. To test this possibility the experiments were repeated, and the effect of adding the cyanide together with the substrate, after the attainment of temperature equilibrium, was compared with that observed when enzyme and inhibitor were mixed initially. The extent of inhibition is slightly, although significantly, less when substrate and cyanide are added at the same time as shown in Fig. 7, but the results leave no doubt that the Dixon type of inhibition accounts for but a fraction of the total inhibition observed. If the Dixon effect were wholly responsible, there would be no inhibition at all when the cyanide was added at the same time as the substrate. It can therefore be concluded that the cyanide-sensitivity of liver xanthine oxidase is due mainly to the inhibition of its catalase.

Aerobic mixed substrate tests for xanthine oxidase have been carried out by Sen [1931] using hypoxanthine and the non-volatile aldehydes, piperonal and vanillin. It seemed of interest to see whether volatile aldehydes showed a similar competitive inhibition, making, of course, appropriate corrections for autoxidation. The substrates chosen were $M/50$ propaldehyde and $M/200$ xanthine both of which are in sufficiently high concentration to saturate the enzyme. The results obtained are plotted in Fig. 8.

No conclusions can be drawn from these results as to the single or dual nature of the enzyme. Saturation of the enzyme with xanthine appears to inhibit the

oxidation of the aldehyde as initially the rate with the mixed substrate is exactly the same as with xanthine alone. It has also been shown by Andersson [1935] that xanthine inhibits the oxidation of aldehydes. The falling off in the rate with the mixed substrate can be ascribed to the toxic effect of the propaldehyde.

Fig. 7. I, $M/100$ xanthine. II, Inhibition by $M/100$ HCN. III, Inhibition by $M/100$ HCN added with substrate. IV, Self-respiration.

Fig. 8. ○—○ $M/50$ propaldehyde. △—△ $M/200$ xanthine. ×—× Mixed substrate.

SUMMARY.

1. In contradistinction to earlier work by Wieland & Frage the respiration of minced liver proceeds normally without any sudden slowing down.

2. The observed rate of self-respiration of minced liver may be ascribed mainly to purine-base oxidation, and this rate is controlled by the rate of formation of purine base by nucleosidase action.

3. Acetaldehyde has no accelerating influence on the oxygen uptake of minced liver and inhibits its CO_2 output.

4. The kinetics of aldehyde oxidation can be measured manometrically if a correction is made for the non-enzymic autoxidation of the aldehydes on the surface of the KOH. For acetaldehyde K_m is probably of the order of 0.005 M . Propaldehyde and, to a less extent, acetaldehyde destroy the enzyme which catalyses their oxidation.

5. Liver xanthine oxidase contains sufficient catalase to protect it against H_2O_2 .

6. Cyanide inhibits xanthine oxidation indirectly by poisoning the catalase.

7. Aerobically a mixture of xanthine and propaldehyde is oxidized at the same rate as xanthine alone.

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