92. LIVER ALDEHYDE OXIDASE

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In the course of an attempt to isolate the xanthine oxidase of pig liver we observed that the ratio $\frac{\text{aldehyde activity}}{\text{xanthine activity}}$ dropped sharply after the first stages in the purification and then remained constant [cf. Corran *et al.* 1939, 1]. This initial fall in the ratio was interpreted at the time to mean that an enzyme inactive towards xanthine but active towards aldehydes was eliminated in the early purification of liver xanthine oxidase. Further investigation has now confirmed the presence in liver of an aldehyde enzyme other than the xanthine oxidase, and the present communication deals with its purification and properties. There have been hints in the literature of a specific aldehyde enzyme in liver [e.g. Lemberg *et al.* 1936] though no attempt was made to lend direct support to the claim by separating the aldehyde oxidase from the xanthine oxidase.

The aldehyde oxidase of liver is a flavoprotein whose prosthetic group is flavin adenine dinucleotide. The mechanism of the catalysis involves a cycle of reduction of the flavin group by aldehyde followed by oxidation of the leucoflavin by molecular oxygen. The enzyme contains another coloured group other than flavin though there is no information yet of its chemical nature.

(1) *Method of testing catalytic activity*

The aldehyde enzyme catalyses the oxidation of aldehydes by molecular oxygen and by other hydrogen acceptors such as methylene blue. Both the aerobic manometric method and the anaerobic Thunberg method are suitable for following enzyme activity but in practice we have relied on the anaerobic methylene blue technique.

We have arbitrarily defined as the unit of aldehyde activity an amount of enzyme that in presence of 0·1 ml. of *M* acetaldehyde will catalyse the reduction of 0·1 ml. of 0·0113 *M* methylene blue in 1 min. in *M*/25 phosphate buffer *p*H 7·2. The experiments were carried out at 38° in a total volume of 2·5 ml. The reduction time should not be greater than *ca.* 1 min. Beyond this limit the velocity is not strictly proportional to the activity of the enzyme. The mixture of enzyme and aldehyde was placed in the main tube and methylene blue in the side bulb. After 2 min. incubation at 38° the contents were rapidly mixed and the time required for complete decoloration of the methylene blue determined.

(2) *Method of preparation*

There are four main coloured impurities which must be eliminated before liver aldehyde oxidase can be obtained homogeneous from the point of view of coloured components: (1) haemoglobin, (2) xanthine oxidase flavoprotein, (3) a $Fe(OH)_3$-protein complex and (4) catalase. Haemoglobin offers little difficulty—being soluble in half-saturated $(NH_4)_2SO_4$ in contrast to the aldehyde enzyme which is completely precipitated at that saturation. Several $(NH_4)_2SO_4$

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precipitations suffice to remove all traces of haemoglobin. The xanthine oxidase flavoprotein is largely destroyed by exposure in 25% alcohol to 48° for 5 min. whereas the aldehyde oxidase is stable under these conditions. The traces of xanthine oxidase which survive the heating procedure are removed in the course of further purification without any special efforts being necessary to attain this end. Pig liver contains a comparatively large amount of an orange red colloidal substance which behaves like a $Fe(OH)_3$-protein compound. This is probably identical with the so-called "ferratin". This $Fe(OH)_3$-protein is less soluble in $(NH_4)_2SO_4$ solutions than the aldehyde oxidase though the margin is slight. Some four or five fractionations in $(NH_4)_2SO_4$ solutions between the limits of 20 and 35% saturation are necessary before the enzyme can be completely separated from the $Fe(OH)_3$-protein. The fractionations are best carried out in ammoniacal $(NH_4)_2SO_4$ solutions for the following two reasons: (1) continued exposure of the $Fe(OH)_3$-protein to ammoniacal $(NH_4)_2SO_4$ leads to denaturation and the protein becomes insoluble even in absence of $(NH_4)_2SO_4$; (2) the aldehyde enzyme is unstable in slightly acid or neutral $(NH_4)_2SO_4$ but keeps indefinitely in ammoniacal solutions of $(NH_4)_2SO_4$. The elimination of catalase has proved to be the most difficult task. In fact we have not as yet been able to obtain a preparation of the aldehyde enzyme freed from all traces of catalase. Theoretically $(NH_4)_2SO_4$ fractionation should effectively separate aldehyde oxidase from catalase. The former is precipitated almost completely at one-third saturation of $(NH_4)_2SO_4$ whereas the latter is largely soluble at half saturation. One or two gross $(NH_4)_2SO_4$ fractionations do in fact remove more than 90% of the original catalase present. The residual catalase precipitates more or less within the same range of $(NH_4)_2SO_4$ concentrations as the aldehyde enzyme and as many as ten successive $(NH_4)_2SO_4$ fractionations have failed to accomplish any significant separation. The persistence with which catalase accompanies the aldehyde enzyme in the course of salt fractionations, adsorptions, solvent precipitations, cataphoresis etc. have led us to the view that catalase forms a type of compound with the aldehyde enzyme which cannot be resolved by the methods employed.

The following are the details of the method for preparing aldehyde enzyme in a highly purified state from fresh pig liver.

(1) 1·8 kg. of finely minced liver are mixed with 4 l. water and 1840 ml. 97% ethyl alcohol. The suspension is maintained at 48° for 5 min. and then rapidly cooled to 20° by addition of crushed ice. The heating must be so regulated that the thermometer reaches 48° within 4 min. of application of heat. The solution is heated in a round-bottom flask immersed in a boiling water bath. Stirring must be vigorous to prevent local overheating. The mixture is centrifuged for 10 min. and the precipitate of denatured protein is discarded.

(2) The clear, pale red supernatant fluid (5·6 l.) is treated with 40 ml. 25% basic lead acetate. The precipitate is centrifuged and decomposed by thorough shaking with 400 ml. of saturated Na_2HPO_4. The precipitate of lead phosphate is rejected. The supernatant fluid (570 ml.) is made 40% saturated with respect to $(NH_4)_2SO_4$. The precipitate is dissolved in 100 ml. water. The solution contains *ca.* 1100 units.

(3) Successive ammoniacal $(NH_4)_2SO_4$ fractionations are now employed to remove $Fe(OH)_3$-protein and catalase. The ammoniacal solution is prepared by adding 6 ml. of NH_4OH (sp. gr. 0·880) to 94 ml. of saturated $(NH_4)_2SO_4$. The fractionations are carried out between the limits of 27 and 40% saturation of $(NH_4)_2SO_4$. The flavoprotein which is associated with liver aldehyde oxidase activity is partially bleached by hydrosulphite whereas the colour both of

$Fe(OH)_3$-protein and catalase is not affected by hydrosulphite. The hydrosulphite test thus offers a quick and reliable test of which fraction contains least coloured impurity. If there is little change on addition of hydrosulphite the fraction is poor. If the bulk of the colour is bleached the fraction is good. Furthermore the residual colour after reduction gives a clue as to the coloured impurity present. A green residual colour confirms the presence of catalase; a reddish brown residual colour indicates $Fe(OH)_3$-protein.

The following are the details of a typical first fractionation:

Fraction	Units	
I	400	Reddish brown, largely $Fe(OH)_3$-protein
II	300	Yellow brown, some $Fe(OH)_3$-protein
III	300	Yellow brown, practically no $Fe(OH)_3$-protein
IV	75	Greenish yellow, some catalase
V	25	Green, catalase and colourless impurities

Fraction III contains least coloured impurity and is largely bleached by hydrosulphite. Fractions I, II and IV are now refractionated and the best fractions are amalgamated with fraction III. This procedure constitutes one complete $(NH_4)_2SO_4$ fractionation. At least three repetitions are necessary to effect complete separation of $Fe(OH)_3$-protein and maximum separation of catalase. The yield from 4 lb. of liver is *ca.* 250 units.

The method described above does not lead to the homogeneous enzyme. Our best preparations are probably of the order of 50 % pure.

(3) *Properties of purified enzyme*

Purified preparations of the enzyme are yellowish brown in colour. On reduction with hydrosulphite the colour is largely bleached and shaking with air restores the colour. In a later section evidence will be presented for the flavin nature of the enzyme.

The best preparations of the enzyme contain 0·17 % flavinphosphate. At this level of purity one enzyme unit is equivalent to 0·62 mg. dry weight. At 38° 1 mg. dry weight of the enzyme transfers 2700 μl. H_2 from acetaldehyde to methylene blue per hour ($Q_{M.B.}=2700$). Assuming that flavin represents the prosthetic group of the enzyme and there is one flavin group per molecule of enzyme, it follows that each molecule of the enzyme catalyses the oxidation of 520 molecules of acetaldehyde per min. at 38°. Table 1 compares the properties of the milk xanthine oxidase at the level of *ca.* 80 % homogeneity with those of the liver aldehyde enzyme prepared by the methods described.

Table 1. *Comparison of catalytic constants of liver aldehyde oxidase and milk aldehyde oxidase*

	Turnover no.	$Q_{M.B.}$	% flavin-phosphate	mg. per unit of enzyme activity
Liver*	520	2700	0·17	0·62
Milk†	570	4500	0·27	0·32

* At purity level represented by extinction ratio $\frac{E_{275\,m\mu}}{E_{450\,m\mu}}$ of 15·6.

† At purity level represented by extinction ratio of 6·2.

Purified preparations of the aldehyde enzyme show three absorption bands with maxima at 450, 380 and 280 mμ. The ratio of the extinctions at 280 and 450 mμ respectively is 15·5 in the best preparations. Fig. 1 shows the absorption spectrum of the enzyme at the extinction ratio 15·5 stage. The 450 mμ band

characteristic of flavin compounds is anomalous in that there is no trough on the violet end. There is clearly some interfering substance absorbing at *ca.* 390–440 mμ which accounts for the indefiniteness of the 450 and 360 mμ bands. Since catalase is known to be present in preparations of the enzyme at extinction ratio 15·5 stage and since catalase shows an intense absorption band with a maximum at 410 mμ we are inclined to the view that the deviation of the visible spectrum of the aldehyde enzyme from that of typical flavoproteins is referable to the catalase present in the preparation.

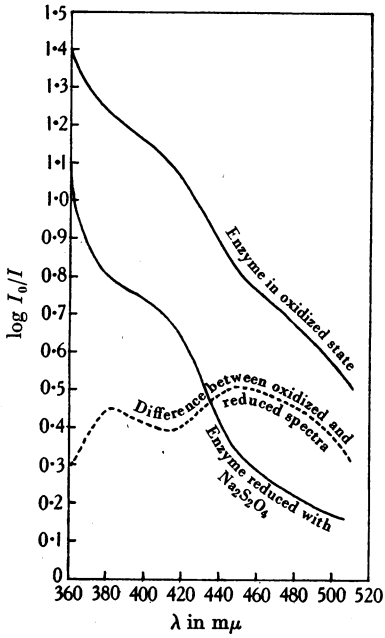

Fig. 1. Absorption spectrum of liver flavoprotein at ratio 15·5 stage. The enzyme solution contained 3·19 mg. per ml. and the length of the cell was 2 cm. For ease of presentation the ultraviolet end of the spectrum with the absorption band at 280 mμ is not shown in the figure.

A large variety of procedures has been explored with a view to advancing the purity of the enzyme beyond the ratio 15·5 stage. But this has so far not proved possible. The enzyme is remarkably stable in ammoniacal $(NH_4)_2SO_4$ but it cannot be further purified by constant repetition of the fractionation procedure. The enzyme is fragile in salt-free solution and in $(NH_4)_2SO_4$ solutions of neutral or acid reaction. Desiccation even at low temperatures completely inactivates the enzyme although no gross denaturation is detectable. Aqueous solutions of alcohol and acetone (>40 %) rapidly denature the enzyme at room temperature though more slowly at 0°.

(4) *Evidence for flavoprotein nature of the enzyme*

The prosthetic group of liver aldehyde oxidase is flavin adenine dinucleotide. On boiling a solution of the enzyme the dinucleotide is liberated from the coagulated protein and can be estimated in the *d*-amino-acid oxidase test system

[cf. Straub 1938, and Corran *et al.* 1939, 2 for full details]. One unit of enzyme contains 1·1 μg. of flavin adenine dinucleotide. The ratio $\frac{\mu g.\ \text{flavin adenine dinucleotide}}{\text{enzyme unit}}$ remains fairly constant from the first clear extract to the highest purity level attained.

The flavin group can be split off from the enzyme by a variety of procedures, e.g. boiling, acidification to *p*H 4, or less, prolonged dialysis against water or dilute buffer, exposure to fat solvents etc. The splitting is irreversible in all these cases.

The following procedure was employed in the isolation of the dinucleotide [cf. Corran *et al.* 1939, 2]. A purified enzyme preparation was heated at 70° for 5 min. in presence of 15 % $(NH_4)_2SO_4$ and acetic acid (*p*H 3·8). After filtration the protein-free solution was saturated with $(NH_4)_2SO_4$ and extracted at *ca.* 70° with molten phenol. Water was added and the phenol was extracted with ether. The dinucleotide was then purified by precipitation as the Ag salt in dilute HNO_3 (*p*H 2). A solution of the decomposed Ag salt showed the typical absorption spectrum of flavin compounds with bands at 265, 370 and 450 mμ respectively. The ratio of the extinction of the three bands was 3·75 : 1·06 : 1 whereas the ratio for the pure dinucleotide is 3·2 : 0·8 : 1 [cf. Warburg & Christian, 1938]. Insufficient material set a limit to further purification of the flavin prosthetic group of the aldehyde enzyme but its identity with yeast flavin adenine dinucleotide could be established by another method. Comparison was made between the catalytic activities of the two dinucleotides in the *d*-amino-acid oxidase test:

	μl. O_2/10 min./μg. flavin phosphate
Yeast	132
Aldehyde oxidase (1)	135
Aldehyde oxidase (2)	126

Thus far no flavin compound other than flavin adenine dinucleotide has been found to be active in the *d*-amino-acid oxidase test.

On treatment of the enzyme with hydrosulphite the yellow-brown colour is only partially bleached. Flavin compounds whether free or combined with protein are completely bleached by hydrosulphite and indeed the prosthetic flavin group which has been separated from the protein part of the enzyme leaves no residue of colour after hydrosulphite treatment. It follows therefore that the flavoprotein must contain some coloured grouping other than flavin. From the extinction of the absorption band at 450 mμ and assuming a β value of $2{\cdot}4 \times 10^7$ as for other flavoprotein one can calculate how much flavin should be present in a given solution of the enzyme in order to account for the observed extinction. The flavin can then be split off from the enzyme and its concentration estimated spectrophotometrically. The results of many experiments agree that only 33 % of the total absorption at 450 mμ can be referred to flavin. The rest is due to some coloured group or groupings non-flavin in nature. It is interesting to note that Corran *et al.* [1939, 1] found that milk flavoprotein contained non-flavin coloured components accounting for the same proportion of the absorption at 450 mμ.

(5) *Mechanism of the reaction*

On addition of acetaldehyde to the enzyme under anaerobic conditions the enzyme is at once partially decolorized. On admission of air the colour is rapidly restored. The enzyme therefore undergoes a cycle of reduction by the substrate

and of oxidation by molecular oxygen. The question arises whether it is the flavin portion of the molecule which undergoes this cyclical process. Spectrophotometric analysis of the enzyme solutions reduced anaerobically by acetaldehyde shows that 33 % of the absorption at 450 mμ has disappeared. In other words the drop in light absorption corresponds precisely to the proportion of the total light absorption due to flavin. Furthermore subtraction of the absorption spectrum of the enzyme reduced with acetaldehyde from that of the oxidized enzyme yields an absorption spectrum of a flavin compound with bands at 450 and 380 mμ respectively. There can be no doubt therefore that it is the flavin group which is alternately reduced and oxidized and hence the justification for considering flavin adenine denucleotide as the prosthetic group of the enzyme.

The reduction of the enzyme by acetaldehyde under anaerobic conditions is for all practical purposes instantaneous. From the turnover number of 520 per min. it follows that the enzyme should be completely reduced in $\frac{1}{520}$ min. The great speed of the observed reduction offers in fact additional support of the cyclical mechanism of the catalysis.

Hydrosulphite bleaches the enzyme some 10 % more than acetaldehyde at 450 mμ. Part of the non-flavin groups must be therefore reducible by hydrosulphite but not by acetaldehyde.

(6) *Kinetics*

Fig. 2 shows how the rate of reduction of methylene blue by acetaldehyde varies with the concentration of enzyme. It is only over a restricted range of enzyme concentrations that a linear relation obtains. This fact must be taken into consideration in carrying out tests of enzyme activity for unless the reduction time of methylene is less than a minute a correction will have to be applied in calculating the number of enzyme units. This falling off in activity with increasing dilution of the enzyme is understandable in terms of destruction of the enzyme. The more dilute the enzyme the larger the time required to reduce a fixed amount of methylene blue. Assuming that the rate of destruction of the enzyme is a function of time only it follows that the ratio

$$\frac{\text{final active enzyme concentration}}{\text{initial active enzyme concentration}}$$

will steadily decrease with dilution of the enzyme. Consistent with this interpretation is the fact that the deviation from linearity progressively increases with dilution of the enzyme. The same phenomenon has been observed with the xanthine oxidase of milk [cf. Booth, 1935].

The relation between velocity and concentration of aldehyde is plotted in Fig. 3. The half-speed concentration of crotonaldehyde (K_m) is *ca.* 0·007 M. Crotonaldehyde was used in preference to acetaldehyde because of its higher boiling point which ensures that there will be no appreciable change in concentration during the evacuation of the Thunberg tubes.

The effect of temperature on the velocity of the reaction is shown in Fig. 4. Above 50° the rate of destruction begins to increase sharply and no simple temperature effect can be observed.

The enzyme is active over the *p*H range 5–11 with a maximum velocity at *ca.* *p*H 7 (cf. Fig. 5). The chemical composition of the buffer did not appear to affect the velocity for a given *p*H.

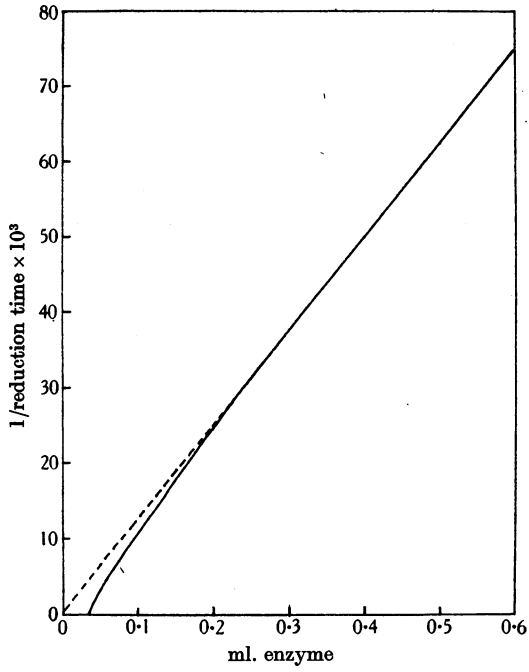

Fig. 2.

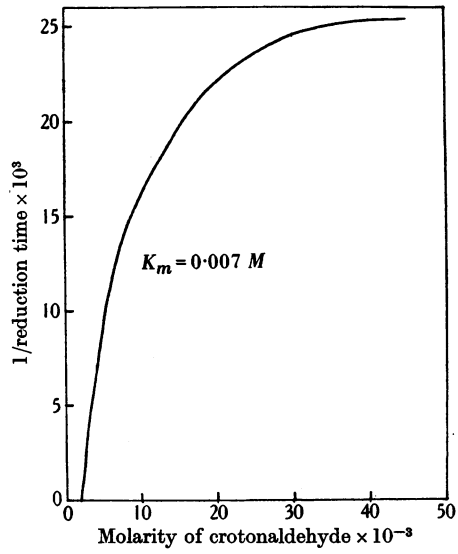

Fig. 3.

Fig. 2. Effect of enzyme concentration on velocity of reduction. The additions in each experiment were 1 ml. $M/10$ phosphate buffer, 0·1 ml. M acetaldehyde, 0·1 ml. 0·011 M methylene blue. Total volume, 2·5 ml. Reduction time in sec. The dotted line is the theoretical slope for a linear relation.

Fig. 3. Effect of aldehyde concentration. The additions in each experiment were 0·3 ml. enzyme, 1 ml. $M/10$ phosphate buffer and 0·1 ml. 0·011 M methylene blue.

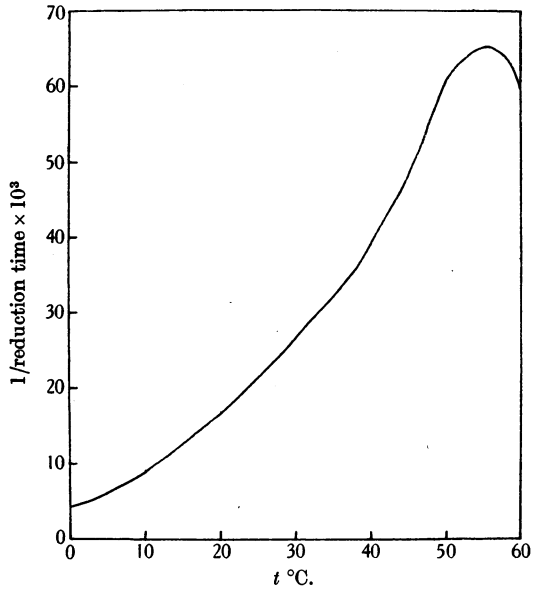

Fig. 4.

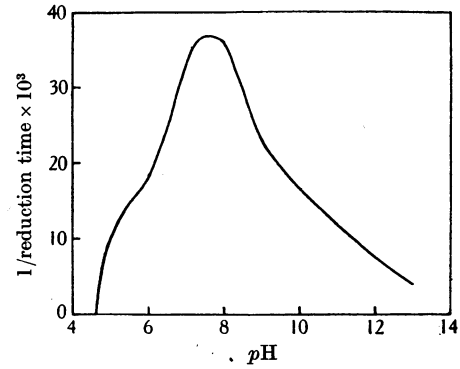

Fig. 5.

Fig. 4. Effect of temperature on velocity of reduction. The additions in each experiment were 0·2 ml. enzyme, 1 ml. $M/10$ phosphate buffer pH 7·2, 0·1 ml. 0·011 M methylene blue and 0·1 ml. M acetaldehyde.

Fig. 5. Effect of pH on velocity of reduction. The additions in each experiment were 0·2 ml. enzyme, 1 ml. buffer 0·1 ml. M acetaldehyde and 0·1 ml. 0·011 M methylene blue.

(7) *Specificity of substrate*

The liver aldehyde enzyme oxidizes a variety of aldehydes. Acetaldehyde, propaldehyde, butaldehyde, crotonaldehyde, benzaldehyde, salicylaldehyde and glycollic aldehyde have been tested and shown to be active as substrates (cf. Table 2). Among aliphatic aldehydes formaldehyde is an apparent exception, but in view of the fact that the enzyme is rapidly denatured in presence of formaldehyde the negative result does not necessarily exclude formaldehyde as substrate. Acetaldehyde is more active for a given concentration than propaldehyde. Increase in the length of the carbon chain decreases the velocity with which the aldehyde is attacked by the enzyme. The unsaturated aldehyde crotonaldehyde is oxidized as rapidly as acetaldehyde. Hydroxyaldehydes such as glycollic aldehyde and salicylaldehyde are less active than the corresponding unsubstituted aldehydes.

Ketones, ketonic acids, acids and alcohols cannot act as substrates. Hypoxanthine is active with crude preparations of the enzyme but this activity disappears when the higher purity levels are attained. The same applies to the oxidation of dihydrocoenzyme I.

Table 2. *Specificity of substrate*

Substrate	Final concentration	Relative activity (acetaldehyde as standard)
Acetaldehyde	*M*/10	100
Propaldehyde	*M*/10	17
Butaldehyde	*M*/10	13
Crotonaldehyde	*M*/10	66
Benzaldehyde	Saturated solution	25
Glycollic aldehyde	*M*/10	8
Salicylaldehyde	Saturated solution	2
Pyruvate	*M*/10	0
Ethyl alcohol	*M*/10	0

(8) *Specificity of hydrogen acceptor*

The enzyme catalyses the oxidation of aldehydes by molecular oxygen, oxidation-reduction indicators such as methylene blue, nitrate and cytochrome *c*. The catalytic reductions of nitrate and cytochrome *c* are comparatively slow reactions and are probably of little physiological interest. The reaction with oxygen is rapid and there can be little doubt that oxygen is the physiological hydrogen acceptor. Fig. 6 shows the rate of oxidation of crotonaldehyde by molecular oxygen as a function of time. Methylene blue in high concentration increases the velocity *ca.* 50 %. The velocity with oxygen in the gas space is the same as with air.

(9) *Product of reaction*

When a limited quantity of aldehyde is allowed to react to completion with oxygen in presence of the enzyme the oxygen absorbed corresponds to 1 atom of oxygen per molecule of aldehyde (cf. Fig. 7). The theoretical oxygen uptakes are reached only with quantities of the order of 0·1 ml. *M*/10 aldehyde. With higher concentrations of aldehyde the reaction time is longer than 30 min. and the enzyme becomes inactivated before the reaction is complete. The stoichiometric proportions suggest that the aldehyde is oxidized to the corresponding acid:

(1) Crotonaldehyde $+ \frac{1}{2}O_2 \rightarrow$ crotonic acid.

The reaction between aldehyde and oxygen is not direct but proceeds through the intermediation of the enzyme in the following way:

(2) Crotonaldehyde + enzyme → crotonic acid + reduced enzyme.
(3) Reduced enzyme + O_2 → enzyme + H_2O_2.

We should expect therefore that each molecule of crotonaldehyde would react with two atoms of oxygen—the oxidation products being crotonic acid and

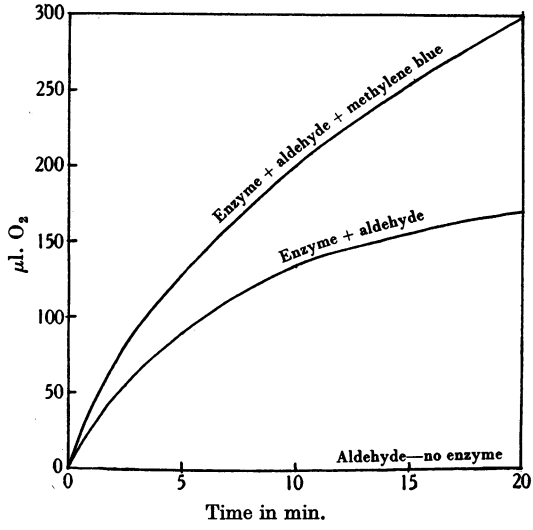

Fig. 6.

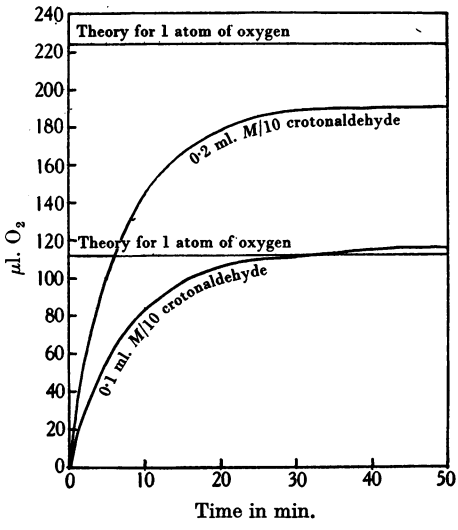

Fig. 7.

Fig. 6. The reaction of the enzyme system with oxygen in presence and absence of methylene blue. The additions were 1 ml. enzyme, 1 ml. $M/10$ phosphate buffer, 0·5 ml. $M/10$ crotonaldehyde and 0·2 ml. 0·5% methylene blue. Air in gas space, 38°.

Fig. 7. The oxygen equivalence of crotonaldehyde. The additions were 1·5 ml. enzyme, 0·5 ml. $M/2$ Na_2HPO_4 and 0·2 ml. 0·5% methylene blue.

H_2O_2. However the presence of catalase in the enzyme preparation ensures that all the H_2O_2 formed by autoxidation of the enzyme (or methylene blue) would be decomposed into O_2 and water:

$$(4)\quad 2H_2O_2 \rightarrow 2H_2O + O_2.$$

Reaction (1) therefore represents the balance of reactions (2), (3) and (4).

For the demonstration of the product of reaction we have carried out our experiments with acetaldehyde as substrate. A mixture containing 25 ml. enzyme, 27 ml. $M/3$ phosphate buffer pH 8, 3 ml. 0·5% methylene blue and 3 ml. M acetaldehyde was shaken aerobically at 38° for 60 min. From the oxygen uptake determined on an aliquot portion of the mixture 0·88 mM of acetic acid should have been formed. The distillate of the acidified mixture was found to contain 0·82 mM of acid and gave a positive reaction in the lanthanum nitrate test for acetic acid.

The method of Dyer [1916] was used for demonstrating that acetic acid was the only volatile acid in the distillate. The method involves steam distillation

at constant volume—the distillates being collected in 100 ml. lots and titrated against $N/10$ alkali. The following is an example of a typical experiment:

	Observed titre ml.	Theoretical titre ml.
1st 100 ml.	2·45	2·45
2nd 100 ml.	1·50	1·60
3rd 100 ml.	1·00	1·10

The values for the theoretical titres were determined in the apparatus with approximately the same quantity of pure acetic acid. The evidence is clear that acetic acid is the sole product of the oxidation.

(10) *Liver aldehyde oxidase and milk xanthine oxidase*

Xanthine oxidase of milk is a flavoprotein which catalyses the oxidation of purines, aldehydes and dihydrocoenzyme I [cf. Ball, 1939; Corran *et al.* 1939, 2]. The liver aldehyde oxidase is a flavoprotein with only one of the three functions of the milk compound. It is of considerable theoretical interest therefore to compare the properties of these two enzymes. We shall first list the similarities:

(*a*) Both contain in addition to flavin adenine dinucleotide some coloured substance or substances which account for some two-thirds of the light absorption at 450 mμ. In that respect they differ from all the other known flavoproteins.

(*b*) The catalytic efficiencies of the two enzymes with aldehydes as substrate are practically identical.

(*c*) Incubation with dilute cyanide and desiccation irreversibly inactivate the two enzymes as far as oxidation of aldehyde is concerned.

There are several differences other than that of substrate specificity:

(*a*) Whereas cyclical reduction and oxidation of the flavin group in the liver enzyme can be easily demonstrated there is no clear evidence of the operation of such a mechanism in the case of the milk enzyme.

(*b*) The liver enzyme is somewhat less soluble in $(NH_4)_2SO_4$ solutions than the milk enzyme—the precipitation range of the former being 25–35 % saturation of $(NH_4)_2SO_4$ and that of the latter 33–43 % saturation.

(*c*) The liver enzyme is rapidly and irreversibly inactivated by 24 hr. dialysis against distilled water at 0° whereas the milk enzyme is stable over a period of days.

The fact that milk flavoprotein apparently catalyses the oxidation of three entirely different substrates, viz. purines, aldehydes and dihydrocoenzyme I, has always been difficult to reconcile with the extreme specificity shown by enzymes in general. The isolation of a flavoprotein from liver which is specific for aldehyde oxidation and whose properties closely resemble its counterpart in milk gives new force to the suggestion that the milk enzyme may after all be a close association of three enzymes which cannot be resolved by the methods hitherto employed.

Liver contains at least three enzymes which catalyse the oxidation of aldehydes: (1) the aldehyde mutase of Dixon & Lutwak-Mann [1937]; (2) the xanthine-aldehyde-dihydrocoenzyme I oxidase of Corran *et al.* [1939, 2], and (3) the aldehyde oxidase described above. The first enzyme belongs to the class of the pyridinoproteins whereas the last two are flavoproteins.

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