CLXIX. ALDEHYDE MUTASE¹

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THE name "aldehyde mutase" is given to the enzyme which catalyses the so-called Cannizzaro reaction (reaction (1) below), a type of reaction which is probably of importance in intermediary metabolism. Batelli & Stern [1910] were the first to show that this reaction was catalysed by animal tissues, but satisfactory quantitative chemical studies were first carried out by Parnas [1910], who showed that the catalyst was a soluble enzyme, to which he gave the name of aldehyde mutase. The mutase was believed to be a separate enzyme until Wieland [1914] claimed to have shown that it was identical with the Schardinger enzyme or aldehyde oxidase, which catalyses the oxidation of aldehydes in accordance with reaction (2):

where A may be O_2 or some other hydrogen acceptor, such as methylene blue. Wieland suggested that the oxidase normally uses a hydrogen acceptor to produce an oxidation of the aldehyde, but when no other acceptor is present it uses a second molecule of aldehyde as acceptor, reducing it to alcohol and so producing a dismutation of aldehyde (reaction (1)). This view has been almost universally held up to the present time.

The mutase has been much less thoroughly studied than the aldehyde oxidase. The main contributions to our knowledge of the enzyme are due to Euler and his co-workers [e.g. Euler & Brunius, 1928], who showed that yeast mutase requires cozymase for its activity, and to Reichel & Köhle [1935], who have recently carried out systematic studies on purified liver mutase. They found that their mutase preparations also catalysed the oxidation of aldehydes under certain conditions and they believed that the same enzyme was responsible for both reactions.

In the course of a systematic study of the Schardinger enzyme we obtained evidence that this enzyme was distinct from aldehyde mutase and we therefore decided to reinvestigate the question of the identity of aldehyde mutase and oxidase.

EXPERIMENTS ON THE MILK ENZYME

Materials

Preparations of the Schardinger oxidase made by the methods of Dixon & Thurlow [1924, 1] and Dixon & Kodama [1926] were used. The acetaldehyde, with which most of the experiments were carried out, was purified by preparing the crystalline aldehyde ammonia and decomposing this with acid and redistilling in the usual way.

¹ A preliminary account of some of the results of this work was communicated to *Nature* [Dixon & Lutwak-Mann, 1937, 1].

Methods

The dismutation of aldehyde can be followed in two ways, namely by chemical estimation of the reactants or by a manometric method described below. Previous work has been carried out entirely by chemical methods. Considerable difficulties were however met with in attempting to study the milk enzyme by chemical methods. The estimation of aldehyde, alcohol and acid in presence of one another in a tissue extract or enzyme preparation is in itself a difficult problem and the existing methods do not give satisfactory results. The estimation of one of the reactants alone is usually insufficient for studying the mutase reaction; for example, the disappearance of aldehyde cannot be taken as a measure of the reaction, for it may be caused by several different processes. It may be due (a) to a dismutation of the aldehyde to acid and alcohol, (b) to an oxidation to acid by aldehyde oxidase and a hydrogen acceptor, (c) to a reduction to alcohol by alcohol dehydrogenase (which is a reversible enzyme) and a hydrogen donor, (d) to a combination with proteins, apart from any enzyme action, and (e) to a loss of aldehyde vapour during the necessary manipulations (in the case of volatile aldehydes). The retention of aldehydes by combination with protein is very troublesome; if a known amount of aldehyde is added to a boiled enzyme preparation from milk and estimated immediately it is found that as much as 50 % of the aldehyde may remain in combination with the protein and so escape estimation. This occurs in any case, but particularly when deproteinizing reagents are used. A similar effect occurs with lower aliphatic acids and alcohols. If a known amount of such an acid is added to milk it cannot be quantitatively recovered by adding phosphoric acid and distilling in steam *in vacuo* [Welde, 1910]. Wieland's work referred to above was carried out by estimations of the acid and alcohol formed, and he encountered the same difficulty and was compelled to introduce considerable corrections. This procedure is impossible if a number of different preparations are to be used, as the retention will vary from case to case.

In order to follow the mutase reaction one must ensure that no oxidase reaction takes place, otherwise a simple oxidation, or at most a mixed dismutation and oxidation, will be obtained. If aldehyde oxidase is present oxygen must therefore be rigidly excluded, and this involves either evacuation of the vessels or the passage of a large volume of oxygen-free nitrogen, which in turn results in a considerable loss of aldehyde if a volatile aldehyde is used. Unfortunately it appears that the most volatile aldehydes such as acetaldehyde are just those which undergo dismutation most readily; aromatic aldehydes appear to be practically inactive. Owing to these difficulties most of our work has been done, not by chemical estimations, but by the manometric method, which however was controlled by chemical estimations.

The manometric method used depends on the measurement of the CO_2 displaced from bicarbonate by the acid produced by the reaction. This well-known principle in manometry has been used by Lohmann [1932] to study a somewhat similar enzyme reaction, namely the conversion of methylglyoxal into lactic acid by glyoxalase. The reaction is caused to take place in Barcroft differential manometers in bicarbonate solutions in equilibrium with a CO_2 -containing gas mixture under rigidly anaerobic conditions. The amounts of bicarbonate and CO_2 were chosen so as to give the *pH* desired (7.7 in most cases). Since it was necessary to pass the gas mixture through the flasks we have used the type of flask illustrated by Keilin & Hartree [1935, Fig. 2] provided with side bulbs and gas outlets. The aldehyde was placed in the side bulbs and was only mixed with

the enzyme solution after the flasks had been freed from oxygen by passing the gas. The gas mixture contained 95 % N_2 + 5 % CO_2 , and it was completely freed from oxygen by passing it through a tube filled with copper heated to dull redness, the copper being prepared by the reduction of CuO wire containing a small amount of palladium.

It was first necessary to determine the conditions for the complete removal of O_2 from the flasks. Measurements of the amounts of O_2 remaining in the flasks after passing the gas for different periods were made as follows. 3 ml. of $M/2$ $NaOH$ were placed in the flasks of four manometers and 0.3 ml. of 1 % pyrogallol in the right-hand side bulbs. Nitrogen was then passed at the standard rate of 200 ml. per min. through each flask and the flow of gas continued for different lengths of time in the different manometers. After shaking for some time to obtain thorough equilibration, the pyrogallol was mixed with the alkali and the absorption of oxygen observed manometrically. Table I shows the amounts of oxygen in the flasks (volume about 35 ml.) remaining at different stages of the treatment with gas.

Table I

Time (min.)	$\mu l.$ O_2
2	46
5	18
10	5.5
20	1

In our experiments we therefore passed the gas for 15 min. so that the amount of oxygen remaining was insignificant, being less than 1 part in 10,000. After the passage of the gas the manometers were shaken for a further 5 min. with the gas delivery tubes still attached and the taps open, in order to obtain complete equilibration, before starting the reaction. The temperature was 38° throughout.

Attention must be paid to a number of possible sources of error inherent in the method if reliable results are to be obtained, and these will now be dealt with in turn.

(1) During the passage of the large volume of gas a certain loss of volatile aldehyde is unavoidable; this is, however, fairly constant and can be allowed for. With acetaldehyde under our standard conditions it amounts to about 20 %. If necessary the loss may be reduced to a minimum by placing the aldehyde solution in Keilin tubes in which the exposed surface of the solution is less.

(2) For technical reasons it is necessary to place the enzyme solution in both flasks and aldehyde in the right-hand flask only, and there will therefore be an uncompensated aldehyde vapour pressure which will be registered on the manometer. This must be determined by control experiments and subtracted from the experimental readings before converting them into volumes of CO_2 . This vapour pressure correction only amounts to a few per cent, and Fig. 1 shows its magnitude with different initial amounts of acetaldehyde.

(3) All the reactants should be adjusted initially to the correct pH as far as possible, but it is hardly possible to do so with sufficient accuracy to avoid small changes on adding them to the bicarbonate solution. It is therefore always necessary to do control experiments by adding them to bicarbonate solution without enzyme.

(4) Owing to the protein content of the enzyme solution a part of the acid and the CO_2 is buffered or "retained" by the protein and is therefore not shown on the manometer. This retention must be allowed for, and this can be done in two ways, either by constructing a retention curve for the enzyme solution, as

described by Dixon [1937], or more simply by adding small known amounts of acid and comparing the amounts of CO_2 evolved with the amounts which should theoretically be obtained. With fairly strong (4 %) enzyme solutions it was found that the result of allowing for the effect of retention was to increase the readings by about 30 %, but with the more purified preparations later obtained much more dilute solutions could be used and the effect was thereby considerably reduced.

(5) Warburg found [1925] that even slight dilution of a solution containing bicarbonate and protein in equilibrium with a CO_2 -containing gas mixture produces a manometric displacement. This is due to the dilution of the bicarbonate without a corresponding dilution of the carbonic acid (which is kept almost constant by the gas mixture), so that the pH, and therefore the amount of CO_2 retained by the protein, is altered. In order to avoid this effect our experiments were arranged as symmetrically as possible, i.e. the enzyme solution and any reactants other than the substrate were added to both flasks of the manometer, and an equal volume of water was always added from the left-hand side bulb to counterbalance the addition of the substrate from the right-hand bulb.

Provided that attention is given to these points the manometric method has proved to be completely reliable, and it has great advantages over the chemical method, for it makes it possible to follow quantitatively every stage of the reaction on small amounts of material with a great saving of time compared with the chemical method.

Results

Numerous experiments were carried out on various preparations of the aldehyde oxidase of milk, using acetaldehyde, propionic, isovaleric and salicylic aldehydes and piperonal. Although these aldehydes are all very readily oxidized by these enzyme preparations in presence of oxygen or methylene blue, no trace of dismutation could be detected in any experiment, even in presence of cozymase. Curve A of Fig. 2 represents the results obtained.

As this negative result was unexpected a search for possible explanations was made. It is known that the oxidase undergoes destruction in presence of aldehydes aerobically, but this is usually attributed to the formation of peroxides. We found, however, that the enzyme is also slowly destroyed in presence of aldehyde under strictly anaerobic conditions where peroxide formation is excluded. Nevertheless this cannot account for the negative results, as the destruction is fairly slow, and strong solutions of the enzyme were used so that the amount of destruction was unimportant and the solutions were always found to be highly active with methylene blue at the conclusion of the experiments. The

Fig. 1. Vapour pressure correction with acetaldehyde.

The amounts shown are those present initially; after passing the gas the amounts present are some 20 % less. The volume of the aldehyde solution in the side bulbs was 1 ml. throughout and the total liquid volume was 3.3 ml. In our routine experiments the amount of aldehyde was less than 1 mg.

aldehyde was found to be present unchanged at the end. Milk is known to contain enzymes which destroy cozymase, but this cannot account for the absence of dismutation, for the destruction is relatively slow and in order to minimize it the cozymase was always added simultaneously with the aldehyde, so that there was no time for its destruction before the reaction should have begun.

It is therefore clear that no dismutation occurs, even though active enzyme, cozymase and aldehyde are all present together, and the conclusion is therefore inevitable that the aldehyde oxidase of milk is not an aldehyde mutase.

Fig. 2.

Fig. 3.

Fig. 2. Anaerobic acid production from various aldehydes by aldehyde oxidase of milk.

Curve A represents the practically identical results obtained with the five aldehydes mentioned in the text, both with and without cozymase. 160 mg. of casein preparation or 40 mg. of whey preparation, 0.5 ml. *M/50* aldehyde and 0.2 ml. cozymase solution were used. Curve B gives the results with 1.12 mg. methylglyoxal.

Fig. 3. Action of boiled and unboiled oxidase preparations on methylglyoxal (0.5 mg.).

In view of our results it is somewhat difficult to account for the positive results obtained by Wieland. Our experiments differ from his in several respects. (a) His experiments were carried out at 60°, whereas ours were done at 38°. In view of the fact that the enzyme is destroyed at about 65° we do not consider 60° a suitable temperature for work on this system. Wieland & Macrae [1930], however, later carried out experiments at 37° by allowing the reaction to take place in the presence of $\text{Ba}(\text{OH})_2$ and estimating the acid produced by back titration. (b) Wieland used salicylic aldehyde, whereas most of our experiments were carried out on acetaldehyde. As stated above, however, we have also obtained negative results with salicylic aldehyde, and it is of interest that Parnas found that among several aldehydes which he tested salicylic aldehyde was completely inactive towards mutase. Wieland & Macrae later used acetaldehyde also and observed an anaerobic acid formation. The reaction was, however, allowed to proceed for as long as 4000 min. and even after this time only about 10% of the aldehyde added had been transformed. With salicylic aldehyde also only 10% of the amount added was transformed. Further (c) no cozymase was added in Wieland's experiments, whereas it is now known

from Euler's work that cozymase is necessary for the action of aldehyde mutase. Finally (*d*) our experiments were done on preparations of the oxidase made as described above, whereas Wieland's experiments were carried out either on whole milk or on preparations made by other methods. We therefore tested numerous samples of fresh milk and obtained negative results in all cases but one. In this one case a rapid dismutation, accelerated by cozymase, was observed, but it was found that this reaction was not enzymic, for it occurred equally rapidly after boiling the milk. We were, however, never able to obtain another sample of milk showing this effect.

The only aldehyde with which we have obtained positive results, using preparations of the aldehyde oxidase, is methylglyoxal (see curve *B* of Fig. 2). But it is to be observed that the amount of CO_2 produced is twice that which would be expected for a dismutation, i.e. every molecule of aldehyde produces one molecule of acid. Moreover, the product was identified as lactic acid and not pyruvic acid, so that the reaction may be described as a glyoxalase rather than a mutase reaction. It is, however, completely unaffected by the addition of glutathione, the coenzyme of glyoxalase. (In the absence of the enzyme preparation the acid production from methylglyoxal was exceedingly slow.) On boiling the enzyme preparation it was found that the reaction was not enzymic (Fig. 3), but was due to a catalytic action of the proteins. A similar effect has been observed with casein peptone by Neuberg [1927]. We may mention that this effect is not observed in preparations made from tissues, but appears to be a characteristic property of milk proteins.

EXPERIMENTS ON LIVER MUTASE

Having shown that the aldehyde oxidase does not act as an aldehyde mutase, we investigated the aldehyde mutase of liver, in order to ascertain whether it acts as an aldehyde oxidase.

Materials

At first preparations made from horse and pig liver by the method of Reichel & Köhle [1935] were used. Their method consists essentially in alternate precipitation by acetone-ether mixtures and extraction with water, repeated several times in succession. It was found that these mutase preparations usually had some oxidase activity, in accordance with the above authors' observations. We found, however, that the following method gave preparations with a high mutase activity but no aldehyde oxidase activity.

1 kg. of horse liver is passed twice through an ordinary mincer, 1200 ml. of water are added and the mixture is allowed to stand for an hour. The suspension is centrifuged, and to every litre of the resulting fluid 550 ml. of 95 % alcohol are added. After leaving overnight at room temperature the precipitate is centrifuged or filtered off, and the fluid is precipitated with 3 vol. of an acetone-ether mixture (3 : 1). The crude preparation thus obtained (about 15 g.) is washed with acetone and dried over CaCl_2 *in vacuo*. It is in nearly all cases completely free from the aldehyde oxidase, if horse or dog liver is used, but not always if pig liver is used. In order to purify it further it is dissolved in a small volume of water, dialysed for 24 hr. against running water, centrifuged and the fluid heated for 10 min. at 55° , in order to get rid of as much inactive protein as possible. The resulting precipitate is removed by filtration or centrifuging and for routine work the fluid was precipitated with acetone-ether as described above (yield about 1 g.). For further purification it may be adsorbed on freshly prepared tricalcium phosphate, made by precipitating CaCl_2 with Na_3PO_4 and washing until chloride-free. The mutase may be eluted from the calcium phosphate with $M/10 \text{ Na}_2\text{HPO}_4$, dialysed and precipitated with acetone-ether in the usual way. By this means it is possible to separate completely the mutase and the oxidase, the latter being relatively slightly adsorbed by calcium phosphate.

The resulting preparation is completely free from both aldehyde oxidase and alcohol dehydrogenase, but contains a very active aldehyde mutase. The mutase is soluble and completely stable both in the dried form and in solution.

Results

The dismutation of acetaldehyde by a mutase preparation completely free from oxidase is shown in Fig. 4, and it will be seen that the reaction is very rapid and that the amount of CO_2 produced corresponds with the amount calculated on the assumption that the process is a pure dismutation. This agreement must only be taken as approximate, as it is difficult to determine accurately the correction for the amount of aldehyde lost during the treatment with gas. The reaction is clearly enzymic, as is shown by the curve obtained in the control experiment with boiled enzyme. The slight initial liberation of CO_2 is always obtained with boiled enzyme solutions, and is apparently due to a combination of aldehyde with amino-groups and consequent slight increase of acidity, as in the formaldehyde titration. A much larger effect of this kind can be obtained by adding aldehyde to amino-acids in bicarbonate solutions.

The total amount of CO_2 evolved is proportional to the amount of aldehyde taken, as shown in Fig. 5, and it is independent of the amount of enzyme. The addition of a further quantity of enzyme from the side bulb at the end of the experiment produces no effect, showing that the cessation of the reaction is not due to destruction of the enzyme. As a matter of fact the mutase was found to be much more stable than the Schardinger enzyme and does not undergo destruction in presence of aldehyde.

The initial velocity is seen to depend on the aldehyde concentration, and this is shown more clearly in Fig. 6, from which it appears that under our usual conditions the enzyme is far from being saturated with aldehyde, for which it must have a rather low affinity.

Although, as mentioned above, the final amount of CO_2 evolved is independent of the amount of enzyme, the initial velocity is proportional to it, as shown in Fig. 7.

The quantities of bicarbonate and CO_2 in our experiments were usually selected so as to give a $p\text{H}$ of 7.7, this being the optimum $p\text{H}$ of the mutase according to Reichel & Köhle [1935, Fig. 1]. We have also varied the $p\text{H}$ between 5.5 and 8.0 by altering the bicarbonate concentration and the CO_2 pressure and have confirmed the $p\text{H}$ curve of these authors within these limits. It is not practicable to go beyond these limits by the manometric method used by us.

Chemical estimations. In order to verify the production of alcohol by the dismutation and at the same time to compare the manometric and chemical methods a number of experiments were carried out in which parallel estimations of aldehyde and alcohol by chemical methods and of acid by manometric measurements were made. After a number of trials the following procedure for estimating aldehyde and alcohol was adopted as giving reasonably satisfactory results.

The solution was first acidified with one-third of its volume of $M \text{H}_3\text{PO}_4$ and distilled in steam *in vacuo* (the apparatus of Parnas & Heller [1924] as used for ammonia estimations was found to be convenient if the usual receiver was replaced by a filter-flask cooled efficiently in a freezing mixture). During the distillation the temperature of the solution did not rise above 35° . The distillation was allowed to proceed until about 30 ml. of distillate from 3 ml. of solution were collected (about 20 min.). An aliquot part of the distillate was taken for iodimetric aldehyde estimation as used for lactic acid determinations by the method of Friedemann *et al.* [1927]. For the

Fig. 4.

Fig. 5.

Fig. 4. *Dismutation of acetaldehyde by horse liver mutase.*

Curve A: 36 mg. purified mutase preparation in 0.5% bicarbonate with 0.55 mg. acetaldehyde (added at arrow). Curve B: control with boiled enzyme.

Fig. 5. *Course of reaction with different amounts of acetaldehyde.*

100 mg. horse liver preparation. At the arrow a further 36 mg. of enzyme was added from the side bulb. The aldehyde was added from hanging tubes in this experiment.

Fig. 6.

Fig. 7.

Fig. 6. *Effect of aldehyde concentration on initial velocity.*

18 mg. purified horse liver preparation in all cases. The velocities were obtained from the readings for the first 10 min., during which the reaction curves were linear.

Fig. 7. *Effect of varying concentration of enzyme.*

Curve A with 96 mg., curve B with 48 mg. of horse liver mutase, 0.5 mg. acetaldehyde in both cases.

alcohol estimation the aldehyde was removed from the remainder of the distillate by boiling for 15 min. under reflux on a water-bath with freshly precipitated silver oxide and a little talcum in order to oxidize it to acid. The solution was then made alkaline with NaOH and distilled, the receiver being cooled in ice. The distillate was then distilled to dryness *in vacuo* at 60–70°, the alcohol being received in $N/10$ $K_2Cr_2O_7$, which was then heated to 100° in a closed vessel for 2 hr. After cooling, the solution was titrated with $N/20$ ferrous ammonium sulphate, using $K_3Fe(CN)_6$ as external indicator.

A number of control estimations on known amounts of aldehyde and alcohol were first carried out in order to test the methods, as shown in Table II. In all these control experiments enzyme action was excluded by adding the phosphoric acid to the enzyme before the aldehyde and alcohol. In section A of the table the acidified solutions were used directly (without distillation) for the aldehyde estimations. It will be seen that the enzyme preparations alone contain neither aldehyde nor alcohol and that the retention of aldehyde by these preparations, unlike the milk proteins, is only slight. In section B of the table the estimations were carried out with the preliminary distillation exactly as described above. The results show that no retention of alcohol by the proteins takes place, but that a slight unavoidable loss of both alcohol and aldehyde occurs during the distillation. The cozymase solution was found to contain small amounts of alcohol. In section C the procedure of the manometric experiments was duplicated, except that, as stated above, the enzyme was first acidified with phosphoric acid. The reagents were placed in manometers in the usual way and mixed after the standard amount of gas had been passed through. The solutions were then removed from the flasks and the estimations carried out as in section B (except in the first case, in which the distillation was omitted). Thus the figures in section C include the losses during the treatment with gas.

Table II. *Control estimations for testing methods*

	Added		Found	
	Alcohol mg.	Aldehyde mg.	Alcohol mg.	Aldehyde mg.
A. Enzyme	0	0	0	0
Enzyme	0	10	0	9.2
Boiled enzyme	0	10	0	9.0
B. Enzyme	5	0	4.7	0
Water	5	0	4.3	0
Water	5	5	4.75	3.9
Enzyme + cozymase	0	0	0.09	Trace?
C. Enzyme	0	10	—	8.2
Enzyme	5	0	4.42	—
Enzyme	5.5	5	4.08	3.15
Enzyme	5	5	3.90	3.42

The actual dismutation experiments were carried out in manometers, the procedure being the same as that described earlier, except that in order to obtain satisfactory estimations it was necessary to use amounts of aldehyde larger than those used in the ordinary manometric experiments. The amounts of acid produced were consequently too large for measurement by the ordinary manometers, and it was necessary to use Clerici solution instead of paraffin in the manometer tubes in order to keep the readings within the range of the scale. In other respects the experiments were carried out as usual; the acid production was followed by the manometric readings, and after a sufficient period of time the manometers were removed from the thermostat and the flasks were rapidly

cooled in ice-water before opening to minimize losses of aldehyde and alcohol. The alcohol and aldehyde in the contents of the flasks were then estimated as described above.

The results are shown in Table III, section A. The amounts of aldehyde remaining after passing the gas (col. 5) must not be taken as very accurate, as they could only be determined in parallel control experiments. In the last four columns of the table the amounts of alcohol and acid have been reduced to their equivalents in mg. of aldehyde, in order to compare the various quantities. In calculating these figures from the actual observations given in cols. 6 to 8 account has been taken of the fact that the true values are always slightly larger than the estimated values, as shown in Table II, and the figures have been corrected accordingly. The acid produced (col. 11) is of course calculated from the manometric CO_2 measurements (col. 8). It will be seen that the amounts of alcohol produced, as found by chemical estimations, are approximately the same as the amounts of acid measured manometrically, showing that the reaction is a simple dismutation. The agreement leaves something to be desired, but we attribute this to the technical difficulties of the chemical method rather than to errors in the manometric method, which we consider gives the more reliable results.

Some experiments were also carried out in phosphate, in the absence of bicarbonate and CO_2 , and in air instead of nitrogen, and one of these is shown in section B. In this case, of course, the acid produced cannot be estimated manometrically, but it was estimated by titrating the first distillate with NaOH . The enzyme preparation itself contained a considerable amount of volatile acid, as shown in the blank experiment, and this was subtracted from the experimental titration figure to obtain the acid produced by the dismutation.

Table III. *Dismutation of acetaldehyde*

Preparation	Co- zymase added	Duration of exp. hr.	Aldehyde added mg.	Aldehyde left after passing gas	Estimated at end			Finally present (in mg. of aldehyde)			Total aldehyde accounted for mg.
					Aldehyde mg.	Alcohol mg.	CO ₂ μl.	Aldehyde	Alcohol	Acid	
A. Bicarbonate and N ₂ + CO ₂											
Horse liver	+	1	5.5	4.5	0.9	1.0	768	1.1	1.10	1.51	3.71
	+	3	5.5	4.5	Trace	1.92	1145	Trace	2.08	2.25	4.33
	+	2	11.0	8.5	0.46	3.3	1790	0.55	3.58	3.51	7.64
	+	4	8.8	7.0	0	3.2	1206	0	3.48	2.37	5.85
Dog liver	+	3	5.5	4.5	Trace	2.1	1451	Trace	2.28	2.85	5.13
	—	3	5.5	4.5	2.1	0.09	67	2.5	0.10	0.13	2.73
B. Phosphate and air											
							Acid ml. N/100				
Dog liver	+	3½	6.0	5.0	0.04	2.13	5.68	0.04	2.30	1.64	3.98
	+	Blank	0	0	—	Trace	2.02	—	—	—	—

Tests for oxidase. Many experiments were carried out on the oxygen uptake of aldehyde in presence of the purified mutase preparations. These experiments were done in the usual way in ordinary Barcroft manometers in the absence of bicarbonate, the flasks being filled with air. (The dismutation was shown to proceed equally well in phosphate buffer without bicarbonate and CO_2 .) No trace of oxygen uptake could ever be detected even with the addition of cozymase, flavin, flavoprotein, cytochrome *c* and indophenol oxidase. With these preparations the dismutation of aldehyde can actually be carried out equally well in the presence of oxygen, as shown in Table III.

Similarly, tests with methylene blue were also entirely negative. The enzyme preparations do not reduce methylene blue even in many hours and the addition of aldehyde produces no increase of reducing power. The alcohol dehydrogenase was also found to be completely absent from these preparations—a point to which we shall return later. The crude preparations on the other hand contain both these enzymes in addition to the mutase and their presence is readily shown by the methylene blue technique. They can, however, be completely removed from these preparations by the method of purification previously described. We suggest that the oxidations of aldehyde with methylene blue and oxygen which were observed by Reichel & Köhle were due to the presence of traces of aldehyde oxidase in their mutase preparations.

INHIBITORS

Further evidence for the non-identity of the mutase and oxidase is provided by the effect of inhibitors, in particular iodoacetate and cyanide. *M/100* iodoacetate has no appreciable action on the aldehyde oxidase, as shown in Table IV. In these experiments the enzyme was incubated with the iodoacetate for half an hour before testing. On the other hand iodoacetate inhibits

Table IV. *Effect of M/100 iodoacetate on reduction of methylene blue*

Enzyme	Control without aldehyde	Reduction time	
		With aldehyde	With aldehyde and iodoacetate
Milk oxidase	∞	6 min. 45 sec.	7 min. 45 sec.
Milk oxidase	∞	3 " 15 "	4 " 15 "
<i>Acetobacter suboxidans</i> * (resting)	∞	20 " 0 "	20 " 0 "
Kidney mutase (crude)	∞	20 " 0 "	20 " 0 "
Liver mutase (crude)	∞	2 hr.	2 hr.
		With hypoxanthine	With hypoxanthine and iodoacetate
Milk oxidase	∞	53 sec.	54 sec.

* This was tested because the acetic bacteria generally are known to produce acetic acid and alcohol in equimolecular proportions. It was thought that this reaction might be due to aldehyde mutase, but although an aldehyde oxidase was present we could detect no trace of mutase in this strain.

the mutase completely, as shown in Fig. 8. It will be seen that, as usual, the action of the iodoacetate takes a few minutes to develop. In this experiment the iodoacetate was added simultaneously with the aldehyde and cozymase at the point marked by the arrow. In presence of glutathione less complete inhibition is obtained; crude preparations therefore show less effect.

Dixon & Keilin [1936] found that aldehyde oxidase is completely and irreversibly destroyed by incubation with cyanide, and this effect was shown by Leloir & Dixon [1937] to be a characteristic property of this enzyme, not shared by other oxidizing enzymes. In general it is not possible to study the influence of cyanide in presence of aldehyde, since they combine to form a cyanohydrin; but, as the inactivation by cyanide is irreversible in this case, the effect may be used as a means of selective destruction of the oxidase, for the enzyme preparation may be treated with cyanide and the cyanide removed before testing with aldehyde. It was therefore thought that it might be possible to make use of this effect in order to get rid of the aldehyde oxidase from the mutase preparation. A crude mutase preparation containing a small quantity of aldehyde oxidase was therefore treated in accordance with Dixon & Keilin's procedure as follows.

A 10% solution of crude horse liver mutase preparation was divided into two parts. To one part neutralized cyanide was added to give a concentration of $M/100$ and both portions were allowed to stand for half an hour. They were then precipitated with acetone-ether in the usual way, washed thoroughly with acetone and dried *in vacuo*. The two fractions were then redissolved and tested

Fig. 8.

Fig. 9.

Fig. 8. Action of $M/100$ iodoacetate on dismutation of acetaldehyde.

18 mg. purified horse liver mutase dialysed 18 hr. (practically glutathione-free). 0.8 mg. acetaldehyde + 0.04 mg. cozymase (ACo 600,000) + iodoacetate (in curve B only) added at arrow.

Fig. 9. Effect of cyanide treatment on mutase.

180 mg. crude horse-liver mutase with 0.8 mg. acetaldehyde. Curve A with untreated enzyme, curve B with enzyme previously treated with cyanide as described in the text.

as usual for mutase and oxidase activity. It will be seen from Fig. 9 that the mutase activity is quite unaffected by this treatment, whereas the oxidase had been completely destroyed (see Table V).

Table V. Effect of cyanide treatment on oxidase

	Reduction time of methylene blue	
	Without aldehyde	With aldehyde
Control	∞	2 hr.
Cyanide-treated	∞	No detectable reduction in 24 hr.

The use of cyanide therefore gives an additional method of preparing oxidase-free mutase and provides further proof of the non-identity of the two enzymes. Thus by using iodoacetate or cyanide it is possible to poison either enzyme independently of the other.

$M/100$ phloridzin and fluoride were found to have no action on the mutase.

COENZYMES

A further point of difference between the two enzymes is connected with the question of coenzymes. It is well known that the aldehyde oxidase does

not require the addition of any coenzyme; in fact the addition of cozymase slows the reaction, as shown in Table VI, which gives an experiment on the milk enzyme. The inhibition is probably due to the fact that cozymase is an adenine compound and, as Dixon & Thurlow [1924, 2] first showed, adenine compounds strongly inhibit this enzyme.

Table VI. *Effect of cozymase on the milk oxidase*

Enzyme alone	Reduction time of
	methylene blue
Enzyme alone	∞
„ + cozymase	4 hr.
„ + hypoxanthine	40 sec.
„ + hypoxanthine + cozymase	50 „
„ + aldehyde	2 min. 20 „
„ + aldehyde + cozymase	5 „ 25 „

On the other hand we have confirmed the statement of Euler that cozymase is necessary for the action of the aldehyde mutase. This is not immediately obvious with crude horse liver preparations, which are active without the addition of any cozymase, even after long dialysis. Fig. 10 shows the effect of adding cozymase to dialysed and undialysed preparations. It will be seen that the addition of cozymase produces no effect with the undialysed preparation. Dialysis considerably reduces the activity, and after the dialysis the addition of cozymase produces a marked acceleration, which indicates that the reduction in velocity is due to the removal of a large part of the cozymase by dialysis. The effect is seen still more clearly in Fig. 11.

In all these cases although the activity is reduced considerably by dialysis it is never reduced to zero. We believe that the residual activity is still due to traces of cozymase which are very difficult to remove by dialysis. This view is supported by experiments on dog liver preparations.¹ Mutase preparations from dog liver are almost always completely inactive without the addition of cozymase, as shown in the first part of Fig. 12. The difference in the behaviour of horse and dog liver preparations is apparently due to the fact that the latter contain an enzyme which destroys cozymase. This is easily shown by incubating cozymase with the enzyme preparations for some time before adding the aldehyde. In this case no dismutation occurs, although on the subsequent addition of a further quantity of cozymase a rapid reaction takes place.

The cozymase used in most of our experiments was prepared from baker's yeast. Boiled extract prepared in the usual way was first precipitated with normal lead acetate, the precipitate discarded and the cozymase then precipitated successively with mercury, phosphotungstic acid and silver. This preparation naturally contained other substances in addition to the cozymase, e.g. glutathione, but no differences were observed in comparison with a more highly purified preparation kindly given to us by Dr D. E. Green, and moreover through the kindness of Dr Greville we were able to test the mutase preparations with a cozymase preparation of ACo 600,000, which was found to act on the mutase in exactly the same way as our cozymase preparations.

The manner in which the initial velocity of dismutation of acetaldehyde varies with the amount of cozymase added is shown in Fig. 13. This experiment was carried out with the pure cozymase (ACo 600,000).

¹ Dog liver was tried in the first place because it was stated [Jones & Austrian, 1906; Schittenhelm, 1909; Morgan, 1926] to contain no xanthine oxidase (now identified with aldehyde oxidase) and it was therefore hoped to obtain an oxidase-free mutase preparation from it. We found, however, that crude preparations from dog livers always show xanthine and aldehyde oxidase activity.

Fig. 10.

Fig. 11.

Fig. 10. *Effect of cozymase on dialysed and undialysed preparations.*

96 mg. horse liver mutase with 0.5 mg. acetaldehyde. Curve *A* with undialysed enzyme, curve *B* with enzyme dialysed 18 hr. Cozymase added at arrows.

Fig. 11. *Effect of cozymase on dialysed and undialysed preparations.*

In each case curve *A* shows the reaction without cozymase, curve *B* with cozymase added initially and curve *C* with cozymase added at the arrow.

Fig. 12.

Fig. 12. *Effect of various coenzymes on mutase.*

18 mg. purified dog liver mutase with 0.8 mg. acetaldehyde. Curve *A* is with cozymase, curve *B* with coenzyme II, curve *C* with adenylypyrophosphate and trigonellin, curve *D* with reduced glutathione (0.02 mg.). In all cases the coenzymes were added at the second arrow.

Fig. 13.

Fig. 13. *Effect of cozymase concentration on initial velocity.*

18 mg. purified horse liver mutase dialysed 15 hr. with 0.8 mg. acetaldehyde. Pure cozymase (ACo 600,000) was used in this experiment.

A number of other possible coenzymes were also tested with dog-liver preparations, these being the most suitable for this purpose, since they are entirely inactive without coenzyme. Although closely related chemically to cozymase, the Warburg coenzyme (coenzyme II) (a cozymase-free preparation of which was kindly given to us by Dr Green) was found to be completely inactive, as were also adenylypyrophosphate, glutathione and trigonellin (see Fig. 12). The coenzyme action is thus quite specific.

OTHER ALDEHYDES

A further point of difference between the mutase and the oxidase is shown by their behaviour towards different aldehydes. The oxidase acts on both aliphatic and aromatic aldehydes; of the former, the lower members of the series (formic, acetic, propionic) are especially readily oxidized; of the latter, benzaldehyde, salicylic aldehyde and, best of all, piperonal, which has a very high affinity for the enzyme. The mutase, on the other hand, acts best on acetaldehyde and propionic aldehyde, less readily on the higher members of the series, and practically not at all on aromatic aldehydes. Parnas found no dismutation with benzaldehyde or salicylic aldehyde; we have obtained an insignificant effect with salicylic aldehyde and none at all with piperonal. Glyceric aldehyde (*dl*-) showed a rather slow but steady dismutation with our preparations: Embden [1912] had shown the same reaction with liver extracts. We found, however, that *d*-glyceric aldehyde (obtained from Prof. H. Fischer through the kindness of Drs J. Needham and Lehmann) underwent dismutation more than five times as rapidly as the *dl*- form, which suggests that the *l*-glyceric aldehyde has an inhibitory action.

We have also carried out a number of experiments with methylglyoxal, but a final conclusion was not reached. The main difficulty is the presence of glyoxalase in our mutase preparations, so that the methylglyoxal is rapidly converted into lactic acid instead of pyruvic acid and acetol, which would presumably be the products of mutase action. This could be avoided either by freeing the mutase from glyoxalase or by removing all traces of glutathione, without which the glyoxalase is inactive. (Lohmann has shown that pure cozymase does not activate glyoxalase.) Attempts were made to separate the mutase and glyoxalase by various means, such as fractional adsorption, fractional precipitation by salts and by chloroform, alcohol or acetone, and treatment by heat, acid and alkali. Although by several of these methods we were able to obtain very active preparations of glyoxalase free from mutase, we were unable to obtain mutase free from glyoxalase. Glyoxalase appears to be a very resistant enzyme in comparison with mutase, and highly purified preparations may be made either by precipitation with chloroform and alcohol, according to the procedure of Tsuchihashi [1930], or by fractional precipitation with alcohol alone. Alternatively, the greater part of the proteins may be precipitated with very dilute CuSO_4 , and after prolonged dialysis of the filtrate the enzyme may be precipitated with acetone. This procedure destroys the mutase completely, but gives a highly active glyoxalase preparation.

As we were unable to obtain the mutase without glyoxalase, we attempted to attain our object by removing all traces of glutathione from the system. Exceedingly small traces are sufficient to activate the glyoxalase, but by efficient dialysis it is possible to reduce the velocity of acid production from methylglyoxal without added cozymase to a very low level. We then found that ordinary cozymase preparations contain quite enough glutathione to activate the glyoxalase, as could be shown by testing with the mutase-free glyoxalase preparations. We found, however, that the pure cozymase (A Co 600,000) was

quite free from glutathione (see Fig. 14). We therefore tested the effect of this pure cozymase on the action of a dialysed mutase preparation on methylglyoxal. As shown in Fig. 15 the addition of the cozymase increased the reaction velocity by several hundred per cent. As this particular cozymase activated the mutase, but was quite incapable of activating the glyoxalase, the acceleration may be taken as evidence that aldehyde mutase can act on methylglyoxal. The rate of the reaction is, however, not very great and under normal conditions it would probably be completely overshadowed by the glyoxalase reaction.

Fig. 14.

Fig. 15.

Fig. 14. Activation of glyoxalase by crude and pure cozymase.

18 mg. purified glyoxalase (free from mutase) with 1.6 mg. methylglyoxal. Curve *A* without cozymase, curve *B* with 0.2 mg. pure cozymase, curve *C* with the usual amount of the ordinary cozymase solution.

Fig. 15. Action of horse liver preparation on methylglyoxal.

18 mg. mutase with 1.6 mg. methylglyoxal. Curve *A* with enzyme dialysed 10 hr., curve *B* with undialysed enzyme. 0.04 mg. pure cozymase (curve *A*) and 0.05 mg. reduced glutathione (curve *B*) added at arrows.

Attempts to demonstrate the formation of pyruvic acid from methylglyoxal were unsuccessful. No pyruvic acid could be detected with 2:4-dinitrophenylhydrazine after deproteinizing with trichloroacetic acid. It appears, however, that pyruvic acid itself may undergo a further reaction in presence of the enzyme preparations. In some cases we have observed two phases in the reaction curve with methylglyoxal, first the production of an amount of CO_2 corresponding roughly with a dismutation, and afterwards a slower but steady production of a further quantity of CO_2 . On adding pyruvate itself to the preparations a reaction curve resembling the second phase was obtained, and by applying the manometric method of Dixon & Keilin [1933] it was shown that this was due to the production of newly formed CO_2 from the pyruvate, as distinct from CO_2 liberated from the bicarbonate by acid formation. Some other mutase preparations however, particularly those which had been kept for some time, had no obvious action on pyruvate; but even with these we could not demonstrate pyruvate formation from methylglyoxal. The question of the products of the action of mutase on this aldehyde must therefore remain open.

DISCUSSION

We may sum up the evidence for the view that the aldehyde oxidase and the aldehyde mutase are distinct enzymes as follows.

(1) The Schardinger oxidase has no mutase activity. In this connexion it should be mentioned that Michlin & Severin [1931] have already shown that the aldehydrase of the potato has no mutase activity, but it is well known that this is a quite distinct enzyme from the Schardinger enzyme, for it differs from the latter in the shape of its *pH* curve, it only reacts with methylene blue within a very narrow *pH* range, it probably does not react with oxygen, and it does not activate purine bases [*v. Bernheim*, 1928]. Michlin & Severin still believed in the identity of the mutase with the Schardinger oxidase [*v. Dixon & Lutwak-Mann*, 1937, 2].

(2) The aldehyde mutase of animal tissues has no oxidase activity and when both enzymes are present together they can be separated by various means.

(3) The two enzymes can be inhibited independently: the oxidase can be completely inhibited by cyanide treatment without affecting the mutase, whilst the mutase can be inhibited by iodoacetic acid without affecting the oxidase.

(4) The mutase depends on cozymase for its activity, whereas the oxidase is independent of coenzymes.

(5) The oxidase acts on both aliphatic and aromatic aldehydes, the mutase does not appear to act on aromatic aldehydes.

Taken together, this evidence seems to us to be conclusive.

The fact that the aldehyde mutase is distinct from the mutase raises a number of points of unusual interest relating to the nature and mechanism of action of the mutase. Three hypotheses have been put forward.

(a) The view of Wieland that the mutase is identical with the oxidase. We consider that this view is inconsistent with our results.

(b) The interesting suggestion was made to us by Dr Green that the mutase might be a linked dehydrogenase system, consisting of aldehyde oxidase and alcohol dehydrogenase with cozymase acting as a carrier. It is well known that the alcohol dehydrogenase is a reversible enzyme and reduces aldehyde to alcohol in presence of reduced cozymase. The suggestion was that one molecule of aldehyde activated by the aldehyde oxidase reduced a molecule of cozymase, which in turn reduced a second molecule of aldehyde activated by the alcohol dehydrogenase, in accordance with the following scheme:

This attractive suggestion, however, could not be verified and appears to be inconsistent with some of the above-described results. In the first place all attempts to reconstruct such a system from isolated aldehyde oxidase and alcohol dehydrogenase proved unsuccessful. We were unable to detect any trace of dismutation using cozymase, flavin, flavoprotein, methylene blue or benzylviologen (either separately or in combination) as carriers. It is however always possible that the carriers used were unsuitable, and that with other carriers the reaction could be brought about. In the second place, as stated above crude mutase preparations, containing both aldehyde oxidase and alcohol dehydrogenase, could be completely freed from these two enzymes without affecting the mutase activity. There is no difficulty in detecting small amounts of these enzymes when present in the mutase preparations, and the conclusion is inevitable that they do not form components of the mutase system. This is also

shown very clearly for the case of the oxidase by the results of cyanide treatment.

(c) Our present view is that aldehyde mutase is an enzyme or enzyme system of a distinct type, and we consider that this is shown by the results given in this paper. There are reasons for thinking that it may not be the only example of this type, but that other dismutation reactions may be brought about in the tissues by other enzyme systems of the same class.

While we cannot say with certainty what is the mechanism of the mutase reaction, some suggestions may be made with a certain degree of probability. As all the known actions of cozymase are due to its power of acting as a hydrogen carrier it may be assumed to act in this way in the mutase system, in which case the dismutation would consist of two consecutive reactions. It seems clear that the function of the cozymase is to transport hydrogen from the aldehyde molecule which undergoes oxidation to the aldehyde molecule which undergoes reduction. We may suppose that these two aldehyde molecules are activated by two different active centres. One of these centres presumably activates the aldehyde so that it can undergo oxidation to the acid, thus resembling the aldehyde oxidase, from which however it differs in being able to react with cozymase and being unable to react with oxygen or methylene blue, like some of the anaerobic dehydrogenases. The other active centre presumably activates the other molecule of aldehyde in such a way that it undergoes reduction to alcohol in presence of reduced cozymase, thus resembling the alcohol dehydrogenase, from which however it differs in being unable to catalyse the oxidation of alcohol. It is impossible at present to say whether both active centres are situated on one and the same enzyme or whether they are situated on two different enzymes. Up to the present, however, we have been unable to resolve aldehyde mutase into two fractions. The question whether the mutase is a single enzyme or a system of enzymes must at present be left open.

SUMMARY

Aldehyde oxidase (Schardinger enzyme) does not dismute aldehydes, does not depend on coenzymes for its activity, is inactivated by cyanide but not by iodoacetate and acts on both aliphatic and aromatic aldehydes.

Aldehyde mutase dismutates but does not oxidize aldehydes, depends on cozymase for its activity, is inactivated by iodoacetate but not by cyanide and acts on aliphatic but not on aromatic aldehydes.

It is concluded that aldehyde mutase is a distinct enzyme or enzyme system.

Neither aldehyde oxidase nor alcohol dehydrogenase is a component of the mutase system.

Purified cozymase (ACo 600,000) acts very efficiently as comutase. Coenzyme II (Warburg coenzyme), adenylypyrophosphate, trigonellin and glutathione are inactive.

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