CXLVII. THE SPECIFICITY OF THE DEHYDRASES.

THE SEPARATION OF THE CITRIC ACID DEHYDRASE FROM LIVER AND OF THE LACTIC ACID DEHYDRASE FROM YEAST.

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Since Thunberg [1920] showed by means of the methylene blue technique that tissues could oxidise a variety of substances, there has been a question as to the specificity of the systems involved. It seemed unnecessarily complicated to assume a separate enzyme for each acid oxidised. Yet a high degree of specificity is the characteristic of most enzymes, and it is therefore unjustified, until definite proof is available, to assume that one system is responsible for the oxidation of, say, citric and lactic acids. Thunberg believed that each substance oxidised had a specific enzyme, but this "is rather unconvincingly inferred from their unequal resistances to thermal changes" [Dakin, 1921].

In addition to this evidence, there is the extraction from muscle of the succinic acid dehydrase [Ohlsson, 1921], which has recently been separated from fumarase [Alwall, 1928]. Wishart [1923] extracted the succinic acid enzyme from liver, and Stephenson [1928] extracted the lactic acid dehydrase from bacteria. It has been shown [Bernheim, 1928] that the aldehyde oxidase in potato can reduce methylene blue and this enzyme can thus be classed as a dehydrase, as can the xanthine oxidase. Although the latter enzyme has been obtained chiefly from milk it is also possible to separate it from liver [Bernheim and Dixon, 1928] and from spleen [Morgan, 1926].

Investigation of the specificities of these four enzymes has shown that their action is almost entirely confined to their respective substrates. In the present paper the separation and properties of two further dehydrases are described, and these have also proved to be highly specific.

The citric acid dehydrase.

The oxidation of citric acid in the body has not been extensively studied. Battelli and Stern [1911] showed that the addition of citric acid to the liver increased the carbon dioxide output, and that this increase occurred just the same when the liver and the citrate were incubated *in vacuo* as when oxygen

was present. Meyerhof [1919] did a few experiments on the oxidation of citric acid by frog's muscle. The addition of methylene blue to the tissue and fumaric acid (which he regards as belonging to the same class as citric acid in respect to its oxidation by muscle) causes as much as a 30 % increase in the oxygen uptake. This may mean two things: (1) that the system reduces methylene blue more readily than it takes up oxygen, and the reoxidation of the methylene white gives the observed increase in the oxygen uptake, or (2) the system is unable to use oxygen directly but reduces a hydrogen acceptor in the tissue which is then reoxidised by oxygen. The increase of the oxygen uptake on addition of methylene blue would be due to a greater concentration of hydrogen acceptor being available.

The ability to utilise citric acid seems to be greater in herbivora than in carnivora, for Salant and Wise [1907] have shown that injected citrate disappears more quickly from the blood of the former than from that of the latter. Finally Amberg and McClure [1924] showed that about 100 mg. of citric acid were excreted in the urine every 24 hours.

In the following an enzyme has been separated from liver which will reduce methylene blue in the presence of sodium citrate but will not take up oxygen in a Barcroft apparatus. This is also true of the lactic acid dehydrase separated from *B. coli* by Stephenson, and from yeast in work described below. The product which citric acid yields has not been identified, but in the case of the lactic acid enzyme pyruvic acid is formed and has been definitely identified, so that there is no doubt that experiments carried out with methylene blue represent a true oxidation. In the intact animal, some of the citric acid may be directly oxidised to carbon dioxide and water, but there is some evidence that a part may be converted into sugars [Greenwald, 1915].

Preparation of the enzyme.

One pound of fresh liver, of pig, ox or sheep, is minced as finely as possible and treated four times with 300 cc. of acetone, each 300 cc. being filtered off with suction before the next is added. The resulting preparation is put in a desiccator and the acetone evaporated off *in vacuo*. The dried powder will then keep for several weeks in the air. To obtain a solution of the citric acid dehydrase, 30 g. of acetone liver are ground in a mortar with 100 cc. of water, and allowed to stand 2 to 3 hours at room temperature with intermittent grinding. The mixture is then squeezed through muslin and centrifuged, and the clear red solution is placed in a collodion sac and dialysed against distilled water for 6 or 7 hours. As the salts dialyse out a precipitate appears which after the dialysis is finished is centrifuged off. The clear solution containing haemoglobin is then used for the experiments.

To get rid of the haemoglobin the solution is half saturated with ammonium sulphate and the precipitate filtered off. The filtrate contains the haemoglobin but not the enzyme. The precipitate is washed with half saturated ammonium sulphate and filtered again. It is dried on the filter paper in a desiccator.

The dry powder thus obtained is dissolved in distilled water giving a clear light brown solution. The colour is due to traces of methaemoglobin that have been adsorbed on to the precipitate. The filtration in this process is slow, and the resulting enzyme has lost some of its activity. Therefore unless otherwise specified the solution containing the haemoglobin is used.

This solution will keep for several days in the ice chest. During this time a small amount of precipitate may settle out which is then centrifuged off without affecting the activity of the enzyme. This may also occur occasionally during an experiment when the solution is in a vacuum tube. The precipitate is probably a remnant of the protein which settles out during dialysis. The enzyme can be obtained in a powdered form from the haemoglobin solution by fully saturating with ammonium sulphate. The filtration of the resulting precipitate is very rapid and the precipitate can be dried in a desiccator. The powder gives a clear red solution with approximately the original activity of the enzyme. The activity of any given solution, however, depends on the liver used and the method of preparing the acetone liver. Quick treatment with acetone and thorough drying *in vacuo* is desirable.

If $M/15$ disodium hydrogen phosphate is used instead of water, the enzyme comes into solution in the same way but is accompanied by the succinic and xanthine dehydrases. None of the other common dehydrases is, however, present. When the clear centrifugate is dialysed a precipitate appears which is centrifuged off. The succinoxidase can thus be got into a clear solution whereas Ohlsson's preparation is turbid. The enzyme is not very active, and more work is necessary on the details of its preparation.

Other tissues have been tried as well as liver with similar results although the enzymes extracted were not very active. Ordinary muscle and the liver of embryo rats behave in the same way when treated with acetone and extracted with water or alkaline phosphate solution.

The specificity and properties of the enzyme.

Oxygen uptake of the enzyme. 3 cc. of a clear solution, an amount which will reduce 1 cc. of 1 : 5000 methylene blue solution in 20–30 minutes, were placed in a Barcroft apparatus and the oxygen uptake measured after 0·1 cc. of molar sodium citrate solution was added. No appreciable uptake occurred in 5 hours. The enzyme was then tested for its ability to reduce methylene blue, and it was found that the velocity of reduction had decreased, indicating a partial destruction of the enzyme due probably to the shaking in the air. This explains why the theoretical amount of oxygen is not taken up when methylene blue is added to the solution in the Barcroft apparatus.

The inability to take up oxygen by itself seems to be the property of the enzyme in the liver, at any rate under the conditions of the experiment. The acetone liver and the fresh liver from which the solution was made both reduce methylene blue more rapidly in the presence of citrate. Neither the acetone liver nor the fresh liver will take up more oxygen in the presence of citrate

than in its absence. The reason for this is not clear. Further work is being done on the general question of the oxygen uptake of the dehydrases.

Inorganic nitrate and *m*-dinitrobenzene [Lipschitz, 1921] were tried as possible hydrogen acceptors. The former was not reduced but the latter was. 0·1 g. of *m*-dinitrobenzene was added to solutions of enzyme with and without citrate. After incubation *in vacuo* at 37° for 2 hours, a few drops of soda were added to each. A deep purplish red colour developed immediately in the tube containing the citrate while a much lighter colour developed slowly in the control. Because of the original colour of the solution it was impossible to detect the yellow colour which develops before adding the soda.

The clear solution of the enzyme is specific for citric acid. None of the other possible hydrogen donators tried was oxidised. They included the following acids as sodium salts: succinic, malic, fumaric, lactic, tartaric, formic, glutamic, maleic, acetic, α-hydroxybutyric, oxalic, saccharic, and acetaldehyde. These were made up in 5 % solutions of which 0·1 cc. was used for each experiment. Thunberg vacuum tubes were used containing 3 cc. of the solution to be tested, 2 cc. of buffer, and 1 cc. of 1 : 5000 methylene blue. The time for complete reduction of the dye at 37° was noted.

The effect of aconitic acid was then tried. This is the unsaturated acid corresponding to citric acid. It is conceivable that under the influence of the enzyme it might add water to form citric acid which then could be utilised to reduce the methylene blue. Incubation of the enzyme with sodium aconitate showed that this was not the case. But because of the similarity of structure with citric acid, aconitic acid inhibits the reduction of methylene blue by the citric acid-enzyme system. The curve obtained seems to indicate that the aconitic acid is adsorbed on the enzyme surface so that part of the surface is unavailable for the citric acid. It is probable that increasing concentrations of aconitic acid act like increasing concentrations of citric acid, namely, in keeping the methylene blue from being accessible to the active centres. This will be discussed more fully below.

The effect of aconitic acid is specific, for the additions of acids like succinic, lactic or tartaric do not increase the reduction time at all. Fig. 1 shows the effect of varying amounts of aconitic acid on the reduction of methylene blue by the enzyme-citric acid system.

The reduction time of methylene blue was obtained when varying amounts of citric acid were present. In this experiment the haemoglobin-free solution was used. As is shown in Fig. 2 the activity, *i.e.* the reciprocal of the reduction time multiplied by 100, increases rapidly with increasing concentrations of citric acid and then falls off. The activity drops sharply from 0·01 cc. of 3*N* citric acid to 0·001 cc., *i.e.* *N*/200 and *N*/2000 respectively. *N*/2000 represents about twice the theoretical quantity of citric acid necessary to reduce the methylene blue present as calculated for two hydrogen atoms for every citric acid molecule. The rate thus falls off at a comparatively high concentration, namely, *N*/200.

The significance of this maximum is probably associated with the saturation of the enzyme surface with an amount of citric acid which enables an easy access of methylene blue, and yet is concentrated enough to ensure a quick

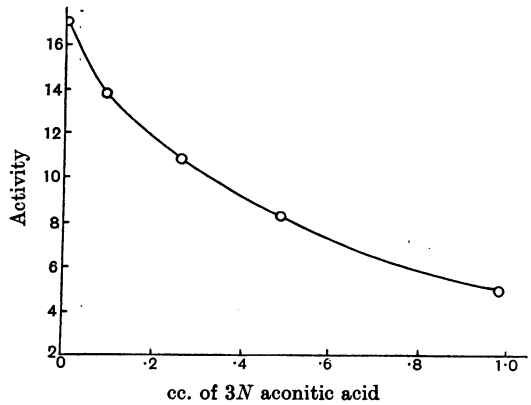

Fig. 1. Effect of aconitic acid on the reduction of methylene blue by citric acid.

reduction. When more citric acid is present the surface becomes supersaturated so that the methylene blue is no longer easily available. The inhibition is not a salt effect for equal amounts of *N* lactic acid or succinic acid do not inhibit. This inhibition at high concentrations of substrate has also been observed with the xanthine oxidase [Dixon and Thurlow, 1924]. In the following curve the concentration of citric acid is given in cc. per 6 cc.

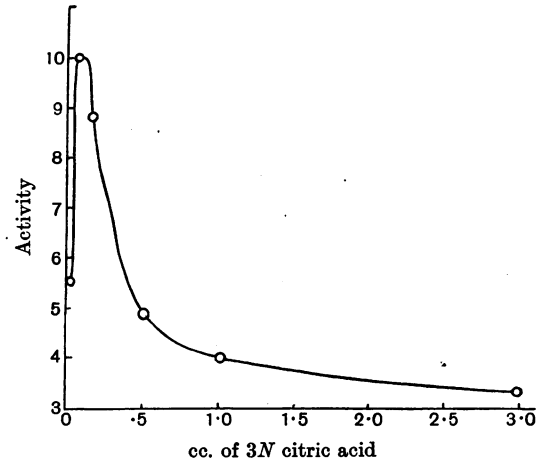

Fig. 2. Effect of varying concentrations of citric acid on the reduction of methylene blue.

The lactic acid dehydrase.

Lactic acid is not considered to be an intermediate product in the fermentation of sugars by yeast. It can, however, always be detected in yeast by extracting with acids or alkalis and applying the thiophen test to the extract. It must, therefore, play some part in the general metabolism. What its function is, is still doubtful. It is oxidised with difficulty. Fürth and Lieben [1922, 1, 2] found that a vigorous stream of oxygen through the yeast suspension was required before any appreciable oxidation occurred. They measured the oxidation by the increase of carbon dioxide produced when lactic acid was added to the suspension. The carbon dioxide produced was not however equivalent to the lactic acid present. They found that the lactic acid went partly into an ether-soluble substance and partly into the cell protoplasm. Hoffert [1926] showed that the lactic acid was not converted into unhydrolysable sugars or sugar-containing proteins. These workers have shown pyruvic acid to be present after allowing yeast to act on lactic acid. Harden and Norris [1915] showed that dried yeast plus lactic acid will readily reduce methylene blue and that pyruvic acid is quantitatively formed. This has been confirmed using untreated yeast and zymin (acetone yeast) by the following work, and is interesting in view of the observations of Fürth and Lieben that the addition of methylene blue made no appreciable difference to the amount of carbon dioxide arising from the oxidation of the lactic acid.

It is easy to show that whole yeast as well as dried yeast and zymin will reduce methylene blue more rapidly when lactic acid is present than without it, and that pyruvic acid is formed. It might therefore be expected that the pyruvic acid formed by the addition of methylene blue to the yeast lactic acid mixture would become decarboxylated and cause a rise in the carbon dioxide output. That Fürth and Lieben did not find this to be the case indicates the inability of the yeast to decarboxylate all the pyruvic acid formed.

But the following anomaly remains. Yeast contains a large amount of the lactic acid dehydrase as measured by the ability of yeast, dried yeast, or zymin to reduce methylene blue in the presence of lactic acid. For instance, 0·1 g. of washed zymin in 6 cc. of buffer, p_H 7·3, will reduce 1 cc. of a 1 : 5000 solution of methylene blue in 1 minute at room temperature. Without lactic acid no reduction occurs. The same is true for yeast. It is able to reduce methylene blue very quickly in the presence of lactic acid. Without the lactic acid the dye is reduced much more slowly. Yet despite this easy and rapid reduction of methylene blue, a vigorous stream of oxygen is necessary before any lactic acid is oxidised in the absence of the dye. This aeration seems a highly artificial condition which does not occur normally in the life of the yeast. The conclusion is that this dehydrase is unable to utilise oxygen and has very little to do with the oxidation of lactic acid unless methylene blue or some other hydrogen acceptor is present. The lactic acid must be oxidised by a system present in smaller concentration or less available so that vigorous aeration is necessary.

The inability of the dehydrase to utilise oxygen may be due to a lack of coenzyme to activate the oxygen. The existence of such a coenzyme seems indicated in muscle. In the presence of lactic acid washed muscle will reduce methylene blue. Szent-Györgyi [1925] has investigated the oxygen uptake of muscle in the presence of lactic acid. By extracting rat's muscle with ice-cold water he finds that the residue is able to reduce methylene blue with lactic acid, but unlike the unextracted muscle, it is unable to take up oxygen. This power is restored if the extract is added to the residue. Thus the extract contains some coenzyme which is able to activate oxygen so that it can be utilised by the lactic acid enzyme. The coenzyme is obviously unnecessary for the methylene blue which does not need activation.

In the following the lactic acid dehydrase has been separated from Delft baker's yeast. In the presence of methylene blue and lactic acid pyruvic acid is formed. This system will take up oxygen because the methylene blue is reduced to methylene white which then takes up oxygen on being reoxidised. No oxygen is taken up or pyruvic acid formed if methylene blue is absent. This fact confirms the inference that the enzyme in yeast is unable to utilise oxygen.

Preparation of the enzyme.

One pound of baker's yeast is broken up into small pieces and allowed to remain 8 to 10 hours exposed to the air. This allows water to evaporate and makes the treatment with acetone easier and more effective. It is then treated four times with 250 cc. of acetone, each portion being sucked off on a filter pump before the addition of the next. After the last treatment the zymin is dried as quickly as possible either with filter papers or in a vacuum. The resulting fine white powder will keep for months. It is important, however, that the zymin should be prepared quickly for a brown lumpy product does not yield a very active enzyme on extraction. 30 g. of the zymin are then ground up with 100 cc. of $M/15$ disodium hydrogen phosphate and allowed to stand from $3\frac{1}{2}$ to 4 hours at room temperature with intermittent grinding. The zymin is then centrifuged off and the solution which is almost clear is dialysed against distilled water for 6 or 7 hours until the solution in the bag gives a negative nitroprusside test for SH compounds. 3 cc. of the enzyme solution are then used for each experiment. The solution will keep in the ice chest for 24 hours but loses its activity rather quickly after that length of time.

If the extraction is continued much longer than 4 hours or if a stronger phosphate solution is used the succinic acid dehydrase which is present in yeast comes into solution with the lactic acid enzyme. The slightly cloudy solution gives only faint protein tests and only a slight precipitation when 2 % sulphosalicylic acid is added. The faint turbidity is not affected by filtering and the activity of the enzyme remains unchanged. Treatment with charcoal or kaolin resulting in a perfectly clear solution entirely inactivates the enzyme.

The cloudiness is increased when the solution is dialysed and disappears to a great extent when 2 cc. of buffer, p_H 7·3, are added to 3 cc. of the dialysed enzyme. The enzyme however is precipitated after standing for a short time at 37° with a solution of methylene blue. This fact may influence the rate of reduction of the dye to a small extent.

Specificity and properties of the enzyme.

The solution of the enzyme is specific for lactic and α-hydroxybutyric acids. None of the other possible hydrogen donators was activated. These included β-hydroxybutyric, malic, maleic, formic, citric, glutamic, succinic, fumaric, oxalic, acetic, tartaric, and pyruvic acids, and acetaldehyde and glucose. Glyceric acid which was also tried will be discussed later. Oxalic acid, however, has a marked inhibitory effect on the rate of reduction of methylene blue by the enzyme lactic acid system, as little as 0·1 cc. of a 5 % solution in 7 cc. causing an almost complete inhibition. That this is not a property of oxalic acid in general is shown by its entirely negative effect on the citric acid enzyme. Its molecule has in the juxtaposition of two CO groups a structure similar in an important respect to lactic acid, which may enable it to become adsorbed on the enzyme surface and thus keep the lactic acid from being activated.

The importance of this juxtaposition of the CO groups is shown by the fact that α-hydroxybutyric acid is oxidised whereas β-hydroxybutyric acid is not. The oxidation of α-hydroxybutyric acid is not quite as rapid as that of lactic acid. A solution of the enzyme which will reduce 1 cc. of a 1 : 5000 solution of methylene blue in the presence of 1 cc. of normal lactic acid in $5\frac{1}{2}$ minutes will reduce the same amount of methylene blue with 1 cc. of normal α-hydroxybutyric acid in 8 minutes. The presence of an extra carbon atom in the α-hydroxybutyric acid almost doubles the reduction time of methylene blue. β-hydroxybutyric acid when added to α-hydroxybutyric acid or to lactic acid does not increase the reduction time of the dye by the last two substances, showing that it is not adsorbed on the enzyme surface.

That pyruvic acid is the product of the action of the enzyme on lactic acid in the presence of methylene blue can be shown by adding to the reduced system a small amount of kaolin, shaking and filtering. The kaolin adsorbs the methylene blue and the clear colourless filtrate then gives the nitroprusside test for pyruvic acid. Pyruvic acid is not further oxidised by the enzyme in the presence of methylene blue so that additions of it to the lactic acid enzyme system ought to cause an inhibition. This is actually found to be the case (Fig. 3).

The inhibition, however, is not due entirely to the presence in excess of the product of the reaction and the consequent slowing of the velocity at which equilibrium is reached, because the pyruvic acid also inhibits the rate at which α-hydroxybutyric acid is oxidised. In this case it is not the product of the reaction. The most probable explanation is that pyruvic acid is adsorbed

on to the enzyme surface, and not being activated keeps the lactic and α-hydroxybutyric acids from being oxidised. This might be expected from the fact that oxalic acid is such a powerful poison and pyruvic acid resembles it in the juxtaposition of two CO groups.

Another example of this is glyceric acid. It is not oxidised by the lactic acid enzyme but inhibits the oxidation of lactic acid. Washed zymin is able to oxidise glyceric acid readily, and occasionally preparations of the separated enzyme will oxidise it slowly, indicating that there is a specific enzyme in yeast that will oxidise glyceric acid a part of which may be extracted with the lactic acid enzyme. The difference between the two enzymes is definitely proved by the fact that toluene does not affect the oxidation of the lactic acid at all, whereas the oxidation of the glyceric acid is markedly inhibited. The conclusion is that glyceric acid is adsorbed to a certain extent on the lactic acid enzyme but is not activated, thus causing an inhibition, and this is confirmed by the fact that enzyme extracts, which will not oxidise glyceric acid at all, are still inhibited by glyceric acid.

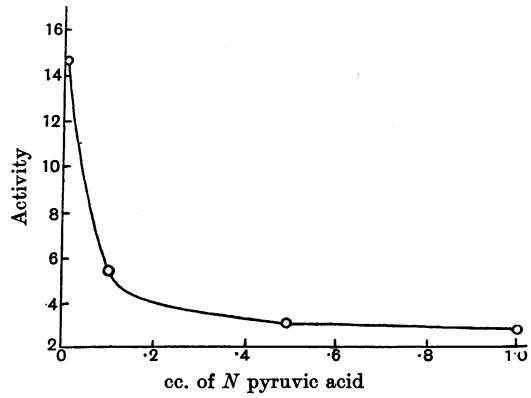

Fig. 3. Effect of pyruvic acid on the reduction time of methylene blue by lactic acid.

These results confirm those of Quastel and Wooldridge [1928] on the effect of various substances containing CO groups in juxtaposition on the oxidation of lactic acid by *B. coli*. Thus the idea that a molecule containing two contiguous CO groups is adsorbed on the enzyme and either inhibits the oxidation of the lactic acid or is oxidised itself seems valid. Exp. 1 shows the effect of glyceric acid on the oxidation of lactic acid, and Exp. 2 shows the effect of pyruvic acid on the oxidation of α-hydroxybutyric acid. Exp. 3 shows the effect of toluene on the oxidation of lactic and glyceric acids.

Exp. 1. 3 cc. of the isolated enzyme, 2 cc. of buffer, p_H 7·3, and 1 cc. of 1 : 5000 methylene blue were placed in each of three tubes. Tube A also contained 1 cc. $N/2$ lactic acid and 1 cc. water, tube B 1 cc. $N/2$ lactic acid and 1 cc. $N/2$ glyceric acid, and tube C 1 cc. $N/2$ glyceric acid and 1 cc. water. The acids were added as sodium salts, and the experiment was carried out *in vacuo* at 37°.

Tube	Reduction time in minutes
A	8
B	17
C	40

Exp. 2. 3 cc. of separated enzyme, 2 cc. of buffer, p_H 7·3, and 1 cc. of 1 : 5000 methylene blue were placed in each of four tubes. Tube A also contained 1 cc. $N/2$ lactic acid and 1 cc. water, tube B 1 cc. $N/2$ lactic acid and 1 cc. $N/2$ pyruvic acid, tube C 1 cc. $N/2$ α-hydroxybutyric acid and 1 cc. of water, and tube D 1 cc. $N/2$ α-hydroxybutyric acid and 1 cc. $N/2$ pyruvic acid. The conditions were the same as in Exp. 1.

Tube	Reduction time in minutes
A	12
B	20
C	18
D	30

Exp. 3. 3 cc. of separated enzyme, 2 cc. of buffer, p_H 7·3, and 1 cc. of 1 : 5000 methylene blue were placed in each of four tubes. Tube A also contained 1 cc. $N/2$ lactic acid, tube B the same plus 0·1 cc. toluene, tube C 1 cc. $N/2$ glyceric acid instead of the lactic acid, and tube D the same plus 0·1 cc. toluene. The conditions were the same as in the other experiments.

Tube	Reduction time in minutes
A	9
B	8
C	40
D	90

The rate of reduction of methylene blue was measured when varying quantities of lactic acid were present. The resulting curve (Fig. 4) differs markedly from that obtained for the citric acid enzyme in that increasing quantities of the substrate do not cause an inhibition. This is true both for lactic acid and for α-hydroxybutyric acid (Fig. 5). It might be possible to differentiate the dehydrases on this basis: on the one hand, the citric acid and xanthine enzymes which are inhibited by an excess of substrate, and on the other the lactic and succinic enzymes which are not. The curves for lactic and α-hydroxybutyric acids are similar. The velocity of reduction falls off in both cases when just under 0·5 cc. N acid is present. The figures are given in Figs. 4 and 5 as cc. in 7 cc. Consequently the velocity falls off when the concentration becomes less than $N/14$, a concentration which is far above the theoretical necessary to reduce the methylene blue present. In this respect too the difference from the citric acid enzyme should be noted, for the maximum concentration of substrate for the latter is about $N/200$.

Besides methylene blue oxygen was tried as a hydrogen acceptor. No uptake could be detected when the enzyme was shaken in a Barcroft apparatus in the presence of lactic acid. If methylene blue were added to the solution an oxygen uptake occurred because the dye was reduced by the enzyme-lactic

acid system and was reoxidised again by the oxygen present. In this case pyruvic acid can be shown to be present whereas no test can be obtained if the enzyme and lactic acid are shaken in oxygen without methylene blue. The enzyme suffers a certain loss of activity after being shaken in air for a length of time. As there is plenty of catalase present in the solution this loss cannot be due to a destruction by hydrogen peroxide. It is probably caused by a partial coagulation of the colloidal particles in the solution to form larger particles with less enzyme surface. An increasing turbidity is observed after shaking in air, suggesting that this may be the case.

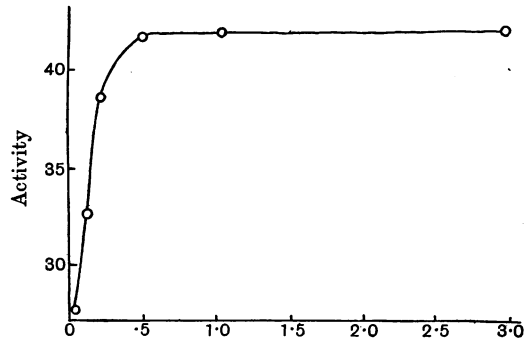

Fig. 4. Effect of varying concentrations of lactic acid on the reduction of methylene blue.

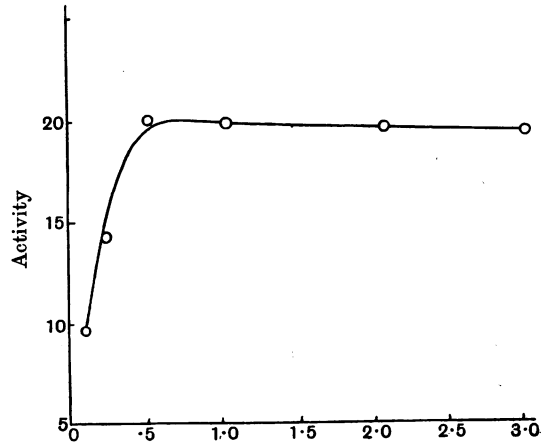

Fig. 5. Effect of varying amounts of α-hydroxybutyric acid on the reduction of methylene blue.

An attempt was made to supply active oxygen to the enzyme-lactic acid system on the possibility that pyruvic acid might be formed without the intervention of methylene blue. In order to do this a solution of xanthine oxidase, hypoxanthine, and a strong solution of peroxidase were added to the lactic acid enzyme and lactic acid. Oxygen was then bubbled through the

solution for several hours at 37°. The solution was then tested for pyruvic acid but with negative results. The peroxidase solution was made strong so that it could successfully compete with the catalase for the hydrogen peroxide.

Inorganic nitrate is not reduced. *m*-Dinitrobenzene gives a yellow colour when placed in a vacuum tube with the enzyme-lactic acid system, but this does not change to a purple on the addition of soda. If the reduction is allowed to go on for a long time, 5 or 6 hours, with a strong enzyme solution which will reduce 1 cc. of 1 : 5000 methylene blue in 10 minutes, a faint purple colour is seen on the addition of soda. It is obvious from the yellow colour produced that a reduction does take place but it seems to differ from the ordinary one found by Lipschitz for the dehydrases of tissue, the lactic acid one included. The possible explanation is that another reduction product is formed along with traces of the usual nitrophenylhydroxylamine. These traces would account for the faint purple colour with soda, and the other reduction product for the deep yellow.

Discussion.

The work done on the reduction of methylene blue by tissues and bacteria is summarised in Table I. Frog's muscle (Thunberg) and *B. coli* [Quastel and Whetham, 1925; Quastel and Wooldridge, 1925] have been most extensively studied in regard to their ability to reduce methylene blue in the presence of various substances. Quastel and Wooldridge [1927] have treated *B. coli* in various ways showing that specific activations are destroyed by various treatments. These observations are very extensive and are not included in the table. The specificity of the alkaline phosphate extracts of washed muscle and liver is taken chiefly from Wishart. He found that a few substances such as formic acid and ketoglutaric acid were activated to a small extent by the extract, but the rates of reduction were so small compared to the rate with succinic acid that they are put as negative in the table. The data for the milk are taken from Dixon [1926], for the potato from Bernheim, and for dried yeast from Harden and Norris. Rat's muscle has been studied but not so completely (Bernheim and Dixon). The list of substances in the following table includes most of those that are active with either *B. coli* or frog's muscle. A + sign indicates that the substance will reduce methylene blue rapidly in the presence of the tissue or extract, a − sign that it will not reduce at a detectable rate. A blank space means that the substance still remains to be tested. Fumarase which is not a dehydrase has been included in the table.

From a consideration of the table there are three types of evidence for the specificity of the dehydrases. The first is the separation of the enzyme from the tissue. This evidence is fairly conclusive for the separation depends on differences in physical properties of the enzymes, *i.e.* the centres responsible for the activations are attached to different colloids. It would be difficult to explain these facts on the assumption of one enzyme in the tissue which was originally capable of effecting all the activations but which during the process of

Table I.

Tissue or extract	Acetaldehyde	Other aldehydes	Hypoxanthine	Alanine	Tryptophan	Histidine	Formic acid	Normal fatty acids to capronic*	*iso*-Valeric acid	Citric acid	Glutamic acid	Glyceric acid	Hydroxyglutaric acid	Lactic acid	α-Hydroxybutyric acid	β-Hydroxybutyric acid	Maleic acid	Malic acid	Succinic acid	Fumaric to malic acid	Tartaric acid	Glycollic acid	Certain hexoses and pentoses	Certain alcohols	Certain disaccharides
Washed frog's muscle	−	−	−	+	−	−	+	+	−	+	+	−	+	+	+	+	−	+	+	+	−	−			
Frog's muscle treated with liquid air ...	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+	−				
Washed rat's muscle	−	−	−				+	+	−	+	+			+			+	+	+	+			−		
M/14 Na_2HPO_4 extract of washed ox's muscle	−	−	−	−			−	−	−	−	−		−	−	−	−	−	−	+	+	−				
Same heated ½ hour at 50°	−	−	−	−			−	−	−	−	−		−	−	−	−	−	−	+	−	−				
M/14 Na_2HPO_4 extract of washed ox's liver	+	+	+				−	−	−	−	−		−	−	−	−	−	−	+		−				
Water extract of acetone liver	−	−	−				−	−	−	+				−			−	−	−	−	−		−		
Resting *B. coli*	+		−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
Water extract of autolysed *B. coli*	−	−	−	−			−	−				+		+	+	−		−	−				−		
Dried yeast (Lebedeff)	−	+		+			−	−				+		+				−	+		−	+			
M/14 Na_2HPO_4 extract of acetone yeast ...	−	−	−				−	−		−	−	−		+	+	−	−	−	−	−	−		−		
Milk	+	+	+	−	−	−	−	−		−	−			−					−				−	−	−
Potato	+	+	−					−		−				−			−	−	−		−		−	−	−

* Dixon (unpublished) has found that propionic acid is oxidised by rat's muscle and Thunberg (privately communicated) has found the same for frog's muscle. In his original paper Thunberg [1920] states that it is not oxidised.

extraction has been so altered as to appear specific for one substrate. Four dehydrases have been separated in this way: the succinic, lactic, citric and xanthine.

The second type of evidence is suggestive but not conclusive. It consists of treating the tissue in various ways, destroying certain activations and leaving others. Thus frog's muscle after treatment with liquid air will only activate succinic and hydroxyglutaric acids. The succinic acid dehydrase has by extraction been obtained free from the hydroxyglutaric acid enzyme, therefore it is justifiable to assume that the oxidation of the latter acid is due to a distinct enzyme. Then the water extract of autolysed bacteria will only oxidise lactic, glyceric and α-hydroxybutyric acids. As the original *B. coli* will oxidise β-hydroxybutyric acid this proves that there are two different enzymes responsible for the oxidation of α- and β-hydroxybutyric acids. Similarly zymin will oxidise lactic, glyceric and α-hydroxybutyric acids, but extracts can be obtained that will only oxidise lactic and α-hydroxybutyric acids, indicating that the oxidation of glyceric acid is due to a distinct enzyme. These arguments are not conclusive, however, until extracts are obtained which will oxidise β-hydroxybutyric or glyceric acid and not lactic and α-hydroxybutyric acids. Another type of argument is also possible. The enzyme from milk will oxidise purines and aldehydes, that from potato only aldehydes, and therefore the milk enzyme may be really composed of two specific entities. But as the xanthine oxidase from milk has been concentrated 4000 times and still retains the ability to oxidise aldehydes [Dixon and Kodama, 1926] it would be unsafe to assume this until further evidence is available. From these facts, then, the existence of three further specific dehydrases is indicated, namely, the enzymes that will oxidise hydroxyglutaric, β-hydroxybutyric and glyceric acids.

Summary.

1. Methods for the separation of the citric acid dehydrase from liver and the lactic acid dehydrase from yeast have been described.

2. The specificity and properties of these two enzymes have been studied.

3. The general question of the specificity of the dehydrases has been discussed.

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