CXXV. THE β-HYDROXYBUTYRIC DEHYDROGENASE OF ANIMAL TISSUES

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WAKEMAN & DAKIN [1909] described an enzyme prepared from liver which catalysed the oxidation of β-hydroxybutyric acid to acetoacetic acid. Apart from the confirmation by other workers of the existence of this enzyme, the problem has remained stationary for the last 18 years. Green & Brosteaux [1936], in their study of the lactic dehydrogenase of heart muscle, observed that their enzyme preparation contained an active β-hydroxybutyric dehydrogenase. The present communication deals with the components of the β-hydroxybutyric dehydrogenase system and with the mechanism of the reaction with molecular oxygen.

Dehydrogenase systems have been classified by Green & Brosteaux [1936] in three categories: aerobic oxidases, cytochrome systems and coenzyme systems. The β-hydroxybutyric dehydrogenase satisfies all the criteria of a coenzyme system and is very similar in behaviour to the lactic and malic dehydrogenases of animal source. That is to say the collaboration of a coenzyme is required for catalysing the oxidation of the substrate; the reaction with molecular oxygen is not direct but proceeds through carriers like flavin, flavoprotein and adrenochrome; finally cytochrome *c* cannot function efficiently as a link between the dehydrogenase system and molecular oxygen.

There is one striking difference between the β-hydroxybutyric dehydrogenase and the lactic and malic dehydrogenases. Whereas traces of the keto-acid formed on oxidation inhibit completely the aerobic oxidation of either lactate or malate, the oxidation of β-hydroxybutyrate is relatively insensitive to the presence of the corresponding keto-acid. The use of ketone fixatives which is obligatory for the study of the lactic and malic systems is not essential for the β-hydroxybutyric system. The opportunity therefore was presented of determining to what extent the ketone fixative may interfere with the normal functioning of a coenzyme dehydrogenase system. The result of this inquiry has been the discovery of an enzyme system which catalyses the oxidation of reduced coenzyme and which is completely inhibited by high concentrations of cyanide. The presence of the coenzyme oxidase was not detected in the previous studies on the lactic and malic systems respectively since cyanide in high concentrations was used as the ketone-fixing agent.

I. PREPARATION OF THE COMPONENTS

The preparation of the β-hydroxybutyric dehydrogenase from the heart muscle of pig follows the procedure employed in the preparation of the lactic enzyme. Two hearts are divested of fat and connective tissue, and minced in a Latapie mincer. The mince, after thorough washing with tap water, is mixed with

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sand and 400 ml. *M*/50 phosphate buffer *p*H 7·2 and ground to a fine homogeneous paste in a mechanical mortar. The sand and insoluble debris are filtered off through muslin. After clarifying the filtrate by centrifuging for 20 min., the supernatant fluid is acidified with 10 % acetic acid to *p*H 4·6 and the flocculent precipitate centrifuged. The supernatant is discarded. The precipitate is washed with *M*/50 acetate buffer *p*H 4·6 and resuspended in 80 ml. *M*/10 phosphate buffer *p*H 7·2. The enzyme is stable for at least a week when kept in solution at 0°. Drying partially inactivates the enzyme.

Some modifications have been introduced in the method of preparation of coenzyme I described by Green & Brosteaux [1936]. Cakes of baker's or brewer's yeast (9 kg.) are crumbled into 6 l. of 1 % sulphuric acid maintained at 80°. The yeast is filtered off. Excess lead acetate is added and the precipitate is filtered and discarded. The filtrate is made alkaline with 6 *N* NaOH (just blue to thymolphthalein) and the precipitate is rapidly filtered. The precipitate is then suspended in 1 l. of water and 10 *N* sulphuric acid added in slight excess of the amount required to decompose the lead salts. The end-point is indicated by a red colour with thymol blue. The lead sulphate is filtered off and the filtrate is treated with slight excess of 25 % phosphotungstic acid (Analar reagent). The precipitate is centrifuged and washed with 5 % sulphuric acid. The phosphotungstic precipitate in some cases tends to come out colloidal. Addition of salts such as ammonium sulphate assists the process of aggregation. After decomposing the phosphotungstic precipitate with amyl alcohol and ether by the method of Myrbäck [1933], the aqueous layer is filtered. Saturated hot baryta is added sufficient to bring the *p*H to 8·0. The barium precipitate is discarded. The filtrate is treated with excess $AgNO_3$ and dilute ammonia added to keep the *p*H between 7·5 and 8·0. The precipitate is centrifuged and decomposed with H_2S. The Ag_2S precipitate is filtered and the filtrate is concentrated *in vacuo* to about 20 ml. By addition of 5 vol. of acetone, the coenzyme is precipitated. The precipitate is centrifuged, washed with acetone and dried *in vacuo*. The yield is about 500 mg. from 9 kg. of yeast. The powder is white if the preparation is made from baker's yeast, whereas a yellow powder is obtained from a brewer's top yeast.

The state of purity of the coenzyme at this stage is about 50 %. Complete purification can be attained by the well-known methods of Euler and Warburg.

II. The reaction with molecular oxygen

A mixture of the dehydrogenase, coenzyme I and *dl*-β-hydroxybutyrate takes up oxygen directly in absence of any added carrier. The rate of oxidation is not increased by the addition of carriers such as methylene blue, flavin, flavoprotein or cytochrome *c* (cf. Table I).

Table I. *The effect of carriers on the direct reaction with oxygen*

The system contained 1·5 ml. enzyme, 1·0 ml. 0·15 % coenzyme and 0·2 ml. *M dl*-β-hydroxybutyrate. The total volume was 3·3 ml.

	μl. O_2 in 10 min.
β-Hydroxybutyrate system	54
Control with no coenzyme	0
Control with no substrate	0
System + 0·6 mg. flavin	51
System + 3 mg. cytochrome *c*	53
System + 15 mg. flavoprotein	54
System + 1·5 mg. methylene blue	54

Ketone fixatives such as hydrazine do not affect the initial velocity of oxidation but tend to prevent the rapid falling off in velocity which occurs in the absence of any fixatives (cf. Table II). Hydrazine has been found to be a

Table II. *The effect of fixatives*

The system contained 1 ml. enzyme, 0·5 ml. coenzyme, 0·5 ml. *M*/10 pyrophosphate and 0·2 ml. *M dl*-β-hydroxybutyrate. The fixative solutions were neutralized.

	μl. O_2		
	10 min.	20 min.	30 min.
β-Hydroxybutyrate system	29	45	57
+0·3 ml. *M*/2 semicarbazide	32	54	68
+0·3 ml. *M*/2 hydrazine	49	93	131

more satisfactory fixative than either semicarbazide or hydroxylamine. It has been used in the bulk of the experiments. Acetoacetic acid inhibits the oxidation of β-hydroxybutyric acid only when present in considerable concentration. The use of fixatives therefore is not essential for the study of the oxidation but is advisable for obtaining regular time-velocity relations.

Cyanide in high concentrations completely inhibits the oxidation of β-hydroxybutyrate by molecular oxygen (cf. Table III). The simultaneous addition

Table III. *Effect of cyanide*

The system contained 1·5 ml. enzyme, 1 ml. 0·15% coenzyme I, 0·3 ml. *M*/10 pyrophosphate, 0·2 ml. *M dl*-β-hydroxybutyrate.

	μl. O_2	
	15 min.	45 min.
β-Hydroxybutyrate system	92	171
+0·5 mg. methylene blue	92	165
+0·3 *M* HCN (final concentration)	0	0
+0·3 *M* HCN +0·5 mg. methylene blue	2	8
+0·06 *M* HCN	8	29
+0·06 *M* HCN +0·5 mg. methylene blue	31	88
+0·03 *M* HCN	7	36
+0·03 *M* HCN +0·5 mg. methylene blue	70	159
+0·006 *M* HCN	29	81
+0·006 *M* HCN +0·5 mg. methylene blue	110	195

of methylene blue reduces the inhibition at high concentrations of cyanide and abolishes it at lower concentrations. These results are in harmony with the following interpretation. Cyanide is inhibiting two components, viz. the dehydrogenase and an enzyme which is concerned in the mechanism for the direct utilization of molecular oxygen. The dehydrogenase is less sensitive to cyanide than the second component. Hence at certain concentrations of cyanide, the reaction between the dehydrogenase system and molecular oxygen can be renewed by adding methylene blue. At higher concentrations of cyanide there is no restoring action of methylene blue since not only is the second component inhibited but the dehydrogenase as well.

The explanation of the cyanide effect becomes clearer from a consideration of the lactic and malic dehydrogenases. These enzymes are completely insensitive to all concentrations of cyanide. A mixture of the dehydrogenase preparation, coenzyme I, malate or lactate and strong cyanide (*M*/5 final concentration) fails to take up any oxygen. With addition of any suitable carrier a vigorous uptake ensues. But if instead of cyanide, semicarbazide or hydrazine

Table IV. *Oxidation of lactate and malate without carriers*

All manometers contained 1·5 ml. enzyme, 1·0 ml. coenzyme and 0·4 ml. *M*/2 hydrazine (neutral).

	μl. O_2 in 10 min.
Enzyme + coenzyme + hydrazine	3
+ 0·2 ml. *M* lactate	115
+ 0·2 ml. *M* malate	96
+ 0·2 ml. *M* β-hydroxybutyrate	73

is used, then no carrier is essential. The reaction with molecular oxygen takes place directly (cf. Table IV). The evidence is clear that in addition to the dehydrogenases present in the enzyme preparation there is another factor which is concerned in the mechanism by which oxygen is directly utilized in the oxidation of lactate, malate or β-hydroxybutyrate.

The best proof of the existence of this factor lies in the properties of the lactic, malic and β-hydroxybutyric enzymes prepared from a powder of acetone-dried heart muscle. This preparation does not show any direct utilization of molecular oxygen in presence of the three substrates unless some carrier is provided (cf. Table V). Obviously acetone-treatment destroys the factor which

Table V. *Oxidation of lactate, malate and β-hydroxybutyrate by an extract of acetone-dried heart muscle*

The system contained 2 ml. enzyme, 0·5 ml. 0·15% coenzyme I, 0·3 ml. *M*/2 hydrazine and 0·3 ml. *M*/10 pyrophosphate. The enzyme was prepared by mincing 2 hearts and mixing the mince with 3 vol. of acetone. Extraction with acetone was repeated several times. The preparation was then dried in a current of air at room temperature. The pulverized powder was extracted with phosphate buffer and the insoluble residue centrifuged.

	μl. O_2 in 30 min.
System with no substrate	0
+ lactate	0
+ lactate + 0·5 mg. methylene blue	112
+ malate	0
+ malate + 0·5 mg. methylene blue	40
+ β-hydroxybutyrate	0
+ β-hydroxybutyrate + 0·5 mg. methylene blue	23

normally accompanies these enzymes. The same type of effect, though not as pronounced, is obtained by drying the enzyme at the acetic acid precipitate stage (cf. Section I). After drying *in vacuo*, the powder yields an extract which can oxidize lactate, malate and β-hydroxybutyrate only in presence of a carrier such as methylene blue (cf. Table VI). There is a small blank in absence of methylene blue which indicates that the additional factor was not completely destroyed.

Table VI. *Oxidation of lactate, malate and β-hydroxybutyrate by an extract of dried enzyme*

The system contained 1·5 ml. enzyme, 0·5 ml. coenzyme, 0·3 ml. *M*/2 hydrazine and 0·3 ml. *M*/10 pyrophosphate. Semicarbazide was used as the fixative in the case of malate.

	μl. O_2 in 60 min.
System with no substrate	0
+ β-hydroxybutyrate	18
+ β-hydroxybutyrate + 0·5 mg. methylene blue	32
+ lactate	33
+ lactate + 0·5 mg. methylene blue	244
+ malate	24
+ malate + 0·5 mg. methylene blue	96

Methylene blue has little or no effect on the lactate, β-hydroxybutyrate and malate dehydrogenases prepared as in Section I (cf. Table VII). Apparently carriers have no influence in presence of the additional factor. Presumably they compete unfavourably with the factor for the reduced coenzyme.

Table VII. *The effect of methylene blue on the untreated enzyme preparation*

The system contained 1·5 ml. enzyme, 0·5 ml. 0·15% coenzyme I, 0·3 ml. *M*/2 hydrazine and 0·3 ml. *M*/10 pyrophosphate. The amount of substrate used was 0·2 ml. of a *M* solution.

	μl. O_2 in 30 min.
System	8
+lactate	80
+lactate+0·5 mg. methylene blue	80
+malate	33
+malate+0·5 mg. methylene blue	34
+β-hydroxybutyrate	75
+β-hydroxybutyrate+0·5 mg. methylene blue	65

In the oxidation of the substrate of any coenzyme system the following reaction takes place:

Substrate + coenzyme → oxidation product + reduced coenzyme.

The reduced coenzyme is non-autoxidizable in the physiological range of *p*H. The reaction between reduced coenzyme and molecular oxygen is not direct and requires some carrier which can be alternately reduced by the coenzyme and oxidized by molecular oxygen. The factor in the heart preparation must either be a carrier like flavoprotein or an enzyme which catalyses the oxidation of reduced coenzyme by molecular oxygen. Examination of the enzyme solution fails to disclose any detectable amounts of flavoprotein. In addition there are two considerations which militate against flavoprotein being responsible for the direct reaction with oxygen; first, flavoprotein has been shown to be very inefficient as a carrier in coenzyme I systems; second, the partial pressure of oxygen has no influence on the oxidation of β-hydroxybutyrate, lactate and malate, whereas reactions involving flavoprotein are very sensitive to the oxygen tension. There is in fact no oxidation carrier as yet described in animal tissues which is sensitive to cyanide and destroyed by acetone or drying. The more reasonable interpretation is to assume that the factor is an enzyme which catalyses the oxidation of reduced coenzyme by molecular oxygen in the same way as the indophenol oxidase catalyses the oxidation of reduced cytochrome by molecular oxygen. The properties of the coenzyme oxidase are very similar to those of the indophenol oxidase except that the former is sensitive to cyanide in high concentrations only, whereas extremely dilute cyanide (*M*/10,000) completely inhibits the latter. No systematic investigation has yet been made of the coenzyme oxidase. It appears to be present in most tissues. It is non-dialysable.

Green & Dewan, in unpublished experiments, have shown spectrophotometrically that the heart enzyme preparation catalyses the rapid oxidation of reduced coenzyme by molecular oxygen.

III. The product of oxidation

That acetoacetic acid is the sole product of the oxidation of β-hydroxybutyric acid can be shown by three independent methods.

Acetoacetic acid can be estimated manometrically as CO_2 by the aniline method of Ostern [1933]. Since there is 1 mol. of acetoacetic formed for each

atom of oxygen absorbed, the ratio of the CO_2 liberated from acetoacetic acid to the volume of O_2 absorbed should be 2. Table VIII shows that the observed ratio is in close agreement with the theoretical.

Table VIII. *The acetoacetic-oxygen ratio*

The oxygen uptake was measured manometrically at 38° for a given period of time. The manometer cups were then disengaged and 0·3 ml. of glacial acetic acid added to stop the reaction. After re-equilibrating the manometers, aniline citrate was tipped in from a side-bulb and the CO_2 estimated.

	μl. CO_2 evolved	μl. O_2 absorbed	Ratio
(1)	143	68	2·1
(2)	123	61	2·0
(3)	63	31	2·0

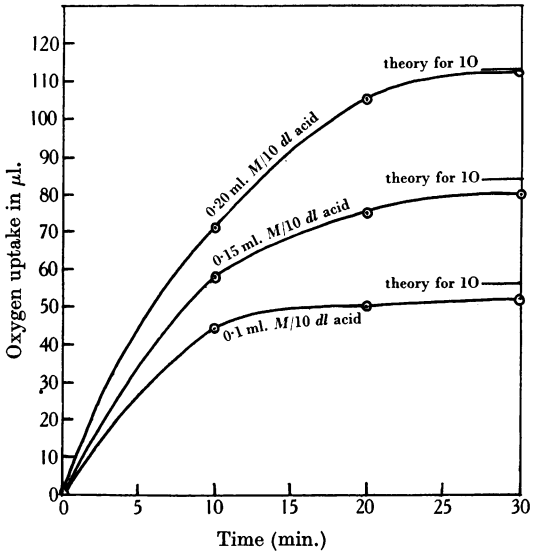


Fig. 1.

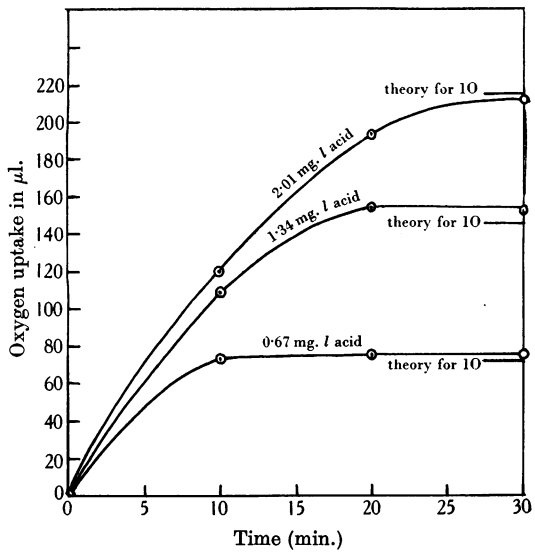


Fig. 2.

Fig. 1. *The oxygen equivalence of* dl-*β-hydroxybutyrate.*

Each manometer contained 1·5 ml. enzyme, 1 ml. coenzyme, 0·3 ml. *M*/2 hydrazine and 0·5 ml. *M*/10 pyrophosphate. The substrate was placed in Keilin cups which were introduced into the main body of fluid after equilibration. The control uptake without substrate has been subtracted from the experimental numbers. The theoretical uptake for 1 atom of oxygen has been calculated on the basis that only one optical isomeride is oxidized.

Fig. 2. *The oxygen equivalence of* l-*β-hydroxybutyrate.*

Manometric details as for Fig. 1. The *l*-β-hydroxybutyric acid was isolated from the urine of a diabetic in the form of the calcium zinc double salt. After decomposing the salt with Na_2CO_3 and filtering off the insoluble carbonates, the strength of *l*-β-hydroxybutyric acid was estimated polarimetrically.

The instability of acetoacetic acid and its 2:4-dinitrophenylhydrazone renders isolation in the pure state very difficult. However, by converting acetoacetic acid into acetone, almost quantitative recovery is possible. A mixture containing 50 ml. enzyme solution, 25 ml. 0·15 % coenzyme I, 10 ml. *M*/2 hydrazine chloride, 5 ml. *M*/10 pyrophosphate and 5 ml. *M dl*-β-hydroxybutyrate was aerated vigorously at 37° for 60 min. The mixture was then deproteinized with trichloroacetic acid and centrifuged. The supernatant was concentrated *in vacuo* to 15 ml.

and clarified by centrifuging. Strong alkali was added sufficient to bring the *p*H to about 10. Under these conditions the hydrazone of acetoacetic acid is decomposed into hydrazine and acetone. The solution was acidified and mixed with 0·5 g. of 2:4-dinitrophenylhydrazine dissolved in 50 ml. of 2 *N* HCl. The 2:4-dinitrophenylhydrazone settled out within a few minutes. The precipitate was washed with 2 *N* HCl and dried *in vacuo*; yield, 200 mg. For purification of the derivative, the substance was dissolved in ethyleneglycol monoethyl ether and the insoluble residue filtered off. Addition of 3 vol. of water reprecipitated the dinitrophenylhydrazone in a crystalline form. The precipitate was dried and recrystallized from hot *iso*butyl alcohol. The sample melted at 123°; the authentic sample of acetone-2:4-dinitrophenylhydrazone melted at 124°, mixed M.P. 123°. (Found (Weiler): C, 45·68; N, 23·4 (mean of 2 estimations); H, 4·21 %. $C_9H_{10}O_4N_4$ requires C, 45·36; N, 23·53; H, 4·23 %.)

If acetoacetic acid is the sole product of oxidation there should be 1 atom of oxygen absorbed for each molecule of β-hydroxybutyrate oxidized. Figs. 1 and 2 show that the observed uptakes are in close agreement with theory. *dl*-β-Hydroxybutyrate is oxidized to the extent of 50 %. Since *l*-β-hydroxybutyrate is completely oxidized, it must be concluded that the *d*-enantiomorph is not oxidized.

IV. Kinetics

Fig. 3 shows the dependence of the rate of oxidation of β-hydroxybutyrate on the *p*H of the solution. The maximum velocity is observed at about *p*H 7·3. The rates fall off rapidly below *p*H 6 and above *p*H 9.

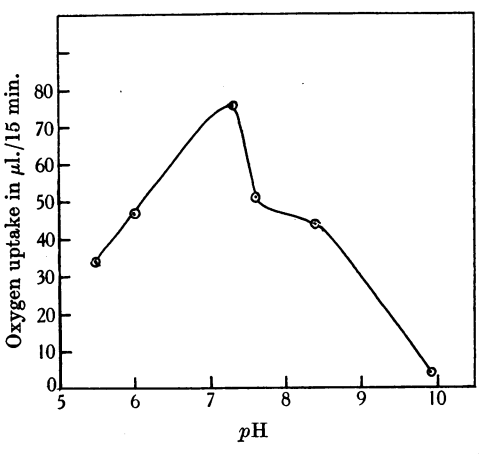


Fig. 3.

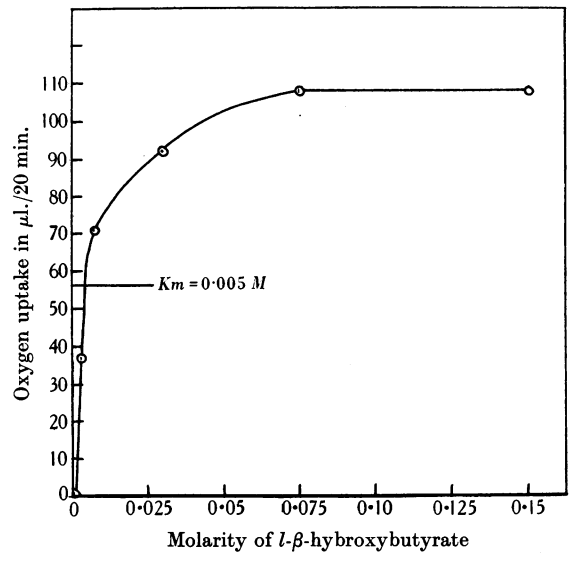


Fig. 4.

Fig. 3. *The effect of* p*H*.

Each manometer contained 1·3 ml. enzyme, 0·8 ml. coenzyme, 0·1 ml. *M dl*-β-hydroxybutyrate and 1 ml. *M*/2 buffer. The enzyme was suspended in water after acetic acid precipitation and neutralized with NaOH.

Fig. 4. *The effect of the concentration of substrate.*

Each manometer contained 1·5 ml. enzyme and 1·0 ml. coenzyme. The total volume was 3·3 ml.

The Michaelis constant, i.e. the substrate concentration at which half the maximum velocity is reached is about $M/200$ (cf. Fig. 4). This value is considerably lower than those for the malic, lactic and α-glycerophosphate enzymes. In presence of hydrazine as fixative the Km is about $M/500$.

Fig. 5 shows the dependence of the rate of oxidation on the concentration of coenzyme. The relation observed is typical for coenzyme systems. That is to say there is a linear relation only at high dilutions of the coenzyme. With

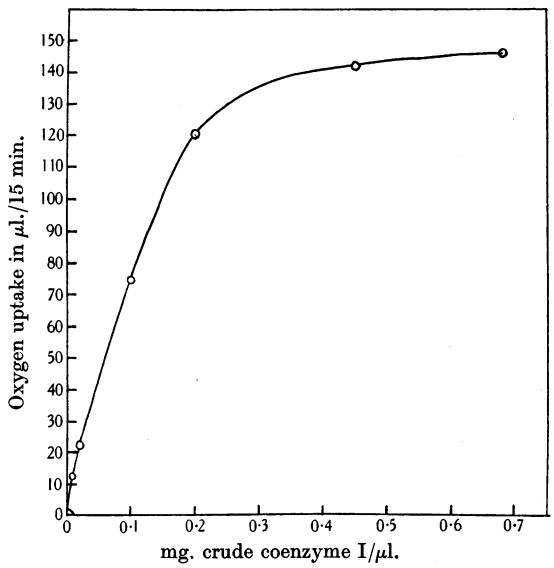


Fig. 5. *The effect of the concentration of coenzyme I.*

Each manometer contained 1·5 ml. enzyme, 0·3 ml. $M/10$ pyrophosphate, 0·3 ml. $M/2$ hydrazine and 0·2 ml. M *dl*-β-hydroxybutyrate.

increasing concentration of the coenzyme, the rate remains constant at a definite asymptotic value. The half-speed concentration for the coenzyme is about 0·05 mg./ml. (allowing for the state of purity of the sample tested).

Pyrophosphate produces a very interesting acceleration of the rate of oxidation (cf. Fig. 6). The effect is more correctly one of stabilization rather than acceleration. In presence of $M/100$ pyrophosphate the sharp fall in activity at the end of 10–15 min. is postponed for at least the same length of time. Table IX shows that pyrophosphate is not replacing either the coenzyme or the fixative, since the effect disappears in the absence of the other components. This stabilizing action of pyrophosphate has been taken advantage of for experimental purposes—small amounts of the reagent being used in most of the experiments. Green & Brosteaux [1936] and Green [1936] observed similar effects of pyrophosphate on the lactic and malic dehydrogenase systems. The mechanism of the pyrophosphate effect still awaits explanation.

A summary of the effects of various inhibitors is given in Table X. *n*-Octyl alcohol is the most effective inhibitor. Pyruvate, oxaloacetate and acetoacetate produce relatively small inhibitions compared with their inhibitions of the lactic and malic oxidations.

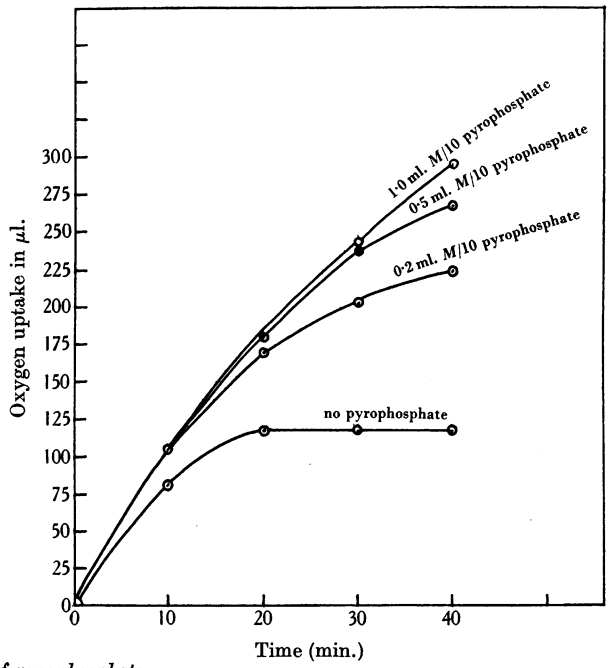


Fig. 6. *Effect of pyrophosphate.*

Each manometer contained 1 ml. enzyme, 1 ml. coenzyme, 0·2 ml. *dl*-β-hydroxybutyrate and 0·3 ml. *M*/2 hydrazine.

Table IX. *Controls for the pyrophosphate effect*

The complete system contained 1 ml. enzyme, 1 ml. 0·15% coenzyme I, 0·5 ml. *M*/10 pyrophosphate, 0·3 ml. *M*/2 hydrazine and 0·2 ml. of *M* β-hydroxybutyrate.

	μl. O_2 in 10 min.
Complete system with 0·5 ml. *M*/10 pyrophosphate	93
Control with no β-hydroxybutyrate	0
Control with no coenzyme	0
Control with no hydrazine	50

Table X. *Inhibitors*

Inhibitor	Final concentration	% inhibition
Iodoacetate	0·03 *M*	56
Arsenious acid	0·03 *M*	69
Malonate	0·06 *M*	29
Urethane	0·17 *M*	0
n-Octyl alcohol	Saturated solution	100
Animal adenylic acid	0·0007 *M*	41
Pyruvate	0·03 *M*	48
	0·006 *M*	32
Oxaloacetate	0·03 *M*	71
	0·006 *M*	42
Acetoacetate	0·012 *M*	55

V. Specificity of substrate

The dehydrogenase specifically catalyses the oxidation of *l*-β-hydroxybutyric acid to acetoacetic acid. β-Hydroxypropionic acid, α-hydroxybutyric acid, crotonic acid, γ-hydroxybutyric acid, butyric and acetic acids are not oxidized

appreciably under the conditions in which a vigorous oxidation is obtained with β-hydroxybutyric acid. Under some conditions oxidation of β-hydroxypropionic acid takes place but this has been found to be unconnected with the β-hydroxybutyric enzyme. Experiments are in progress to test the higher homologues of β-hydroxybutyric acid.

There are several lines of evidence which establish that the β-hydroxybutyric enzyme is distinct from the lactic and malic enzymes. (1) Preparations have been made from tissues other than heart which contain active lactic and malic enzymes but no trace of the β-hydroxybutyric enzyme. (2) The kinetics are very appreciably different. (3) The β-hydroxybutyric enzyme does not have the same general distribution as the lactic and malic enzymes. It is found in fewer tissues and in much smaller concentration.

VI. Specificity of coenzyme

Green & Brosteaux [1936] have already demonstrated that coenzyme II is not active with the β-hydroxybutyric enzyme. We have been able to confirm this inactivity of coenzyme II. The preparation of coenzyme I from yeast was purified further by the procedures of Euler [1936] and Warburg *et al.* [1935]. Pure coenzyme I was found to be fully as active as the crude preparation (cf. Table XI). Furthermore the crude yeast preparation was found to be weak with

Table XI. *Purified coenzyme I compared with crude coenzymes I and II*

The system contained 1 ml. enzyme, 0·2 ml. *M* β-hydroxybutyrate, 0·3 ml. *M*/10 pyrophosphate and 0·3 ml. *M*/2 hydrazine.

	μl. O_2 in 10 min.
System with no coenzyme	0
+0·7 mg. crude coenzyme I	90
+0·35 mg. purified coenzyme I	90
+100 mg. crude coenzyme II	5
+0·35 mg. purified coenzyme II	5

respect to coenzyme II—showing only a small fraction of the activity of a crude horse blood preparation. Conversely the blood preparation had only negligible coenzyme I activity

VII. Carriers

Thus far only the mechanism for the direct utilization of molecular oxygen has been considered. But this mechanism is independent of the dehydrogenase. In Section II it was shown that a special enzyme is necessary to catalyse the reaction between reduced coenzyme and molecular oxygen. In the absence of this coenzyme oxidase carriers must be provided to enable the reaction with molecular oxygen to take place. In order to test the action of carriers, the

Table XII. *The effect of flavin and flavoprotein on cyanide-treated enzyme*

The system contained 1·5 ml. enzyme, 0·5 ml. 0·15% coenzyme I, 0·2 ml. *M*/5 HCN, 0·5 ml. *M*/10 pyrophosphate and 0·2 ml. *M dl*-β-hydroxybutyrate.

	μl. O_2	
	10 min.	20 min.
System + cyanide	18	27
+0·5 mg. flavin	44	77
+0·5 mg. methylene blue	33	61
+50 mg. flavoprotein	20	35

coenzyme oxidase was inactivated with cyanide. Table XII shows that flavin, and to a very slight extent flavoprotein, can act as carriers. Green & Richter [1937] have shown that adrenochrome can also function in that capacity.

VIII. Distribution

Attempts were made to determine the distribution of the β-hydroxybutyric dehydrogenase in the tissues of various animals. It was found that the concentration of this enzyme was in general too small to permit accurate estimation. The enzyme was detected in the heart muscle, skeletal muscle, liver and kidney of pig. There was no trace found in brain. The hearts of several animals (pig, ox, guinea-pig, rabbit and rat) were found to be the richest sources of the enzyme. Liver and kidney yielded much less active preparations.

The enzymes previously studied in this series, viz. the lactic, malic and α-glycerophosphate dehydrogenases, were shown to be widely distributed in animal tissues. The high concentrations of their occurrence argued an active part in essential oxidation processes. The oxidation of β-hydroxybutyric acid to acetoacetic acid does not belong to this category. It is an oxidation which is peculiar to a limited number of tissues. Quantitatively this oxidation can account only for a small fraction of the total oxidations.

IX. Reversibility

The reversibility of the oxidation of β-hydroxybutyrate to acetoacetate was demonstrated as follows. A mixture of enzyme (*p*H 7·5), coenzyme, benzylviologen and 1·5 mg. of *l*-β-hydroxybutyrate was placed in the main limb of a Thunberg tube. The hollow stopper of the tube was filled with 15 mg. of acetoacetate in buffer of *p*H 7·5. After evacuation the tube was incubated at 38°. A deep violet colour developed in a few minutes. The contents of the hollow stopper were then introduced into the main limb. The violet colour gradually became less intense and at the end of 10 min. represented about 10% of the original intensity. A control with buffer tipped in instead of acetoacetate showed no change. When the acetoacetate was replaced by oxaloacetate the change was much more rapid and complete. In about 5 min. the solution became colourless. Pyruvate acted more or less like acetoacetate.

These reactions may be formulated as follows:

(1) β-Hydroxybutyrate + coenzyme I $\rightleftharpoons$ acetoacetate + reduced coenzyme I.
(2) Reduced coenzyme I + 2 benzylviologen $\rightleftharpoons$ coenzyme I + 2 reduced benzylviologen.
(3) Pyruvate + reduced coenzyme I $\rightleftharpoons$ lactate + coenzyme I.
(4) Oxaloacetate + reduced coenzyme I $\rightleftharpoons$ malate + coenzyme I.

The β-hydroxybutyrate reduces the coenzyme; in turn reduced coenzyme reduces the oxidation-reduction indicator, benzylviologen. There are thus three oxidation-reduction systems in equilibrium with one another: (1) β-hydroxybutyrate-acetoacetate, (2) reduced coenzyme I-oxidized coenzyme I and (3) reduced benzylviologen-oxidized benzylviologen. The first system comes into equilibrium with the third through the intermediation of the second. The total concentrations of systems 2 and 3 are made very small in comparison with that of 1. Hence the final equilibrium is determined by the concentration of the β-hydroxybutyrate-acetoacetate system. Starting with the reductant, β-hydroxybutyrate, the equilibrium is in favour of reduced coenzyme and hence reduced benzylviologen. Tipping in acetoacetate, the oxidant, shifts the equilibrium towards oxidized coenzyme and hence towards oxidized benzylviologen.

Pyruvate and oxaloacetate are the oxidants of the lactic and malic systems respectively. Like acetoacetate, they tend to shift the equilibrium in favour of the oxidized coenzyme.

The general experimental details for the potential measurements have been discussed previously by Green [1934] in the study of the reversibility of the xanthine oxidase and will not be considered here. The potential measurements were made at 38° in the Borsook-Thunberg vacuum electrode vessels. The contents of the tubes were made up as follows: 2 ml. enzyme, 2 ml. $M/2$ buffer, 0·5 ml. 0·15% coenzyme, 0·5 ml. 0·002 M benzylviologen, 0·3 ml. $M/10$ pyrophosphate and 1 ml. of a mixture of $M/10$ l-β-hydroxybutyrate and $M/10$ acetoacetate. The pH of the buffers was determined with the hydrogen electrode at 38°. The measurements were made with buffers diluted to the same extent as in the electrode vessels. The reference decinormal calomel half cell was standardized frequently against the quinhydrone electrode in $N/10$ HCl and the hydrogen electrode in $N/10$ HCl, the potentials for which were considered to be +0·6232 and −0·0664 V. respectively at 38°.

Equilibrium values were generally reached in about 20 min. The potentials thereafter remained constant for at least 60 min. Acetoacetic acid decomposes slowly at 38°. It was considered unreliable therefore to continue any experiment for more than 2 hr. The practice was to discard the experiment if the equilibrium value was not reached within 1 hr. In the absence of benzylviologen, the potential level of the β-hydroxybutyrate-acetoacetate system was not reached. This indicates that the coenzyme-reduced coenzyme system does not come into equilibrium with the electrode. The function of the oxidation-reduction indicator is that of bringing the system to be measured into equilibrium with the electrode.

The quantitative criterion of a reversible system is that the potential for a given pH should depend upon the ratio of $\frac{\text{reductant}}{\text{oxidant}}$ according to the following equation:

$$E_h = E_0' - \frac{RT}{2F} \ln \frac{[\text{reductant}]}{[\text{oxidant}]}.$$

Table XIII contains a summary of the potential measurements. The agreement between observed and theoretical potentials is additional evidence in favour of reversibility. The 1:9 ratio of β-hydroxybutyrate to acetoacetate could not be

Table XIII

pH	Ratio $\frac{l\text{-}\beta\text{-hydroxybutyrate}}{\text{acetoacetate}}$	E_h observed in V.	E_h calculated in V.	$\tilde{E}$ in V.
6·71	9 : 1	−0·290	−0·292	+0·148
7·07	9 : 1	−0·315	−0·314	+0·151
7·50	9 : 1	−0·341	−0·341	+0·150
7·50	5 : 5	−0·313	−0·313	+0·150
7·97	9 : 1	−0·371	−0·370	+0·151
7·97	5 : 5	−0·341	−0·342	+0·149
8·31	9 : 1	−0·391	−0·391	+0·150
8·31	5 : 5	−0·363	−0·363	+0·150

Mean $\tilde{E} = +0{\cdot}150$ V.

Mean $E_0 = +0{\cdot}176$ V. $= \tilde{E} + \frac{RT}{2F} \ln \frac{Ka}{K_\beta}$.

E_0' at pH 7·0 $= -0{\cdot}282$ V.

tested since the inhibition of the reduction of benzylviologen was almost complete and the enzyme was inactivated before equilibrium could be reached. The variation of potential with the ratio of reductant to oxidant and with the

hydrogen ion concentration could be represented by the following electrode formula of Clark and Borsook:

$$E_h = \tilde{E} - \frac{RT}{2F} \ln \frac{[\text{total } \beta\text{-hydroxybutyric}]}{[\text{total acetoacetic}]} - \frac{RT}{F} \ln \frac{1}{[H^+]} + \frac{RT}{2F} \ln \frac{K_a}{K_\beta} \times \frac{K_\beta + [H^+]}{Ka + [H^+]},$$

where E_h is the observed potential, R is the gas constant, T the absolute temperature, F the Faraday equivalent, K_β the dissociation constant of β-hydroxybutyric acid ($3{\cdot}9 \times 10^{-5}$) and K_a the dissociation constant of acetoacetic acid ($2{\cdot}6 \times 10^{-4}$). The values for the dissociation constants are those given by Michaelis [1914] and Ljunggren [1925]. The assumption has been made that concentrations of the reactants are equal to activities.

The mean $\tilde{E}$ which is the electrode potential against the normal hydrogen electrode for the reaction, β-hydroxybutyrate $\rightleftharpoons$ acetoacetate $+ 2H^+ + 2\epsilon$, is $+0{\cdot}150$ V. for 38°.

The free energy change ΔF for the oxidation with all the reactants at unit activity can be calculated from $\tilde{E}$ by the equation, $\Delta F = nEF$. Since $n = 2$ and $F = 23{,}068$, $\Delta F = 6920$ calories.

It is interesting to note that $\tilde{E}$ for the lactate-pyruvate system according to Barron & Hastings [1934] is 0·248 V., i.e. about 100 mV. more positive than the β-hydroxybutyrate-acetoacetate system. These potential measurements provide a satisfactory explanation for the inhibitory action of keto-acids. The oxidations of lactate and of malate are completely inhibited by traces of their respective keto-acids. On the other hand acetoacetate inhibits the oxidation of β-hydroxybutyrate only when present in relatively high concentration. Let us assume that the potential of the coenzyme system lies midway between those of the relatively positive lactate and malate systems and the relatively negative β-hydroxybutyrate system. That is to say even with excess of lactate or malate the equilibrium is in favour of oxidized coenzyme whereas with excess of β-hydroxybutyrate the equilibrium is entirely in favour of reduced coenzyme. Since the rate of oxidation is proportional to the concentration of reduced coenzyme, almost complete inhibition is observed in the lactate and malate systems as soon as any keto-acid accumulates whereas little effect is observed in the β-hydroxybutyrate system. The use of ketone fixatives is made necessary by the fact that the keto-acids force the equilibrium almost completely to the side of oxidized coenzyme. By combining with keto-acids the fixatives maintain the most favourable conditions for the reduction of the coenzyme.

The malic-oxaloacetic system has not as yet been studied potentiometrically, but the evidence is suggestive that it forms a reversible system somewhat more positive in potential than the lactate-pyruvate system.

X. Linked coenzyme reactions

Another line of evidence in favour of the reversibility of coenzyme I systems is found in the study of reactions between dehydrogenase systems via the coenzyme as the carrier. Dewan & Green in unpublished experiments have been able to link the following oxido-reductions:

(1) β-Hydroxybutyrate + pyruvate $\rightleftharpoons$ acetoacetate + lactate;
(2) β-Hydroxybutyrate + fumarate $\longrightarrow$ acetoacetate + succinate;
(3) Malate + fumarate $\longrightarrow$ oxaloacetate + succinate.

The coenzyme is alternately reduced by the donator dehydrogenase system and oxidized by the acceptor dehydrogenase system—thereby linking the two

systems. It is now possible in practice to link any two dehydrogenase systems provided that they can either reduce or oxidize the coenzyme and provided that there is a potential difference between them. Furthermore even if a dehydrogenase is not dependent upon a coenzyme in the normal way, it may like the succinic enzyme be capable of oxidizing the reduced coenzyme or like the yeast lactic enzyme be capable of reducing the oxidized coenzyme. Coenzyme I appears to be the natural carrier which Green *et al.* [1934] looked for in their attempts to link dehydrogenase systems by means of known cellular components. Coenzyme II very probably can act in a similar capacity.

Recently Euler *et al.* [1937] using an extremely delicate spectrophotometric technique demonstrated that the coenzyme-reduced coenzyme system comes into rapid equilibrium with the lactate-pyruvate, alcohol-aldehyde and α-glycerophosphate-triosephosphate dehydrogenase systems. Euler *et al.* have also determined the equilibrium constants for these various interactions.

XI. A SUMMARY OF THE PROPERTIES OF COENZYME I SYSTEMS

The three coenzyme I systems which have been studied thus far in this series, viz. the lactic, malic and β-hydroxybutyric dehydrogenases, are remarkably similar in their general properties. The elements of similarity may be summarized as follows:

(1) The indispensability of coenzyme I for the catalytic activity of the dehydrogenase.

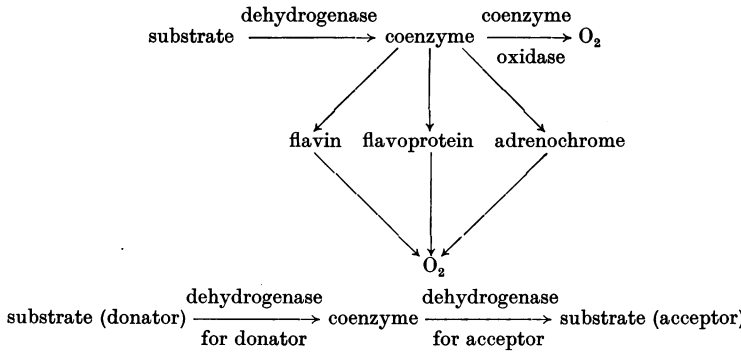
(2) The inability of the dehydrogenase system to react directly with molecular oxygen in absence of either carriers or the coenzyme oxidase.

(3) The ability of flavin, flavoprotein and adrenochrome to act as carriers between the coenzyme and molecular oxygen.

(4) The inactivity of cytochrome *c* as a carrier.

(5) The complete specificity for the optical isomeride which is stereochemically related to *l*(+) lactic acid.

The various mechanisms by which coenzyme I can act catalytically may be represented by the following schematic diagrams.



The arrows represent the direction of transfer of hydrogen. It is clear that the coenzyme occupies a pivotal place in cellular oxidations and is the effective link between anaerobic and aerobic processes.

There are no data available as to the mechanism by which coenzyme systems react with molecular oxygen *in vivo*. The cyanide-sensitivity of the oxidations of lactate, malate and β-hydroxybutyrate in tissue slices indicates that physiologically these oxidations proceed through the cytochrome-indophenol oxidase

chain. It is possible that the coenzyme oxidase is the indophenol oxidase collaborating with a cytochrome component other than *c*. Theorell [1936] has shown that flavoprotein can reduce cytochrome *c* and he suggests that flavoprotein may be the connecting link between cytochrome and the coenzyme systems.

Flavin has been shown to be active as a carrier in the coenzyme I systems which have been reviewed here. This activity is difficult to reconcile with the finding of Haas [1937] that reduced coenzyme I cannot be oxidized by uncombined flavin. On the other hand, flavoprotein which can oxidize reduced coenzyme with extraordinary velocity is comparatively inefficient as a carrier in coenzyme I systems. These discrepancies may mean that the reaction between reduced coenzyme and carriers is complex. It is also possible that reduced coenzyme in presence of the other components of the catalytic system may show quite different properties from reduced coenzyme prepared by reduction with hydrosulphite.

The question of how the coenzyme functions in the catalytic system has now become a matter of great dispute. The concept of "Zwischenferment" introduced by Warburg implies that the coenzyme combines with the dehydrogenase to form the catalytically active complex. What is ordinarily referred to as a dehydrogenase is considered by Warburg to be merely a highly specific protein with no catalytic properties apart from its prosthetic group—the coenzyme. Euler and his school have accepted this view but they prefer to call the active complex the "holodehydrase".

There is a good deal of evidence in favour of the view that the dehydrogenase is the seat of catalytic activity. (1) If the coenzyme is common to dehydrogenases which are involved in the oxidation of substrates as different as glucose, alcohol, lactate, malate and β-hydroxybutyrate, obviously the coenzyme cannot be the active group responsible for the activation of all these substances. It seems improbable that the coenzyme should be at once the mechanism for activating and for oxidizing the substrate. (2) The phenomenon of competitive inhibition (inhibition of an enzymic oxidation by substances structurally related to the substrate) offers impressive proof of the existence of an active group in the dehydrogenase. This effect is understandable only in terms of competition between the substrate and the related substance for the active group of the enzyme. (3) The succinic dehydrogenase catalyses either the oxidation of succinate to fumarate or the reduction of fumarate to succinate. No coenzyme is essential for either process. Yet fumaric acid can oxidize reduced coenzyme in presence of the dehydrogenase. There can be no question here of the coenzyme being the active group since it can be entirely dispensed with. The same reasoning applies to the yeast lactic dehydrogenase which according to Warburg & Christian [1936] can catalyse the reduction of coenzyme I although no coenzyme is essential for the activation of the substrate. In these two cases at least, the coenzyme acts merely as a transporter of hydrogen and has no relevance to the problem of the activation of the substrate.

The classical conception of the dehydrogenase as the actual activating mechanism seems to be in fair agreement with the facts. No doubt the coenzyme combines with the dehydrogenase in the same way that the substrate does. But the function of the coenzyme seems to be that of a highly specific hydrogen acceptor which cannot be replaced by any other substance.

SUMMARY

The preparation and properties of the β-hydroxybutyric dehydrogenase of heart muscle are described. Coenzyme I (diphosphopyridinenucleotide) is an indispensable component of the system.

Evidence is presented for the existence of a coenzyme oxidase which catalyses the oxidation of reduced coenzyme I by molecular oxygen.

The dehydrogenase specifically catalyses the oxidation of *l*-β-hydroxybutyrate to acetoacetate. The oxidation product was isolated in the form of acetone-2:4-dinitrophenylhydrazone.

The reversibility of the change from β-hydroxybutyrate to acetoacetate has been demonstrated potentiometrically. The E_0' at *p*H 7·0 is −0·282 V. The free energy change was calculated to be 6920 calories.

A résumé of the properties of coenzyme I systems is given.

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