CXLI. COENZYME-LINKED REACTIONS BETWEEN DEHYDROGENASE SYSTEMS

By JOHN GEORGE DEWAN and DAVID EZRA GREEN[1]

From the Institute of Biochemistry, Cambridge

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Green *et al.* [1934], in an attempt to elucidate one of the mechanisms of anaerobic oxidoreductions, investigated the conditions which are essential for the interaction of dehydrogenase systems *in vitro*. They found that dehydrogenase systems cannot react directly with one another but require the intermediation of a carrier which can be alternately reduced by one system and oxidized by the other. Given a suitable carrier it was possible to link the oxidation of many metabolites such as lactate, hypoxanthine, formate etc. with reduction of metabolites such as fumarate, nitrate etc. Artificial oxidation-reduction dyes were used as carriers in these linked reactions, since no naturally occurring cellular component (pyocyanine excepted) could be found to act in the capacity of an anaerobic carrier. Flavin, flavoprotein, glutathione, ascorbic acid and cytochrome *c* were all inactive.

The limitation to the progress possible in these experiments of Green *et al.* was twofold: (1) the dearth of information concerning the preparation and properties of dehydrogenases and (2) the obscurity of the role of coenzymes in dehydrogenase systems. In the last 3 years these deficiencies in our knowledge have been greatly repaired. Systematic investigations have been made of the lactic, malic, α-glycerophosphoric and β-hydroxybutyric dehydrogenases of animal tissues [Green & Brosteaux, 1936; Green, 1936, 1, 2; Green *et al.* 1937]. But most important was the discovery by Warburg *et al.* [1935] that coenzyme II was capable of reversible oxidation and reduction. Later Euler *et al.* [1937, 1, 2] showed that several dehydrogenase systems can either reduce oxidized coenzyme I or oxidize reduced coenzyme. That is to say the reaction of these dehydrogenases with the coenzyme comes to an equilibrium which can be shifted merely by change in the ratios of the various reactants. Finally, the work of Krebs & Johnson [1937] on anaerobic oxidoreductions in tissue slices made it clear that the model reconstructions which Green *et al.* called "carrier-linked reactions" have their counterpart in physiological events.

Using various pairs of dehydrogenase systems with coenzyme I as the carrier we have been able to produce the following reactions:

(1) β-Hydroxybutyrate + pyruvate → acetoacetate + lactate.
(2) β-Hydroxybutyrate + oxaloacetate → acetoacetate + malate.
(3) β-Hydroxybutyrate + aldehyde → acetoacetate + alcohol.
(4) β-Hydroxybutyrate + fumarate → acetoacetate + succinate.
(5) Malate + fumarate → oxaloacetate + succinate.

I. *Preparation of components*

The preparations of the various enzymes used have been fully described in the previous communications of this series. Through the kindness of Dr M. Dixon and Dr C. Lutwak-Mann, we have been able to test highly purified preparations of aldehyde mutase of liver [cf. Dixon & Mann, 1937].

[1] Beit Memorial Research Fellow.

Coenzyme I was prepared by the method of Green *et al.* [1937] which is merely a variant of the original method of Myrbäck [1933].

II. *Methods of estimation*

Acetoacetic acid. The solution containing acetoacetic acid (*ca.* 3 ml.) is pipetted into the Barcroft manometer vessel and mixed with 0·3 ml. glacial acetic acid. After 3 min. equilibration at 37° (150 oscillations per min.) 0·3 ml. of aniline citrate solution (one part aniline to one part 50 % citric acid) is introduced from a Keilin cup and the CO_2 evolved is measured. The reaction is complete in less than 20 min. Rapid equilibration is necessary to minimize the spontaneous decomposition of acetoacetic acid. Ostern [1933] and Edson [1935] have elaborated the method as used in its present form. There is one molecule of CO_2 produced for each molecule of acetoacetic acid decomposed.

Oxaloacetic acid is estimated by this method as well. It is possible, however, to estimate acetoacetic acid in presence of oxaloacetic acid by the simple expedient of adding strong cyanide (0·2 ml. of 2*M* NaCN) to the neutral test solution 10 min. before mixing with the glacial acetic acid. Under these conditions, only acetoacetic acid is decomposed. The cyanohydrin of oxaloacetic acid decomposes extremely slowly (> 8 hr.) and does not interfere within the 10–20 min. necessary for the completion of the acetoacetic acid reaction.

Succinic acid. The oxidation of succinic acid to fumaric acid is the basis of the method of estimation. The succinic dehydrogenase is prepared from ox heart by the method of Ogston & Green [1935]. The deproteinized solution containing succinic acid (*ca.* 3 ml.) is mixed in the manometer cup with 2 ml. enzyme and 0·1 ml. *M* HCN. After equilibration, 0·2 ml. of 0·5 % cresyl blue is introduced from a Keilin cup and the oxygen uptake is measured. The end of the reaction is reached in 60–90 min. depending upon the amount of succinic acid present. The method is most satisfactory when the amount of succinic acid lies between 1 and 2 mg.

The function of the cyanide is to prevent any oxidation of succinic acid before the cresyl blue has been added. Weil-Malherbe [1937] has found cresyl blue to be the most efficient artificial carrier for the succinic oxidation.

There are several precautions which must be considered. A blank with enzyme, cyanide and cresyl blue must be carried out simultaneously and the oxygen uptake of the blank subtracted from those of the experimentals. The enzyme preparation should be tested with a known amount of succinic acid to ascertain that oxidation will be complete within 90 min. Where there is a possibility of coenzyme, either reduced or oxidized, being present in the solution containing succinic acid, the procedure for destroying the coenzyme described by Green [1936, 2] should be followed. Finally the method is not suitable when α-glycerophosphate is present as well as succinate.

It is not necessary to filter the deproteinized solutions before testing. The suspended denatured protein does not interfere appreciably with the estimation.

Lactic acid. A convenient manometric method of estimating *l*(+)lactic acid has been developed with the use of the lactic dehydrogenase of yeast. This is prepared from baker's yeast by grinding in a ball mill [cf. Ogston & Green, 1935]. The procedure is the same as that for the succinic estimation. The neutral coenzyme-free solution of lactate (3 ml.) is mixed with 2 ml. enzyme and 0·1 ml. *M* HCN. After equilibration 0·2 ml. 0·5 % cresyl blue is introduced from a Keilin cup. The reaction is complete in less than 60 min.; α-glycerophosphate is the only interfering substance. Malate, succinate, fumarate, aldehyde, alcohol,

pyruvate, acetoacetate, oxaloacetate and β-hydroxybutyrate are not estimated. The blanks without lactate are very small.

A simple method of preparing the lactic enzyme of yeast is to grind 25 ml. of a plasmolysed suspension of yeast for 15 min. at 2000 r.p.m. and then centrifuge hard. The opalescent supernatant after 12 hours' dialysis is ready for use.

Malic acid. The malic dehydrogenase as prepared by Green [1936, 2] can be used for the manometric estimation of *l*-malic acid. Both lactic and succinic acids are estimated simultaneously. But these two substances if present can be allowed for by independent estimations.

The neutral coenzyme-free and deproteinized solution of malic acid (*ca.* 3 ml.) is mixed with 1·5 ml. enzyme, 1·0 ml. of a 0·075 % solution of coenzyme I and 0·2 ml. 2*M* HCN. After equilibration, 0·2 ml. 0·5 % methylene blue is introduced from a Keilin cup. In absence of the coenzyme, only succinic acid is estimated. The difference between the uptakes in presence of coenzyme and in absence of coenzyme represents the uptake due to malic acid. Where no succinic acid is present, there is no necessity for doing the control without coenzyme.

III. *Reaction between β-hydroxybutyrate and pyruvate*

The reactions were carried out anaerobically in Thunberg tubes at 38° for 1–2 hr. Precautions must be taken to ensure that β-hydroxybutyrate is not introduced from the hollow stopper until oxygen has been removed from the tube.

Anaerobically, β-hydroxybutyrate is not oxidized in presence of the enzyme and coenzyme. In presence of pyruvate, a vigorous reaction ensues whereby acetoacetate and lactate are produced. In absence of the coenzyme or the enzymes, no reaction ensues (cf. Table I). Pyrophosphate only slightly increases the rate. The enzyme preparation contains both the β-hydroxybutyric dehydrogenase and the lactic dehydrogenase.

Table I. *Controls for the β-hydroxybutyrate-pyruvate oxidoreduction*

Heart enzyme (ml.)	2·0	2·0	2·0	2·0	2·0	—
0·15 % coenzyme I (ml.)	1·0	1·0	—	1·0	1·0	1·0
M β-hydroxybutyrate (ml.)	0·2	0·2	0·2	0·2	—	0·2
M pyruvate (ml.)	0·2	0·2	0·2	—	0·2	0·2
M/10 pyrophosphate (ml.)	—	0·5	—	—	—	—
Water (ml.)	0·5	—	1·5	0·7	0·7	2·5
μl. acetoacetic CO_2 in 60 min.	276	316	18	30	0	0

The dependence of the rate of oxidation of β-hydroxybutyrate by pyruvate on the concentration of coenzyme is shown in Fig. 1. Making allowance for the state of purity of the coenzyme used, 0·01 mg. of coenzyme I catalyses the production of 52 μl. of acetoacetic CO_2 in 60 min.

The reaction between β-hydroxybutyrate and pyruvate occurs in two stages.

(6) β-Hydroxybutyrate + coenzyme I $\xrightarrow[\text{dehydrogenase}]{\beta\text{-hydroxybutyric}}$ acetoacetate + reduced coenzyme I.

(7) Reduced coenzyme I + pyruvate $\xrightarrow[\text{dehydrogenase}]{\text{lactic}}$ coenzyme I + lactate.

In another communication, direct proof will be given for the validity of this interpretation.

The reaction is not reversible to any appreciable extent. That is to say, the reaction is entirely in favour of the formation of acetoacetic and lactic acids. If the reaction were significantly reversible, simultaneous addition of large amounts of lactate should inhibit completely the formation of acetoacetic acid. Such is

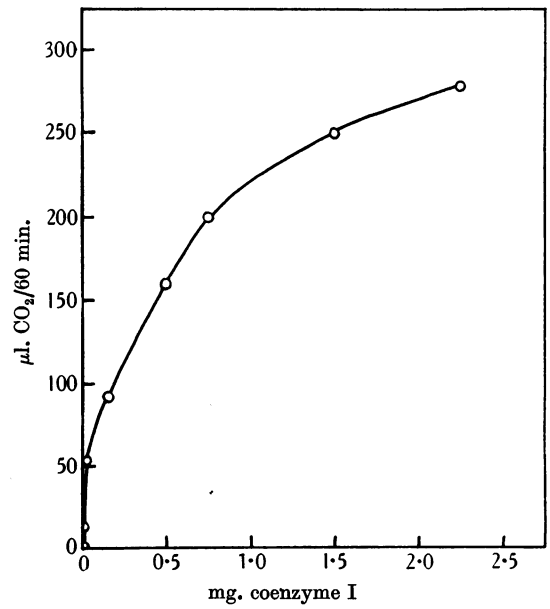

Fig. 1. Effect of coenzyme concentration of the rate of oxidation of β-hydroxybutyrate by pyruvate. Each tube contained 1·5 ml. enzyme, 0·2 ml. *M* β-hydroxybutyrate and 0·7 ml. *M*/5 pyruvate. Total volume, 3·9 ml.

Table II. *Inhibition of β-hydroxybutyrate-pyruvate oxidoreduction by lactate*

Heart enzyme (ml.)	2·0	2·0
0·15% coenzyme (ml.)	1·0	1·0
M β-hydroxybutyrate	0·2	0·2
M pyruvate	0·05	0·05
2*M* lactate	—	0·15
Water	0·15	—
μl. acetoacetic CO_2 in 60 min.	318	268

actually not the case (cf. Table II). When acetoacetate and lactate are incubated anaerobically with the enzymes and the coenzyme, only a small amount of acetoacetic acid disappears, indicating that equilibrium is practically reached when only lactic and acetoacetic acids are present (cf. Table III).

Table III. *The reduction of acetoacetate by lactate*

Heart enzyme (ml.)	2·5	2·5
0·15% coenzyme I (ml.)	1·0	1·0
2*M* lactate (ml.)	0	0·2
1% acetoacetate (ml.)	0·2	0·2
Water (ml.)	0·2	—
μl. acetoacetic CO_2 remaining after 2 hr.	394	374

Fig. 2 shows the effect of the β-hydroxybutyrate concentration on the rate of oxidation by pyruvate. The curve is practically identical with that for the rate of oxidation by molecular oxygen [cf. Green *et al.* 1937].

It can be easily demonstrated that coenzyme I itself and not any possible impurity is the active carrier in the oxidoreduction. Coenzyme I in the oxidized form is destroyed by boiling in alkaline solution. The reduced form is not

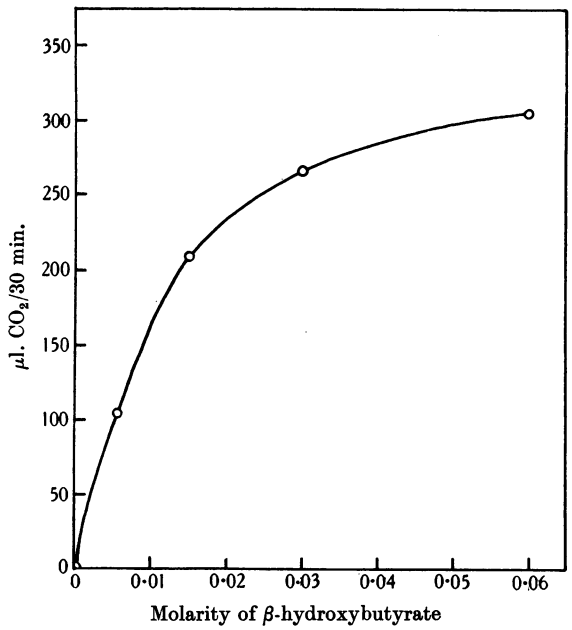

Fig. 2. Effect of concentration of β-hydroxybutyrate on the reaction with pyruvate. Each tube contained 2 ml. enzyme, 1 ml. 0·15% coenzyme I and 0·2 ml. *M* pyruvate. Total volume, 3·7 ml.

Table IV. *Alkaline-boiled oxidized and reduced coenzyme*

Heart enzyme (ml.)	2·0	2·0	2·0
0·15% coenzyme I (ml.)	1·0	—	—
M β-hydroxybutyrate (ml.)	0·2	0·2	0·2
M pyruvate (ml.)	0·2	0·2	0·2
0·15% alkaline-boiled coenzyme I (ml.)	—	1·0	—
0·15% alkaline-boiled reduced coenzyme I (ml.)	—	—	1·0
µl. acetoacetic CO_2 produced in 60 min.	308	25	200

affected by such treatment. Table IV shows that the alkaline-boiled oxidized coenzyme is inactive, whereas similarly treated reduced coenzyme retains its activity.

If every molecule of acetoacetic acid produced is formed at the expense of the reduction of one molecule of pyruvic to lactic acid, the ratio $\frac{\mu l.\ \text{acetoacetic } CO_2}{\mu l.\ \text{lactic } O_2}$ should be exactly 2. The observed ratios are given in Table V. They are in good agreement with theory.

Table V. *Ratio of acetoacetic to lactic acid*

	µl. acetoacetic CO_2	µl. lactic O_2	Ratio
(1)	251	130	1·94
(2)	268	142	1·89
(3)	259	136	1·91

IV. *Reaction of β-hydroxybutyrate and fumarate*

In the previous papers of this series, a scheme for the classification of dehydrogenase systems was proposed. There are three main classes, aerobic oxidases, coenzyme systems and cytochrome systems. The succinic dehydrogenase is an example of a cytochrome system. It does not require a coenzyme and normally reacts with molecular oxygen through the intermediation of cytochrome *c*. The β-hydroxybutyric system is an example of a coenzyme system. No oxidation of the substrate can take place in absence of coenzyme I. It now appears that the distinction between cytochrome and coenzyme systems although still valid is not very rigid. The succinic dehydrogenase, for example, does not require coenzyme I for oxidation of the substrate. However, the dehydrogenase can catalyse the oxidation of reduced coenzyme I by fumaric acid. That is to say a cytochrome system, although not dependent upon coenzymes in the normal way, nevertheless may either reduce oxidized coenzyme or oxidize reduced coenzyme.

The anaerobic oxidation of β-hydroxybutyrate by fumarate does not proceed in absence of either coenzyme I or the respective dehydrogenases (cf. Table VI).

Table VI. *Oxidation of β-hydroxybutyrate by fumarate*

Heart enzyme (ml.)	2·0	2·0	—	2·0	2·0
0·15% coenzyme I (ml.)	1·0	—	1·0	1·0	1·0
M β-hydroxybutyrate (ml.)	0·2	0·2	0·2	—	0·2
M fumarate (ml.)	0·2	0·2	0·2	0·2	—
Water (ml.)	—	1·0	2·0	0·2	0·2
μl. acetoacetic CO_2 in 60 min.	288	21	0	11	0

The observed ratios of $\frac{\mu l.\ \text{acetoacetic } CO_2}{\mu l.\ \text{succinic } O_2}$ are in close agreement with the theoretical ratio of 2 for the oxidation of one molecule of β-hydroxybutyric by one molecule of fumaric acid (cf. Table VII).

Table VII. *Ratio of acetoacetic to succinic acid*

	μl. acetoacetic CO_2	μl. succinic O_2	Ratio
(1)	133	63	2·11
(2)	264	151	1·75
(3)	211	90	2·34
(4)	229	103	2·22

Table VIII. *Effect of concentration of coenzyme I and of β-hydroxybutyrate on the β-hydroxybutyrate-fumarate oxidoreduction*

Enzyme (ml.)	2·0	2·0	2·0	2·0	2·0	2·0
0·15% coenzyme I (ml.)	1·0	1·0	0·2	—	1·0	1·0
0·015% coenzyme I (ml.)	—	—	—	0·2	—	—
M β-hydroxybutyrate (ml.)	—	0·2	0·2	0·2	0·05	0·02
M fumarate (ml.)	0·2	0·2	0·2	0·2	0·2	0·2
Water (ml.)	0·2	—	0·8	0·8	0·15	0·18
μl. acetoacetic CO_2 in 60 min.	0	190	134	42	133	113

Table VIII shows the dependence of the rate of oxidation of β-hydroxybutyrate by fumarate on the concentration of coenzyme I and on the concentration of β-hydroxybutyrate.

None of the oxidoreductions described in this communication is cyanide-sensitive. Furthermore, addition of carriers other than the coenzyme has no appreciable effect. Flavin and flavoprotein tend to inhibit the reactions slightly.

V. *Reaction between β-hydroxybutyrate and oxaloacetate*

This reaction is the most rapid one that has been studied. The controls given in Table IX show that the oxidation of β-hydroxybutyrate by oxaloacetate is catalysed by coenzyme I and by the respective dehydrogenases. The ratio

Table IX. *The oxidation of β-hydroxybutyrate by oxaloacetate*

Heart enzyme (ml.)	2·0	—	2·0	2·0	2·0
0·15% coenzyme (ml.)	2·0	2·0	—	2·0	2·0
M β-hydroxybutyrate (ml.)	0·2	0·2	0·2	—	0·2
M/3 oxaloacetate (ml.)	0·2	0·2	0·2	0·2	—
Water (ml.)	—	2·0	2·0	0·2	0·2
μl. malic O_2 in 60 min.	164	0	0	7	0

$\frac{\mu l.\ \text{acetoacetic } CO_2}{\mu l.\ \text{malic } O_2}$ is in fair agreement with theory assuming that one molecule of malic acid is produced for each molecule of β-hydroxybutyric oxidized to acetoacetic acid (cf. Table X).

Table X. *Ratio of acetoacetic to malic acid*

μl. acetoacetic CO_2	μl. malic O_2	Ratio
414	175	2·37
424	213	1·99
407	171	2·30

Oxaloacetic acid is rather unstable at 38°, decomposing spontaneously into pyruvic acid and CO_2. The possibility was therefore open that pyruvic acid and not oxaloacetic acid was the actual oxidizing agent. This possibility, however, is excluded for the following reasons. In parallel experiments, oxidation by oxaloacetate was much more rapid than by pyruvate. Furthermore, lactic estimations with the yeast enzyme failed to detect any appreciable amount of lactic acid formed. The small amount of pyruvic acid formed in the course of the experiment apparently does not divert the main reaction between β-hydroxybutyric and oxaloacetic acids.

By determining the rates of reaction with small amounts of coenzyme in contact with large excess of enzyme we were able to calculate the turnover number of the coenzyme (which is defined as the number of times per minute that the coenzyme is both reduced and oxidized). The highest value observed was 20. The significance of this number requires some clarification. In the reaction between β-hydroxybutyrate and oxaloacetate, the coenzyme is reduced by the former and oxidized by the latter. The turnover number refers therefore to the slower of the two reactions. We have evidence for assuming that the limiting reaction is the rate of reduction of the coenzyme by β-hydroxybutyric acid. Very probably if our reducing system were more active, turnover numbers of 50–100 could be reached.

VI. *Reaction between β-hydroxybutyrate and aldehyde*

Dixon & Mann [1937] have demonstrated that the aldehyde mutase of liver catalyses the dismutation of aldehyde to alcohol and acid in presence of coenzyme I. Their mutase preparations were completely freed from all traces of both the alcohol and aldehyde dehydrogenases.

Assuming that the mechanism of the action of the mutase consists in the reversible reduction of the coenzyme by one molecule of aldehyde (with production of acid) and in the oxidation of reduced coenzyme by a second molecule of

aldehyde (with production of alcohol), the possibility exists of linking the oxidation of β-hydroxybutyrate with the reduction of aldehyde by means of the coenzyme. Table XI shows that a vigorous reaction between β-hydroxybutyrate

Table XI. *Oxidation of β-hydroxybutyrate by aldehyde*

Heart enzyme (ml.)	2·0	2·0	2·0	—	2·0
0·15% coenzyme I (ml.)	1·0	—	1·0	1·0	1·0
Liver mutase (ml.)	0·5	0·5	—	0·5	0·5
M β-hydroxybutyrate (ml.)	0·2	0·2	0·2	0·2	0·2
M acetaldehyde (ml.)	0·1	0·1	0·1	0·1	—
Water (ml.)	—	1·0	0·5	2·0	0·1
μl. acetoacetic CO_2 in 60 min.	218	121	6	0	24

and aldehyde occurs in presence of the complete system. No oxidoreduction occurs in absence of the liver mutase, the β-hydroxybutyric dehydrogenase or aldehyde. The blank reaction in absence of coenzyme is large since the liver mutase had not been freed completely from coenzyme. Using more purified preparations this blank without coenzyme was practically negative.

Although alcohol was not demonstrated as the product of reduction of aldehyde, analogy with the other oxidoreductions of the same kind renders it certain that this reduction must take place.

VII. *The dismutation of fumarate*

Green [1936, 1] demonstrated the dismutation of fumarate to succinate and oxaloacetate. Methylene blue was used as the carrier in addition to coenzyme I. We have now been able to establish that methylene blue is superfluous for the dismutation and that coenzyme I is the only carrier required provided that it is present in sufficiently high concentration. The following reactions occur in the dismutation of fumarate:

(8) Fumarate $\overset{\text{fumarase}}{\rightleftharpoons}$ malate.

(9) Malate + coenzyme I $\xrightarrow[\text{dehydrogenase}]{\text{malic}}$ oxaloacetate + reduced coenzyme I.

(10) Reduced coenzyme I + fumarate $\xrightarrow[\text{dehydrogenase}]{\text{succinic}}$ coenzyme I + succinate.

It is obvious that whether fumarate or malate is used as starting substance, the same reactions will take place. The test for the dismutation is the production of succinic acid and oxaloacetic acid. Table XII shows that both

Table XII. *Anaerobic production of succinic and oxaloacetic from fumaric or malic acid*

Heart enzyme (ml.)	2·0	2·0	—	2·0	2·0
0·15% coenzyme I (ml.)	2·0	—	2·0	2·0	2·0
M fumarate (ml.)	0·2	0·2	0·2	0·2	—
M malate (ml.)	—	—	—	—	0·2
0·5% methylene blue (ml.)	—	—	—	0·2	—
Water (ml.)	0·2	2·2	2·2	0	0·2
μl. succinic O_2	35	3	0	37	45
μl. oxaloacetic CO_2	70	5	0	70	85

are produced regardless of whether malate or fumarate is used. There is a small blank production of succinic acid in absence of coenzyme I. Oxaloacetic

acid formed in the reaction was fixed with hydrazine and then estimated as CO_2 by the aniline citrate method [cf. Green, 1936, 2].

This dismutation of fumarate is very slow compared with the other oxido-reductions described above. When β-hydroxybutyrate was oxidized by fumarate, it could be shown that all the succinic acid formed was equivalent to the aceto-acetic. This means that malate cannot compete with β-hydroxybutyrate for the reduction of the coenzyme. We shall discuss later the theoretical basis for this observation.

VIII. *Attempted linkages with hypoxanthine and α-glycerophosphate*

The essential condition for the linking of two dehydrogenase systems by means of coenzyme I is that one system should be capable of reducing the coenzyme and the other should be capable of oxidizing the system. Potential considerations determine whether any dehydrogenase system can act as a reducing or as an oxidizing system. Not all dehydrogenase systems can react with coenzyme I. The xanthine oxidase of milk, for example, cannot be used as a reducing system although the potential requirements are fulfilled, since it cannot catalyse the reduction of coenzyme I by hypoxanthine. Negative results were obtained in the attempts to link the reduction of either fumarate or aceto-acetate with the oxidation of xanthine.

The α-glycerophosphate dehydrogenase of animal tissues has been studied in detail by Green [1936, 1]. The reaction with molecular oxygen is not direct but proceeds normally through the cytochromes. Also no coenzyme is required for the oxidation of the substrate. Recently Euler *et al.* [1937, 2] have claimed that the α-glycerophosphate dehydrogenase system can reduce coenzyme I and that the coenzyme is necessary for the actual oxidation of the substrate. They consider that the negative results which Green obtained on adding coenzyme were due to the fact that the enzyme preparations were saturated with respect to coenzyme.

We have repeated our earlier experiments on the α-glycerophosphate dehydrogenase prepared from the skeletal muscle of the rabbit. The method of preparation involving extensive washing of the minced tissue and precipitation of the enzyme at *p*H 4·6 renders highly improbable the presence of any appreciable amounts of coenzyme. The preparation contains in addition to the α-glycerophosphate enzyme a highly active lactic enzyme. The latter is known definitely to require coenzyme I. If the preparation is saturated with respect to coenzyme, there should be no effect on adding coenzyme to the lactic system as well as to the α-glycerophosphate system. Table XIII shows that in absence of added

Table XIII. *Influence of coenzyme on the oxidation of α-glycerophosphate and lactate in presence of rabbit muscle enzyme*

Rabbit skeletal muscle enzyme (ml.)	1·5	1·5	1·5	1·5	1·5
0·15% coenzyme I (ml.)	—	—	0·5	0	0·5
0·5% methylene blue (ml.)	0·3	0·3	0·3	0·3	0·3
M α-glycerophosphate (ml.)	—	0·2	0·2	—	—
M lactate (ml.)	—	—	—	0·2	0·2
2*M* HCN (ml.)	—	—	—	0·1	0·1
Water (ml.)	1·5	1·3	0·8	1·4	0·9
μl. O_2/10 min.	6	140	144	0	232

coenzyme no oxidation of lactate ensues, whereas the rate of oxidation of α-glycerophosphate is not affected by addition of coenzyme. It is quite clear therefore (1) that the enzyme preparation does not contain any measurable

amount of coenzyme I and (2) that no coenzyme is necessary for the functioning of the α-glycerophosphate system. As for the alleged reduction of the coenzyme by the α-glycerophosphate dehydrogenase, Green & Dewan [1937] have been unable to confirm this. It is quite possible that there are two enzymes which can deal with α-glycerophosphate—the one described as a cytochrome system and the other probably a type of mutase which depends upon coenzyme.

We have attempted to link the reduction of acetoacetate, fumarate, pyruvate and oxaloacetate with the oxidation of α-glycerophosphate. But in no case was there any indication of a positive reaction. These negative results are to be expected if the α-glycerophosphate dehydrogenase system cannot reduce coenzyme I.

IX. *The mechanism of coenzyme linked oxidoreductions*

All the reactions described above conform to the following mechanism:

(11) $\text{Reductant}_A + \text{coenzyme I} \xrightarrow{\text{dehydrogenase}_A} \text{oxidant}_A + \text{reduced coenzyme I.}$

(12) $\text{Oxidant}_B + \text{reduced coenzyme I} \xrightarrow{\text{dehydrogenase}_B} \text{reductant}_B + \text{coenzyme I.}$

The evidence for this interpretation is the following. Euler *et al.* [1937] have demonstrated that the lactic and malic systems can either reduce or oxidize the coenzyme depending upon the ratio of reduced to oxidized substrate. We have been able to confirm their observations and also to demonstrate that the β-hydroxybutyrate system comes into true equilibrium with the coenzyme system. Elsewhere evidence is presented that reduced coenzyme can be oxidized by aldehyde in presence of liver mutase and by fumarate in presence of the succinic oxidase. These results leave no possibility of doubt that the coenzyme functions by being reduced by one system and oxidized by the other.

X. *Oxidation-reduction potentials*

Table XIV contains a summary of the oxidation-reduction potentials of the systems employed in this study. Many observations now become clear from a consideration of the potentials. The fact that the lactate system is approximately

Table XIV. *Oxidation-reduction potentials*

System	E_0' at *p*H 7·0	Reference
Succinate-fumarate	0·0	Borsook & Schott [1931]
Alcohol-aldehyde	−0·09	Lehmann [1934]; Wurmser & Filitti [1935]
Malate-oxaloacetate	−0·17	Laki *et al.* [1937]
Lactate-pyruvate	−0·18	Barron & Hastings [1934]
Reduced coenzyme I-coenzyme I	−0·27 approx.	Green & Dewan [1937]
β-Hydroxybutyrate-acetoacetate	−0·28	Green *et al.* [1937]

100 mV. more positive than the β-hydroxybutyrate system indicates that equilibrium will be reached when practically all the β-hydroxybutyric and pyruvic acids are converted into acetoacetic and lactic acids respectively. The reverse reaction can proceed only to a very small degree. It is interesting that all but the β-hydroxybutyric system are more positive in potential than the coenzyme system. Following the convention of Euler, we may refer to those systems more positive as the acceptor systems and those more negative as the donator systems.

The most rapid reactions occur when the potential of the coenzyme lies between that of the acceptor and donator systems. Since all these reactions involve the same mechanism, there is a rough relation between the free energy

difference and the velocity. The reaction between malate and fumarate is very much slower than the reaction between β-hydroxybutyrate and fumarate, although the activity of the β-hydroxybutyric enzyme is only a small fraction of that of the malic enzyme.

XI. *Significance of coenzyme linked reactions*

The recent work of Krebs & Johnson [1937] on anaerobic oxidoreductions in tissue slices shows clearly that coenzyme-linked reactions are not artificial. They succeeded in linking the following oxidoreductions: pyruvate with oxaloacetate, pyruvate with fumarate and pyruvate with acetoacetate. Nothing is yet known about the properties of the pyruvic enzyme, but apparently it can catalyse the reduction of coenzyme I and like the β-hydroxybutyric dehydrogenase probably belongs to the class of donator systems. The tissue slices are saturated with respect to coenzyme I; hence no addition is necessary. It is, of course, an assumption that the coenzyme is the actual linking agent, but since the coenzyme is present in all tissues and since most of the systems tested by Krebs & Johnson normally react with coenzymes, the assumption is to some extent justified.

Thus far we have only considered coenzyme I (diphosphopyridinenucleotide). Experiments are in progress to test whether coenzyme II (triphosphopyridinenucleotide) can play the following role:

(13) $\text{Reductant}_A + \text{coenzyme II} \longrightarrow \text{oxidant}_A + \text{reduced coenzyme II}$.

(14) $\text{Reduced coenzyme II} + \text{coenzyme I} \longrightarrow \text{coenzyme II} + \text{reduced coenzyme I}$.

(15) $\text{Reduced coenzyme I} + \text{oxidant}_B \longrightarrow \text{coenzyme I} + \text{reductant}_B$.

There is also the possibility that a reaction between two dehydrogenase systems, e.g. hexosemonophosphate and fumarate can take place via coenzyme II exclusively.

The oxidoreductions between α-glycerophosphate or triosephosphate and pyruvate, which are considered to be essential reactions in the glycolytic process, have been shown to require coenzyme I [cf. Euler *et al.* 1937]. The assumption is generally made that dehydrogenases are involved in these oxidoreductions, although the evidence is inadequate. A triosephosphate dehydrogenase has been described in animal tissues but no one has succeeded in demonstrating an active triosephosphate dehydrogenase in actively glycolysing extracts. The α-glycerophosphate dehydrogenase similarly is not readily demonstrable in such extracts. Furthermore, this dehydrogenase is incapable of catalysing the reaction of the substrate with the coenzyme. It is conceivable that a special mutase catalyses the reaction between triosephosphate or α-glycerophosphate and pyruvic acid. By mutase is implied an enzyme which activates an oxidant of one substrate system and a reductant of another. The simplest mutase is the aldehyde mutase which activates the same substrate, viz. aldehyde which is at once the oxidant of the alcohol-aldehyde system and the reductant of the aldehyde-acid system. If a mutase is in fact involved in the glycolytic cycle, it must be considered as a mixed mutase.

The mechanism of mutase action is essentially that of a coenzyme-linked reaction. The only difference lies in the fact that the two activating centres instead of being present in two different dehydrogenases are present on the same enzyme. The two activating centres in the mutase cannot be identical otherwise no coenzyme would be essential to act as the link. For example, the xanthine oxidase which catalyses the dismutation of xanthine to hypoxanthine and uric acid does not require a carrier for this reaction since the same active group catalyses both the oxidation and reduction of xanthine [cf. Green, 1934].

SUMMARY

Coenzyme I can act as the carrier for the following oxidoreductions: the oxidation of β-hydroxybutyrate by (1) fumarate, (2) oxaloacetate, (3) pyruvate, (4) acetoacetate and (5) aldehyde, and the dismutation of fumarate to succinate and oxaloacetate. The coenzyme functions by being reduced by the donator system and oxidized by the acceptor system.

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Note added 2 July 1937. In collaboration with Dr D. M. Needham we have succeeded in demonstrating that the reaction between α-glycerophosphate and pyruvate is catalysed by a soluble mutase which is not identical with the α-glycerophosphate dehydrogenase. This mutase or an associated enzyme also catalyses the reactions between triosephosphate or glyceraldehyde on the one hand and pyruvate or oxaloacetate on the other. Coenzyme I is necessary for all these oxidoreductions.