CXL. THE REVERSIBLE OXIDATION AND REDUCTION OF COENZYME I

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Warburg *et al.* [1935] showed that coenzyme II (triphosphopyridinenucleotide) was capable of reversible reduction and oxidation. The reduced coenzyme showed a band at 345 mμ whereas the oxidized coenzyme did not absorb in that region. Euler *et al.* [1936] and Warburg & Christian [1936] found that the reduced form of coenzyme I (diphosphopyridinenucleotide) showed a similar band at 340 mμ. This characteristic absorption band of the reduced coenzyme has been utilized by Warburg and Euler for studying the reversible oxidation and reduction spectrophotometrically.

Dewan & Green [1937], in their study of linked oxido-reductions between pairs of dehydrogenase systems, found that coenzyme I could act as the carrier for many of these reactions. By analogy with the action of artificial carriers in the "carrier-linked reactions" described by Green *et al.* [1934], coenzyme I was considered to be undergoing a cycle of reduction by one dehydrogenase system and oxidation by the other. In this communication, spectrophotometric evidence will be presented that such is actually the case. Also direct proof is given for the existence of the coenzyme oxidase postulated by Green *et al.* [1937].

I. *Method*

A Hilger Spekker spectrophotometer was used for following changes in the absorption spectrum of reduced or oxidized coenzyme I. The source of light was a tungsten spark. The length of the cups was 2 cm.

The coenzyme was prepared from baker's yeast by the method of Green *et al.* [1937]. The purity was not very high. The impurities, however, do not affect the results since only the difference in absorption at 340 mμ between the reduced and oxidized coenzyme is measured.

The methods of preparation of the enzymes used have been fully described in previous communications [cf. Green, 1936, 1, 2; Green & Brosteaux, 1936; Green *et al.* 1937]. It was found necessary after precipitating the enzymes with acetic acid to wash the precipitate thoroughly with acetate buffer *p*H 4·6. This procedure removes residual traces of haemoglobin and other interfering substances.

Reduced coenzyme was prepared as follows. 10 ml. of a 0·15% solution of coenzyme I were mixed with 2·5 ml. of 0·5% $NaHCO_3$ and 2·5 ml. of 0·2% $Na_2S_2O_4$ (freshly prepared). The mixture was protected from air and maintained at 38° for 15 min. It was then vigorously aerated for 15 min. to oxidize excess hydrosulphite. A control without coenzyme was treated in exactly the same way. 1·5 ml. of reduced coenzyme were used for each individual experiment.

In testing enzymic oxidation of reduced coenzyme, the procedure was as follows. 1·5 ml. of reduced coenzyme were mixed with 0·5 ml. enzyme and 0·1 ml. $M/3$ substrate and incubated anaerobically in a Thunberg tube for some

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minutes. The contents of the tube were then brought to the boil and water added to bring the volume to 8 ml. The deproteinized solution was then mixed with a small amount of kieselguhr and filtered. The clear filtrate was used directly for analysis. The control solution containing all the components except the coenzyme was treated in exactly the same fashion.

In testing enzymic reduction of oxidized coenzyme, the above details were also followed except that 1 ml. of 0·15 % oxidized coenzyme I was used.

Fig. 1 shows the curves for reduced and oxidized coenzyme. Only a small part of the band at 290 mμ is included. A concentration of coenzyme was

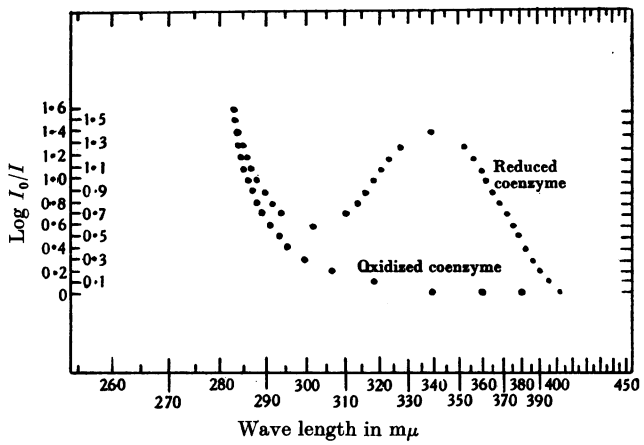

Fig. 1. Absorption spectrum of oxidized and reduced coenzyme I.

selected at which the peak of the band of the reduced coenzyme at 340 mμ reached a density of 1·3–1·5.

II. *Oxidation of reduced coenzyme by pyruvate, oxaloacetate, fumarate and acetoacetate*

Table I shows that in presence of pyruvate, oxaloacetate or fumarate and the respective dehydrogenases, the band of reduced coenzyme at 340 mμ disappears.

Table I. *Oxidation of reduced coenzyme by pyruvate, oxaloacetate, fumarate and acetoacetate*

	$\log \frac{I_0}{I}$ at 340 mμ
Reduced coenzyme and heart enzyme	1·2
Reduced coenzyme + enzyme + oxaloacetate	0·2
As above with boiled enzyme	1·2
Reduced coenzyme + enzyme + pyruvate	0·2
As above with boiled enzyme	1·2
Reduced coenzyme + enzyme + fumarate	0·2
Reduced coenzyme + enzyme + acetoacetate	0·7
As above with boiled enzyme	1·2
Oxidized coenzyme + enzyme	0·2

Time of experiment: 5 min.

The period of anaerobic incubation at 38° was 5 min. Acetoacetate only partially oxidizes reduced coenzyme. Later it will be shown that there is

an equilibrium between the coenzyme system and the β-hydroxybutyric-acetoacetic system, which makes it impossible for the oxidation of reduced coenzyme by acetoacetate to be complete.

Euler *et al.* [1936; 1937] have also observed oxidation of reduced coenzyme I by pyruvate and oxaloacetate.

III. *Reduction of coenzyme by malate, lactate and β-hydroxybutyrate*

The coenzyme system is some 100 mV. more negative than either the lactic or malic system. That means that the equilibrium will be very much in favour of oxidized coenzyme. The equilibrium, however, can be shifted to the side of reduced coenzyme by using ketone fixatives such as cyanide. In the case of the β-hydroxybutyric system, the equilibrium is in favour of reduced coenzyme. Ketone fixatives however, such as hydrazine, shift the equilibrium even more to the side of reduced coenzyme. Table II shows that coenzyme I can be reduced

Table II. *Reduction of coenzyme by lactate, malate and β-hydroxybutyrate*

	$\log \frac{I_0}{I}$ at 340 mμ
Oxidized coenzyme + heart enzyme	0·2
Oxidized coenzyme + enzyme + malate + cyanide	0·9
Oxidized coenzyme + enzyme + lactate + cyanide	0·9
Oxidized coenzyme + enzyme + β-hydroxybutyrate	0·8
Reduced coenzyme + enzyme	1·2

Time of experiment: 10 min.

by malate, lactate and β-hydroxybutyrate. It is interesting that the coenzyme is never fully reduced by the enzymic method. That simply means that an equilibrium is reached or is being approached.

IV. *Reduction of coenzyme by α-glycerophosphate*

Green [1936, 1] described the properties of the α-glycerophosphate dehydrogenase of animal tissues. This enzyme does not require the collaboration of a coenzyme. Recently Euler *et al.* [1937] claimed to have demonstrated the reduction of coenzyme I by α-glycerophosphate in presence of the dehydrogenase. We have been unable to confirm this observation (cf. Table III). The α-glycero-

Table III. *Reduction of coenzyme by α-glycerophosphate*

	$\log \frac{I_0}{I}$ at 340 mμ
Oxidized coenzyme + skeletal muscle enzyme	0·2
Oxidized coenzyme + enzyme + lactate + cyanide	0·9
Oxidized coenzyme + enzyme + α-glycerophosphate ± cyanide	0·2

Time of experiment: 15 min.

phosphate enzyme tested was actually twice as active as the lactic enzyme in the extract prepared from the skeletal muscle of rabbit. Yet no reduction of the coenzyme by α-glycerophosphate could be observed over a period of 15 min., whereas considerable reduction by lactate was obtained. The possibility exists that the reduction of coenzyme observed by Euler *et al.* may have been catalysed by some enzyme other than the α-glycerophosphate dehydrogenase, very probably the mutase postulated by Dewan & Green [1937].

V. *Oxidation of reduced coenzyme by molecular oxygen catalysed by coenzyme oxidase*

Green *et al.* [1937] have described an enzyme which catalyses the oxidation of reduced coenzyme by molecular oxygen. Their evidence was only indirect. We have been able to demonstrate spectrophotometrically that the enzyme extract prepared from the heart muscle of the pig does contain a coenzyme oxidase.

Reduced coenzyme is non-autoxidizable at neutrality. But when aerated for a few minutes at 38° in presence of the heart enzyme, it becomes oxidized (cf. Table IV). The fact that the boiled enzyme control is negative shows that

Table IV. *Oxidation of reduced coenzyme by molecular oxygen*

	$\log \frac{I_0}{I}$ at 340 mμ
Reduced coenzyme + enzyme (boiled on mixing)	1·2
Reduced coenzyme + enzyme (aerated at 38° for 1 min.)	0·4
As above (aerated 5 min.)	0·2
As above (aerated 10 min.)	0·2
Reduced coenzyme + boiled enzyme (aerated 10 min.)	1·2
Reduced coenzyme + enzyme + cyanide (aerated 10 min.)	1·2
Reduced coenzyme + enzyme (incubated anaerobically 10 min.)	1·2

the oxidation is catalysed. Finally, the complete inhibition of the catalysis by $M/50$ cyanide is in harmony with the manometric findings of Green *et al.* [1937].

VI. *Oxidation of reduced coenzyme by aldehyde in presence of liver mutase*

Dewan & Green [1937] have postulated that a mutase is an enzyme with two active centres capable of catalysing an oxidoreduction through the intermediation of the coenzyme. It should therefore be possible to demonstrate in the case of the liver mutase that aldehyde can either reduce oxidized coenzyme or oxidize reduced coenzyme. Through the kindness of Dr M. Dixon and Dr C. Lutwak-Mann we have been able to use a highly purified preparation of liver mutase for testing our hypothesis. Actually only the complete oxidation of reduced coenzyme by aldehyde can be observed. In less than 5 min. the original density at 340 mμ dropped from 1·2 to 0·1. We were unable to demonstrate any reduction of the coenzyme in presence of aldehyde. This negative result, however, does not necessarily mean that aldehyde cannot reduce the coenzyme. More probably the rate of oxidation of reduced coenzyme is so much more rapid than the reduction of oxidized coenzyme that the kinetic equilibrium is entirely in favour of oxidized coenzyme. Unfortunately there is no method of dissociating these two competing processes.

VII. *The potential of the coenzyme*

The β-hydroxybutyric dehydrogenase system has been shown to be thermodynamically reversible [cf. Green *et al.* 1937]. The fact that the coenzyme system lies within the potential range of the β-hydroxybutyric system has made possible a rough estimation of the potential of the coenzyme system. Table V is a summary of an experiment conducted at *p*H 7·07. Reduced coenzyme and enzyme were incubated for 1 hr. in presence of different ratios of β-hydroxybutyrate to acetoacetate. It is clear that with change in the ratio of β-hydroxybutyrate to acetoacetate, there is a corresponding change in the ratio of reduced to oxidized coenzyme. No claim can be made for great accuracy by this method. All that

Table V

Ratio $\frac{\beta\text{-hydroxybutyrate}}{\text{acetoacetate}}$	E_h	$\log \frac{I_0}{I}$ at 340 mμ	% reduced coenzyme	% oxidized coenzyme	E_0'
9 : 1	−0·314	1·1	92	8	−0·282
5 : 5	−0·286	0·9	81	19	−0·267
1 : 9	−0·258	0·7	36	64	−0·266

Completely reduced coenzyme had a density of 1·3; completely oxidized coenzyme a density of 0·1 at 340 mμ.

can be said at the moment is that the coenzyme lies at about the same potential level as that of the β-hydroxybutyric system. Reduced coenzyme in presence of the heart enzyme can reduce benzylviologen. Experiments are in progress to measure electrometrically the potential of the coenzyme system.

VIII. *Oxidation of reduced coenzyme by methylene blue*

Green & Brosteaux [1936], in their study of the properties of reduced coenzyme, showed that methylene blue oxidizes reduced coenzyme. Adler *et al.* [1936], however, state that, at neutral or alkaline reaction, reduced coenzyme cannot be oxidized by methylene blue. We have been able to confirm spectrophotometrically the results obtained by Green & Brosteaux with a colorimetric technique.

Reduced coenzyme (1·5 ml.) was rapidly mixed with 0·1 ml. of 0·1 % methylene blue and 6·4 ml. water (final *p*H 8). The mixture was then rapidly filtered with suction through a pad of kieselguhr. The filtrate was colourless. A similar mixture was prepared but allowed to incubate for 15 min. at 38° before being filtered through kieselguhr. The control solution had a density of 1·3 at 340 mμ whereas the incubated experimental solution went down to density 0·3. In other words oxidation was practically complete.

The following is a summary of the reducing and oxidizing agents for the coenzyme:

Agents which can reduce coenzyme I	Agents which can oxidize coenzyme I
Lactate (*e*)	Pyruvate (*e*)
Malate (*e*)	Oxaloacetate (*e*)
β-Hydroxybutyrate (*e*)	Acetoacetate (*e*)
	Aldehyde (*e*)
	Fumarate (*e*)
	Methylene blue
	Flavin
	Flavoprotein
	Oxygen (*e*)

(*e*) Indicates that the reaction is catalysed by an enzyme.

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