C. ON THE CATALYTIC FUNCTION OF HEART FLAVOPROTEIN

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The oxidation in animal tissues of metabolites such as lactate, malate, β-hydroxybutyrate, triosephosphate etc., can be resolved into at least three consecutive reactions:

(1) Metabolite + coenzyme I → oxidized metabolite + reduced coenzyme I.
(2) Reduced coenzyme I + carrier → coenzyme I + reduced carrier.
(3) Reduced carrier + O_2 → carrier.

The first reaction is catalysed by a family of enzymes, each of which is specific for a particular metabolite. For example, the lactic enzyme catalyses the oxidation of lactate by coenzyme I; the malic enzyme the oxidation of malate, etc. There are three components in this reaction, viz. enzyme, metabolite and coenzyme I, and the sole chemical change is the catalysed transfer of hydrogen from the metabolite to the coenzyme. The second reaction is not spontaneous, but requires a special catalyst, referred to as coenzyme factor or diaphorase [Euler & Hellström, 1938; Dewan & Green, 1938]. The mechanism whereby the coenzyme factor catalysed the oxidation of reduced coenzyme was obscure owing to lack of information concerning the chemical nature of the catalyst. Recently Straub [1939, 1, 2] isolated from heart muscle a highly purified flavoprotein compound with properties which at once suggested its identity with the coenzyme factor. This flavoprotein is rapidly reducible by dihydrocoenzyme I and its leuco- or reduced form is rapidly oxidizable by suitable carriers. Reaction (2) therefore can be further resolved into two constituent processes:

(2*a*) Reduced coenzyme I + flavoprotein → coenzyme I + leucoflavoprotein.
(2*b*) Leucoflavoprotein + carrier → flavoprotein + reduced carrier.

To complete our knowledge of how certain metabolites are oxidized by molecular oxygen via coenzyme I, it will be necessary to know what substance or substances in animal tissues can assume the role of carrier. In reconstructed systems artificial carriers such as methylene blue are employed. There is evidence that the cytochromes are the physiological equivalents of methylene blue, but the possibility remains that the hypothetical reaction between dihydrocoenzyme and the cytochromes is complex.

I. Catalytic properties of heart flavoprotein

For the following experiments a preparation of heart flavoprotein at the 0·66 % flavinphosphate level of purity was used (cf. Straub [1939, 2] for the details of the method of isolation and purification). The concentration of the flavoprotein in solution was estimated spectrophotometrically from the light absorption at 450 mμ, the absorption coefficient at 450 mμ being taken as $2{\cdot}4 \times 10^7$. The concentration of flavoprotein was also estimated by an independent method which

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involves splitting off the prosthetic group (adenineflavin dinucleotide) and estimating the concentration of the prosthetic group in the amino-acid oxidase test system. We shall consider this method in greater detail in section III. Both methods of estimating the concentration of flavoprotein agreed to within *ca.* 5%.

Reduced coenzyme I is oxidized extremely slowly by methylene blue. In presence of heart flavoprotein rapid reaction takes place. The reduced coenzyme-methylene blue system thus offers a convenient "test" for measuring quantitatively the catalytic action of heart flavoprotein. Dihydrocoenzyme was prepared either chemically, by reducing coenzyme with hydrosulphite and removing excess hydrosulphite by aeration, or enzymically by reducing coenzyme with the lactic enzyme-lactate system. The velocity of the catalytic oxidation of dihydrocoenzyme I by methylene blue was measured either anaerobically by the rate of decoloration of methylene blue or aerobically by the rate of O_2 absorption (cf. reactions (2) and (3)).

Table I summarizes a manometric experiment designed to show the catalytic effect of heart flavoprotein. The lactic enzyme was prepared from rabbit skeletal muscle by the method of Green *et al.* [1937]. The function of cyanide is to trap

Table I. *Catalytic effect of flavoprotein in the lactic enzyme system*

Lactic enzyme (ml.)	1·5	1·5	1·5	1·5	1·5
M lactate (ml.)	0·2	—	0·2	0·2	0·2
0·075% coenzyme I (ml.)	1·0	1·0	—	1·0	1·0
2*M* HCN (ml.)	0·2	0·2	0·2	0·2	0·2
0·5% methylene blue (ml.)	0·2	0·2	0·2	—	0·2
Flavoprotein (ml.) (17·5 μg./ml. as flavinphosphate)	0·1	0·1	0·1	0·1	—
Water	—	0·2	1·0	0·2	0·1
μl. O_2 in 5 min.	340	14	14	14	14

the product of oxidation, viz. pyruvate, which inhibits the forward reaction. For the oxidation of lactate by molecular O_2 both flavoprotein and methylene blue are necessary. The fact that there is no appreciable O_2 uptake in absence of methylene blue indicates that reduced flavoprotein is not autoxidizable under the conditions of the experiment. Increase of the flavoprotein concentration beyond *ca.* 0·5 μg. flavinphosphate equivalent per 3·6 ml. does not increase the rate of O_2 uptake. The effect of successive dilution of flavoprotein on the rate of O_2 uptake is shown in Table II. A measurable catalytic effect is given by

Table II. *Variation of O_2 uptake of lactic system with flavoprotein concentration*

The complete system contained 1·5 ml. enzyme, 0·2 ml. *M* lactate, 1 ml. 0·075% coenzyme I, 0·2 ml. 2*M* HCN and 0·2 ml. 0·5% methylene blue. Total vol. 3·6 ml., 38°, air in gas space.

Flavoprotein in μg. flavin-phosphate	μl. O_2/5 min.
1·74	385
0·87	385
0·53	360
0·35	267
0·18	160
0·087	90
0·053	70
0·018	37
0	2

0·005 μg. flavinphosphate equivalent/ml. The homogeneous flavoprotein contains 0·66% flavinphosphate. 0·018 μg. of flavinphosphate therefore is equi-

valent to 2·77 μg. of flavoprotein. Since 2·77 μg. catalyse the absorption of 444 μl. O_2/hr. the Q_{O_2} (μl. O_2/hr./mg.) is 160,000. In terms of the flavinphosphate moiety the Q_{O_2} is *ca.* $2{\cdot}5 \times 10^7$. Fig. 1 shows the dependence of the turnover number (T.N.) on the concentration of flavoprotein. The formula for calculating T.N. is

$$\frac{\mu\text{l. } O_2/\text{min.}}{0{\cdot}049 \times \mu\text{g. flavinphosphate}}.$$

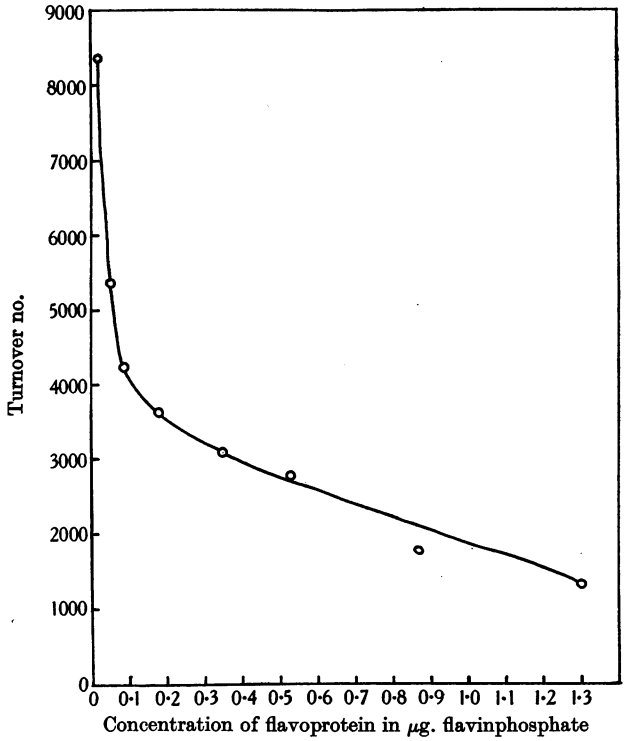


Fig. 1. Dependence of turnover number on flavoprotein concentration. Details as in Table I.

Strictly speaking the numerator should be μl. H_2/min. which is $2 \times \mu$l. O_2/min. But since in the autoxidation of methylene blue an equivalent amount of H_2O_2 is produced, 1 μl. O_2 absorbed is equivalent to 1 μl. H_2 transferred. The limiting value of the T.N. is *ca.* 8000/min.

Comparison of the catalytic activities of the heart and Warburg-Christian yeast flavoprotein shows a tremendous difference. For example, 0·015 μg. flavinphosphate equivalent of heart flavoprotein catalysed the same O_2 uptake as 5·3 μg. flavinphosphate equivalent of Warburg-Christian flavoprotein. The ratio of activities was therefore $5{\cdot}3/0{\cdot}015 = 353$.

The flavoprotein effect is independent of the nature of the enzyme which is concerned in the reduction of the coenzyme. Table III shows that by replacing the lactic enzyme-lactate system by the malic, triosephosphoric or α-glycerophosphoric enzyme systems, the same qualitative results obtain. The quantitative differences are referable to the activities of the different enzymes and the speed with which dihydrocoenzyme is made available by the various enzyme systems.

Table III. *Catalytic effect of heart flavoprotein in the malic, triosephosphoric and α-glycerophosphoric enzyme systems*

The dialysed extract of the acetone powder of rabbit skeletal muscle was used as the source of the three enzymes. Cyanide was used as fixative in the malic and α-glycerophosphoric enzymes. For further experimental details, cf. Green *et al.* [1937]. The enzyme system in all cases contained enzyme, substrate, coenzyme I and methylene blue. The amount of flavoprotein added was 2 μg. flavinphosphate equivalent.

	μl. O_2 in 5 min.
Malic enzyme system	2
Malic enzyme system + flavoprotein	168
α-Glycerophosphoric enzyme system	2
α-Glycerophosphoric enzyme system + flavoprotein	138
Triosephosphoric enzyme system	2
Triosephosphoric enzyme system + flavoprotein	60

A solution of dihydrocoenzyme I was prepared according to the method of Green & Dewan [1937]. Spectrophotometric estimation showed that the solution contained 1900 μg. dihydrocoenzyme per ml. 2 ml. of this solution reduced 0·15 ml. of 0·125 *M* methylene blue in 10 min. In presence of 3 μg. flavinphosphate equivalent of flavoprotein the reduction time was 10 sec. It was of theoretical interest to compare the T.N. of flavoprotein in this system with those obtained in the lactic enzyme system. The following figures show the T.N. for 0·13 and 0·03 μg. flavinphosphate equivalent of flavoprotein in the two systems.

	Turnover number	
	Hyposulphite reduced coenzyme	Enzymically reduced coenzyme
0·13 μg. flavinphosphate	3700	3850
0·03 μg. flavinphosphate	6200	6250

The correspondence is remarkably close and offers convincing proof that the enzyme system has no influence on the catalysed reaction between dihydrocoenzyme and methylene blue except in so far as it regulates the velocity with which dihydrocoenzyme I is formed.

Some anaerobic experiments have been carried out on the catalysed reaction between methylene blue and dihydrocoenzyme I prepared *in situ* by the lactic enzyme system. Table IV summarizes a controlled experiment. Increase of the flavoprotein concentration beyond 1 μg. flavinphosphate equivalent does not increase the rate of reduction of methylene blue. In other words, above 1 μg. flavinphosphate equivalent the limiting factor under the conditions of the experiment is the enzymic rate of formation of dihydrocoenzyme and not the concentration of flavoprotein. We should expect on theoretical grounds that the

Table IV. *Flavoprotein effect in the lactic enzyme system under anaerobic conditions*

The experiment was carried out in evacuated Thunberg tubes at 38°. The complete system contained 1 ml. enzyme, 0·5 ml. 0·075 % coenzyme I, 0·2 ml. 2 *M* HCN, 0·2 ml. 0·0125 *M* methylene blue and 0·1 ml. flavoprotein solution containing 17·5 μg. flavinphosphate per ml.

	Reduction time
Complete system	45 sec.
Complete without flavoprotein	∞
Complete wihout lactate	∞
Complete without coenzyme	∞

Table V. *Turnover number of flavoprotein in lactic system under anaerobic conditions*

Details as in Table IV.

Flavoprotein concentration in μg. flavin-phosphate	Reduction time in min.	T.N.
3·50	0·75	435
1·75	0·75	870
0·87	1·08	1180
0·53	1·58	1360
0·35	3·33	975
0·18	10	625

efficiency of flavoprotein as a catalyst would progressively increase on dilution beyond the saturating concentration. Table V shows that the T.N. first increases and then falls with successive dilutions of flavoprotein. This fall in the T.N. can only mean destruction of flavoprotein under the conditions of the experiment. Below 0·05 μg. flavinphosphate equivalent per ml. no catalytic effect can be demonstrated under anaerobic conditions, whereas aerobically a measurable effect can be obtained with 0·005 μg. We have no explanation for the anaerobic destruction of flavoprotein.

Heart flavoprotein also catalyses the oxidation of dihydrocoenzyme II by methylene blue. The hexosemonophosphoric enzyme system was used as the reducing system for coenzyme II. The flavoprotein-free enzyme was prepared from rabbit skeletal muscle by the following unpublished method of one of us (H. S. C.). Acetone powder of rabbit skeletal muscle was mixed with water (2 vol.), stirred to a homogeneous paste for 20 min., and the mixture was strained through muslin. The filtrate was centrifuged and dialysed. Coenzyme II was prepared from horse red blood corpuscles by the method of Warburg & Christian [1933]. One of us (H. S. C.) has established that the hexosemonophosphoric enzyme of rabbit skeletal muscle specifically collaborates with coenzyme II. Table VI shows the catalytic effect of heart flavoprotein on the reaction between

Table VI. *Effect of flavoprotein on hexosemonophosphoric enzyme system*

Enzyme (ml.)	2·0	2·0	2·0	2·0
0·1% crude coenzyme II (ml.)	0·5	—	0·5	0·5
0·3 *M* hexosemonophosphate (ml.)	0·5	0·5	—	0·5
Flavoprotein (ml.) (17 μg. flavinphosphate/ml.)	0·2	0·2	0·2	—
0·0125 *M* methylene blue (ml.)	0·1	0·1	0·1	0·1
M/2 phosphate buffer *p*H 7·2 (ml.)	0·3	0·3	0·3	0·3
Water	—	0·5	0·5	0·2
Reduction time of methylene blue in min.	4	∞	∞	∞

dihydrocoenzyme II and methylene blue. The activity of the enzyme preparation is too low to permit a more extensive investigation of the kinetics of coenzyme II systems. We have not therefore pursued this line further.

II. Mechanism of the catalysis

On addition of a comparatively minute amount of the lactic enzyme system (i.e. enzyme, lactate, cyanide and coenzyme I) to a concentrated solution of heart flavoprotein the yellowish green colour is discharged within a few minutes at 16°. If any of the components of the lactic system is eliminated no decoloration is observed. The reduced leuco form is slowly autoxidizable as shown by the fact that shaking with air gradually restores the original colour (within a minute). Hydrosulphite also bleaches the colour of heart flavoprotein. Shaking

with air restores the colour as soon as excess hydrosulphite is removed. There is an apparent discrepancy in that leucoflavoprotein prepared enzymically is sluggishly autoxidizable, whereas when prepared by hydrosulphite reduction it is practically instantaneously oxidized by molecular oxygen. Keilin & Hartree [1936], in their study of catalase, observed that hydrosulphite yields H_2O_2 when oxidized by air. The production of H_2O_2 might well explain the difference in the rates of autoxidation under the two sets of conditions. To decide the question whether flavoprotein is reduced and oxidized in the course of its catalysis the following experiment was carried out. Two Thunberg tubes were filled with 1·0 ml. of a flavoprotein solution containing 31 μg. flavinphosphate equivalent and 0·5 ml. of the enzyme system (0·1 ml. purified enzyme, 0·1 ml. M lactate, 0·1 ml. 0·05 % coenzyme I and 0·1 ml. M HCN). One of the tubes also contained 0·05 ml. of 0·125M methylene blue. The times for the anaerobic decoloration of flavoprotein and methylene blue were 6 and 61 min. respectively at 18°. If the catalytic action of heart flavoprotein involves a cycle of reduction by dihydrocoenzyme and oxidation by methylene blue, the ratio

$$\frac{\mu\text{l. } H_2 \text{ transferred to methylene blue}}{\mu\text{l. } H_2 \text{ equivalent of flavoprotein}}$$

should be equal to the ratio

$$\frac{\text{reduction time of methylene blue}}{\text{reduction time of flavoprotein}}.$$

The value of the first ratio was $14/1{\cdot}52 = 9{\cdot}2$ and that of the second was $61/6 = 10{\cdot}2$. Clearly the catalytic reduction of methylene blue can be entirely accounted for on the basis of the cyclical reduction and oxidation of flavoprotein.

Oxidized heart flavoprotein shows an intense greenish fluorescence on irradiation with ultraviolet light. Addition of the lactic enzyme system leads to the gradual disappearance of the fluorescence. Disappearance of fluorescence runs parallel with the formation of the reduced form. The fluorescence method is eminently suitable for following the reduction of heart flavoprotein.

The use of comparatively large concentrations of flavoprotein and minute amounts of the reducing enzyme system is merely a device to obtain a "slow motion" picture of the reduction of flavoprotein. Under physiological conditions the concentration of flavoprotein is minute compared with the concentration of coenzyme I. On mixing 30 μg. flavinphosphate equivalent of flavoprotein with 190 μg. of dihydrocoenzyme I at room temperature the yellow-green colour and green fluorescence of the oxidized form disappear almost instantaneously.

III. Identity of heart flavoprotein and coenzyme factor

Coenzyme factor (diaphorase) is characterized by the following properties [cf. Euler & Hellström, 1938; Dewan & Green, 1938]. It occurs in association with insoluble particles from which it cannot be removed merely by washing the particles with water or salt solutions at neutral pH. Above 55° or in solutions below pH 4·6 and above pH 9 the enzyme is rapidly inactivated. Furthermore, fairly active and purified preparations of the enzyme seem not to contain any appreciable amounts of flavin. On the other hand heart flavoprotein is a soluble flavoprotein which is destroyed by temperatures not lower than 80°. It is also unstable below pH 4 and above pH 9. At first sight the resemblance between coenzyme factor and heart flavoprotein seems meagre, to say the least. In fact from the brief description of their respective properties one would be tempted to conclude that they were two distinct compounds. These differences however are more apparent than real.

The starting point of the isolation of heart flavoprotein is the standard preparation of coenzyme factor. That is to say, the flavoprotein is found initially associated with insoluble particles from which it cannot be removed by exhaustive washing with water. By exposure of this enzyme suspension to 2% $(NH_4)_2SO_4$ and 3% ethyl alcohol at *p*H 4·6 and 43° the flavoprotein becomes separated from the particles (cf. Straub [1939, 2] for complete details) and behaves thereafter as a soluble protein. Significantly, when the flavoprotein is brought into solution the soluble extract shows very high coenzyme factor activity.

The prosthetic group of heart flavoprotein is flavinadenine dinucleotide. On boiling a solution of the flavoprotein the dinucleotide is liberated from the coagulated protein and can be estimated in the amino-acid test system. We should expect that if the coenzyme factor is identical with heart flavoprotein the ratio $\frac{\text{coenzyme factor activity}}{\text{dinucleotide concentration}}$ would be of the same order of magnitude for both. The following is the protocol of a typical experiment. 0·5 ml. of a pig heart flavoprotein solution containing 1·64 μg. flavinphosphate equivalent per ml. was found to have approximately the same activity in catalysing the oxidation of reduced coenzyme I by methylene blue (lactic enzyme system) as 0·5 ml. of a standard coenzyme factor preparation from pig heart. The respective O_2 uptakes were 238 and 231 μl. O_2 in 5 min. When the same amounts of boiled flavoprotein and factor solutions were tested with the amino-acid oxidase the respective oxygen uptakes were 82 and 79 μl. O_2. Taking the oxygen uptake in the lactic system as a measure of coenzyme factor activity and the O_2 uptake in the amino-acid oxidase test as a measure of the dinucleotide concentration, the ratio

$$\frac{\text{coenzyme factor activity}}{\text{dinucleotide concentration}}$$

can be equated with the ratio

$$\frac{\mu\text{l. } O_2 \text{ in lactic system}}{\mu\text{l. } O_2 \text{ in amino-acid oxidase system}}.$$

The respective ratios for coenzyme factor and flavoprotein were 2·92 and 2·90. Clearly there was sufficient flavoprotein in the heart factor preparation to account for all the catalytic activity shown in the lactic enzyme system.

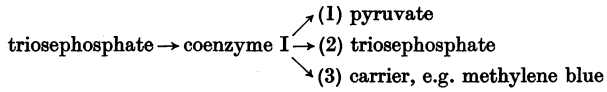
The amino-acid oxidase was prepared free of flavin dinucleotide by the following modification of the method of Straub [1938]. Acetone powder of pig kidney was rubbed up with 10 vol. water and the mixture centrifuged after 20 min. standing. The supernatant was made 33% saturated with respect to $(NH_4)_2SO_4$, cooled to 0° and brought to *p*H 4·6. The precipitate was centrifuged and redissolved in water. This procedure was repeated twice. The extract of 10 g. was finally made up to 30 ml. in *M*/5 phosphate buffer, *p*H 7·2. One ml. of this enzyme takes up *ca.* 150 μl. O_2/10 min. at 38° in presence of excess flavinadenine dinucleotide (3 μg. per manometric cup). In absence of dinucleotide there is a blank of *ca.* 20–30 μl. O_2/10 min. which disappears after the enzyme preparation has been kept at 0° for about 24 hr. The enzyme is stable for weeks at 0° and can be kept free of bacterial infection by saturation with octyl alcohol. 1 ml. of the enzyme works at half the maximum rate in presence of *ca.* 1 μg. of dinucleotide. For purposes of estimation the concentration of dinucleotide should be such as to cover the range from *ca.* 40 to 80 μl. O_2/10 min. The following quantities were used: 1 ml. enzyme, 1 ml. buffer, *p*H 7·2 and 0·2 ml. *M dl*-alanine. The gas space contained air.

The inability of various investigators to detect flavin in the factor preparations of various tissues is easily explained by the following considerations. The standard coenzyme factor preparations contain on the average *ca.* 1·5 μg. of flavinphosphate per ml. The method of detecting flavin involves treating the enzyme preparation with 3 vol. methyl alcohol and centrifuging off the denatured

protein. The supernatant fluid should be yellow if flavin is present. It is clear that even if all the flavin were split off by the methyl alcohol treatment, the concentration of flavin in the methyl alcohol solution (*ca.* 0·4 μg. flavinphosphate per ml.) would be beyond the limit of visual detection. Heart flavoprotein solutions diluted so as to contain the same catalytic activity per ml. as the standard coenzyme factor preparations do not show any detectable colour and the presence of flavin dinucleotide is just detectable in the amino-acid oxidase test system.

The ratio $\frac{\mu\text{l. } O_2 \text{ in lactic system}}{\mu\text{l. } O_2 \text{ in amino-acid oxidase system}}$ has been determined for coenzyme factor preparations from *Bact. coli*, rabbit kidney and liver. The respective ratios were 3·0, 2·8 and 1·9, as compared with a ratio of 2·9 for heart flavoprotein. The low value of the ratio for liver coenzyme factor preparations points to the presence of one or more additional flavoproteins which do not catalyse the oxidation of reduced coenzyme I. A coenzyme factor preparation made from yeast by the method of Dewan & Green [1938] was anomalous in that the amount of flavin dinucleotide was barely measurable although high activity was shown in the lactic enzyme test. This can mean either that the coenzyme factor of yeast is a flavoprotein with higher catalytic activity than that of animal tissues or that the active principle in the yeast preparations is not a flavoprotein.

Triosephosphate in presence of the aqueous extract of the acetone powder of rabbit skeletal muscle can undergo oxidation via three different mechanisms:



The arrows indicate the direction of transfer of hydrogen. In mechanisms (1) and (2) reduced coenzyme becomes oxidized by pyruvate in presence of the lactic enzyme or by triosephosphate in presence of the α-glycerophosphoric enzyme. The net changes are in (1) the oxidation of triosephosphate by pyruvate, and in (2) the dismutation of triosephosphate to the corresponding alcohol and acid. In mechanism (3) reduced coenzyme I is oxidized by some carrier—the net change being the oxidation of triosephosphate by oxygen via the coenzyme and carrier. Whether mechanism (3) will be realized at the expense of (1) and (2) depends on the relative velocities with which reduced coenzyme I is oxidized in the different reactions. In presence of the coenzyme factor and methylene blue, it is possible to obtain a comparatively rapid reaction between triosephosphate and molecular O_2. The Warburg-Christian flavoprotein shows practically no activity in the system even in high concentration. Presumably the coenzyme factor is able to catalyse the oxidation of reduced coenzyme I by methylene blue with a velocity of the same order of magnitude as that with which the lactic or glycerophosphoric enzymes catalyse the reduction of their respective substrates by dihydrocoenzyme I. In other words, the coenzyme factor must be as efficient a catalyst as the lactic and α-glycerophosphoric enzymes in order to compete with them for the available dihydrocoenzyme I. The inactivity of the Warburg-Christian flavoprotein in the triosephosphoric system simply means that its catalytic efficiency is not high enough to compete with the other enzymes for dihydrocoenzyme. It is significant that heart flavoprotein can replace coenzyme factor in the triosephosphoric test system and shows the same order of catalytic efficiency.

Both coenzyme factor and heart flavoprotein agree in the following properties. They are both destroyed to the extent of *ca.* 50 % by exposure to

solutions of *p*H 3·8 for 7 min. at 18°, and to the extent of *ca.* 90 % by exposure to solutions of *p*H 9·2 for 7 min. at 18°. At 55° and *p*H 7·0 a preparation of coenzyme factor loses 55 % of its activity after 10 min. exposure, whereas heart flavoprotein is half destroyed at about 85°. Were it not for the almost complete correspondence in other details between the properties of the two compounds, this formidable discrepancy in temperature lability could be used as definite proof of their non-identity. The coenzyme factor is associated with particles whereas the flavoprotein is soluble. Change in physical state might account for some difference in temperature-lability. It is also conceivable that the destruction of the coenzyme factor at 55° might be due to causes other than denaturation, e.g. hydrolysis by proteolytic enzymes associated with the insoluble particles. There is in fact evidence for this view. The instability of preparations of coenzyme factor extends even to temperatures as low as 38°. Exposure of the factor preparations of pig heart to 38° for 60 min. at *p*H 7·0 destroys some 30 % of their activity.

We may summarize the comparison of coenzyme factor and flavoprotein of heart as follows. The catalytic activity of both is proportional to the concentration of flavin dinucleotide. The respective catalytic activities are qualitatively and quantitatively identical. The starting point for the preparation of the soluble flavoprotein is the suspension of insoluble particles with which coenzyme factor is associated. Thus flavoprotein becomes soluble only as the result of special treatment of the suspension of insoluble particles. The two catalysts are destroyed below *p*H 4 and above *p*H 9 with equal velocities. They differ however in temperature-lability. On the whole the evidence is strongly in favour of the view that they are one and the same compound in two different physical states.

Summary

Heart flavoprotein catalyses the oxidation of the dihydropyridinenucleotides by "carriers" such as methylene blue. The mechanism of the catalysis involves a cycle of reduction of the flavoprotein by dihydrocoenzyme and oxidation of its leuco form by the "carrier". Leucoflavoprotein is sluggishly autoxidizable. Under optimum conditions each molecule of flavoprotein catalyses the oxidation of 8500 molecules of dihydrocoenzyme I per min.

Coenzyme factor (diaphorase) is considered to be identical with heart flavoprotein. Whereas the former is found in association with insoluble particles, the latter has been separated from the particles and brought into solution.

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