LXXXIII. COENZYME FACTOR—A NEW OXIDATION CATALYST¹

BY JOHN GEORGE DEWAN AND DAVID EZRA GREEN²

From the Institute of Biochemistry, Cambridge

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REDUCED coenzyme I reacts slowly with carriers such as methylene blue, flavin, cytochrome *c*, etc. In presence of flavoprotein these reactions are considerably accelerated. Our previous studies on the lactic, malic and β -hydroxybutyric dehydrogenases of animal tissues [cf. Green & Brosteaux, 1936; Green, 1936; Green *et al.* 1937, 1] have shown that the reaction between reduced coenzyme and carriers was extremely rapid in presence of the dehydrogenase solutions. Euler and his colleagues in a long series of papers [cf. Euler, 1936 for a resumé] have maintained that flavoprotein was the catalyst in dehydrogenase preparations of animal origin and that flavoprotein was in fact an indispensable component of all coenzyme dehydrogenase systems. Their conviction of the essential nature of flavoprotein led them to classify all coenzyme dehydrogenase systems as "Flavinenzym Systemen". Our own experience has been in direct contradiction to this interpretation of the Euler school. We have consistently failed to obtain any evidence either of the presence of any detectable amounts of flavoprotein in any of our preparations or of any significant catalytic action of flavoprotein when added to the complete dehydrogenase systems. In the analysis of the β -hydroxybutyric dehydrogenase of pig heart [cf. Green *et al.* 1937, 1] we were able to show indirectly that the dehydrogenase preparation contained an enzyme which catalysed the oxidation of reduced coenzyme by molecular oxygen. Later the existence of the "coenzyme oxidase" as this enzyme was called, was established beyond any doubt by a direct spectrophotometric method [cf. Green & Dewan, 1937].

The full implications of the "coenzyme oxidase" were not clear to us until we had occasion to compare the properties of mutases and dehydrogenases. The mutases are enzymes which catalyse the oxidation of their substrates by means of α -ketonic acids [cf. Green *et al.* 1937, 2]. Coenzyme I is an essential component of all the known mutase systems. The triosephosphate mutase for example catalyses the oxidation of triosephosphate by oxaloacetate. The mechanism of this oxidoreduction consists in the coenzyme undergoing a cycle of reduction by triosephosphate and oxidation by the ketonic acid. This is in effect a coenzyme-linked reaction between two dehydrogenase systems, viz. the triosephosphate and the malic dehydrogenases. It should therefore be possible to use the triosephosphate mutase as a typical coenzyme dehydrogenase and, by replacing oxaloacetic acid as oxidant with methylene blue or flavin, to obtain the oxidation of reduced coenzyme by these carriers. However, the results of such experiments were invariably negative. It was thus clear that the mutase preparations lacked the agent necessary for catalysing the oxidation of reduced coenzyme. When small amounts of the usual dehydrogenase preparations were added to that of the mutase, vigorous oxidation of triosephosphate via coenzyme and carrier took

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² Beit Memorial Research Fellow.

place. Clearly our dehydrogenase preparations supplied the missing coenzyme factor. The question naturally arose whether dehydrogenases freed from the factor would be incapable of catalysing the oxidation of reduced coenzyme by carriers. Such was indeed found to be the case.

The oxidation of metabolites in coenzyme-dehydrogenase systems may be regarded as a two-stage process: (1) oxidation of the substrate and reduction of coenzyme, and (2) oxidation of the coenzyme and reduction of the carrier. The dehydrogenase catalyses the oxidation of the substrate; the coenzyme factor catalyses the oxidation of the reduced coenzyme.

The relation between the coenzyme factor and the "coenzyme oxidase" referred to above can now be easily understood. "Coenzyme oxidase" is a complex system containing the following components: (1) coenzyme factor, (2) cytochromes *a* and *b* and (3) cytochrome oxidase. The coenzyme factor catalyses the oxidation of reduced coenzyme by cytochromes *a* and *b*, and the cytochrome oxidase catalyses the oxidation of the reduced cytochromes by molecular oxygen. Thus in the reaction of coenzyme dehydrogenase systems with molecular oxygen three catalysts are involved in the following sequence:

The arrows indicate the direction of transfer of hydrogen. Since the last stage is cyanide-sensitive the overall reaction is also cyanide-sensitive.

Recently Adler *et al.* [1937], following on the original observations of Green *et al.* [1937, 1] and Green & Dewan [1937], have presented confirmatory evidence of the existence of the coenzyme factor in animal tissues. Adler *et al.* seem to have made the discovery quite independently; from their preliminary report they appear to have made substantial progress in the study of the coenzyme factor.

I. The catalytic oxidation of reduced coenzyme I

A solution of reduced coenzyme I can be prepared by adding hydrosulphite to a bicarbonate solution of the oxidized coenzyme, incubating for 15 min. at 38° and then aerating vigorously for several hours to convert the residual hydrosulphite or sulphite to sulphate [for details of procedure cf. Green & Brosteaux, 1936; and Green & Dewan, 1937]. The reaction between reduced coenzyme and methylene blue carried out anaerobically in Thunberg tubes is rather slow (Table I). On addition of a phosphate extract of washed and ground

Table I. *The reaction between reduced coenzyme I and hydrogen acceptors*

	Reduction time in min.
Reduced coenzyme (10 mg.) + methylene blue (1 mg.)	14
Reduced coenzyme + methylene blue + crude muscle extract (2.5 mg.)	0.5
Reduced coenzyme + methylene blue + boiled muscle extract (2.5 mg.)	12
Reduced coenzyme + methylene blue + flavoprotein (2 mg.) (2 mg. $\approx$ 12 γ lactoflavin phosphate)	1.0
Reduced coenzyme (10 mg.) + flavin (1 mg.)	40
Reduced coenzyme + flavin + muscle extract (2.5 mg.)	4

skeletal or cardiac muscle, the reduction of methylene blue is a matter of a few seconds. The catalytic effect disappears when the extract is previously boiled. Flavoprotein shows the same effect, but there is no means of comparing the relative efficiencies of the two catalysts in absence of data on the molecular

strength of the coenzyme factor. Methylene blue can be replaced with flavin or cytochrome and the catalytic effect of the factor is substantially the same.

II. *Properties of the factor*

The most convenient sources for preparation of the coenzyme factor are rabbit skeletal muscle and pig heart. The following procedure is employed in the two cases. The fresh muscle tissue (*ca.* 400 g.) is minced in a Latapie and washed exhaustively with tap water. For this purpose it is best to suspend the mince in about 10 l. of water and to stir vigorously for 5 min. The suspension is filtered through muslin and the wash water discarded. The process is repeated six times. The thoroughly washed mince is then mixed with sand and 300 ml. *M*/50 phosphate buffer *pH* 7.2, and ground to a fine paste in a mechanical mortar. The mixture is squeezed through muslin—the pulp being discarded. The filtrate is centrifuged for a minute to sediment fine particles of sand or cellular debris. The solution is then brought to *pH* 4.6 (just yellow to chlorophenol red) by cautious addition of acetic acid. The precipitate is centrifuged and the supernatant fluid is discarded. If the precipitation is carried out correctly the sedimentation is sharp and no difficulty is experienced in pouring off the supernatant. The precipitate is suspended in 500 ml. *M*/50 phosphate buffer and ground in a mortar to break up any lumps. The suspension is then centrifuged at 3000 r.p.m. for 20 min. The supernatant fluid is discarded. This washing procedure is repeated at least three times in order to remove the soluble dehydrogenases which adhere mechanically to the coenzyme factor. The thrice washed sediment is finally suspended in 100 ml. *M*/10 phosphate buffer *pH* 7.2.

As the method of preparation indicates, the coenzyme factor is associated with insoluble particles which can be easily centrifuged out of suspension and thus separated from the soluble dehydrogenases. Successive washing by suspension and centrifuging is necessary because of the tendency of the soluble dehydrogenases to adhere to the insoluble particles. The final suspension contains high coenzyme factor activity and little or no dehydrogenase activity. Unfortunately with prolonged washing there is a fall in factor activity—thus setting a limit to the amount of purification which can be effected by the above procedure.

Comparison of the activities of the suspensions before and after washing shows that about 60% of the original activity has disappeared in the course of the manipulations. This loss is not due to destruction. Just as is the case for other insoluble enzymes, the particles with which the coenzyme factor is associated show all gradations of size. The gross particles are easily centrifuged whereas some of the smaller particles are so highly peptized that only prolonged centrifuging at 20,000 r.p.m. suffices to sediment them.

The separation of the soluble dehydrogenases from the coenzyme factor is technically much simpler than the converse. Fractional centrifuging or filtration is ineffectual because of the tendency of the coenzyme factor to form highly peptized suspensions. Thus far we have been unable to find any one procedure which is successful in all cases. But in general the following treatments either alone or in combination have been used to separate the coenzyme factor from the soluble dehydrogenases: (1) treating for 15–30 min. at 55°, (2) acetone precipitation and drying and (3) filtration through a pad of charcoal and kieselguhr. The efficiencies of these three methods depend upon the relative proportions of the dehydrogenase and the factor. If the dehydrogenase is in excess, destruction of the dehydrogenase is small compared with that of the factor. But with a relatively small amount of dehydrogenase these treatments are obviously unsuitable. Heating to 52–55° for at least 15 min. invariably destroys

the coenzyme factor. It is inadvisable to centrifuge off the precipitate which forms during the heat treatment as there is a tendency for active dehydrogenase to be carried down as well. Precipitation of the dehydrogenase solution with acetone at 0° does not destroy the factor so much as render it insoluble. The dried acetone power is rubbed up with buffer and the suspension is centrifuged hard. This procedure effects considerable separation. Unfortunately, some of the dehydrogenases are destroyed by acetone precipitation. This treatment is not therefore of general application. Filtration through a pad of charcoal and Kieselguhr (equal proportions) clarifies crude dehydrogenase solutions and removes a great deal of the factor activity. But in some cases the dehydrogenase is correspondingly removed.

III. The factor effect with the mutases

The triosephosphate and triose mutases are prepared by acetone precipitation of the aqueous extracts of minced but unground rabbit skeletal muscle (cf. Green *et al.* [1937, 2] for details). The virtue of these mutases as experimental material lies in the fact that the original extract from which they are prepared contains at most traces of the coenzyme factor.

Triosephosphate mutase.

The mutase solution in presence of coenzyme I, hexosediphosphate (i.e. triosephosphate) and methylene blue does not absorb any oxygen. The factor solution in presence of the same components is similarly inactive. But with mutase and factor solution together a vigorous oxidation ensues (cf. Fig. 1).

Fig. 1. Factor effect with triosephosphate mutase.

The complete system contained 1.5 ml. mutase (skeletal muscle of rabbit), 0.7 ml. coenzyme factor (pig heart), 0.7 ml. 0.15% coenzyme I, 0.2 ml. 0.5% methylene blue and 0.3 ml. $M/10$ hexosediphosphate. The centre pot of the manometer was filled with KOH paper to absorb CO_2 .

The mutase solution provides the catalyst for the reduction of the coenzyme; the factor solution provides the catalyst for the oxidation of reduced coenzyme at the expense of the reduction of methylene blue. The significance of the factor effect can be demonstrated in a striking way by carrying out the experiment in Thunberg tubes (cf. Table II).

Table II. *Factor effect with the triosephosphate mutase*

The complete system contained 1 ml. factor, 1 ml. mutase, 1 ml. 0.15% coenzyme I, 0.2 ml. *M*/10 hexosediphosphate and 0.3 ml. 0.5% methylene blue.

	Methylene blue, reduction time in min.
Complete system	2
Without mutase	∞
Without factor	∞
Without substrate	∞
Without coenzyme	80

Fig. 2. The effect of varying the amount of coenzyme factor.

The following quantities were used in each manometer: 0.5 ml. mutase, 1 ml. 0.15% coenzyme I, 0.3 ml. 0.5% methylene blue, 0.4 ml. *M*/10 pyrophosphate and 0.3 ml. *M*/10 hexosediphosphate. The centre pots were filled with KOH paper to absorb CO₂. Both factor and mutase were prepared from rabbit skeletal muscle.

Fig. 3. The effect of varying the amount of mutase.

Each manometer contained 1.5 ml. factor, 1 ml. 0.15% coenzyme I, 0.3 ml. 0.5% methylene blue, 0.4 ml. *M*/10 pyrophosphate and 0.3 ml. *M*/10 hexosediphosphate. The centre pots were filled with KOH paper to absorb CO₂. Both factor and mutase were prepared from rabbit skeletal muscle.

If there are two catalysts involved in the oxidation of triosephosphate by methylene blue, we should expect that with a constant amount of dehydrogenase the velocity of oxidation would be proportional to the amount of coenzyme factor and vice versa. Figs. 2 and 3 show that such is indeed the case. It is obvious from the curves that the maximum activity of a given amount of dehydrogenase is independent of the amount of factor beyond a critical saturation concentration. In other words, these two catalysts although collaborating in series are independent of one another as far as their catalytic activity is concerned.

The factor solution used in the above experiments was tested for the presence of flavin as follows. The solution was centrifuged hard until the supernatant was water clear. The sediment was then shaken mechanically for 24 hr. with a mixture of 3 parts methyl alcohol to 1 part of water. The methyl alcohol extract of an amount of factor solution equivalent to 300 g. of fresh tissue contained no trace of flavin. As a control a solution of flavoprotein was tested for flavin under the same conditions and the extraction of flavin was complete. There can be no question therefore of the factor effect being due to traces of flavoprotein. But more important is the fact that flavoprotein has little activity as factor in the reconstructed system (cf. Table III). 1 mg. of crude heart factor has the same

Table III. *Flavoprotein as factor*

The system contained 1 ml. 0.15% coenzyme I, 0.3 ml. *M*/10 hexosediphosphate and 0.3 ml. 0.5% methylene blue. The other quantities were 1 ml. mutase, 1 crude heart factor and 1 ml. 2.5 mg. pure flavoprotein.

	Methylene blue, reduction time in min.
Mutase + system	∞
Mutase + factor + system	2
Mutase + flavoprotein + system	60
Mutase + factor + flavoprotein + system	2

activity as 70 mg. pure flavoprotein in the triosephosphate system and as 20 mg. in the malic system. Making the not unreasonable assumption that the purity of the crude factor solution is not > 1%, weight for weight the factor is at least 2000–7000 times as active.

That the coenzyme factor is not identical with flavoprotein is quite definite. But it would be dogmatic to conclude from the inability of flavoprotein to replace the factor under the conditions of our experiments that flavoprotein plays no catalytic role at all. Before anything can be decided about the role of flavoprotein in the oxidative events of animal tissues, it would be well to know whether the flavoprotein which has been isolated from bottom yeast by Warburg & Christian [1932] has any counterpart in animal tissues. Free flavin is indeed found in abundance but there is not the slightest evidence yet presented that the Warburg-Christian flavoprotein is distinct from unspecifically "bound flavin" exists in animal tissues.

Triose.

The triose mutase catalyses the oxidation of glyceraldehyde to glyceric acid [cf. Green *et al.* 1937, 2]. The following experiment shows the effect of factor. The complete system containing 0.5 ml. mutase, 1 ml. factor, 1 ml. coenzyme I and 0.5 ml. 1% glyceraldehyde reduced 1 mg. methylene blue in 5 min. Without mutase, triose or coenzyme no reduction took place. Without factor complete reduction was reached in 50 min. Although the triose mutase in our preparations

is extremely active when tested by the usual anaerobic mutase oxidoreductions, poor results are obtained in aerobic experiments with the full system which consists of mutase coenzyme, factor and methylene blue. It is possible that traces of peroxide formed during the aerobic oxidation destroy the enzyme. In a later section the results are given of aerobic experiments in which methylene blue is replaced by the cytochromes.

Lactic and malic.

Both the triosephosphate and triose mutases catalyse the oxidation of their substrates by means of α -ketonic acids such as pyruvic and oxaloacetic acids. Since it is possible by means of the factor to study the triosephosphate mutase as a triosephosphate dehydrogenase, it should similarly be possible to study the oxidizing half of the mutase as a lactic or malic dehydrogenase. Table IV contains

Table IV. *Factor effect with lactic and malic enzymes associated with the muscle mutase*

The complete system contained 1.5 ml. mutase, 0.5 ml. factor, 1.0 ml. 0.15% coenzyme I, 0.2 ml. 0.5% methylene blue, 0.2 ml. 2 *M* HCN and 0.2 ml. *M* lactate or malate. Factor prepared from pig heart was used in the lactic experiments whereas factor prepared from rabbit skeletal muscle was used in the malic experiments.

Oxygen uptake in μ l./10 min.

	Lactic	Malic
Complete system	276	78
Without mutase	5	0
Without factor	0	10
Without substrate	17	0
Without coenzyme	5	0

the results of typical experiments. When dealing with the lactic enzyme, heart factor was employed. But in the case of the malic enzyme, skeletal muscle factor was found to be more suitable. The choice of the factor solution has no relevance to the activity of the enzyme or to any possible differences between the factors prepared from the two sources. The factor solutions are always found to contain small amounts of dehydrogenases which defy the washing process. The amount in the final factor solution depends upon the concentration of the dehydrogenase in the original extract from the ground tissue. Thus since pig heart is relatively weak in the lactic dehydrogenase, the factor solution obtained from this source shows only a very slight lactic enzyme activity. Similarly, since the skeletal muscle of rabbit is relatively weak in the malic enzyme, the factor solution obtained from this source is suitable for tests with a malic enzyme. It is possible therefore by judicious selection of the source of factor to eliminate the blanks occasioned by the factor solution containing the particular dehydrogenase or mutase under study.

IV. *The factor effect with coenzyme dehydrogenases*

When rabbit skeletal muscle is minced and mixed with water, the extract is found to be rich in the mutases referred to above, but practically free of the coenzyme factor. Mere washing of the tissue is insufficient to extract the factor. The essential requirement for extraction is that the tissue should be finely ground. The same is true of the coenzyme dehydrogenases, e.g. the lactic, malic and β -hydroxybutyric enzymes. It was natural therefore that for a long time the coenzyme dehydrogenases were credited with properties which were really

those of the coenzyme factor. The study of the mutases showed that the properties of, for example, the lactic enzyme prepared from ground muscle were different from those of the lactic enzyme associated with the mutase. The former apparently could catalyse the oxidation of reduced coenzyme by methylene blue whereas the latter could not except in presence of the factor. But experiment soon showed that this difference was not real and that the lactic enzyme regardless of the method of preparation lacked the power of catalysing the oxidation of reduced coenzyme.

Tables V and VI show that the lactic, malic, β -hydroxybutyric, alcohol and hexosemonophosphate dehydrogenases when separated from the coenzyme

Table V. *Factor effect with lactic and malic dehydrogenases*

The malic dehydrogenase was prepared from pig heart as follows. The washed heart mince was ground with sand and phosphate. The mixture was filtered through muslin. The filtrate was cooled to 0° and mixed with 3 vol. cold acetone. The precipitate was dried with acetone and ether and resuspended in phosphate buffer. The mixture was centrifuged and the supernatant filtered with suction through a pad of charcoal and kieselguhr. The factor was prepared by the usual method from rabbit skeletal muscle.

The lactic dehydrogenase was prepared from rabbit skeletal muscle by the method of Green & Brosteaux [1936]. The preparation was centrifuged hard and the supernatant heated at 55° for 30 min. The factor used was prepared from pig heart.

The following quantities were used in both the lactic and malic experiments: 1 ml. dehydrogenase, 1 ml. factor, 0.2 ml. 2 *M* HCN, 0.4 ml. *M*/10 pyrophosphate, 0.05 ml. 0.5% methylene blue and 0.2 ml. *M* substrate.

Oxygen uptake in μ l./40 min.

	Lactic	Malic
Complete system	282	260
Without dehydrogenase	15	30
Without factor	40	30
Without substrate	0	0
Without coenzyme	0	0

Table VI. *The factor effect with the β -hydroxybutyric, alcohol and hexosemonophosphate dehydrogenases*

The β -hydroxybutyric dehydrogenase was prepared from pig heart by the usual method and heated at 55° for 15 min. The factor used was that of rabbit skeletal muscle. The complete system contained the following components: 1 ml. dehydrogenase, 1 ml. factor, 1 ml. 0.15% coenzyme I, 0.2 ml. *M*/2 hydrazine, 0.4 ml. *M*/10 pyrophosphate, 0.2 ml. *M dl*- β -hydroxybutyrate and 0.05 ml. 0.5% methylene blue.

The alcohol dehydrogenase was prepared from horse liver by the method of Dixon & Lutwak-Mann [1937]. The factor used was that of rabbit skeletal muscle. The complete system contained 1.5 ml. dehydrogenase, 1 ml. factor, 1 ml. 0.15% coenzyme I, 0.2 ml. 0.5% methylene blue and 0.4 ml. 98% ethyl alcohol.

The hexosemonophosphate dehydrogenase was prepared from the skeletal muscle of the rabbit by the same procedure as for the lactic enzyme. The factor used was that of rabbit skeletal muscle. The complete system contained 1 ml. dehydrogenase, 1.5 ml. factor, 0.2 ml. 0.5% methylene blue, 0.3 ml. *M*/3 hexosemonophosphate and 1 ml. 3% crude coenzyme II prepared from horse corpuscles.

Methylene blue, reduction time in min.

	β -Hydroxy- butyric	Alcohol	Hexosemono- phosphate
Complete system	5	0.5	10
Without factor	∞	∞	∞
Without dehydrogenase	∞	∞	∞
Without substrate	∞	25	∞
Without coenzyme	∞	∞	∞

factor are unable to catalyse the oxidation of their respective substrates by means of methylene blue. The lactic and hexosemonophosphate enzymes were prepared from ground and washed rabbit skeletal muscle by the methods described in previous communications, the malic and β -hydroxybutyric enzymes were prepared from pig heart muscle and the alcohol enzyme was obtained from horse liver by a method similar to that of Dixon & Lutwak-Mann [1937]. The detailed purification necessary for eliminating the factor is given in connexion with the appropriate table. Oxygen uptakes were measured whenever the enzyme studied was sufficiently active. With enzymes of comparatively weak activity, the methylene blue technique was employed. The hexosemonophosphate dehydrogenase of skeletal muscle requires the presence of coenzyme II. The strikingly positive factor effect shows clearly that the factor catalyses the oxidation of reduced coenzyme II, and that even in the case of coenzyme II dehydrogenases flavoprotein is not an essential component.

It will be observed that pyrophosphate is added in many cases in addition to the enzymes, coenzyme and methylene blue. Green *et al.* [1937, 1] showed that pyrophosphate exerted a stabilizing effect on the β -hydroxybutyric dehydrogenase in the sense that the initial velocity was maintained for a longer period in presence of the reagent than in its absence. We have found that this peculiar effect of pyrophosphate is shown in the cases of practically all coenzyme-dehydrogenase systems. Pyrophosphate is, of course, not necessary for demonstrating the factor effect but it is of value in prolonging the period during which the enzymes still retain catalytic activity.

V. *Distribution of the factor*

We have not as yet carried out any systematic investigations of the distribution of the coenzyme factor in animal tissues. Some experiments with the tissues of the rabbit show that the enzyme is present in brain, heart, skeletal muscle, kidney and liver. The first three tissues were rich in the coenzyme factor, whereas liver and kidney contained smaller amounts.

The factor is prepared from the various tissues by the method outlined in section II. The presence or absence of the factor is determined by the catalytic effect on the reaction between reduced coenzyme and methylene blue (cf. section I).

VI. *The reaction of coenzyme systems with cytochromes a and b*

The triosephosphate mutase system in presence of the factor from pig heart takes up oxygen in absence of any added carrier. On addition of methylene blue or pyocyanine this uptake is doubled or trebled. These facts are consistent with the view that the factor solution contains a natural carrier albeit in small concentration. Green *et al.* [1937, 1] have already observed this direct reaction with molecular oxygen in absence of added carrier in the cases of the lactic, malic and β -hydroxybutyric dehydrogenases. They used the preparation from pig heart as the source of these enzymes. The direct reaction with oxygen was inhibited completely by $M/100$ HCN. Furthermore the enzyme preparation after precipitation with acetone failed to react with oxygen unless some carrier was added.

Spectroscopic examination of the factor solution prepared from pig heart discloses the presence of cytochromes *a* and *b*. A mixture of the triosephosphate mutase, heart factor and coenzyme I shows no bands of the reduced cytochromes. On addition of triosephosphate the bands of reduced cytochromes *a* and *b* appear instantaneously (cf. Table VII). This experiment can be repeated with lactic and

Table VII. *Reduction of cytochromes a and b by the triosephosphate mutase-factor system*

The experiment was carried out in evacuated Thunberg tubes at room temperature. The reduction was observed with a microscope-spectroscope using a Pointolite as source of illumination.

	1	2	3	4
Mutase	0.1	—	0.1	0.1
Factor	2.0	2.0	2.0	2.0
Coenzyme	0.2	0.2	0.2	—
M/10 hexosediphosphate	0.3	0.3	—	0.3
Water	—	0.1	0.3	0.2
Reduction time of cytochromes a and b	Instantaneous	Partial reduction in 10 sec.	∞	∞

malic acids and glyceraldehyde as substrates with the same results. The mutase solution provides the enzyme for reducing the coenzyme while the factor catalyses the reaction between reduced coenzyme and the cytochromes.

The factor solution prepared as in section II is relatively weak in the cytochromes. Prof. D. Keilin and Dr Hartree have kindly informed us of a simple method of obtaining a factor solution extremely rich in cytochromes *a* and *b*. The thoroughly washed and minced heart muscle is ground in a mechanical mortar for *ca.* 2 hr. until a completely homogeneous cream is formed. Instead of straining the mixture through muslin as in the usual preparation, the cream is centrifuged and the top fluid part is poured off and brought to pH 4.6. The sediment after precipitation is resuspended in buffer and used directly. Since this method does not involve prolonged washing of the insoluble particles the final preparation has high lactic, malic and β -hydroxybutyric activities. Fortunately, triosephosphate and triose activities are practically nil. It should be pointed out that cytochromes *a* and *b* as well as the cytochrome oxidase and the factor are all associated with insoluble particles and invariably accompany one another.

Figs. 4 and 5 show the complete cytochrome reconstructions for the triosephosphate and triose systems respectively. The velocities of these reactions are much higher than those in presence of methylene blue, flavin or pyocyanine as carriers. Furthermore, the reactions are more linear with time and fail to show the rapid falling off in activity which characterizes experiments with artificial carriers. From a rough estimation of the amount of cytochrome present in the factor preparation, it would appear that each molecule of cytochrome is reduced and oxidized at least a thousand times per minute.

Cytochrome *c* appears to take no part in the reaction between reduced coenzyme and oxygen. Addition of comparatively huge amounts to the reconstructed system has no effect on the velocity. This is in agreement with the fact that the reaction between reduced coenzyme and cytochrome *c* is extremely slow compared with the reaction with cytochromes *a* and *b*. When the concentration employed of cytochromes *a* and *b* is considerable as in the reconstruction experiments, the addition of other carriers such as methylene blue has no influence on the velocity.

Table VIII shows that the other coenzyme systems can react vigorously with oxygen via the cytochrome path. Since the factor solution already contains the malic and β -hydroxybutyric enzymes, only substrate, coenzyme and ketone fixative need be added. In the case of the experiment on the lactic enzyme mutase solution is also added in order to ensure an adequate concentration of the lactic enzyme.

The diagnostic test of the working of the cytochrome-cytochrome oxidase system is the sensitivity to low concentrations of cyanide. It is obviously impossible to use cyanide as an inhibitor in presence of glyceraldehyde or triosephosphate because of irreversible cyanohydrin formation and consequent

Fig. 4. The direct reaction between the triosephosphate system and molecular oxygen in absence of added carrier.

The following quantities were used in the complete system: 1 ml. mutase, 1.5 ml. factor, 1 ml. 0.15% coenzyme I, 0.4 ml. *M*/10 pyrophosphate and 0.3 ml. *M*/10 hexosediphosphate. The centre pot of each manometer was filled with KOH paper to absorb CO_2 . The mutase was prepared from rabbit skeletal muscle and the factor from pig heart by the special method of Keilin and Hartree.

Fig. 5. The direct reaction between the triose system and molecular oxygen in absence of added carrier.

Details as for Fig. 4 except that 0.2 ml. 5% *dl*-glyceraldehyde was used as substrate.

removal of free cyanide. *M*/100 cyanide completely inhibits the oxidation of lactic acid whereas *M*/500 inhibits to the extent of about 65%. The cytochrome oxidase, however, should be completely poisoned at *M*/1000 cyanide concen-

Table VIII. *The reaction of coenzyme systems with molecular oxygen via cytochromes a and b*

The complete system in all cases contained 1.5 ml. factor, 1 ml. 0.15% coenzyme I, 0.4 ml. *M*/10 pyrophosphate, 0.2 ml. *M*/2 hydrazine and 0.2 ml. *M* substrate. In the case of the lactic experiment 0.5 ml. of muscle mutase was also added to provide additional enzyme.

	$\mu\text{l. O}_2$ in 40 min.
Complete lactic system	542
Without coenzyme	0
Without substrate	10
With <i>M</i> /100 HCN	40
With <i>M</i> /500 HCN	181
Complete malic system	290
Without coenzyme	0
With <i>M</i> /100 HCN	25
Complete β -hydroxybutyric system	340
Without coenzyme	0
With <i>M</i> /100 HCN	27

tration. For some time we considered this discrepancy to mean that the cytochrome oxidase was not involved in the direct reaction with oxygen. But further consideration has disclosed a valid reason for this discrepancy. Lactic acid on oxidation yields the corresponding ketonic acid which can combine with cyanide. The oxidation of lactic acid by the coenzyme is a process which proceeds independently of the oxidation of reduced coenzyme by the cytochromes. That is to say, in presence of a given quantity of coenzyme a certain amount of pyruvic acid must be formed depending upon the equilibrium. Using *M*/1000 final concentration of inhibitor, the amount of cyanide available in the manometer is of the order of magnitude of the pyruvic acid formed. The effective concentration of cyanide is therefore much lower and the inhibition is correspondingly less. Furthermore, if the reduced coenzyme can be oxidized even to the extent of 2-3 $\mu\text{l.}$ of oxygen equivalence by traces of an oxidizing agent in the preparation, an amount of pyruvic acid would be formed sufficient to reduce the effective cyanide concentration to a level at which inhibition would only be partial. Unfortunately all the coenzyme systems available for study involve either the oxidation of an aldehyde or the production of a ketonic acid. In both cases the difficulty of cyanide fixation cannot be avoided.

When the cytochrome-containing factor solution is precipitated with acetone at 0°, dried and resuspended in buffer, no direct reaction with oxygen takes place except in presence of added carriers. It is obvious that acetone treatment destroys the components necessary for the cyanide-sensitive reaction. Spectroscopic examination discloses considerable destruction of components *a* and *b*. Furthermore, all cytochrome oxidase activity is lost. This correspondence between the disappearance of the direct reaction and the destruction of the cytochrome oxidase system may be fortuitous. But there are other striking illustrations of this parallelism. For example, factor preparations such as that of rabbit skeletal muscle which are relatively deficient in the cytochromes show only a weak reaction with oxygen in absence of added carriers.

We may summarize the various lines of evidence in favour of the view that cytochromes *a* and *b* are the carriers in our preparation which account for the direct reaction with molecular oxygen.

(1) Cytochromes *a* and *b* are rapidly reduced by all coenzyme systems in presence of the factor.

(2) The direct reaction is poisoned completely by $M/100$ cyanide and to the extent of 65 % by $M/500$ cyanide. The cytochrome oxidase is the only cyanide-sensitive enzyme known to be present in the reconstructed system.

(3) Procedures which destroy the cytochromes or the oxidase eliminate the direct reaction with oxygen.

(4) The velocity of the direct reaction is proportional to the amount of cytochromes a and b in the factor solution.

VII. *General considerations*

Strictly speaking, the coenzyme factor is a dehydrogenase which catalyses the oxidation of its substrate, i.e. reduced coenzyme by means of various hydrogen acceptors. But to refer to the coenzyme factor as "coenzyme dehydrogenase" would introduce confusion with "coenzyme dehydrogenase systems". It is preferable that the term dehydrogenase should be applied only to enzymes which are concerned in catalysing the oxidation of metabolites. For the moment at any rate, in lieu of a more suitable name, the enzyme which catalyses the oxidation of reduced coenzymes I and II will be referred to as the coenzyme factor.

There are various lines of evidence that the coenzyme factor is definitely an enzyme. It is destroyed by 15 min. exposure to 55° and by a few minutes exposure to strong acid or alkali. It is non-dialysable and is associated with insoluble particles of a protein nature. Incubation of the factor solution with trypsin at 38° gradually destroys all enzymic activity. As yet there is nothing known of the mechanism whereby the coenzyme factor produces its catalytic effect. Purified factor solutions from rabbit skeletal muscle show little colour apart from that due to traces of the cytochromes. There is therefore no basis as yet for postulating that the factor like flavoprotein undergoes reversible oxidation and reduction.

Flavoprotein has $<1/1000$ the activity of coenzyme factor in any of the systems which we have studied in animal tissues. But flavoprotein definitely can act in the capacity of a carrier in the same way as flavin or adrenochrome. The catalytic efficiency of flavoprotein is about of the same order as that of adrenochrome (turnover number 5) but much less than that of cytochromes a and b , the turnover number of which lies in the thousands [cf. Green & Richter, 1937; Haas, 1934; Warburg, 1934; Warburg & Christian, 1933].

That the cytochromes are the normal physiological carriers for the coenzyme systems is borne out by the following facts. The oxidation of the substrates of coenzyme systems in tissue slices is inhibited completely by dilute cyanide [cf. Green & Brosteaux, 1936; van Heyningen, 1935]. The same cyanide-sensitivity is true of yeast [Ogston & Green, 1935]. Furthermore, Haas [1934] has calculated the percentage of the total respiration of yeast which takes the cytochrome path, and from his data it would appear that within the limits of experimental error the entire respiration involves the reversible reduction and oxidation of the cytochromes.

Prof. D. Keilin has informed us that he has invariably observed in yeast and in animal tissues rapid reduction of the cytochromes by the substrates of coenzyme systems. It was in fact Prof. Keilin's insistence on the participation of the cytochromes in coenzyme phenomena that induced us to correlate the cyanide-sensitive reaction with the cytochrome system.

The cytochrome class of dehydrogenases has been defined by Green & Brosteaux [1936] as one which catalyses the direct oxidation of the substrate by the cytochromes (a , b , or c). The coenzyme dehydrogenases are sharply

separated from this class in that the reaction between substrate and cytochrome is indirect—the coenzyme being the intermediary. Furthermore, the coenzyme dehydrogenases are not concerned with the reduction of cytochrome—that being the function of the coenzyme factor. The distinction drawn between the cytochrome and coenzyme systems is still valid. In the former class cytochrome is the oxidant for the substrates; in the latter the coenzyme specifically fulfils that function.

SUMMARY

1. The isolation and properties of the coenzyme factor are described. This enzyme catalyses the oxidation of reduced coenzymes I or II by hydrogen acceptors such as flavin, cytochrome, adrenochrome etc.

2. Coenzyme dehydrogenases from animal tissues have been shown to take part only in the reaction between the substrate and the coenzyme. In absence of coenzyme factor no reaction with oxygen via carriers is possible.

3. Flavoprotein has $< 1/1000$ the activity of the factor in coenzyme I systems.

4. A system in which the substrates of coenzyme dehydrogenases are oxidized by way of cytochromes *a* and *b* has been reconstructed from natural components.

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