CXCIII. CYTOCHROME *c* AS A CARRIER WITH THE GLUCOSE DEHYDROGENASE SYSTEM

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A FEW dehydrogenases of the aerobic oxidase type can apparently catalyse the direct oxidation of their substrates by molecular oxygen, but in the majority of cases the aerobic oxidation of metabolites by dehydrogenase systems occurs only in presence of some suitable carrier system to act as an intermediary between oxygen and the hydrogen of the substrate undergoing oxidation.

The first experiments with cytochrome *c* as carrier with a dehydrogenase system *in vitro* were carried out by Harrison [1931], who showed that addition of cytochrome *c* and cytochrome oxidase to the glucose dehydrogenase preparation obtained by him from liver resulted in a system capable of bringing about the aerobic oxidation of glucose. Ogston & Green [1935], however, were unable to confirm Harrison's results with glucose dehydrogenase and concluded from their experiments that of a number of dehydrogenase systems tested only the succinic dehydrogenase and the yeast lactic dehydrogenase reacted with the cytochrome *c*-cytochrome oxidase system. Green [1936] later showed that the α -glycerophosphoric dehydrogenase is similar in this respect to these two dehydrogenases.

As a result of Ogston & Green's work, Green & Brosteaux [1936] suggested that the group of coenzyme dehydrogenases (which includes glucose dehydrogenase) was unable to utilize the cytochromes as carriers. Recently, however, Dewan & Green [1938] have demonstrated that the aerobic oxidation of substrates by several coenzyme dehydrogenases can occur with cytochromes *a* and *b* as carriers, provided that in addition to the cytochromes and cytochrome oxidase another factor (diaphorase) is present to act as intermediary between the coenzyme and the cytochromes. However, in these experiments also, addition of cytochrome *c* to the combined enzyme systems had no effect on the rate of oxidation of the substrate, and Dewan & Green therefore concluded that the aerobic oxidation of substrates by coenzyme dehydrogenases is normally brought about with the diaphorase-cytochrome *a* and *b*-cytochrome oxidase system as carrier mechanism, and that cytochrome *c* is unable to act as intermediate carrier with such dehydrogenase systems.

In the course of an investigation of the oxidation of glucose by enzyme systems present in animal tissues we have found, as might be expected from Dewan & Green's work, that the aerobic oxidation of glucose is brought about by the glucose dehydrogenase system *plus* the cytochrome *a* and *b* system. In carrying out this work we had an opportunity to reinvestigate the carrier action of cytochrome *c* with the glucose dehydrogenase and have found that under suitable conditions addition of cytochrome *c* to the combined dehydrogenase and cytochrome oxidase systems does increase the O_2 uptake due to glucose oxidation. Further, we have been able to show spectroscopically that the glucose dehydrogenase system can reduce cytochrome *c*. Harrison's claim that cytochrome *c*

acts as carrier with this dehydrogenase system is therefore substantiated. In the course of these experiments evidence has been obtained that some factor, which may be the coenzyme factor diaphorase, is necessary for the reduction of cytochrome *c*, and it seems possible that a deficiency of this factor in the oxidase preparations of Ogston & Green may explain the inability of these workers to confirm Harrison's results.

EXPERIMENTAL

(1) *Preparation of material*

Glucose dehydrogenase was prepared from ox liver by the second method of Harrison [1933], the precipitation by ammonium sulphate being repeated a number of times until a preparation sufficiently free from residual substrates was obtained. The enzyme solutions were prepared by dissolving a weighed amount of the dry preparation as completely as possible in phosphate buffer and centrifuging off inactive material. The activity of the solutions was always tested by the Thunberg methylene blue technique before use in the aerobic experiments.

Coenzyme I (cozymase) prepared from baker's yeast by the method of Green *et al.* [1937] was used instead of the coenzyme preparation from liver described by Harrison.

Cytochrome *c* was extracted from ox heart by the method of Keilin & Hartree [1937]; the precipitate obtained with 20 % trichloroacetic acid, after washing with saturated ammonium sulphate solution, was dissolved in ammonia (2 ml. of concentrated ammonia solution diluted to 400 ml.) and dialysed in parchment sacs in the ice-chest for 2 days against frequent changes of dilute ammonia. After removal of the ammonia *in vacuo* the residue was dissolved in water and diluted to a strength of 3×10^{-4} *M*. A number of different preparations were used in this work and fresh solutions were made up at frequent intervals.

In most cases cytochrome oxidase prepared from pig heart by the method of Keilin & Hartree [1938] was used. The preparations were purified by repeating three times the precipitation from phosphate by addition of acetate buffer. The preparation probably contained little if any cytochrome *c* since neither the final supernatant fluid, the preparation itself, nor a trichloroacetic acid extract of the preparation made by the Keilin & Hartree method for preparing cytochrome *c* showed any absorption bands of reduced cytochrome *c* on addition of sodium hydrosulphite. Stock solutions of the oxidase were made by suspending 5.6 g. of the preparation in *M*/10 phosphate buffer *pH* 7.4 and diluting to 10 ml. These solutions were stored in the ice chest and were diluted with phosphate buffer to the required strength before use. Fresh preparations of oxidase were made up every few days.

The aerobic experiments were carried out in Barcroft differential manometers. The various solutions, diluted to a total volume of 3 ml., were placed in the right-hand flasks, while the left-hand flasks received an equal volume of distilled water. In the centre compartment of each flask was placed 0.2 ml. of 5 % NaOH soaked on a roll of filter paper to absorb any CO₂, except in the experiments on cyanide inhibition, when, in order to avoid errors due to absorption of HCN by the NaOH, the solutions recommended by Krebs [1935] were used instead. The manometers were shaken at 100 oscillations per min. at a constant temperature of 37°.

Anaerobic experiments were carried out in Thunberg tubes which were evacuated and filled with N₂ twice, then re-evacuated and incubated at 37°.

(2) *Aerobic oxidation of glucose with the cytochromes as carriers*

The oxidation of glucose by the glucose dehydrogenase system *plus* the diaphorase-cytochrome *a* and *b*-cytochrome oxidase system, and the effect of addition of cytochrome *c* are shown in Table I.

Cytochrome oxidase preparations made by the Keilin & Hartree method, although probably free from cytochrome *c* (as indicated by the failure to detect the absorption bands of reduced cytochrome *c* on treatment with hydrosulphite) contain cytochromes *a* and *b* and diaphorase in addition to cytochrome oxidase.

Table I. *Glucose oxidation in presence of the cytochromes*

No. of manometer	1	2	3	4	5	6	7	8
Dehydrogenase (ml.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Coenzyme I (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glucose (ml.)	—	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Cytochrome <i>c</i> (ml.)	1.0	1.0	—	1.0	—	1.0	—	1.0
Oxidase (ml.)	0.2	—	0.2	0.2	0.2*	0.2*	0.2†	0.2†
Distilled water (ml.)	0.3	0.2	1.0	—	1.0	—	1.0	—
O ₂ absorbed (μl) after 90 min.	19	14	751	750	297	485	188	274

The dehydrogenase solution contained 55 mg. of the dry preparation (equivalent to 5 g. acetone liver) in 1 ml. *M*/10 phosphate buffer pH 7.4; coenzyme I, 4 mg. per ml.; glucose, 2*M*; cytochrome *c*, 3×10^{-4} *M*; oxidase, Keilin & Hartree's preparation, stock solution; * diluted 1:20 with *M*/10 phosphate buffer pH 7.4; † diluted 1:40.

The activity of the dehydrogenase was such that 0.2 ml. of the enzyme solution + 0.2 ml. coenzyme I (4 mg. per ml.) + 0.2 ml. 2*M* glucose reduced 0.25 ml. 1/5000 methylene blue (in a total volume of 1.65 ml.) in 5 min., while without coenzyme or without glucose the times of reduction were 30 min. and 35 min. respectively.

As was expected from Dewan & Green's work, addition of such an oxidase preparation to a mixture of glucose dehydrogenase, coenzyme I and glucose forms a complete enzyme system capable of bringing about the aerobic oxidation of the glucose. This is shown by comparing manometer 3 (or manometers 5 or 7) with the controls, manometers 1 and 2. Comparing the O₂ uptakes in the pairs of manometers 3 and 4 in which a high concentration of the oxidase-cytochrome *a* and *b* preparation was used, it can be seen that addition of cytochrome *c* produced no increase in O₂ uptake. On the other hand, comparing manometers 5 and 6 where a much more dilute oxidase preparation was added, it is evident that in this case addition of cytochrome *c* resulted in a large increase in the amount of O₂ absorbed as compared with the amount absorbed by the combined dehydrogenase-cytochrome *a* and *b* system without cytochrome *c*. A similar result was obtained on further decreasing the amount of oxidase-cytochrome *a* and *b* preparation used as can be seen by comparing manometers 7 and 8. Apparently, therefore, in presence of relatively large amounts of the oxidase-cytochrome *a* and *b* preparation, the total hydrogen transport proceeds via cytochromes *a* and *b*, since under these conditions addition of cytochrome *c* has no effect on the rate of oxidation of glucose. If, however, the amount of cytochrome oxidase preparation added is decreased so that the concentration of the cytochrome carrier becomes the limiting factor in the reaction then addition of cytochrome *c* leads to an increased O₂ uptake. Under these conditions, therefore, cytochrome *c* can act as carrier with the glucose dehydrogenase system.

The effect of cytochrome *c* in increasing the rate of oxidation of glucose by the dehydrogenase system *plus* the cytochrome *a* and *b* and oxidase system has

been confirmed in a large number of experiments, the results of some of which are summarized in Table II. The final amounts of O_2 absorbed by the following four systems are shown:

A	B	C	D
Dehydrogenase	Dehydrogenase	Dehydrogenase	Dehydrogenase
Coenzyme	Coenzyme	Coenzyme	Coenzyme
—	Glucose	Glucose	Glucose
Cytochrome <i>c</i>	Cytochrome <i>c</i>	—	Cytochrome <i>c</i>
Oxidase	—	Oxidase	Oxidase

Table II. *Glucose oxidation in presence of cytochrome c*

Time of experiment min.	Dilution of stock oxidase	Oxygen uptakes (μ l.)			
		A	B	C	D
120	1:20	33	36	266	388
90	1:10	33	26	417	543
135	1:15	49	68	379	588
180	1:15	79	79	279	564
180	1:20	25	24	382	551
75	1:15	37	49	371	520
120	1:60	21	38	190	344
150	1:30	25	24	361	499

The conditions varied slightly in different experiments but as a general rule the following quantities were used: dehydrogenase (55–90 mg.) equivalent to 5 g. acetone liver dissolved in 1 ml. *M*/10 phosphate buffer pH 7.4; coenzyme I, 2 mg.; glucose, final concentration 0.2 *M*; cytochrome *c*, final concentration 10^{-4} *M*; oxidase, 0.2 ml. of a solution prepared by diluting the stock solution with *M*/10 phosphate buffer pH 7.4 as shown in the second column. The activities of the dehydrogenase solutions used were such that 0.2 ml. dehydrogenase + 0.2 ml. coenzyme I (4 mg. per ml.) + 0.2 ml. 2*M* glucose reduced 0.25 ml. 1/5000 methylene blue (in a total volume of 1.65 ml.) in from 5 to 7 min., while without glucose or without coenzyme the time required for the reduction of the methylene blue was at least five times as great.

In many cases controls without dehydrogenase or without coenzyme were set up in addition to the above. Actually the O_2 uptake in the systems either without glucose or without oxidase (A and B respectively) was found to be due almost entirely to the dehydrogenase solution alone, and this figure is therefore common to all four systems. It can be seen from Table II that while addition of the cytochrome *a* and *b*-cytochrome oxidase preparation alone to the dehydrogenase system resulted in an aerobic oxidation of glucose (column C), addition of cytochrome *c* led to a further increase in the amount of O_2 absorbed (column D). Exactly similar results have been obtained using cytochrome oxidase preparations made from sheep heart by a slight modification of the older method of Keilin [1930], or preparations from pig heart made by the method used by Dewan & Green [1938] for preparing "coenzyme factor".

The increase in O_2 uptake on addition of cytochrome *c*, like the O_2 uptake which results on adding the cytochromes *a* and *b*-cytochrome oxidase preparation alone to the dehydrogenase system is completely inhibited by *M*/1000 cyanide as shown in Table III.

Comparing manometers 5 and 6 with manometers 3 and 4 it is clear that cyanide completely inhibits the oxidation of glucose, the O_2 uptakes with cyanide being reduced to those of the controls without glucose or without oxidase (manometers 1 and 2). As is well known the activity of the cytochrome oxidase system is inhibited by dilute cyanide. This experiment affords evidence, therefore, that the oxidation of glucose is brought about by the cytochromes and not by other carriers present in the enzyme preparations.

Table III. *Inhibition by cyanide of glucose oxidation*

No. of manometer	1	2	3	4	5	6	7
Dehydrogenase (ml.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Coenzyme I (ml.)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
2 <i>M</i> glucose (ml.)	—	0.3	0.3	0.3	0.3	0.3	0.3
Cytochrome <i>c</i> (ml.)	1.0	1.0	—	1.0	—	1.0	—
Oxidase (ml.)	0.2	—	0.2	0.2	0.2	0.2	—
<i>M</i> /100 KCN (ml.)	—	—	—	—	0.3	0.3	1.0
1/500 methylene blue (ml.)	—	—	—	—	—	—	0.5
Distilled water (ml.)	0.6	0.5	1.3	0.3	1.0	—	—
Oxygen absorbed (μ l.) after 180 min.	48	43	312	418	39	49	429

The dehydrogenase solution contained 50 mg. (equivalent to 5 g. acetone liver) in 1 ml. *M*/10 phosphate buffer pH 7.4; coenzyme I, 10 mg. per ml.; cytochrome *c*, 3×10^{-4} *M*; oxidase prepared by the Keilin & Hartree method, diluted 1:20. The cyanide was brought to pH 7.4 with HCl and made up to *M*/100.

The activity of the dehydrogenase was such that 0.2 ml. of the solution + 0.2 ml. coenzyme I (4 mg. per ml.) + 0.2 ml. 2*M* glucose reduced 0.25 ml. 1/5000 methylene blue (total volume 1.65 ml.) in 8 min., while without coenzyme or without glucose more than an hour was required.

This result is not due to inhibition of the dehydrogenase itself by cyanide, for cyanide in final concentration of even *M*/300 does not inhibit the activity of the dehydrogenase as is shown by adding methylene blue as carrier. It can be seen from the last column of Table III that with methylene blue added the full O_2 uptake is obtained even in presence of cyanide.

In his original experiments, on the aerobic oxidation of glucose by glucose dehydrogenase *plus* cytochrome *c* and cytochrome oxidase, Harrison noted the O_2 uptake caused on addition of cytochrome oxidase alone to the dehydrogenase system. This he attributed to small amounts of cytochrome *c* in the oxidase preparations, whereas it now seems clear from the above experiments, taken in conjunction with the work of Green and his co-workers, that it is due to the cytochromes *a* and *b* present in the oxidase preparation. The claim by Harrison, however, that cytochrome *c* can act as carrier with the glucose dehydrogenase system is completely confirmed by this investigation.

It is surprising that Ogston & Green were not only unable to repeat Harrison's results with cytochrome *c* but observed no O_2 uptake on addition of cytochrome oxidase preparations alone to any of the coenzyme I-dehydrogenase systems tested. Since these authors themselves pointed out that the cytochrome oxidase preparations used by them contained also cytochromes *a* and *b*, the only explanation of their results appears to be that the preparations were deficient in diaphorase, the factor which is required as intermediate carrier between the coenzyme and the cytochromes. We carried out some experiments similar to those described above, using the indophenol (cytochrome) oxidase preparations described in Ogston & Green's paper, but the results were unsatisfactory; in some cases an O_2 uptake on addition of the cytochrome oxidase to the glucose dehydrogenase system was observed, but it was always considerably smaller than that obtained with an oxidase preparation made from the same heart by Keilin's method.

(3) *Anaerobic reduction of cytochrome c by the glucose dehydrogenase system*

The ability of cytochrome *c* to act as carrier with the glucose dehydrogenase system was confirmed by studying the reduction of cytochrome *c* by the system under anaerobic conditions.

A mixture of glucose dehydrogenase, coenzyme I and glucose rapidly reduces methylene blue under anaerobic conditions, but although glucose dehydrogenase

has a very low (negative) reduction potential and therefore from the thermodynamic point of view would be expected to be capable of reducing the much more positive cytochrome *c*, yet if the methylene blue in the above system is replaced by cytochrome *c*, only very slight reduction of the cytochrome actually occurs. This reduction is independent of both glucose and coenzyme I and is probably due to small amounts of other enzyme systems in the dehydrogenase preparation. It was found, however, that in presence of an additional factor contained in cytochrome oxidase preparations made by either the Keilin or the Keilin & Hartree method, the cytochrome *c* is rapidly and fully reduced by the glucose dehydrogenase system. This is shown in Table IV in which is given the time required for complete reduction of the cytochrome *c*.

Table IV. *Anaerobic reduction of cytochrome c*

No. of Thunberg tube	1	2	3	4	5
Dehydrogenase (ml.)	—	1.0	1.0	1.0	1.0
Coenzyme I (ml.)	0.2	—	0.2	0.2	0.2
Glucose (ml.)	0.2	0.2	—	0.2	0.2
Oxidase (factor) (ml.)	0.2	0.2	0.2	—	0.2
Cytochrome <i>c</i> (ml.)	0.5	0.5	0.5	0.5	0.5
Time (min.)	>30	>30	>30	>30	5

The dehydrogenase solution contained 9 mg. of the dry preparation (equivalent to 1 g. acetone liver) in 1 ml. *M*/20 phosphate buffer pH 7.4; coenzyme I, 4 mg. per ml.; glucose, 2*M*; cytochrome *c*, 3×10^{-4} *M*; oxidase, stock solution prepared by Keilin's method. The total volume in each tube was made up to 2.1 ml. by addition of distilled water where required.

In this experiment, in order to decrease the residual reducing power of the dehydrogenase solution as far as possible, a mixture of 10 ml. of the dehydrogenase solution, 1 ml. cytochrome *c* and 0.5 ml. of cytochrome oxidase was placed in a water bath at 37° and aerated for 20 min. to allow residual substrates to become oxidized. The cytochrome oxidase was then removed by centrifuging for 10 min. The activity of the dehydrogenase was then tested with methylene blue before use in the experiments on the reduction of cytochrome. 1 ml. of the dehydrogenase solution + 0.2 ml. coenzyme I (4 mg. per ml.) + 0.2 ml. 2*M* glucose reduced 0.25 ml. 1/5000 methylene blue in 8 min., while without glucose or without coenzyme more than 30 min. were required.

The reduction of the cytochrome *c* was carried out in Thunberg tubes which were evacuated and filled with nitrogen twice, re-evacuated and incubated at 37°. Reduction of the cytochrome was easily followed by the change in colour. For comparative purposes two standard tubes were used, the first containing a sample of cytochrome *c* reduced by hydrosulphite to which were added the other components as in tube 5, while the second contained a system identical with that in tube 5 but incubated under aerobic conditions so that the cytochrome was maintained in the oxidized form by the cytochrome oxidase. In addition, the solutions were examined spectroscopically before opening the tubes. After 5 min. incubation the solution in tube 5 showed strong bands of reduced cytochrome *c*, while in the other tubes the α -band of reduced cytochrome *c* could only just be detected after 30 min. incubation. This experiment has been repeated a number of times with similar results.

It is clear, therefore, that cytochrome *c* is not directly reduced by the dehydrogenase system but that the reaction is catalysed by a factor contained in heart muscle and found in cytochrome oxidase preparations made from it. (In these experiments the cytochrome oxidase as such plays no part, of course, in the reaction since this is carried out under anaerobic conditions. The oxidase preparation is added solely as source of the factor.)

In view of the well-known effect of yeast flavoprotein in catalysing the reduction by coenzyme dehydrogenase systems of carriers such as methylene blue it seemed possible that the factor required for the reduction of cytochrome *c* might be identical with this Warburg-Christian flavoprotein. We found, however, that while flavoprotein prepared from yeast showed some catalytic action, it was very much less active than the cytochrome *c* factor in the oxidase preparations. Since these experiments were completed, diaphorase, the factor required for the reduction of cytochromes *a* and *b* by dehydrogenase systems, has been identified with a flavoprotein isolated by Straub [1939, 1, 2] from heart muscle [Straub *et al.* 1939; Corran *et al.* 1939] and it is interesting that Corran *et al.* have found that yeast flavoprotein has a small, though relatively negligible, effect in the cytochrome *a* and *b* system.

It seems possible that the factor required for the reduction of cytochrome *c* by the glucose dehydrogenase is identical with diaphorase. On the other hand, Hopkins *et al.* [1939] and Dixon & Zervas [1939] have demonstrated that the reduction of cytochrome *c* by the succinic dehydrogenase and yeast lactic dehydrogenase occurs only in presence of a factor which is probably not diaphorase, and Potter & Lockhart [1939] have recently suggested in the case of the succinic enzyme that the factor may be cytochrome *b*. Until more evidence is available, it appears undesirable, therefore, to speculate further on the nature of the factor required with the glucose dehydrogenase system.

SUMMARY

Cytochrome *c* in presence of cytochrome oxidase preparations can act as a carrier and bring about the aerobic oxidation of glucose by the glucose dehydrogenase system. The cytochrome oxidase preparations can, by themselves, induce some oxidation of glucose in presence of the dehydrogenase system. This is apparently due to the other carriers, cytochromes *a* and *b*, present in the oxidase preparations.

In acting as a carrier in this oxidation cytochrome *c* is not directly reduced by the dehydrogenase-coenzyme system but requires for its reduction an additional factor which is present in heart muscle and in the cytochrome oxidase preparations made from heart muscle.

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