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Oxidation Path of the *d*-Glucose Dehydrogenase

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In the mammalian liver, Harrison [1,2] discovered a dehydrogenase protein, which in presence of coenzyme 1 or 2 (Andersson [3], Das [4]), oxidizes *d*-glucose. The enzyme cannot use oxygen directly, but methylene blue can function as a hydrogen acceptor, and so can the cytochrome system. Hawthorne and Harrison [5] showed the participation of cytochrome c, and suggested that diaphorase (Straub's [6] heart flavoprotein), is necessary for the reaction. The full system is supposed to comprise: O_2 + cytochrome oxidase + cytochrome c + diaphorase + (dehydrogenase protein + coenzyme) + *d*-glucose. Eichel and Wainio [7] re-examined the function of the system, but were not able to decide if the diaphorase participates or not.

In the present investigation the advantage of a highly purified dehydrogenase protein preparation (Brunelli and Wainio [8]) was taken for spectrophotometric experiments, in order to examine the rôle of the diaphorase for the function of the dehydrogenase *. — The necessity of cytochrome c for the full system has been re-examined in manometric experiments.

MATERIAL AND METHODS

d-Glucose dehydrogenase protein. In manometric experiments a crude preparation according to Harrison [9] was employed. For spectrophotometrical experiments the dehydrogenase was further purified according to Brunelli and Wainio's method [8]. *Coenzyme 1* was prepared from baker's yeast by the method of Williamson and Green [10]. *Diaphorase* was obtained from pig heart muscle according to Straub's procedure [6]. *Cytochrome c* was prepared from horse heart muscle according to Keilin and Hartree's procedure [11, 12]. The convention that the pigment contains 0.34 per cent Fe is adopted. *Cytochrome oxidase* was obtained from pig heart muscle, by following, with minor alterations, the method of

* The term dehydrogenase is used to denote the holoenzyme: *d*-glucose dehydrogenase protein + coenzyme.

Keilin and Hartree[13]. The grinding with sand was substituted by a 2 min Waring Blendor mincing. Used amounts of oxidase are referred to dry weight.

Oxygen uptake. All manometric experiments (Warburg technique) were performed at 30° C. Total flask charge: 3.8 ml in the main compartment + 0.3 ml. KOH in the bottom centre tube. Phosphate buffer of pH 7.1 and a final concentration of 0.066 *M* was used throughout. Under extreme conditions the formed gluconic acid may change the pH towards the end of the experiment to 6.9. Gas phase: air. For further experimental details see Borei[14].

Spectrophotometric measurements: The reduction of ferricytochrome c was observed by the rise of extinction at 549 mμ (Beckman spectrophotometer).

RESULTS

A. Participation of cytochrome oxidase and cytochrome c

The cyanide inhibitability of the full system (Hawthorne and Harrison[5] and others) points to the participation of the cytochrome oxidase. Similar experiments in this investigation are in full accordance with these results. In addition the full system has been found strongly inhibitable by NaN_3. On the other hand, if the cytochrome c complemented oxidase preparation is replaced by methylene blue, no inhibition is achieved either by KCN or NaN_3 additions. Instead a slight augmentation is observed.

The used cytochrome oxidase preparation supports maximal glucose dehydrogenase activity even when no cytochrome c is added. This suggests that sufficient cytochrome c is present in the oxidase preparation.

Keilin and Hartree[15] have shown that repeated freezing of cytochrome oxidase preparations in liquid air and thawing at room temperature impairs the accessibility of endogenous cytochrome c. The activity of the oxidase is, however, completely restored by addition of external cytochrome c. Oxidase treated in this way gives a very low oxidation rate with the glucose dehydrogenase system if no external cytochrome c is present. The effect of addition of cytochrome c is seen from Fig. 1.

Lineweaver-Burk[16] analysis applied to experiments, in which the amount of added cytochrome c was varied, shows a straight line relationship between the inverse function of reaction velocity and cytochrome c concentration.

Cytochrome c complemented cytochrome oxidase prepared according to Haas[17] is unable to mediate between oxygen and glucose dehydrogenase. Addition of diaphorase (see below) is of no effect in this case.

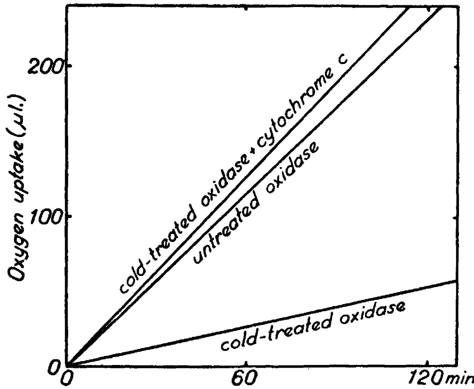

Fig. 1. Necessity of cytochrome c for the function of the d-glucose dehydrogenase system. Cytochrome oxidase preparation (untreated or 3 times frozen) (0.604 mg) + coenzyme 1 (0.8 mg) + d-glucose dehydrogenase protein (6.3 mg) + glucose (final concentration 0.16 M). Final concentration of cytochrome c, when added, 1.6 × 10^{-6} M. The addition of the same amount of cytochrome c to the untreated oxidase causes only a very slight augmentation.

B. Participation of diaphorase

If a complete system, containing the Keilin and Hartree cytochrome oxidase preparation, is tested manometrically, the omission of diaphorase proves to be of no significance for the activity, *cf.* Table 1. This is probably due to the content of diaphorase in the enzyme preparations.

Table 1. Significance of different components in manometric experiments with cytochrome oxidase preparations. The amounts or final concentrations of the various additions are denoted in the table.

Additions:	1	2	3	4	5	6	7
Cytochrome oxidase preparation (7.4 mg)	+	+	+	+	+	—	—
Cytochrome c (16 × 10^{-6} *M*)	+	+	+	+	+	—	—
Diaphorase (1 mg)	+	—	+	+	+	+	—
Coenzyme 1 (0.8 mg)	+	+	—	+	+	+	+
Glucose dehydrogenase protein (12.6 mg)	+	+	+	—	+	+	+
Glucose (0.16 *M*)	+	+	+	+	—	+	+
Oxygen uptake (µl)	450	447	12	— 2	15	72	4

If, on the other hand, the cytochrome oxidase and the cytochrome c are replaced by methylene blue, the oxidation rate is strongly dependent on the presence of added diaphorase. Moreover, the decolourization rate of methylene blue in Thunberg experiments is increased several hundred per cent if diaphorase is added.

It is obvious from Table 1 that the diaphorase content of the oxidase preparation excludes the possibility to decide from manometric experiments if the diaphorase is essential as a mediator between the cytochrome part of the full system and the dehydrogenase.

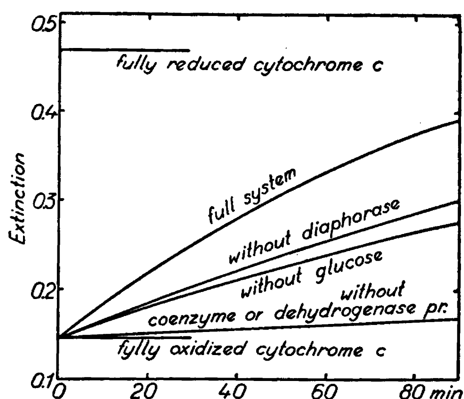

Fig. 2. Reduction of cytochrome c in the d-glucose dehydrogenase system. The rise in extinction at 549 mμ is followed spectrophotometrically. Full system: Cytochrome c (1 mg) + diaphorase (2.4 mg) + coenzyme 1 (0.8 mg) + d-glucose dehydrogenase protein (2.2 mg) + glucose (108 mg). Test volume 3.8 ml. Length of optical cell 1 cm. Room temperature.

It is, however, possible to follow spectrophotometrically the reduction of cytochrome c, and to study in such experiments, the effect of diaphorase addition. In those experiments, which are represented in Fig. 2, the diaphorase content of the test system itself was further depressed by using the purified glucose dehydrogenase preparation of Brunelli and Wainio [8]. Though this preparation is comparatively poor in both diaphorase and substrate, the controls with omitted external addition of these two reactants give quite appreciable reduction rates. Addition of diaphorase, however, doubles the activity of the system, thus establishing the significance of this carrier for the reaction.

DISCUSSION

The inhibition experiments with CN′ and N_3' support conclusively the view (Hawthorne and Harrison [5]) that the glucose dehydrogenase can function over the cytochrome system. The inhibitability by the named ions is bound to the presence of cytochrome oxidase and is not to be attributed to the dehydrogenase itself. This is shown by the noninhibitability of systems, where the cytochrome oxidase preparation is replaced by methylene blue.

The participation of cytochrome c as a mediator between the cytochrome oxidase and the dehydrogenase seems to be evident from Hawthorne and Harrison's [5] experiments. Eichel and Wainio [7] had, however, difficulties in confirming these results. Only with extremely diluted oxidase preparations was it possible for them to show any influence of added cytochrome c. In the present investigation it was found that the oxidase preparation (Keilin and Hartree's procedure) itself contains enough endogenous cytochrome c to give a maximal catalysis over the dehydrogenase system. External cytochrome c is

without influence. If, however, the accessibility of the endogenous cytochrome c is impaired through treatment in liquid air (procedure of Keilin and Hartree [15]) addition of external cytochrome c augments the oxidation strongly (*cf.* Fig. 1). The necessity of cytochrome c for the system used is thus clearly established. This result is furthermore supported by the fact that Lineweaver-Burk analysis shows that the cytochrome oxidase-cytochrome c complex, normally found in cytochrome oxidase-mediated systems (*cf.* Borei [14]), is functioning also in the glucose dehydrogenase system.

Yeast flavoprotein was found by Ogston and Green [18] and others, to mediate between oxygen and the dehydrogenase, and by Adler and Euler [19] to augment the decolourisation of methylene blue. The participation of diaphorase as a mediator between the cytochrome system and the dehydrogenase was suggested by Hawthorne and Harrison [5]. As the cytochrome oxidase preparations themselves apparently contain enough flavoprotein to maintain full activity in the system, it was not possible for Eichel and Wainio [7] to decide if the diaphorase is a necessary link or not.

In the present investigation, where Keilin and Hartree's cytochrome oxidase preparation was used, the same phenomenon was found (*cf.* Table 1, experiments 1—5). The addition of diaphorase is of no influence on the oxidation rate. This must mean either that diaphorase is not a link in the system, or that the enzyme preparations contain enough diaphorase themselves to support full activity. Furthermore it is confirmed that the diaphorase itself can mediate between oxygen and the dehydrogenase (Table 1, experiments 6 and 7). In this case, however, the oxidation rate is much lower than when cytochrome oxidase is present.

In methylene blue experiments, a positive effect was invariably found when diaphorase was added. This must imply a carrier function, of diaphorase between the dehydrogenase and the dye stuff. These findings agree with Adler and Euler's [19] results, but are in apparent contradiction to Eichel and Wainio's [7]. This discrepancy is probably due to high endogenous flavoprotein content of the dehydrogenase preparation used by the latter workers.

The spectrophotometric experiments on the reduction of oxidized cytochrome c through the dehydrogenase (Fig. 2) conclusively show, however, that diaphorase acts as a link between the two reactants.

SUMMARY

1. It is confirmed that the cytochrome system can mediate between the *d*-glucose dehydrogenase and oxygen (inhibition experiments with KCN and NaN_3).

2. Cytochrome c is necessary for this mediation (experiments with cytochrome oxidase preparations with impaired activity of endogenous cytochrome c).

3. Diaphorase is necessary as a link between cytochrome c or methylene blue and the dehydrogenase (spectrophotometric experiments on reduction of cytochrome c; Thunberg experiments).

4. The reaction path for *d*-glucose dehydrogenase activity thus comprises: $O_2 \rightarrow$ cytochrome oxidase $\rightarrow$ cytochrome c $\rightarrow$ diaphorase $\rightarrow$ (coenzyme 1 + *d*-glucose dehydrogenase protein) $\rightarrow$ *d*-glucose.

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