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The Dihydrocozymase-cytochrome *c* Reductase Activity of Heart-muscle Preparation

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The dihydrocozymase-cytochrome *c* reductase may be defined, following Potter & Albaum (1943), as that portion of the Co I H₂-oxidase system which is concerned with the reduction of cytochrome *c* by Co I H₂. According to the scheme proposed in the previous paper, it may be represented thus

This paper is concerned with some properties of this enzyme complex in Keilin & Hartree's (1947) heart-muscle preparation. No attempt has been made to obtain it in solution and purify it.

METHODS

Cytochrome c reductase activity was measured by following the reduction of cytochrome *c* in a Beckman spectrophotometer at 550 mμ., using 1 cm. cells. The reoxidation of the cytochrome *c* was prevented by adding cyanide (0.001 M). The diluted heart-muscle preparation was added to cytochrome *c* in phosphate buffer, pH 7.3 (final concentration 0.125 M) in the Beckman cell several minutes before zero

time in order completely to oxidize the cytochrome *c*. At zero time, the cyanide and Co I H₂ solutions were added and the optical density at 550 mμ. measured at approximately 20 sec. intervals. The rates of reduction of cytochrome *c* were sufficiently rapid so that only a small amount of oxidized cytochrome *c* combined with cyanide during the course of the reaction (Potter, 1941; Horecker & Kornberg,

1946). The 'blank' cuvette contained cytochrome *c*, heart-muscle preparation, cyanide and phosphate buffer. Corrections for temperature were made as described in the previous paper (Slater, 1950).

Optical densities at 550 mμ. were converted to molar concentrations of reduced cytochrome *c* by multiplying by the factor 5.35×10^{-5} . This factor was calculated from the molar extinction coefficient at 550 mμ. of reduced cytochrome *c*, reported by Theorell & Akesson (1941), and the observation that, under the conditions of the experiments described in this paper, the optical density of oxidized cytochrome *c* at 550 mμ. was 0.34 times that of reduced cytochrome *c*.

Other measurements were made as described in the previous paper (Slater, 1950). The Co I H₂ was prepared by enzymic reduction. Optical densities at 340 mμ. were con-

verted to molar concentrations of Co I H_2 by multiplying by the factor 16.1×10^{-5} (calculated from Horecker & Kornberg, 1948).

EXPERIMENTS

In Fig. 1, curve *A*, the course of the reduction of cytochrome *c* by Co I H_2 in the presence of very dilute heart-muscle preparation (0.021 mg. of fat-free dry wt./ml.) is shown. This reduction follows practically the same course as that obtained when succinate was the reducing agent (curve *B*). Thus, at the temperature of this experiment (27°), the Co I H_2 -cytochrome *c* reductase activity of the heart-muscle preparation was practically the same as its succinate-cytochrome *c* reductase activity. Curve *C* shows the course of the oxidation of Co I H_2 in the absence of added cytochrome *c* or cyanide.

Fig. 1. Reduction of cytochrome *c* by succinate and Co I H_2 in presence of heart-muscle preparation. Phosphate buffer (pH 7.3), 0.125M ; cytochrome *c*, $2.6 \times 10^{-5}\text{M}$; Co I H_2 , $3.67 \times 10^{-5}\text{M}$; succinate, 0.0133M ; KCN, 0.001M ; heart-muscle preparation, $0.021\text{ mg. of fat-free dry weight/ml.}$ Experiments carried out at 27.0 – 27.5° . No correction for temperature. Curve *A*, formation of reduced cytochrome *c* by Co I H_2 ; curve *B*, formation of reduced cytochrome *c* by succinate; curve *C*, disappearance of Co I H_2 in absence of cyanide or cytochrome *c*.

The reduction of different concentrations of cytochrome *c* by Co I H_2 and the heart-muscle preparation is shown in Fig. 2. In Fig. 3, log. oxidized cytochrome *c* concentration at time *t* is plotted against time *t*. It is apparent that the points do not fall on a straight line, except at very low concentrations of cytochrome *c*. Thus the reduction of cytochrome *c* by Co I H_2 and heart-muscle preparation cannot be described by the simple unimolecular equation, which holds for the dihydrocoenzyme II-cytochrome *c* reductase isolated by Haas, Horecker & Hogness (1940). When the rate of reduction was plotted against the concentration of oxidized cytochrome *c*,

Fig. 2. Reduction of cytochrome *c* by Co I H_2 in presence of heart-muscle preparation (same preparation as in Fig. 1). Phosphate buffer (pH 7.3), 0.125M ; Co I H_2 , $3.67 \times 10^{-5}\text{M}$; KCN, 0.001M ; heart-muscle preparation, $0.021\text{ mg. of fat-free dry wt./ml.}$ No correction for temperature. Curve *A*, $1.04 \times 10^{-5}\text{M}$ cytochrome *c*, 28° ; curve *B*, $2.08 \times 10^{-5}\text{M}$ cytochrome *c*, 28.1° ; curve *C*, $3.64 \times 10^{-5}\text{M}$ cytochrome *c*, 28.5° ; curve *D*, $5.2 \times 10^{-5}\text{M}$ cytochrome *c*, 28.8° . The horizontal arrows show the final value reached.

Fig. 3. Rate of decrease of the logarithm of the oxidized cytochrome *c* concentration, calculated from Fig. 2. Curve numbers as in Fig. 2.

a fairly close fit to a rectangular hyperbola was obtained. The fit was not sufficiently close, however, to allow a reliable extrapolation to infinite cytochrome

c concentration. The maximum rate of reduction of cytochrome *c* found in Fig. 2 was 0.42×10^{-5} mole litre⁻¹, corrected to 20°, when the initial concentration of oxidized cytochrome *c* was 5.2×10^{-5} M.

Using a highly purified sample of cozymase, Hogeboom (1949) found that the amounts of Co I H₂ oxidized and cytochrome *c* reduced when both were added to a preparation from rat liver agreed with the equation

where *c*⁺⁺⁺ and *c*⁺⁺ are oxidized and reduced cytochrome *c* respectively. With the sample of cozymase used in the present investigation, there was good agreement with this equation when an excess of cytochrome *c* was added to the Co I H₂. When, however, the Co I H₂ was in excess, as in Figs. 1 and 2, about 25% more cytochrome *c* was reduced than could be accounted for by the amount of Co I H₂ oxidized, probably due to the presence in the cozymase preparation of impurities capable of reducing cytochrome *c*. The reduction of cytochrome *c* by these impurities does not affect any of the conclusions drawn in this paper.

Applying the above equation, the initial rate of oxidation of Co I H₂ by heart-muscle preparation and 5.2×10^{-5} M-cytochrome *c* would be 0.21×10^{-5} mole litre⁻¹ min.⁻¹ at 20°. This may be compared with the initial rate of oxidation in the absence of added cytochrome *c* or cyanide calculated from curve C, Fig. 1, viz. 0.40×10^{-5} mole litre⁻¹ min.⁻¹. Thus, even this very high concentration of added cytochrome *c* is not as active as the heart muscle alone in bringing about the oxidation of Co I H₂.

The relative rate of reduction of the endogenous cytochrome *c* of the preparation compared with that of added cytochrome *c* was calculated in the following way. It was found from Fig. 3 that at low concentrations of cytochrome *c*, the rate of reduction at 20° was expressed by the equation $-dc/dt = 0.39c$, where *c* is the concentration of added oxidized cytochrome *c*. The concentration of the endogenous cytochrome *c* of the heart-muscle preparation at the dilution employed was calculated from the fat-free dry weight content of the preparation and the figures previously given (Slater, 1949*a*) to be 1.7×10^{-8} M. If this endogenous cytochrome *c* were reduced by the Co I H₂ at the same rate as added cytochrome *c*, the rate of reduction would be $0.39 \times 1.7 \times 10^{-8} = 0.666 \times 10^{-8}$ mole litre⁻¹ min.⁻¹. The rate of reduction of the cytochrome *c*, calculated from the observed rate of disappearance of the Co I H₂ in the absence of cyanide or added cytochrome *c*, was $2 \times 0.40 \times 10^{-5} = 0.8 \times 10^{-5}$ mole litre⁻¹ min.⁻¹. The rate of reduction of the endogenous cytochrome *c* is then 1200 times that of added cytochrome *c*. Thus the endogenous cytochrome *c* of heart-muscle preparation is about a thousand times more active than added cytochrome

c in catalysing the oxidation of Co I H₂. Similarly, it is much more active in the oxidation of succinate and *p*-phenylene-diamine (Slater, 1949*a, b*).

The relation $-dc/dt = 0.39c$ enables a calculation of the 'specific cytochrome reductase activity' of the heart-muscle preparation in the units of Haas *et al.* (1940). This value was 13.7 at 25°.

Fig. 4. Effect of BAL treatment on Co I H₂-cytochrome *c* reductase activity of heart-muscle preparation. Phosphate buffer (pH 7.3), 0.125 M; Co I H₂, 6×10^{-5} M; KCN, 0.001 M; heart-muscle preparation, 0.020 mg. of fat-free dry wt./ml. Temperature 24.0–24.8°. No correction for temperature. Curve A, heart-muscle preparation shaken in air for 15 min. at 37° before dilution; curve B, heart-muscle preparation shaken in air with 0.01 M-BAL for 15 min. at 37° before dilution; curve C, reduction in the absence of heart-muscle preparation.

Treatment of the heart-muscle preparation with 2:3-dimercaptopropanol (BAL) strongly inactivated its Co I H₂-cytochrome *c* reductase (Fig. 4). When the rates of reduction were corrected for the small reduction by the Co I H₂ preparation alone (curve C), it was found that the BAL treatment (0.01 M-BAL for 15 min. at 37°) had inactivated the Co I H₂-cytochrome *c* reductase by 94.6%. The inactivation of the Co I H₂ oxidase was not measured at the same time, but from other experiments with heart-muscle preparations of similar concentration, it would be expected that the inactivation would be about 90–95%. The initial lag in the reduction of cytochrome *c* by the control heart-muscle preparation parallels the lag of the oxidation of Co I H₂ in the absence of cyanide or added cytochrome *c* after shaking heart-muscle preparation at 37° for 15 min. (Fig. 4; Slater, 1950). Although there was some unoxidized BAL left in the heart-muscle preparation after treatment with BAL, the final concentration of this BAL after dilution of the heart-muscle prepara-

tion was so low that it did not cause any reduction of the cytochrome *c* before the addition of the Co I H₂.

DISCUSSION

It was concluded in the previous paper that a factor, or factors, links diaphorase with cytochrome *c*. This factor, or one of them, is the BAL-sensitive factor, which also mediates the oxidation of cytochrome *b* by cytochrome *c* in the succinic oxidase system. According to this conclusion, the reaction between Co I H₂ and cytochrome *c* may be described by the equations

overall reaction

where *D* and *DH*₂ are oxidized and reduced diaphorase, *F*^{···} and *F*^{··} are oxidized and reduced factor (assumed, for purposes of these equations, to be a haematin compound), *c*^{···} and *c*^{··} are oxidized and reduced cytochrome *c*. Each of these equations probably represents a number of reactions.

It has been shown in the previous paper that reaction (1) is more rapid than the overall reaction. The fact, also reported in the previous paper, that added cytochrome *c* has little stimulating effect on the aerobic oxidation of Co I H₂ suggests that reaction (3) is also not limiting when the *c*^{··} formed by reaction (3) is rapidly reoxidized by cytochrome *c* oxidase. Thus it seems likely that, in the absence of cyanide, reaction (2) is the slowest reaction of the series. In the presence of cyanide, however, the oxidation of *c*^{··} is prevented, owing to inhibition of the cytochrome oxidase, so that when the small amount of endogenous cytochrome *c* is reduced, it is removed from the reaction. Reaction (3) can now be restored by adding a large excess of cytochrome *c*, but added cytochrome *c* reacts with the factor very much more slowly than does the endogenous cytochrome *c* of the heart-muscle preparation. Neither added nor endogenous cytochrome *c* reacts directly with diaphorase, since treatment of the heart-muscle preparation with BAL inactivates the aerobic oxidation of Co I H₂ and the Co I H₂-cytochrome *c* reductase without affecting the reaction between Co I H₂ and methylene blue. (There might be a very slow direct reaction between the endogenous cytochrome *c* and diaphorase, since treatment of the heart-muscle preparation with BAL under such conditions that the succinic oxidase activity is completely inactivated, does not completely stop the aerobic oxidation of Co I H₂. The residual oxidation usually amounts to 1–2% that of the untreated preparation.)

The conclusion that at least two enzymes are required for the reduction of cytochrome *c* by Co I H₂ might appear at variance with the findings of several workers who have obtained various cytochrome reductases in solution. There are, however, several possible explanations of this apparent discrepancy.

(i) In no case is there unequivocal evidence that only one enzyme was concerned in the reaction between the coenzyme and cytochrome *c*, under the conditions of test used. The Co I H₂-cytochrome *c* reductase of animal tissues recently obtained in solution by Heppel (1949) and that extracted from yeast by Altschul, Persky & Hogness (1941) have not been completely purified. In this connexion, it is interesting to note that crude solutions of yeast lactic dehydrogenase catalyse the reduction of cytochrome *c* by lactate, but this property is lost on purification, presumably due to the removal of an additional factor (Bach, Dixon & Zerfas, 1946). Dihydrocoenzyme II-cytochrome *c* reductase has been isolated from yeast and highly purified by Haas *et al.* (1940) and Haas, Harrer & Hogness (1942) (see also Ochoa, 1945). However, although as judged by flavine content and other measurements this enzyme was very pure, considerable amounts of crude enzyme preparations have been added in the tests for cytochrome reductase activity which have been published. (I am indebted to Dr C. Liébecq for drawing my attention to this.) It is possible that these crude enzyme preparations provided an additional factor. Horecker (1949) has recently briefly reported the isolation of dihydrocoenzyme II-cytochrome *c* reductase from animal tissues. The specific activity of this enzyme is one-sixth that of the yeast enzyme, i.e. 158/6 = 26, which may be compared with 13.7 for the Co I H₂-cytochrome *c* reductase activity of unpurified heart-muscle preparation found in the present study.

(ii) Diaphorase II, which catalyses the oxidation of dihydrocoenzyme II by methylene blue (Adler, Euler & Günther, 1939) may react directly with cytochrome *c*, although diaphorase I requires an additional factor.

(iii) Different enzyme systems may be involved in yeast and animal tissues.

(iv) Even if an enzyme in solution rapidly reduces a large concentration of cytochrome *c*, the two enzymes may not interact directly when they are attached to granules in the cell. (The converse is equally possible since two enzymes which interact very rapidly *in vivo* when attached to granules may react much more slowly when the two are in solution.) The enormously greater rate of reduction of the endogenous cytochrome *c* of the heart-muscle preparation compared with that of added cytochrome *c* is an illustration of the differences between the two forms of cytochrome *c*. Moreover, cyto-

chrome *c* in solution is reduced very rapidly by a number of reducing agents, e.g. ascorbic acid which reduce the cytochrome *c* in the heart-muscle preparation much more slowly (Slater, 1949*b*). Thus the various 'cytochrome reductases' which have been isolated may be the appropriate diaphorase, which when reduced, can be reoxidized either by methylene blue or cytochrome *c* in solution, but which *in vivo* is not oxidized by cytochrome *c* but by another catalyst, which may be a haematin compound like cytochrome *c* (Slater, 1949*c*).

SUMMARY

1. The Co I H_2 -cytochrome *c* reductase activity of heart-muscle preparation is about the same as its succinate-cytochrome *c* reductase activity at room temperature.

2. The concentration of oxidized cytochrome *c*

does not fall exponentially with time during the course of the reduction. The rate of reduction of cytochrome *c* is approximately a rectangular hyperbolic function of the oxidized cytochrome *c* concentration.

3. The rate of reduction of the endogenous cytochrome *c* of the heart-muscle preparation, calculated from the rate of the aerobic oxidation of Co I H_2 , was 1200 times that of the same concentration of cytochrome *c* added to the heart-muscle preparation.

4. The Co I H_2 -cytochrome *c* reductase activity of heart-muscle preparation is inactivated by treatment with BAL.

5. It is concluded that the Co I H_2 -cytochrome *c* reductase in heart-muscle preparation consists of two or more enzymes.

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On Certain Peptides Occurring in Marine Algae

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In an earlier communication Haas, Hill & Russell-Wells (1938) expressed their intention of investigating the water-soluble peptide occurring in the red alga *Griffithsia flosculosa* Batt. (*G. setacea* C. Ag.). The outbreak of war, however, interrupted the work, and further collaboration became impossible. The present communication is an attempt to amplify the earlier work on *G. flosculosa*, and to provide certain additional data concerning the peptides contained in two species each of the brown alga *Pelvetia* and the encrusted alga *Corallina*, belonging to the Phaeophyceae and Rhodophyceae respectively.

METHODS

The freshly gathered weed was spread out to dry and worked up as required, drying having been found to have no effect on the nature of the extract. For extraction the dried weed was immersed in 4-5 times its weight of water and extracted for about 1 hr. at 60-70° with occasional stirring; after straining the weed, a second extraction was made and the combined extracts were filtered through paper pulp and precipitated by basic Pb acetate; the filtrate from this was freed from Pb by saturation with H_2S and the resultant acid solution was concentrated under reduced pressure at about 60°. The resulting solution was made alkaline with Na_2CO_3 , freed from NH_3 by a rapid current of air, and after neutral-