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The Determination of Cytochrome *c* Reductase Activity in the Keilin and Hartree Beef Heart Preparation

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The succinate-cytochrome *c* reductase activity decreases consistently in reaction mixtures as if it were inhibited by the reaction products. Consequently, in enzyme assays, the activity should be extrapolated to zero time.

Variations in the ferricytochrome *c* concentration within the range 0.15–0.8 mg/ml did not cause any pronounced change in the extrapolated activity.

Succinate-cytochrome *c* reductase is an integrating component of the respiratory chain. It is of particular interest that this enzyme can be specifically inhibited by one of the antibiotics, antimycin A.

The effect of antimycin A on the respiratory chain was first pointed out by Ahmad *et al.*[1,2] Research on this subject has been rather extensive during the last 15 years.[3] The site of antimycin A inhibition has been specified differently for different oxidation-reduction systems. In the succinate-cytochrome *c* reduction system an increasing amount of evidence indicates that the site of action is localized between cytochromes *b* and c_1.[4,5] The inhibitory mechanism, however, is still obscure.

The Keilin and Hartree [6] heart muscle preparation catalyzes the oxidation of succinate. A preparation of this kind was used in the present investigation. It contains the assembly of components necessary for electron transport in the terminal respiration, but it does not have the capability of oxidative phosphorylation. Part of the terminal respiration can be blocked by cyanide. If this is done, the reduction of cytochrome *c* can be followed.

We have taken up a study of the mechanism by which antimycin A exercises its influence on the respiratory chain. A prerequisite is a method for the accurate

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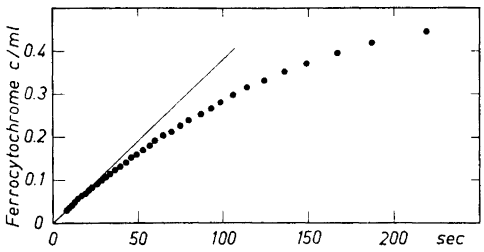

Fig. 1. The concentration of ferrocytochrome *c* (mg/ml) at various times (sec) in a reaction mixture originally containing 0.667 mg ferricytochrome *c* and 0.046 mg enzyme preparation per ml. The tangent was drawn to give the same value for the enzymic activity extrapolated to zero time as is calculated from Figs. 2 and 3.

measurement of the cytochrome *c* reductase activity. It soon appeared necessary to develop a new method, as earlier ones [7–9] were not adequate for our purpose.

A typical picture of the reduction of ferricytochrome *c* in the presence of succinate-cytochrome *c* reductase is shown in Fig. 1, in which the concentration of ferrocytochrome is plotted *versus* time. The reaction rate at any time t corresponds to the tangent of the curve, and it can be seen from Fig. 1 that this rate decreases consistently. That may be a consequence either of the diminishing of the ferricytochrome concentration or of an inhibitory effect of the reaction products. To be able to judge which of these two causes is predominant, we must measure the reaction rate as extrapolated to zero time in reaction mixtures with different ferricytochrome concentrations.

THEORETICAL

The extrapolation of the cytochrome *c* reductase activity can, of course, be done by fitting a tangent at zero ferrocytochrome concentration to a curve like that shown in Fig. 1. However, the graphical fitting of such a tangent involves some uncertainty which can be overcome to a large extent by using more suitable plotting methods. Two such methods have been particularly useful for our extrapolations. In one of these methods, the logarithm of the ferricytochrome concentration is plotted *versus* time, as shown in Fig. 2, and in the other, the inverse value of the ferricytochrome concentration is plotted *versus* time, as shown in Fig. 3. It is obvious that a tangent can be drawn more accurately in Figs. 2 and 3 than in Fig. 1.

The following relationship is valid between the enzymic activity $\mathrm{d}c/\mathrm{d}t$ (*cf.* Fig. 1) and the inclination of the tangent $\mathrm{d}(\log c)/\mathrm{d}t$ of Fig. 2:

$$\frac{\mathrm{d}c}{\mathrm{d}t} = -\frac{c}{\log \mathrm{e}} \frac{\mathrm{d}\log c}{\mathrm{d}t} \tag{1}$$

where c is the ferricytochrome concentration in the reaction mixture at the time t.

For the extrapolation to the conditions at $t=0$, when the ferricytochrome concentration is c_0, Eqn. (1) takes the following form:

$$\frac{\mathrm{d}c}{\mathrm{d}t} = -\frac{c_0}{\log \mathrm{e}} \frac{\mathrm{d}\log c}{\mathrm{d}t} \tag{2}$$

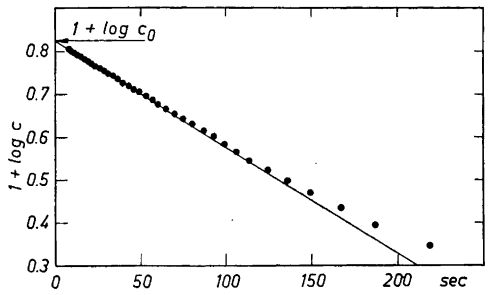

Fig. 2. The logarithm of the concentration of ferricytochrome *c* (mg/ml) *versus* time (sec) in the same experiment as shown in Fig. 1. The enzymic activity in the reaction mixture, extrapolated to zero time (Eqn. 2) is 0.0038 mg·ml^{-1}·sec^{-1}, and is calculated as the product of the inclination of the tangent and the original concentration of ferricytochrome *c*, multiplied with −2.303.

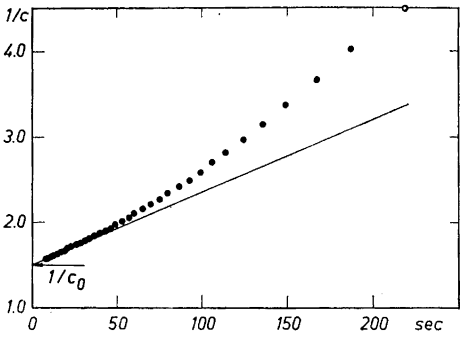

Fig. 3. The inverse value of the concentration of ferricytochrome *c* (mg/ml) *versus* time (sec) in the same experiment as shown in Fig. 1. The enzymic activity in the reaction mixture, extrapolated to zero time (Eqn. 3) is 0.0038 mg·ml^{-1}·sec^{-1}, and is calculated as the product of the inclination of the tangent and the square of the original concentration of ferricytochrome *c*.

The activity of the cytochrome *c* reductase is obtained if the inclination of the tangent is multiplied by the original ferricytochrome concentration and by the factor $-1/\log e = -2.303$. It is not correct to use the inclination of the line as a measure for the enzyme activity as was done according to a recent method,[9] unless it is first multiplied by the ferricytochrome concentration; however, if the reaction mixture is always made up to the same reactant concentration, the inclinations are proportional to the enzyme activities.

In the other method for extrapolation to $t=0$, in which the inverse value of the ferricytochrome concentration is plotted versus time as in Fig. 3, the inclination of the tangent can be written $\mathrm{d}(1/c)/\mathrm{d}t$. The following relationship is valid between the enzymic activity $\mathrm{d}c/\mathrm{d}t$ and this inclination:

$$\frac{\mathrm{d}c}{\mathrm{d}t} = c^2 \times \frac{\mathrm{d}(1/c)}{\mathrm{d}t} \qquad (3)$$

The activity of the cytochrome *c* reductase at zero time is in this case obtained if the inclination of the tangent is multiplied by the square of the original ferricytochrome concentration.

By using both methods one can check the correctness of the calculations.

EXPERIMENTAL

Heart muscle preparation. We used the method of Keilin and Hartree,[6] mainly as described by Shore.[9] A fresh beef heart was trimmed free from fat and ligaments. It was ground and weighed, and the mince was washed with cold water at 0−5°C for one hour with occasional stirring. The material was filtered off, and transferred to a Waring

Blendor. For each gram wet weight of the mince, 3 ml of 0.04 M phosphate buffer pH 7.0 was added. The mixture was disintegrated for 2 min and then centrifuged at 1500 *g* for 20 min in a Sorvall RC2—B centrifuge with angle rotor GSA at 0—5°C. The precipitate was discarded, and the supernatant was adjusted to pH 5.4 by addition of 0.2 M acetate buffer pH 4.6, and it was allowed to stand at 0—5°C for 30 min. The resulting suspension was centrifuged at 1500 *g* for 20 min at 0—5°C. The supernatant was discarded, and the precipitate was suspended in a minimal amount of 0.1 M phosphate buffer pH 7.4 and homogenized for 3 min in a glass tissue homogenizer kept in an ice bath. The protein concentration was calculated from micro Kjeldahl analyses,[10] using 6.25 as the conversion factor. Subsequently, the protein concentration of the suspension was adjusted to 25 mg/ml with a proper amount of 0.1 M phosphate buffer pH 7.4. Thereafter, between 2 and 3 ml of this suspension were dispensed into ampoules and frozen in acetone and solid carbon dioxide. The samples were stored at —60°C.

Cytochrome *c* reductase assays of our enzyme preparation gave the following activity value, extrapolated to zero time at 0.667 mg ferricytochrome *c* per ml: 0.053 mg ferricytochrome per sec per mg protein.

Succinate-cytochrome c *reductase assays.* Our method for the determination of cytochrome *c* reductase activity is a development of the methods published by Sekuzu and by Shore.[7,9] The reduction of ferricytochrome *c* was followed in a Zeiss Spectrophotometer PMQ II at 550 nm. The reaction mixture, the final volume of which was 3 ml, was made up in two steps in a cuvette. First, a solution was prepared which contained all the substances except succinate, and then, when the cuvette with the mixture had been kept in the thermostated (37°C) spectrophotometer sample compartment for 10 min for temperature equilibriation, the succinate, also kept at 37°C, was added. The components of the final reaction mixture were present in the following concentrations: succinate 10.0 mM, cyanide 7×10^{-3} M, and phosphate (pH 7.4) 0.1 M; furthermore, cytochrome *c* and heart muscle preparation were present in concentrations specified in the legends for the figures. Blanks contained all reagents except succinate. At the end of each experiment solid sodium dithionite was added to the reaction mixture in order to provide a reference point of completely reduced cytochrome *c*. Tracings of transmission *versus* time were obtained from a recorder connected to the spectrophotometer. Values read from the tracings were used for the calculation of data plotted in Figs. 1—5.

RESULTS AND DISCUSSION

As already mentioned, the succinate-cytochrome *c* reductase activity in a reaction mixture decreases consistently as the reaction proceeds. This can be concluded from Fig. 1, and is also evident from Fig. 4, in which the enzyme activity is shown at various remaining ferricytochrome concentrations in the reaction mixture. The activity extrapolated to zero time, when no ferrocytochrome *c* was present, is also plotted. It is obvious that the ferricytochrome *c* concentration in the range 0.333—0.667 mg/ml has only a rather small influence on the extrapolated value for the cytochrome *c* reductase activity, and that the decrease in enzyme activity during the course of the reaction is mainly due to some other factor. The only plausible explanation in this case is an inhibitory effect of at least one of the reaction products.

During the enzymic reduction of ferricytochrome *c* the redox potential steadily declines. The difference in redox potential between the two systems succinate-fumarate and ferricytochrome-ferrocytochrome must, from a thermodynamic point of view, be one of the parameters affecting the enzymic activity.

Further measurements were done in the convenient range 0.15—0.8 mg ferricytochrome *c* per ml. The enzyme activity was almost the same; the extra-

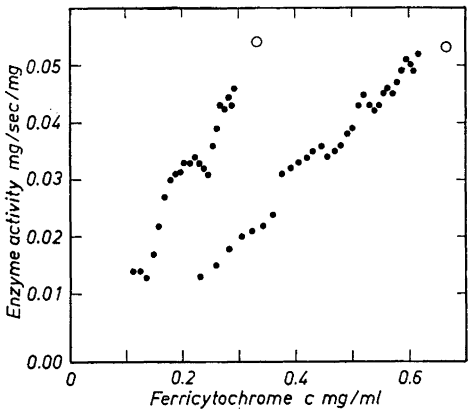

Fig. 4. The activity of the cytochrome *c* reductase preparation ($mg \cdot sec^{-1} \cdot mg^{-1}$) in reaction mixtures of different original concentrations of ferricytochrome *c* (0.667 and 0.333 mg/ml) at various remaining ferricytochrome *c* concentrations (●, zero time value ○).

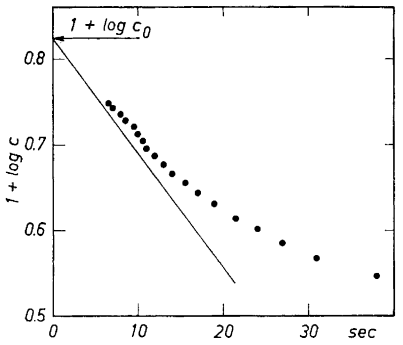

Fig. 5. The logarithm of the concentration of ferricytochrome *c* (mg/ml) *versus* time (sec) in a reaction mixture originally containing 0.667 mg ferricytochrome *c* and 0.56 mg enzyme preparation per ml. A tangent drawn at zero time gives about the same value for the enzyme activity of the preparation as obtained in other experiments, but the accuracy is much lower at this high enzyme concentration.

polated values from the various measurements did not differ sufficiently to be useful for the calculation of constants in mechanism studies.

The enzyme preparation was used in concentrations between 0.05 and 0.6 mg/ml in these experiments. Measurements with concentrations larger than about 0.3 mg/ml and with shorter time than about 3 min for the reduction of 2/3 of the ferricytochrome *c* present were clearly less accurate than measurements taken under more suitable conditions. This is illustrated in Fig. 5 which shows the extrapolation to zero time in an experiment in which the enzyme concentration and the reaction rate were too large to give an accuracy such as was obtained from the experiment corresponding to Figs. 1—3.

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