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The Bioelectrode, a New Concept Explaining the Reaction Mechanism of Beef Heart Cytochrome c Reductase

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The term bioelectrode is suggested for an electric half cell constituting a specific part of an enzyme or enzymic biological unit, to or from which electrons can be transferred.

The bioelectrode concept enables the elucidation of part of the mechanism by which ferricytochrome c is reduced with succinate by a Keilin and Hartree cytochrome c reductase preparation. It is presumed that electrons are transferred to ferricytochrome c molecules in successful electric contact collisions with the individual bioelectrodes which thereby function as redox electrodes. This presumption leads to the following equation: $\text{d ln } [\text{FeCy}^{3+}]/\text{d}t = -V/([\text{FeCy}]+Q_{ox}+(Q_{ox}/Q_{red}-1)[\text{FeCy}^{2+}])$ which is in agreement with the earlier observation that plots of the logarithm of the ferricytochrome c concentration $[\text{FeCy}^{3+}]$ *versus* time t fit a straight line rather closely as long as the ferrocytochrome c concentration $[\text{FeCy}^{2+}]$ is not a large fraction of the total concentration of ferri- and ferrocytochrome c, [FeCy]. V is the theoretical maximum value for the enzyme activity, Q_{ox} and Q_{red} are functions which comprise rates for inbound and outbound collisions in a changing rate kinetics.

It has been known for some time regarding determination of ferricytochrome c reductase activity that the relationship between time and the remaining concentration of ferricytochrome in a reaction mixture is not linear but that plots of the logarithm of the ferricytochrome concentration *versus* time rather well fit straight lines, the inclinations of which are proportional to enzyme concentrations.[1,2] In a recent publication [3] the present authors showed that a correct expression of the enzymic activity is obtained if the inclination of a line so obtained is multiplied with the ferricytochrome c concentration:

$$-\text{d } [\text{FeCy}^{3+}]/\text{d}t = -\frac{[\text{FeCy}^{3+}]}{\log \text{e}} \frac{\text{d } \log [\text{FeCy}^{3+}]}{\text{d}t} \qquad (1)$$

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In this article an explanation will be given for the observation that straight lines do not fit regular plots of ferricytochrome c concentrations *versus* time but fit semilogarithmic plots, however, only as close tangents to a theoretical curve.

THEORETICAL

The bioelectrode hypothesis. For the theoretical discussion a new concept is introduced: the bioelectrode.

The bioelectrode is regarded as an electric half cell in the sense this word is commonly used in physical chemistry. However, the electrode is part of an enzyme, being of biological origin; it shows specificity as to the substances with which it can interact as acceptor or donor of electrons; its area is of atomic size; and electricity is conducted between the anode and the cathode not by metal but otherwise. Thus, the word bioelectrode is used here in a sense entirely different to the sense in which this word was used in a recent conference entitled "Bioelectrodes".[4]

Cytochrome c reductase. For the transfer of electrons to a ferricytochrome c molecule from the bioelectrode segment of a Keilin and Hartree preparation the following mechanism is suggested. The frequency $\Delta n_{ox}\downarrow$ by which ferritochrome c molecules are inward bound to bioelectrode sites within a small time interval Δt is proportional to the number of free bioelectrode places N_f and to the concentration of ferricytochrome c in the medium; and the frequency $\Delta n_{red}\downarrow$ by which ferrocytochrome c molecules are inward bound to bioelectrode places are likwise proportional to the number N_f and to the concentration of ferrocytochrome c in the medium. Let the proportionality factor be k. Then,

$$\Delta n_{ox}\downarrow = k\ [FeCy^{3+}]\ N_f \Delta t \quad (2)$$

$$\Delta n_{red}\downarrow = k\ [FeCy^{2+}]\ N_f \Delta t \quad (3)$$

The frequencies $\Delta n_{ox}\uparrow$ and $\Delta n_{red}\uparrow$ by which ferricytochrome c and ferrocytochrome c, respectively, are outward bound from bioelectrode places within the same small time interval is proportional (factor c) to the number of places N_{ox} occupied by ferricytochrome c molecules, and to the number N_{red} occupied by ferrocytochrome c molecules, respectively. Hence

$$\Delta n_{ox}\uparrow = c\ N_{ox}\Delta t \quad (4)$$

$$\Delta n_{red}\uparrow = c\ N_{red}\Delta t \quad (5)$$

The following quotients are calculated:

$$Q_{ox} = \frac{c\ \Delta n_{ox}\downarrow}{k\ \Delta n_{ox}\uparrow} = \frac{[FeCy^{3+}]\ N_f}{N_{ox}} \quad (6)$$

and

$$Q_{red} = \frac{c\ \Delta n_{red}\downarrow}{k\ \Delta n_{red}\uparrow} = \frac{[FeCy^{2+}]\ N_f}{N_{red}} \quad (7)$$

Let the probability be p for a collision contact to be successful, *i.e.*, to provide an electric contact by which an electron is transferred to a ferricyto-

chrome c molecule. If the probability of electron transfer from a ferrocytochrome molecule when it is in contact with a cytochrome c reductase bioelectrode place is small, and this will be discussed subsequently, the number of successful contacts $\Delta n_{ox\to red}$ during the time interval Δt considered obviously is

$$\Delta n_{ox\to red} = p \ \Delta n_{ox} \downarrow \tag{8}$$

Using eqns (2) and (6) we get

$$\Delta n_{ox\to red} = kQ_{ox}pN_{ox}\Delta t \tag{9}$$

Thus, the observed reaction rate v is proportional to the number of bioelectrode sites in electric contact with a ferricytochrome c molecule. Consequently, the maximum rate V is proportional to the limit value when all bioelectrode sites (which can be written $N_f + N_{ox} + N_{red}$) are in electric contact with ferricytochrome c molecules, provided no other rate limiting reaction has a noticeable effect.

Hence,

$$\frac{V}{v} = \frac{N_{ox} + N_{red} + N_f}{N_{ox}} \tag{10}$$

Using eqns. (6) and (7) we get

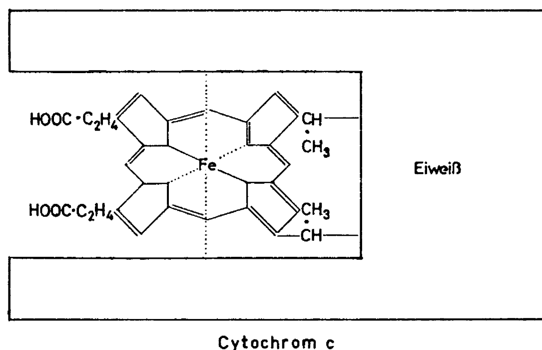
$$\frac{V}{v} = \frac{[FeCy^{3+}] + (Q_{ox}/Q_{red})[FeCy^{2+}] + Q_{ox}}{[FeCy^{3+}]} \tag{11}$$

This equation is rearranged. Q_{ox}/Q_{red} $[FeCy^{2+}]$ is written $[FeCy^{2+}] + (Q_{ox}/Q_{red} - 1)[FeCy^{2+}]$; and $[FeCy^{3+}] + [FeCy^{2+}]$ is written [FeCy].

$$\frac{V}{v} = \frac{[FeCy] + Q_{ox} + (Q_{ox}/Q_{red} - 1)[FeCy^{2+}]}{[FeCy^{3+}]} \tag{12}$$

It is in accordance with modern concepts, first expressed by Theorell,*[,5]

* Die angeführten Tatsachen lassen es berechtigt erscheinen, die folgende Formel für das Cytochrom c aufzustellen:



Cytochrom c

Footnote continued on page 3420.

that only a small fraction of the surface of the molecule has the iron atom accessible to permit the transfer of an electron from the bioelectrode site.

Even if we admit the possibility of some kind of guidance for the ferricytochrome c molecules, it is reasonable to presume (and this presumption is in accordance with experimental results presented here) that only a small fraction of the collisions are successful in providing the necessary electric contact. A consequence of our presumption is that the numerical value of the quotient Q_{ox}/Q_{red} in the preceding equation is rather close to 1; and it is slightly larger than 1 because during the collision contact some of the places N_{ox} turn into N_{red} in eqns. (6) and (7). Consequently, $(Q_{ox}/Q_{red}-1)$ is a small number, and if $[FeCy^{2+}]$ is sufficiently small, the product of them is negligible compared with Q_{ox}. However, during the course of the reaction, the value $[FeCy^{2+}]$ increases, and the product becomes more and more significant.

If we consider that $v=-d[FeCy^{3+}]/dt$, and if we multiply the nominator and the denominator of the right hand side of eqn. (12) with the total concentration of ferri- and ferrocytochrome c, [FeCy], we can rewrite eqn. (12) as follows:

$$-\frac{d \ln [FeCy^{3+}]}{dt} = \frac{V}{[FeCy]} \cdot \frac{[FeCy]}{[FeCy]+Q_{ox}+(Q_{ox}/Q_{red}-1)[FeCy^{2+}]} \quad (13)$$

In a reaction mixture $[FeCy^{2+}]$ increases with time; and in eqn. (13) the right hand side denominator increases with time. However, this increase is almost insignificant as long as $[FeCy^{2+}]$ has not grown to a large fraction of the total cytochrome c concentration, because $(Q_{ox}/Q_{reg}-1)$ is a small number. Thus, the derivative eqn. (13) keeps almost constant with increased values of t; and thereby the tendency of change is in the direction of decreased absolute value with increased time. An illustration of this is given as Fig. 2 in our previous publication.[2]

Extrapolation to zero ferrocytochrome c concentration. According to elementary rules for derivation,

$$-[FeCy^{3+}] \frac{d \ln [FeCy^{3+}]}{dt} = -\frac{d [FeCy^{3+}]}{dt} \quad (14)$$

Hence, the cytochrome c reductase activity v_0, extrapolated to zero ferrocytochrome c concentration, can be written

$$v_0 = -[FeCy] \left(\frac{d \ln [FeCy^{3+}]}{dt}\right)_{[FeCy^{2+}]=0} \quad (15)$$

and we obtain from eqn. (13)

$$v_0 = V \frac{[FeCy]}{[FeCy]+Q_{ox}} \quad (16)$$

Footnote continued from page 3419.

Es ist zur Formel zu bemerken, dass es noch ungewiss ist, ob die Eiweisskomponente an beide oder nur eine von den Seitenketten des „Häms" gekoppelt ist.

Die plane Häm-Scheibe soll nach neueren Anschauungen bei den Eiweisshämochromogenen in einem Spalt des Proteins in radiärer Stellung eingebaut sein.

The calculation of the extrapolated value of the derivative in eqn. (15) can be done graphically as was shown in our previous publication [3] as Fig. 2, and it can also be done numerically by fitting a second degree equation to experimental values according to the method of least squares as described elsewhere.[6]

Further equations and graphs for activity calculations. Eqn. (12) can be rewritten in several ways which are useful for graphic and numeric extrapolations of cytochrome c reductase activity to zero ferrocytochrome concentration. With the notation

$$Q = Q_{ox} + (Q_{ox}/Q_{red} - 1)[FeCy^{2+}] \quad (17)$$

eqn. (12) can be rewritten as follows:

$$v = V \frac{[FeCy]}{[FeCy]+Q} \frac{[FeCy^{3+}]}{[FeCy]} \quad (18)$$

$$\frac{1}{v} = \frac{1}{V} \frac{[FeCy]+Q}{[FeCy]} \frac{[FeCy]}{[FeCy^{3+}]} \quad (19)$$

$$v = V \frac{[FeCy]}{[FeCy]+Q} - v \frac{[FeCy^{2+}]}{[FeCy^{3+}]} \quad (20)$$

$$\frac{1}{v} = \frac{1}{V} \frac{[FeCy]+Q}{[FeCy]} + \frac{1}{V} \frac{[FeCy]+Q}{[FeCy]} \frac{[FeCy^{2+}]}{[FeCy^{3+}]} \quad (21)$$

$$\frac{[FeCy^{3+}]}{v[FeCy^{2+}]} = \frac{1}{V} \frac{[FeCy]+Q}{[FeCy]} + \frac{1}{V} \frac{[FeCy]+Q}{[FeCy]} \frac{[FeCy^{3+}]}{[FeCy^{2+}]} \quad (22)$$

$$-\frac{dt}{d \ln [FeCy^{3+}]} = \frac{1}{V}\left([FeCy]+Q_{ox}\right) + \frac{1}{V}\left(\frac{Q_{ox}}{Q_{red}} - 1\right)[FeCy^{2+}] \quad (23)$$

The use of these equations for graphical evaluations is shown in Figs. 1—5. Because Q is not constant but approaches the limit value of Q_{ox} when $[FeCy^{2+}]$ approaches zero, the straight lines shown in Figs. 1—5 are tangents or asymptotes.

In Fig. 1 (eqn. 18) the straight line is a tangent to the theoretical curve at the abscissa value 1 and passes through origo, in Fig. 2 (eqn. 19) also the straight line is a tangent at the abscissa value 1 and passes through origo, in Fig. 3 (eqn. 20) the straight line has the inclination -1 and is a tangent at the abscissa value 0, in Fig. 4 (eqn. 21) the straight line crosses the axis of abscissas at its value -1 and is a tangent to the theoretical curve at the abscissa value 0, and in Fig. 5 (eqn. 22) the straight line also crosses the line of abscissas at its value -1 but is an asymptote. Eqn. (23) corresponds to Fig. 2 in our previous publication, however, with the axes of coordinates interchanged.[3]

Calculation of the values of V *and* Q_{ox}. In eqn. (16) V and Q_{ox} are the unknown quantities to be calculated. This equation can be rearranged to

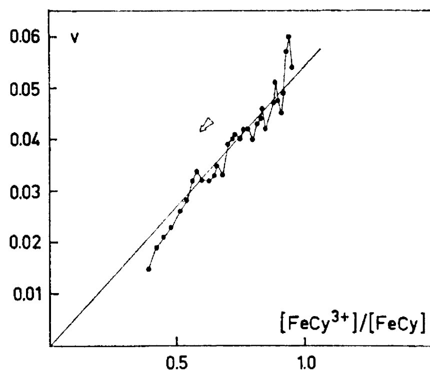


Fig. 1. The cytochrome c reductase activity v (mg mg^{-1}sec^{-1}) *versus* the quotient between the concentration of ferricytochrome c and the total cytochrome c concentration (*cf.* eqn. 18) in an experiment shown in Figs. 1–4 of a previous publication.[3] The direction of time is indicated with an arrow. The enzymic activity v_0 in the reaction mixture, extrapolated to zero ferrocytochrome concentration can be read as the inclination of a line through origo which fits the points as a tangent at the abscissa value 1. The activity is 0.0553 mg mg^{-1} sec^{-1} as calculated numerically [6] from eqn. 15, and the line is drawn accordingly.

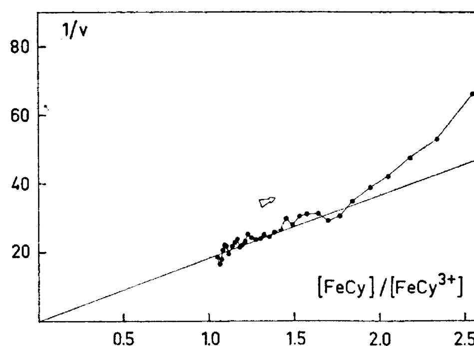


Fig. 2. The inverse value of the cytochrome c reductase activity v *versus* the quotient between the total cytochrome c concentration and the concentration of ferricytochrome c (*cf.* eqn. 19) in the same experiment as shown in Fig. 1. The enzymic activity v_0 can be read as the inverse value of the inclination of the tangent at the abscissa value 1.

equations which are convenient for graphical and numerical calculations, and such equations are similar to those used for the calculation of Michaelis' constants.[7–9] Statistical discussions on the use of such equations and on the accuracy of the results have been published during the last few years.[10–12]

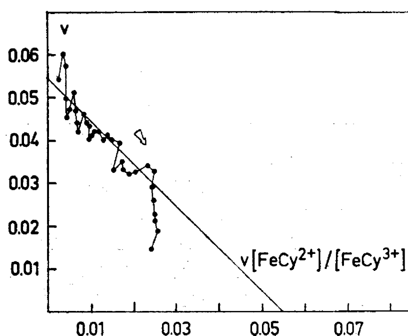


Fig. 3. The cytochrome c reductase activity v *versus* the product of v and quotient between the concentration of ferrocytochrome c and the concentration of ferricytochrome c (*cf.* eqn. 20) in the same experiment as shown in Fig. 1. The enzymic activity v_0 can be read as the intersection of the tangent at zero abscissa value with either of the coordinate axes; the inclination of the line is -1.

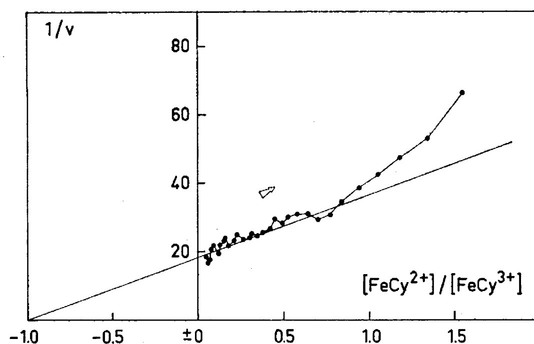


Fig. 4. The inverse value of the cytochrome c reductase activity v *versus* the quotient between the concentration of ferrocytochrome c and the concentration of ferricytochrome c (*cf.* eqn. 21) in the same experiment as shown in Fig. 1. The enzymic activity v_0 can be read as the inverse value of the intersection of the tangent at zero abscissa value with the axis of ordinates, and, with the same result, as the inverse value of the inclination of the line, because this line crosses the axis of abscissas at the point -1 (*cf.* Fig. 2).

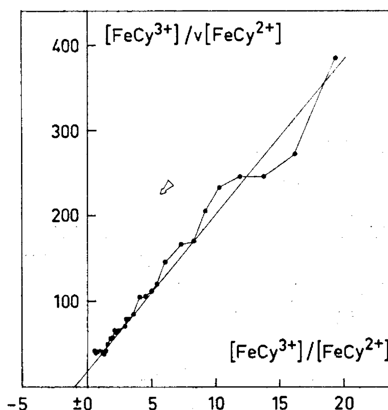


Fig. 5. The product of the inverse value of the cytochrome c reductase activity v and the quotient between the concentration of ferricytochrome c and ferrocytochrome c *versus* the quotient between the concentration of ferricytochrome c and the concentration of ferrocytochrome c (*cf.* eqn. 22) in the same experiment as shown in Fig. 1. The enzymic activity v_0 can be read as the inverse value of the intersection of the asymptote with the axis of ordinates, and with the same result as the inverse value of the inclination of the line, because this line crosses the axis of abscissas at the point -1.

EXPERIMENTAL

Enzyme preparation. We used a Keilin and Hartree beef heart preparation, described in our previous publication.[3,13]

Succinate-cytochrome c reductase assays. Our method which was developed from the methods used by Sekuzu and by Shore was described in our previous paper.[1-3] * The reduction of ferricytochrome c was followed in a spectrophotometer connected to a recorder. Readings from the tracings were used for our calculations. Figs. 1–4 of our previous article [3] and Figs. 1–5 of this article contain plots from the same experiment. The values of v used for Figs. 1–5 were calculated as the mean values of three consecu-

* Correction to Ref. 3: The cyanide concentration was 7×10^{-3} mM, and the enzyme preparation concentration (Fig. 1) was 0.070 mg/ml.

tive original measurements of v which were obtained as the quotients between the difference in ferricytochrome c concentration and the corresponding difference in time at consecutive crossing points between the tracing curve and parallel transmission lines of the recorder paper.

RESULTS AND DISCUSSION

The classical theory of enzyme action considers the transfer of atoms or groups of atoms by means of the formation and the disruption of chemical bonds between atoms, as originally suggested by Henri and by Brown; this theory has been further developed and is the basis for the calculation of Michaelis constants.[14-22] The bioelectrode theory, on the contrary, concerns the transfer of electrons with the specificity characteristic for enzymes.

Whereas Michaelis constants apply to a steady-state kinetics and comprise rate constants for the formation and dissolution of chemical bonds, the functions Q_{ox} and Q_{red} suggested here apply to a changing rate kinetics and comprise quotients of rates for inbound and outbound collisions, some of which may give electric contacts by which the chemical nature of the colliding molecule is changed without the formation and disruption of chemical bonds. Consequently, Q_{ox} and Q_{red} are not Michaelis constants but represent a new kind of functions, expressed in units of concentration.

Nevertheless, it should be pointed out here that there are considerable mathematical similarities between the theoretical deductions in this article and Henri's theoretical treatment of the enzymatic hydrolysis of sucrose.[14,23]

The normal redox potential at pH 7 is for cytochrome c 0.22 V, and for the succinate and fumarate system 0.03 V.[24] The equilibrium constant corresponding to this potential difference is $10^{3.2}$ for the transfer of one electron. Although the over-all mechanism is still obscure, we can conclude that for the experiments and limited theory discussed in this paper any transfer of electrons from ferrocytochrome c to the cytochrome c reductase may be disregarded. In the theoretical deduction here the possibility of a reaction in a direction opposite to the one described was disregarded, and consequently the theory presented here on a changing rate kinetics also in this respect is different to the theory for a changing rate when a reaction approaches its equilibrium, as is well-known from work on esterases.

As mentioned in our previous article,[3] measurements were done at pH 7.4 in range 0.15—0.8 mg/ml ferricytochrome c which is convenient for spectrophotometric measurements. The activity v_0 of equal dilutions of a ferricytochrome c reductase preparation did not differ significantly in this series of experiments, and the conclusion is that the limit value of Q_{ox} is much smaller than 0.15 mg/ml ferricytochrome c. This is not surprising, considering that physiological total concentrations of cytochrome c are much smaller than the concentrations used in our experiments.

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