The crystals of the Michaelis complex were prepared using D-alanine as substrate as reported previously[1]. The crystal suspension was injected into the cell and centrifuged at 3000 × *g* for 15 min. By the centrifugation, the crystals were packed in the capillary part of the cell. ESR measurement was performed at room temperature (25°) under the following conditions: modulation, 100 kcycles, 6 Gauss; sensitivity, 2000; power, 5 db; response, 0.3 sec; magnetic-field scanning speed, 42 Gauss/min; chart speed, 4 cm/min.

As shown in Fig. 2 A, a typical signal was observed with crystal, the *g* value being 2.004.

The crystals were then removed and the mother liquid was injected into the capillary part of the cell. No ESR signal was demonstrable (Fig. 2 B). Elevating the field modulation to be 100 kcycles, 15 Gauss, however, revealed a typical signal with the same *g* value as that of the crystal.

These results suggest that both the crystal and the mother liquid contain a free radical. Considering this fact together with the former results[1], it is concluded that the crystal is a complex of the semiquinoid form of the holoenzyme and the activated or partially modified substrate; *viz.*, an intermediate complex formed during the enzymic catalysis, which was theoretically assumed by MICHAELIS AND MENTEN[3].

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The reaction kinetics of respiratory enzymes studied by electron-spin-resonance absorption

We have already reported on the free-radical signals observed during reactions catalysed by fungal glucose oxidase (EC 1.1.3.4) and pig-heart succinate dehydrogenase (EC 1.3.99.1)[1]. In this paper the reaction kinetics of these enzymes are described.

As an acceptor methylene blue was used, with 1–100 mM β-glucose and 10 to 100 mM succinate as substrate. The experimental conditions of the ESR apparatus were: X-band, low-frequency (380 cycles/sec) field modulation (modulation width: 0.22–0.8 Gauss) and ambient air temperature (27°). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0).

Abbreviation: ESR, electron-spin resonance.

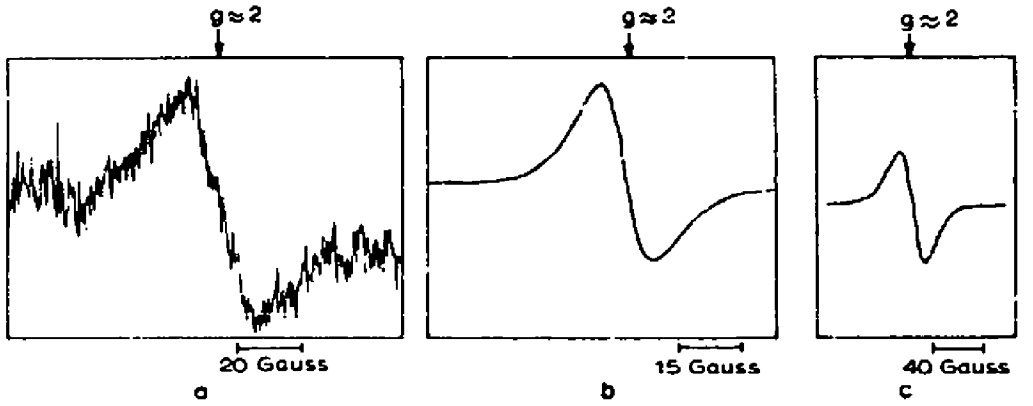

Fig. 1. The ESR spectra obtained for the radical in the glucose oxidase reaction. a, O_2 as an acceptor; b, methylene blue as an acceptor; c, radical produced during reduction of methylene blue by $Na_2S_2O_4$.

The singlet ESR signals of free radicals are observed only in the complete systems and the g value is always about 2 and line width is always about 15 Gauss and 20 Gauss, respectively (Fig. 1a and b).

Whereas in the reduction of methylene blue by $Na_2S_2O_4$ a singlet ESR signal having a g value of about 2 and a line width (peak to peak) of about 20 Gauss (Fig. 1c)

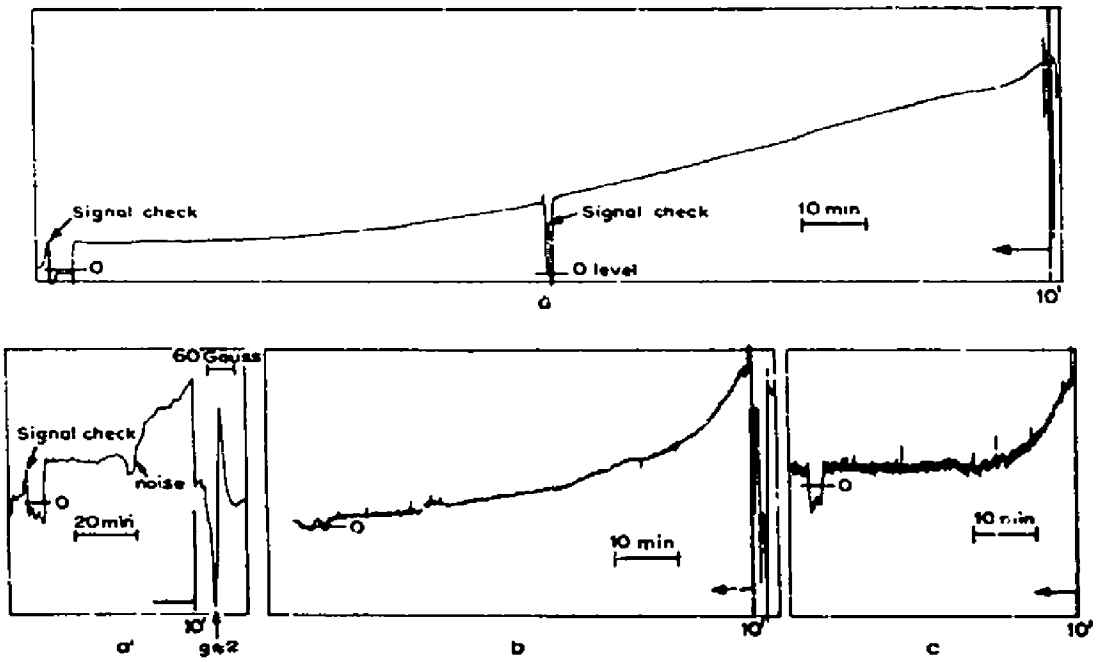

Fig. 2. The decay curves of the signal height (in 100 mM phosphate buffer, pH 7.0). a, 40 mM β-glucose + 40 mM methylene blue (MB) + 10 μM glucose oxidase; a', 10 mM succinate + 10 mM MB + succinate dehydrogenase; b, 60 mM β-glucose + 20 mM MB + 10 μM glucose oxidase; c, 60 mM β-glucose + 10 mM MB + 20 μM glucose oxidase.

is observed in viscous water solution containing 90% of glycerol, identification of these radicals can not be determined by a simple ESR technique. However, free radicals are produced in the complete enzymic reaction in such a way that the decay curves of the ESR signals seem to be related to the intermediate free radicals.

Random mixture of these systems show decay curves typical of non-linear equations (Fig. 2a, a').

When one of the substrates (acceptor or donor) is in excess, the concentration of the other decays exponentially. In this condition we can resolve the decay curves of free radicals as a quasi-steady state (Fig. 2b, c).

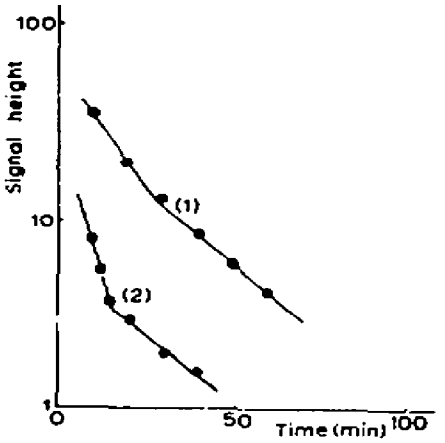

Fig. 3. Semilogarithmic plots of the decay curves of Fig. 2 b and c. Curves 1 and 2 correspond to Figs. 2 b and c, respectively.

The semilogarithmic plots of signal height in Figs. 2b and c show that the two exponential terms overlap (Fig. 3).

The usually accepted reaction scheme for glucose oxidase is

$$\beta\text{-glucose} \rightarrow \text{gluconolactone} \quad \underset{E\text{-}Fl}{\overset{E\text{-}FlH_2}{\rightleftharpoons}} \quad \underset{MB}{\overset{MBH_2}{\rightleftharpoons}}$$

where *E-Fl* is the enzyme and MB methylene blue. When all the oxidation–reduction steps include the free-radical states, we can only observe the free radical when the free radical-forming rate constant (k_1) is greater than its destroying rate constant (k_2).

Decay curves of Fig. 3 include 2 dominant exponential terms. So we may write generally

$$EA + B \overset{k_1}{\rightarrow} R\cdot \overset{k_2}{\rightarrow} E + A' + B'$$

where E is enzyme, $A \cdot B$ is acceptor or donor, A', B' are reaction products, and $R\cdot$ is the free radical observed, and put

$$\frac{dR\cdot}{dt} = -k_2 R\cdot + Ke^{-k_1 t} \qquad (1)$$

(destroyed) (formed)

where

$$K = k_1[EA_1][B_0]$$

and integrated (initial condition $t = 0$, $R\cdot = 0$)

$$R\cdot = \frac{k_1[EA_1][B_0]}{k_2 - k_1}(1 - e^{-(k_2 - k_1)t})e^{-k_1 t} \qquad (2)$$

Eqn. 2 agrees well with the experimental results (Fig. 2b, c and 3) indicating that the assumption underlying Eqn. 1 may be valid.

In the down phase of the decay curves, Eqns. 1 and 2 indicate that the steeper decline depends chiefly on k_1 and the other on k_2. We obtain the values of k_2 as $3{\cdot}4 \cdot 10^{-4}$ per sec from Fig. 3. And the fact that these are the only two dominant steps in Fig. 3 show that the species of observable free radical in this reaction is single.

As the reaction of the first term in Eqn. 1 is first order, it is thought that the observable free radical combines with an other unknown free radical having a high transfer activity such as H·.

The identification of observable free radicals should be achieved by nuclear g factor determined by Endor method, and temperature control of the sample from dielectric heating is required in our experiments.

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