

*FREE RADICALS IN HEART MUSCLE MITOCHONDRIAL PARTICLES:
GENERAL CHARACTERISTICS AND LOCALIZATION IN THE
ELECTRON TRANSPORT SYSTEM**

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Introduction.—The technique of electron spin resonance (ESR) spectrometry has enabled a new approach to the study of free radicals in biological and biochemical systems. There are good theoretical grounds for expecting free radicals to occur as intermediates in a number of biochemical processes, and the new technique presents an opportunity for testing these theories.

Ideally what is required is information regarding the identity of free radicals which occur in biochemical systems, and quantitative data which relate the free radical concentration to the kinetic properties of the system. Since the ESR technique is new and still in the process of development, the degree to which this ideal is approached is very largely determined by the characteristics of the available spectrometers.

In the initial discovery by Commoner, Townsend, and Pake¹ of ESR signals due to free radicals in biological materials, the spectrometer available at the time precluded the examination of samples containing more than a trace of liquid water, so that it was necessary to lyophilize the tissue samples before analysis. Measurements of this type yield useful information regarding the presence or absence of free radicals, but such static data provide only indirect evidence regarding the relationship between the free radicals and metabolic processes. In more recent studies of enzyme systems by Kubo *et al.*² and Bray *et al.*³ the problem presented by the aqueous character of functional enzyme systems was solved by examining samples in the frozen state. As in the case of lyophilized samples, this approach also precludes kinetic data.

With improvements in spectrometer design it is possible to obtain ESR signals from samples containing 0.1–0.15 ml of liquid water.^{4–8} The presence of liquid water, which tends to absorb the microwave radiation incident on the sample, increases the difficulty of achieving an instrument sensitivity sufficient to detect the very low concentrations of free radicals that occur in biological and biochemical systems. One way to achieve sensitivity is to scan the ESR signal relatively slowly. Ehrenberg and Ludwig⁹ have reported an ESR signal in a functional aqueous system containing yellow enzyme using a scanning time of 12 min. However, such a time scale imposes a serious limitation on kinetic experiments.

With the development by Dr. Jonathan Townsend of the Department of Physics at Washington University of an ESR spectrometer of extreme sensitivity, it is now possible to detect free radicals in aqueous solutions at concentrations of 10^{-6} to 10^{-7} M, using 1.5–2 min scanning periods. Investigation of a number of functional enzyme systems by means of this spectrometer has led to evidence that free radicals occur in them, and in certain intact living cells.^{5, 6, 8} However, these experiments, like the preceding ones, lack sufficient quantitative detail to permit

conclusions regarding the biochemical mechanisms which give rise to the observed free radicals.

In the present paper, and one which follows,¹⁰ we report quantitative observations on the kinetic properties of free radicals which occur in particles derived from the sarcosomes of pig-heart muscle. The data lead to conclusions which localize the free radicals in a particular segment of the heart particle electron transport system and which describe the biochemical mechanisms in which the free radicals participate. On the basis of these conclusions, it becomes possible to deduce from the ESR data the redox potential of succinic dehydrogenase (as distinct from the redox potential of the enzyme-substrate system) and the equilibrium constant for the enzyme-substrate reaction—values hitherto unavailable from biochemical investigations of this enzyme system.

Experimental Methods.—Reagents: Cytochrome *c* was purchased from the Sigma Chemical Company and Antimycin A was obtained through the University of Wisconsin Alumni Research Foundation. Antimycin A solutions were prepared in 50 per cent to 95 per cent ethanol. All buffered solutions of salts and other reagents were prepared to contain 10^{-4} to 10^{-2} *M* Tris or phosphate buffer at a final pH of 7.4.

Heart particle preparations: Pig hearts were chilled immediately after slaughter. The ventricles were removed, cleaned of connective and adipose tissues, and ground twice. The resulting macerate was washed by stirring 200 gm. of macerate with 2 liters of water for 10 min, the supernatant liquid being removed by decantation and discarded. This washing procedure was repeated four more times. Finally the washed macerate was homogenized in a Waring blender for 7 min with 500 ml of 0.1 *M* potassium phosphate buffer at pH 7.4. The resulting homogenate was centrifuged for 30 min at $1800 \text{ RCF} \times g$. The supernatant was retained and the sediment was further homogenized in the blender for 3 min with 300 ml of fresh buffer. This second homogenate was centrifuged as above and the first and second supernates were combined. The combined supernatants were then centrifuged at 16 to $20 \times 10^3 \text{ RCF} \times g$ for about 1 hr. The resulting sediment, after having been resuspended in a minimal amount of 0.1 *M* potassium phosphate buffer, pH 7.4, comprises the heart particle preparation. Preparations were stored at -20°C until used. After grinding the heart, all subsequent operations were performed at $2-4^\circ\text{C}$.

Apparatus: The ESR spectrometer was designed by Dr. Jonathan Townsend of the Washington University Physics Department and constructed under his supervision. A brief description has been published elsewhere.^{4, 8} The apparatus can detect free radicals in aqueous media at concentrations of 10^{-6} to 10^{-7} *M* or higher.

For most ESR observations, samples were contained in closed Pyrex cells which were inserted through a slot into the resonance cavity of the ESR spectrometer. These cells, which have been described earlier,^{4, 8} accommodate 0.1 to 0.2 ml of sample.

In some experiments it was desirable to control the gaseous environment of the sample. This was accomplished by means of a flow system composed of a Sigma T4 peristaltic pump, glass gas exchanger, narrow bore Tygon and rubber tubing, and a U-shaped Pyrex cell which is accommodated by the slot in the resonance cavity. The sample flows through the gas exchanger into the cell, from the cell to

the receiving side of the pump, and finally is returned to the gas exchanger. The system accommodates 1 to 2 ml of liquid at variable flow rates up to 20 ml min⁻¹, depending on pump speed. ESR signals may be determined while the sample flows through the ESR cell.

The control of sample temperature during ESR studies was accomplished by the flow of gas at a desired temperature past the sample cell. Buffered solutions of potassium peroxyamine disulfonate were used as the magnetic field marker. Spectrophotometric observations were made with a Cary Model-11 recording spectrophotometer.

Results.—General properties of heart particles: The preparations of heart particles described above were found to contain 0.4 to 0.6 gm. ml⁻¹ of nondialyzable material on a wet weight basis and approximately 0.07 to 0.10 gm. ml⁻¹ on a dry weight basis. Upon storage the preparations exhibit succinoxidase activity and other properties relevant to the purposes of this paper for at least several days at 0°C and three weeks at -20°C.

FIG. 1.—Difference spectra between reduced and oxidized heart particles. The ordinate represents the optical density of the reduced system under anaerobic conditions minus the optical density of the oxidized system under aerobic conditions. The reducing agents are sodium hyposulfite (solid curve) and 10⁻² M succinate (broken curve). The heart particle preparation is diluted 3 fold with 0.1 M potassium phosphate buffer, pH 7.4.

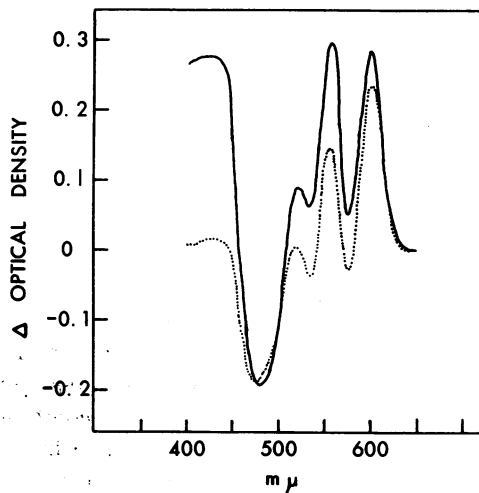


Figure 1 illustrates difference spectra between an aerobic heart particle suspension and the same suspension reduced anaerobically by succinate and sodium hyposulfite. The spectra indicate that the heart particles contain the well-known components normally found in mammalian electron transport systems. The spectrum includes the 605 m μ band of cytochromes of the α group, the 560 m μ peak representative of the fused α bands of other cytochromes, and the strongly overlapping and mutually repressed peaks at lower wavelengths due to flavins and the γ bands of the cytochromes.

The heart particles exhibit succinoxidase and succinate-cytochrome c reductase activities, and therefore those components of the electron transport system which effect the transfer of electrons from succinate to cytochrome c and to oxygen are functional. These components include succinic dehydrogenase, cytochromes and, in the case of succinoxidase activity, cytochrome oxidase. Observation of succinoxidase activity of heart particles does not require addition of cytochrome c . These heart particle preparations appear to be similar to those described by Keilin and

Hartree¹¹ with regard to method of preparation, spectral properties, and oxidative activity.

The perceived color of dense heart particle suspensions changes from red-brown to green-brown under anaerobic conditions in the presence of succinate. After sufficient oxygenation, the reverse color change is observed, marking the exhaustion of succinate. These reversible changes in color, due mainly to the appearance and disappearance of the 605 m μ absorption band, were found to be useful indices in establishing the times required, under aerobic conditions, for the complete oxidation of succinate.

The electron spin resonance of heart particles: When a sample cell, containing about 0.05 gm. of heart particles suspended in 0.15 ml of aerated phosphate or tris

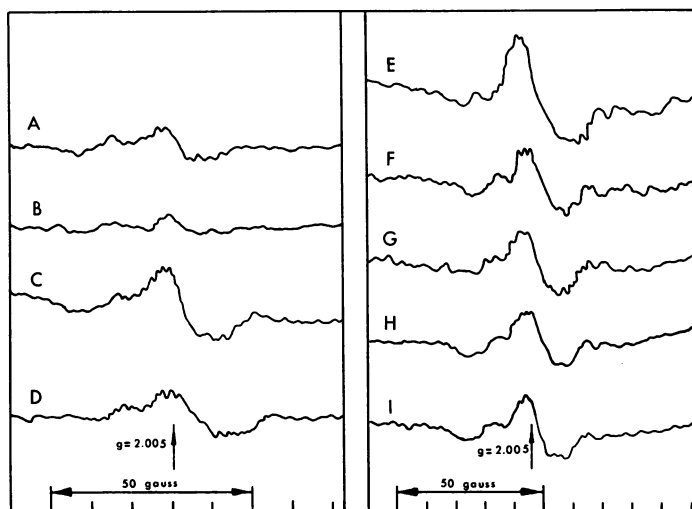


FIG. 2.—ESR signals obtained under anaerobic conditions in closed cells in the system containing heart particles and $10^{-2} M$ succinate under different instrumental conditions. The curves represent the derivative of resonance absorption with respect to magnetic field as a function of magnetic field, which increases to the right along the abscissa. The magnetic field modulation amplitude in gauss, the spectrometer's minimum response time in seconds, and the time in minutes required to scan a 100-gauss segment of the magnetic field are, respectively, the following: (a) 2, 1, 3.3; (b) 1, 1, 3.3; (c and d) 4, 1, 3.3; (e) 8, 0.1, 0.6; (f) 4, 0.1, 0.6; (g) 4, 0.1, 1.3; (h) 4, 1, 1.3; (i) 4, 1, 2.5. Gas phase was nitrogen. Initial oxygen concentration in aqueous phase was that of dissolved oxygen under atmospheric conditions and 25°C.

buffer, pH 7.4, is examined in the ESR spectrometer at magnetic fields in which signals due to organic free radicals are observable, no signals are detected other than the small effects attributable to the glass of the sample cell. Succinate solutions also show no ESR signal under aerobic or anaerobic conditions. However, Figure 2 shows that the addition of relatively low concentrations of succinate to aerated heart particle suspensions contained in closed cells produces readily detectable ESR signals. Due to the high succinoxidase activity of the particles, the system is anaerobic at the time ESR observations are made.

The ESR signal is centered at $g = 2.003 \pm 0.002$, and the width between regions of zero slope in the first derivative curves shown in Figure 2 corresponds to 15 ± 1.5

gauss. Figure 2 shows that the signal width and shape are not affected appreciably by variations in the amplitude of the magnetic field modulation between 1 and 8 gauss, so that the observed width can be regarded as characteristic of the signal. In the studies reported below, modulation amplitudes of the order of 8 to 11 gauss were commonly employed.

The signals shown in Figure 2 are not due to a simple Gaussian absorption. A small but reproducible asymmetry can be seen on the low field side of the main signal which suggests that more than one species of unpaired electron may be present. However, since symmetrical sub-peaks due to hyperfine splitting are not always equally resolved on both sides of the resonance center, it is possible that the signal is due to a single, complex resonance. In any case, in what follows we shall be concerned with the behavior of the main part of the signal, the magnitude of which may

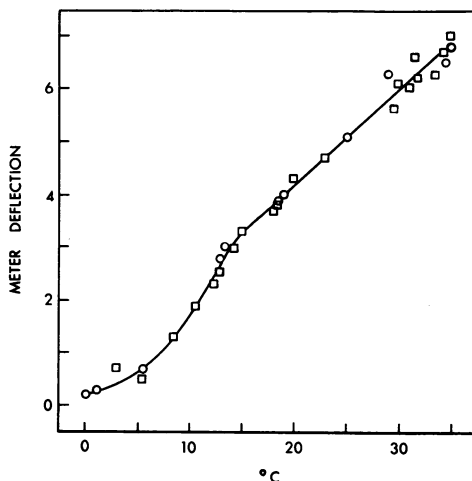


FIG. 3.—The reversible effect of temperature on the size of the ESR signals from heart particles in the presence of fumarate and succinate under anaerobic conditions in a closed cell. Fumarate/succinate mole ratio of 1; fumarate plus succinate concentration is $20 \times 10^{-3} M$. All points are corrected for any resonance in glass of cell. Open circles: decreasing temperature. Open squares: increasing temperature. Gas phase was nitrogen.

be estimated from the meter deflections at points of zero slope in the ESR derivative curve. This estimate provides a relative value for the concentrations of the unpaired electrons in the sample.

Comparison of the observed meter deflections with those given by standard aqueous solutions of a stable free radical indicates that heart particles contain a maximum of about 10^{-8} moles of unpaired electrons per gm. wet weight.

When heart particle suspensions are centrifuged in the presence of succinate, the ESR signal is found in the sedimented particles and not in the supernatant fluid. The signal is therefore associated with the heart particles themselves.

The g value and signal shape suggest that the observed ESR signal is due to organic free radicals.¹² The low concentrations of free radicals observed and the fact that the only known interaction between succinate and the heart particles involves the succinoxidase system suggest that the free radicals are associated with the catalytic activity of the heart particle and, in particular, with one or more components of the succinoxidase system. The data which follow serve to specify further the biochemical origin of the ESR signal and to establish that it results from free radicals associated with redox activity of the heart particles.

Metabolic properties of heart particle free radicals: The free radicals observed in heart muscle particles upon the addition of succinate appear to be stable in the ab-

scence of oxygen. Under this condition no diminution has been noted in the size of ESR signals from a given sample for a period of at least 7 hr at about 30 °C. Storage at 4 °C for several days also appears to have no effect on the signal size as measured at about 30 °C.

Figure 3 illustrates the effect of temperature on the size of the ESR signal observed under anaerobic conditions in the presence of $10^{-2} M$ succinate. Since the signal shape is not affected by temperature, the meter deflection between points of zero slope can be regarded as a measure of free radical concentration. The free radical concentration diminishes with decreasing temperature in the range from

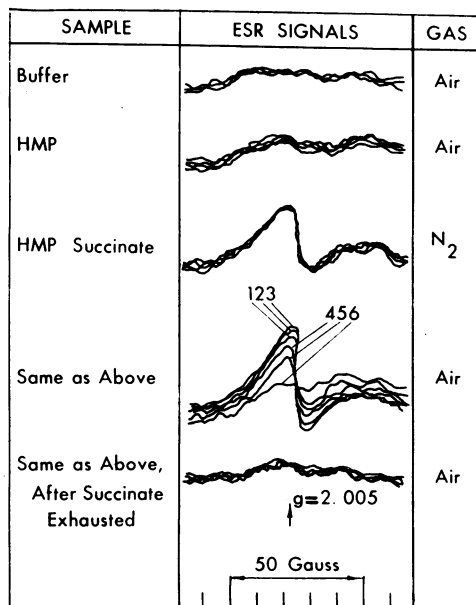


FIG. 4a.—ESR signals from a flowing suspension of heart muscle particles. About 2 ml. of fluid was circulated through a closed flow system consisting of a peristaltic pump, a gas exchanger, and a U-shaped ESR cell (effective volume about 0.1 ml) connected by tygon tubing. Rate of liquid pumping was about 10 ml/min. Rate of gas flow through the gas exchanger was about 100 ml/min. Uppermost figure represents tracings of 3 successive ESR runs with the system containing 0.1 M phosphate buffer, pH 7.4, the gas phase in the exchanger being air. The second figure shows the signals obtained when the system contains a suspension of heart muscle particles (about 0.5 gm./ml) in phosphate buffer, in equilibrium with air. The third figure represents tracings of 4 successive ESR runs after succinate ($10^{-2}M$) is added to the foregoing system, with nitrogen as the gas phase. The fourth figure represents tracings of 6 successive ESR runs, over a 20-minute period following the admission of air into the previous system. The lowermost figure shows the ESR signal observed after the succinate in the above system has been exhausted.

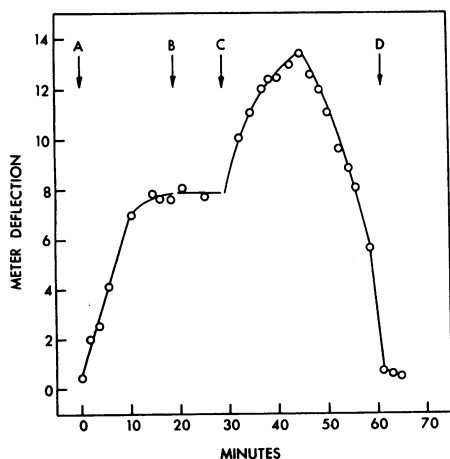


FIG. 4b.—Changes in the size of the ESR signal from heart particles associated with the oxidation of succinate by oxygen. Flow system contains 2 ml of heart particle preparation. Liquid and gas flow rates are 15 ml min^{-1} and 100 ml min^{-1} , respectively. (a) pump and air on, $10^{-2} M$ in succinate; (b) pump off, air off; (c) pump on, air on; (d) pump off. All points are corrected for the dilution of heart particles and for any resonance in glass of cell. Aqueous phase contained initially dissolved oxygen under atmospheric conditions at 25 °C.

35° to 0 °C, being very nearly undetectable around 0° to 2 °C. This effect is completely reversible. Contrasting with this reversible change is the irreversible effect of higher temperatures. Heating a sample to 70 °C for a few minutes or to 100 °C for a few seconds, which destroys the enzymatic activities of heart particles, causes

the permanent loss of the ESR signal. These results suggest that the observed free radicals result from a temperature-dependent process associated with the redox enzymes present in heart particles.

When succinate is added to heart particles under aerobic conditions in a circulating system, results such as those illustrated in Figures 4*a* and *b* are obtained. In the presence of oxygen the signal changes in size, first increasing and then decreasing. During the time when the signal declines in size, the color of the heart particle suspension changes from green to red; this indicates the exhaustion of succinate in the preparation. The ESR signal can be restored by adding fresh succinate, the size of the regenerated signal being a function of the initial succinate concentration and of the concentration of fresh succinate. This effect is shown in Figure 5.

If oxygen is withdrawn from the system, either by stopping the circulation pump or by introducing nitrogen into the gas exchanger, the ESR signal becomes stabilized with respect to size, as shown in Figure 4*b*. Regardless of its size at the time of

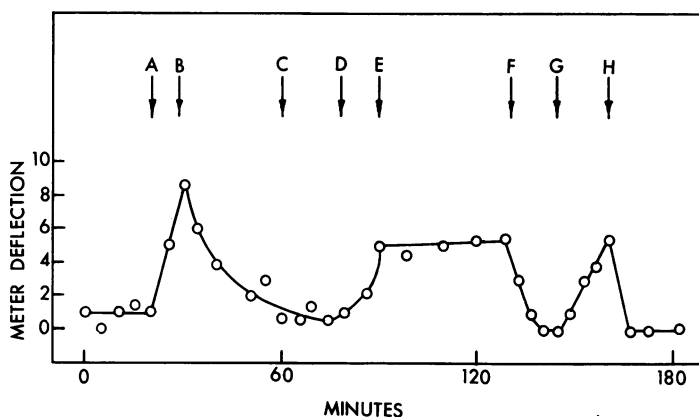


FIG. 5.—The effect of cyanide on the stability of the free radical of heart particles toward oxygen and potassium ferricyanide. Flow system contains 2 ml of heart particle preparation. Liquid and gas flow rates are 12 ml min^{-1} and 150 ml min^{-1} , respectively. (Time zero to *a*) pump and air on; (*a*) $10^{-2} M$ in succinate; (*b*) air off, oxygen on; (*c*) oxygen off, $10^{-3} M$ in cyanide; (*d*) $5 \times 10^{-3} M$ in succinate; (*e*) oxygen on; (*f*) oxygen off, nitrogen on, $15 \times 10^{-3} M$ in ferricyanide; (*g*) $5 \times 10^{-3} M$ in succinate; (*h*) $15 \times 10^{-3} M$ in ferricyanide. All points are corrected for the dilution of heart particles and for any resonance in glass of cell.

anaerobiosis, the signal maintains this amplitude indefinitely thereafter. The changes in signal size upon the oxidation of succinate, therefore, are not transitory effects associated with net electron transport. The free radicals apparently arise from some enzymatic transformation involving succinate which occurs in the absence of oxygen and over-all electron transport.

The point of origin of heart particle free radicals: In order to localize the redox processes which give rise to the observed free radicals in the succinoxidase system, the effects of altering the electron transport system by means of inhibitors and artificial oxidants were determined.

Figure 5 shows the effect of cyanide on the stability of the free radicals toward oxygen and toward an alternate oxidant, potassium ferricyanide, in a circulating system. As shown in Figure 5, under aerobic conditions addition of succinate (at time

A) causes the free radical concentration to rise briefly and then fall. When cyanide and fresh succinate are added (times C and D) the ESR signal becomes stable in the presence of oxygen for an indefinite period. Thus, cyanide in the presence of oxygen has the same effect as anaerobic conditions (See Fig. 4a and b).

The addition (at time F), under nitrogen, of an amount of potassium ferricyanide in excess of the redox equivalents of succinate present in the cyanide-inhibited system results in the rapid decay of the ESR signal. However, when succinate is again added to achieve a concentration which exceeds the redox equivalents of ferricyanide present (time G), the signal again rises. Finally, the ferricyanide-induced reduction in free radical concentration is again exhibited when ferricyanide is added, in excess to the succinate, at time H.

These results show that when electron transport in the succinoxidase system is inhibited by cyanide, oxygen cannot diminish the free radical concentration. The

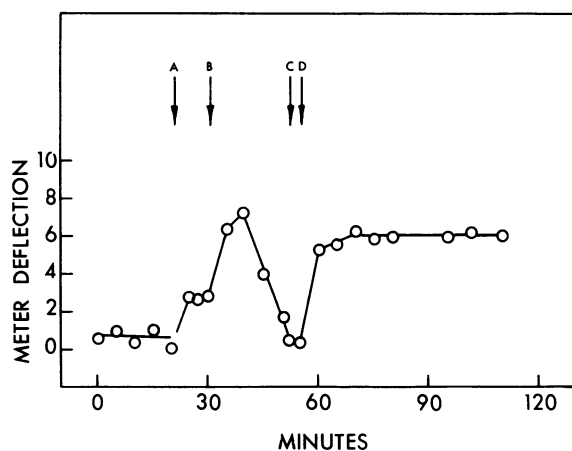


FIG. 6.—The effect of Antimycin A on the stability of the free radical of heart particles toward oxygen. Flow system contains 2 ml of heart particle preparation. Liquid and gas flow rates are 12 ml min^{-1} and 100 to 150 ml min^{-1} , respectively. (Time zero to a) pump on, nitrogen on; (a) $1.25 \times 10^{-2} M$ in succinate; (b) nitrogen off, oxygen on; (c) pump and oxygen off; (d) $1.25 \times 10^{-2} M$ in succinate, $10 \mu\text{g ml}^{-1}$ in Antimycin A, pump and oxygen on. All points are corrected for dilution of heart particles and for any resonance in glass of cell. Aqueous phase initially contained dissolved oxygen under atmospheric conditions at 25°C .

events which produce the free radicals therefore lie on the succinate or reducing side of the site of cyanide inhibition, which is at the level of cytochrome oxidase.¹¹ This result also shows that the free radicals are not destroyed by reacting directly with oxygen.

The reduction of free radical concentration by ferricyanide in the presence of cyanide, which is counteracted by excess succinate, appears to be due to oxidative depletion of succinate. Ferricyanide can bypass the site of cyanide inhibition in the succinoxidase system and thereby oxidize succinate despite the presence of the inhibitor. The effect of ferricyanide resembles that of a redox titration and substantiates the conclusion, indicated with regard to Figure 4, that the presence of succinate is essential for the occurrence of detectable amounts of free radical.

Similar studies with Antimycin A are described in Figures 6 and 7. Antimycin inhibits both the succinoxidase and succinate-cytochrome *c* reductase activities of

heart particles at a site between succinic dehydrogenase and cytochrome *c*.¹³⁻¹⁵

Figure 6 shows that, like cyanide, Antimycin A (added at time D) brings about a stabilization of the succinate-induced ESR signal in the presence of oxygen. Figure 7 illustrates the effect of oxidized cytochrome *c* on the ESR signal from the heart particle system under various conditions. In these systems neither reduced or oxidized cytochrome *c* alone, nor mixtures of these, exhibits a detectable ESR signal under the spectrometer conditions employed in these experiments.

Figure 7 shows that addition of more than 12 mg of oxidized cytochrome *c* abolishes the ESR signals in the heart particle system (within 3-5 min). The same effect is observed when cyanide is present. However, in the presence of Antimycin A the ESR signal is not immediately affected by the addition of cytochrome *c*. In this case the cytochrome *c* effect does occur after a delay of 30 to 60 min. Ap-

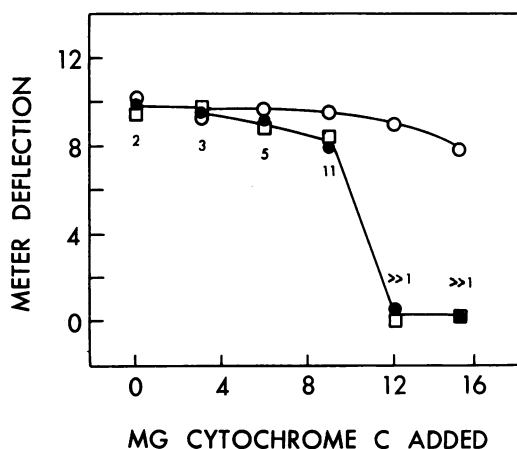


FIG. 7.—The effect of the oxidation of succinate by oxidized cytochrome *c* on the ESR signal from heart particles in the presence and absence of electron transport inhibitors. Signals are from 0.1 ml heart particle preparation in closed glass cells under nitrogen, containing 0.5×10^{-6} mole succinate and a fumarate to succinate mole ratio of 2. Solid circles: no inhibitor. Open squares: $5 \times 10^{-4} M$ in cyanide. Open circles: $2 \mu g$. Antimycin A per ml heart particle preparation. The latter curve reached zero meter deflection about 45 min after the addition of cytochrome *c* at the 12 to 15 mg level. Meter deflection values were recorded 2 to 10 min after the addition of solid cytochrome *c*. The numbers represent an estimate of the fumarate/succinate ratio based on a molecular weight of 13,000 for cytochrome *c*. All points are corrected for any resonance in glass of cell and represent the average of 3 to 9 values.

parently the amount of Antimycin A employed does not completely inhibit the succinate-cytochrome *c* reductase system.

The stoichiometry of the redox reactions depicted in Figure 7 substantiates further the conclusion that the disappearance of the ESR signals coincides with the complete oxidation of succinate. The systems originally contain 0.5μ mole or 1μ redox equivalent of succinate under anaerobic conditions. The level of cytochrome *c* required to abolish the ESR signal is about 12 mg which, on the basis of a molecular weight of 13,000 and one redox equivalent per mole,¹⁶ corresponds also to about 1μ redox equivalent, and is just sufficient to oxidize all of the succinate present.

The foregoing results indicate that the formation of free radicals by succinate does not require the activity of any part of the succinoxidase system which lies on the

oxidizing side of the site of Antimycin A inhibition. The only redox components known to participate in the succinoxidase system on the reducing side of the Antimycin A-sensitive point are succinic dehydrogenase and possibly a cytochrome, which may be cytochrome *b*.^{17, 18}

Figure 8 illustrates the effect of malonate, a specific competitive inhibitor of succinic dehydrogenase,¹⁹ on the succinate-induced ESR signal. When malonate is added, under anaerobic conditions, to achieve a concentration of $10^{-2} M$, to a heart particle suspension which is $1.25 \times 10^{-2} M$, with respect to succinate and $0.5 \times 10^{-2} M$ with respect to fumarate, the ESR signal is reduced about 50 per cent (time C). The effect of $1.5 \times 10^{-2} M$ malonate is slightly greater. At these concentrations of malonate, succinoxidase activity is not completely inhibited, and when

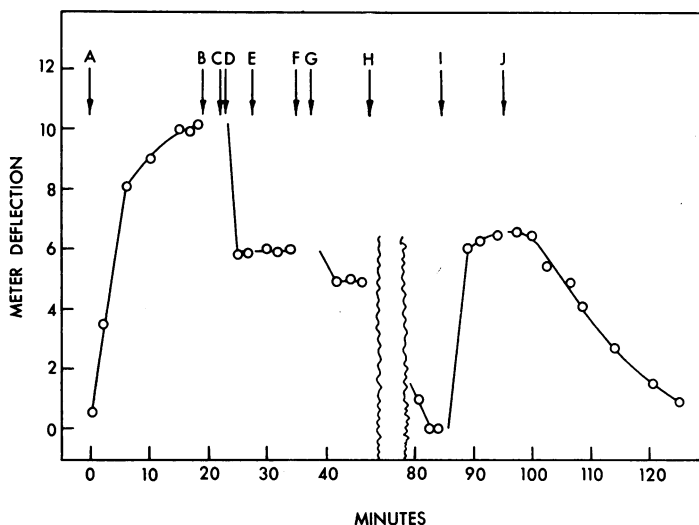


FIG. 8.—The effect of malonate on the ERS signals from heart particles. The flow system contains 2 ml of heart particle preparation. Liquid and gas flow rates are 15 ml min^{-1} and 100 to 150 ml min^{-1} , respectively. (a) pump and nitrogen on, $1.25 \times 10^{-2} M$ and $0.5 \times 10^{-2} M$ in succinate and fumarate, respectively; (b) pump and nitrogen off; (c) $10^{-2} M$ in malonate; (d) pump and nitrogen on; (e) pump off; (f) $1.5 \times 10^{-2} M$ in malonate; (g) pump on; (h) nitrogen off, oxygen on; (i) oxygen off, nitrogen on, $0.86 \times 10^{-2} M$ and $1.5 \times 10^{-2} M$ in succinate and fumarate, respectively; (j) nitrogen off, oxygen on. All points are corrected for the dilution of heart particles and for any resonance in glass of cell. Aqueous phase initially contained dissolved oxygen under atmospheric conditions at 25°C .

oxygen is readmitted to the system (time H), the signal gradually declines to zero. When more succinate is added under nitrogen, at time I, the signal is restored, but it again disappears when oxygen is readmitted and the succinate is oxidized (time J).

Table 1 shows that the degree to which malonate reduces the free radical concentration is a function of the concurrent concentration of succinate. This competitive effect is analogous to that observed when succinic dehydrogenase activity is determined biochemically in the presence of various concentrations of malonate and succinate.

Malonate, Antimycin A, and cyanide all inhibit succinate oxidation by the electron transport system of heart particles. Of these three inhibitors, however, only

TABLE 1

THE COMPETITIVE EFFECTS OF MALONATE AND SUCCINATE CONCENTRATIONS ON THE SIZE OF THE ESR SIGNAL FROM HEART PARTICLES UNDER ANAEROBIC CONDITIONS

Malonate, $M \times 10^3$ ↓ Succinate, $M \times 10^3$	Meter Deflection			
	2	4	6	10
0	5.2	5.9	6.2	6.6
10	2.3	3.9	4.3	4.8
20	1.4	2.4	3.2	4.3

The systems contained heart muscle preparation, succinate, and malonate as indicated, and a mole ratio of fumarate to succinate of 2. Final pH was 7.4. ESR measurements were made at 35°C in closed glass cells. Meter deflections are proportional to free radical concentration.

malonate is capable of diminishing the free radical concentration under conditions which preclude net oxidation of succinate. Cyanide and Antimycin A appear to have no direct effect on the reaction which produces the free radicals.

These observations are in keeping with the conclusion that malonate inhibits free radical formation in the heart particle systems by virtue of its well-known competitive inhibition of succinic dehydrogenase. Our data show, therefore, that succinic dehydrogenase activity is at least in part responsible for the appearance of free radicals in the heart particle system.

Discussion.—The foregoing experiments show that addition of succinate to heart particles produces organic free radicals which require for their formation the presence of succinate and the activity of the components of the succinoxidase system on the reducing side of the site of Antimycin A inhibition. These components include succinic dehydrogenase, and possibly cytochrome *b*.^{13-15, 17, 18} The presence of free radicals does not depend on net electron transport through the succinoxidase system or any portion of this system, and the ESR signal is best observed under conditions which preclude net electron transport.

The free radicals give the superficial impression of considerable stability. They may be detected for extended periods as long as succinate is present, and they are not destroyed directly by oxygen. However, this stability is only apparent.

That the observed ESR signal is not due to a chemically stable free radical is shown by the reversible effects of temperature and of changes in concentrations of substrate and malonate. The effects of malonate are specific evidence that the free radicals are associated with the activity of succinic dehydrogenase.

That the observed free radicals are reactive intermediates, despite their persistence under anaerobic conditions, is evident from a consideration of the properties of succinic dehydrogenase.

This enzyme catalyzes reversible redox reactions between succinate and its oxidized product, fumarate.²⁰ Enzyme-catalyzed oxidation of succinate and reduction of fumarate will proceed in the absence of oxygen, so that free radical redox intermediates, if they occur, may be observed under these conditions. In the absence of oxygen there is no net oxidation of succinate and the system exists in equilibrium. Hence the parameters which govern the enzyme redox reactions will remain unchanged with time, and we may therefore expect the concentration of free radical intermediates to remain constant as well.

In the presence of oxygen, which results in net oxidation of succinate to fumarate, at least one of the relevant parameters, the fumarate/succinate ratio, varies and may in turn affect the concentration of free radical intermediates. As shown by Figure 4, when oxygen is admitted into a heart particle system containing succinate,

thereby initiating a rapid change in the fumarate/succinate ratio, the free radical concentration increases to a maximum and then declines to zero

Our results suggest, therefore, that the free radicals observed in heart particles arise from equilibrium processes catalyzed by succinic dehydrogenase and that their steady-state concentration is governed by the parameters which affect this equilibrium. The paper which follows presents quantitative data on the relation between these parameters and the free radical concentration, which confirm the foregoing conclusion and characterize the reaction mechanisms involved in free radical formation.

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¹ Commoner, B., J. Townsend, and G. E. Pake, *Nature*, **174**, 689 (1954).

² Kubo, H., H. Watari, and T. Shiga, *Bull. Soc. Chim. Biol.*, **41**, 981 (1959).

³ Bray, R. C., B. C. Malström, and T. Vänngård, *Biochem. J.*, **73**, 193 (1959).

⁴ Commoner, B., J. J. Heise, and J. Townsend, these PROCEEDINGS, **42**, 710 (1956).

⁵ Commoner, B., J. J. Heise, B. B. Lippincott, R. E. Norberg, J. V. Passonneau, and J. Townsend, *Science*, **126**, 57 (1957).

⁶ Commoner, B., B. B. Lippincott, and J. V. Passonneau, these PROCEEDINGS, **44**, 1099 (1958).

⁷ Commoner, B., and B. B. Lippincott, these PROCEEDINGS, **44**, 1110 (1958).

⁸ Lippincott, B. B., Ph.D. thesis, Washington University (1959).

⁹ Ehrenberg, A., and G. D. Ludwig, *Science*, **127**, 1177 (1958).

¹⁰ Hollocher, T. C., Jr., and B. Commoner, these PROCEEDINGS, **46**, 416 (1960).

¹¹ Keilin, D., and E. F. Hartree, *Proc. Roy. Soc. London*, **B125**, 171 (1938); **127**, 167 (1939); **129**, 277 (1940).

¹² Wertz, J. E., *Chem. Rev.*, **55**, 829 (1955).

¹³ Potter, V. R., and A. E. Reif, *J. Biol. Chem.*, **194**, 287 (1952).

¹⁴ Reif, A. E., and V. R. Potter, *Arch. Biochem. Biophys.*, **48**, 1 (1954).

¹⁵ Thorn, M. B., *Biochem. J.*, **63**, 420 (1956).

¹⁶ Theorell, H., and A. Akesson, *J. Am. Chem. Soc.*, **63**, 1804, 1812, 1818 (1941).

¹⁷ Slater, E. C., *Adv. Enzymol.*, **20**, 147 (1958).

¹⁸ Green, D. E., *Adv. Enzymol.*, **21**, 73 (1959).

¹⁹ Quastel, J. H., and A. H. M. Wheatley, *Biochem. J.*, **25**, 117 (1931).

²⁰ Quastel, J. H., and M. D. Whetham, *Biochem. J.*, **18**, 519 (1924).

FREE RADICALS IN HEART MUSCLE MITOCHONDRIAL PARTICLES: MECHANISM OF FORMATION*

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A preceding paper¹ describes electron spin resonance (ESR) experiments on the free radicals associated with the succinoxidase system of heart particles. The results indicate that the free radicals arise from redox equilibria involving succinate and one or more components of the succinoxidase system which function on the reducing side of the site of Antimycin A inhibition. Of these components, succinic dehydrogenase, a flavin enzyme^{2, 3} appears to be involved specifically in free radical formation.