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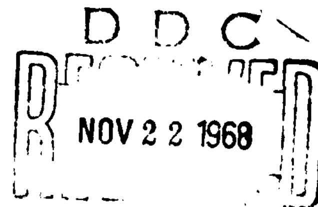


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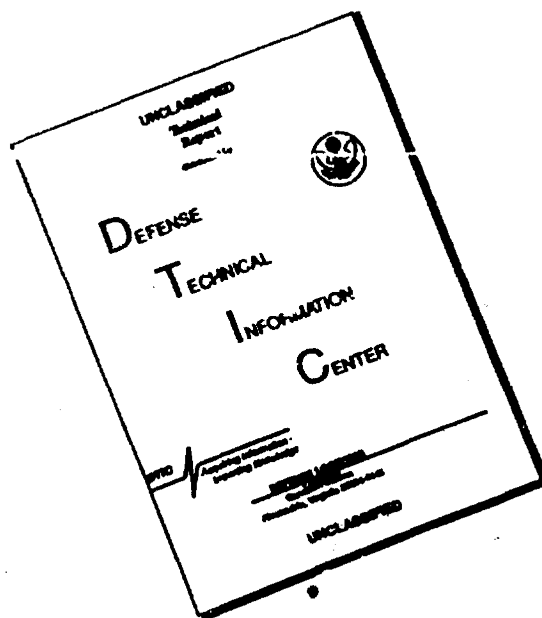


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# FREE-RADICAL STATES OF A CELL AND OF SUBCELLULAR STRUCTURES

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It is known that tissues in the native state and a number of model systems give a slightly asymmetrical singlet signal of electron paramagnetic resonance (EPR) with a g factor close to the g factor of a free electron and a width of 6-8 gausses between the points of maximum slope (1-4). In article (5) it was shown that the signal in tissue conforms in all characteristics to the signals from free radicals of the semiquinone type adsorbed on the surfaces of protein molecules. Naturally the signal from an intact cell is the sum of the signals from the subcellular components. At present intact tissues and model systems have been investigated in detail and several articles have been entirely devoted to intracellular structures (6-9). We investigated the characteristics of the EPR signals from the intact cell, nuclei and mitochondria, the contribution of the nucleus and mitochondria to the total number of free-radical centers of the cell and the connection of the free-radical states of the mitochondria with the intensity of oxidation and phosphorylation.

## METHOD

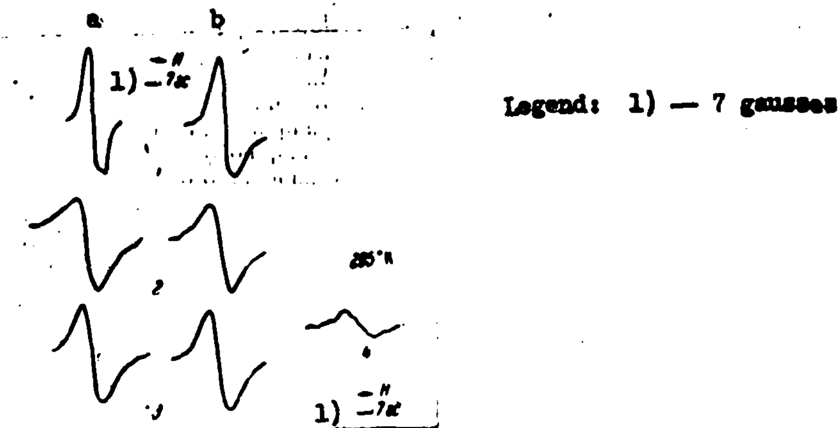
Tissue and subcellular fractions of rat liver have been used as the items studied. The preparations of isolated nuclei and mitochondria were obtained by the methods described in (10, 11). The oxidation rate was measured on the polarograph according to the decrease of the oxygen content in the incubation mixture; the phosphorylation rate was measured according to the diminution of inorganic phosphate.

The lyophilically dried specimens were measured on an EPR spectrometer. The measurements were made immediately after lyophilization. All the numerical data are averaged values from a series of experiments. The

relative error is 10-15 percent.

## RESULTS

Both at room temperature (295°K) and at the temperature of liquid nitrogen (77°K) a singlet EPR signal with a g factor of about 2 was observed in the intact cells and subcellular fractions. In Fig. 1a are presented the signals from the tissue, nuclei, mitochondria, and the centrifugate remaining after precipitation of the nuclei and mitochondria, which are recorded at room temperature. The shape and width of the signals at 77°K conform to the shape and width of the signals at 295°K (Fig. 1); the increase in intensity takes place according to Curie's law. It is seen from Fig. 1 that the EPR signal in intact cells differs both in shape and width from the signals in the nuclei and mitochondria. The signal in the tissue is clearly asymmetrical; the signals in the nuclei and mitochondria are almost symmetrical. The width between the points of maximum slope are: 8 gausses for tissue and 10-12 gausses for the nuclei and mitochondria.




Legend: 1) — 7 gausses

Fig. 1. EPR signals from intact cells and subcellular fractions at room temperature 295°K (a) and 77°K (b).

1 — liver tissue; 2 — nuclei; 3 — mitochondria;
4 — centrifugate (remaining after precipitation of the nuclei and mitochondria)

In Fig. 2 are given the linear anamorphoses for the signals from the tissue, nuclei and mitochondria. As is seen from Fig. 2b and 2c, the shape of the signal in the nuclei is almost indistinguishable from the shape of the signal in the mitochondria. The central portions of all three signals are described by the Lawrence formula; the wings, by the Gauss formula. The transition from the Lawrence form to the Gauss one takes place with a

departure of 4.5-5 gausses from the center of the line for the signal from the tissue and 10-12 gausses for signals from the nuclei and mitochondria.

The saturation curves of the signals from the tissue, nuclei and mitochondria practically coincide. The saturation curve at 295°K is depicted in Fig. 3a, and the one at 77°K, in Fig. 3b. Saturation at 77°K appears considerably earlier. The shape and width of the signals at saturation do not change. The absence of a drop in the saturation curves and the preservation of the shape and width testify to the non-uniform broadening of the signals from the tissue, nuclei and mitochondria.

We further investigated the distribution of free-radical centers in each of the cell components (Table 1).

TABLE 1

| System | Intensity of EPR signal (n. e./g) | Number of free-radical centers (per cell) |
| --- | --- | --- |
| Whole cells | $(1-1.5)\cdot 10^{17}$ | $(1.6 \pm 0.5)\cdot 10^{8}$ |
| Mitochondria | $(3-4)\cdot 10^{16}$ | $(4 \pm 1)\cdot 10^{6}$ |
| Nuclei | $(8-10)\cdot 10^{16}$ | $(2 \pm 0.5)\cdot 10^{5}$ |
| Centrifugate (after precipitation of nuclei and mitochondria) | $(2-3)\cdot 10^{16}$ | $(3 \pm 1)\cdot 10^{7}$ |

As is seen from Table 1, the contribution of the mitochondria to the total number of free-radical centers of the cell is very small, and it is doubtful whether the EPR signal recorded in the tissue is connected with the oxidation-reduction processes taking place directly in the mitochondria. The contribution of the nuclei is even smaller. Consequently the bulk of the free-radical states observed in the intact cells arises in the oxidation-reduction processes taking place outside the nuclei and mitochondria -- in the cell plasma. The process of isolating the subcellular fractions apparently disrupts the conditions for the formation of free-radical states in the cell plasma, since the signal in the centrifugate remaining after precipitation of the nuclei and mitochondria is smaller than the signal in the tissue.

The appearance of a comparatively intense EPR signal in the nuclei is of interest. It is possible that this signal testifies to the fact that oxidation-reduction processes also take place intensively in the nuclei. The preliminary experiments we carried out showed that the signal in the nuclei is extremely stable. The long incubation of the isolated nuclei under aerobic conditions, the pH of the incubation medium, and the presence of deoxyribonuclease and ribonuclease in the mixture had practically no affect on the intensity of the signal. Only the addition of a strong oxidising

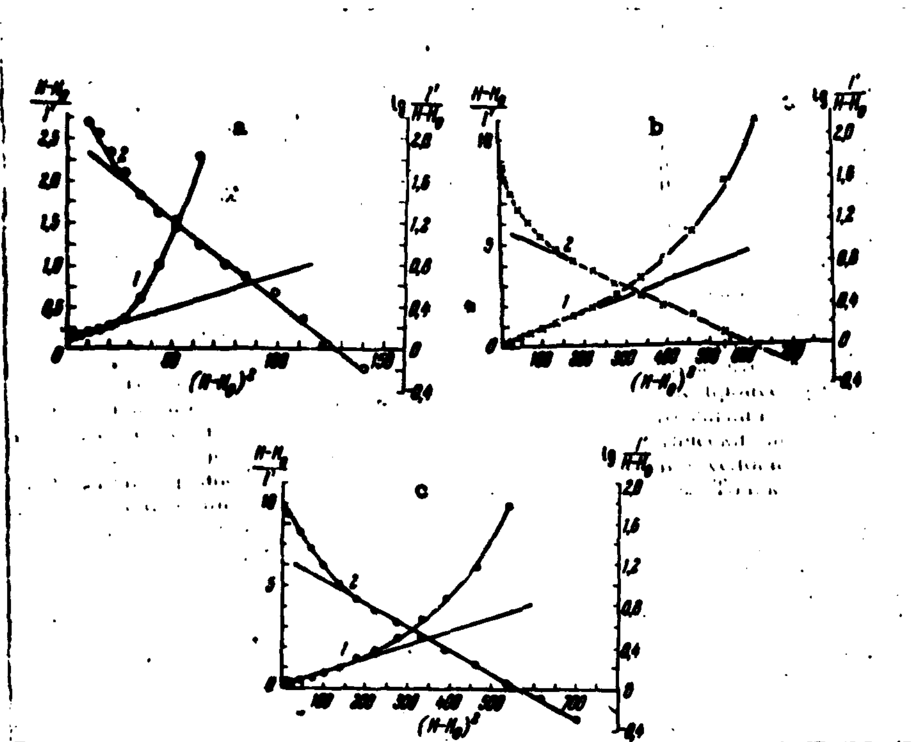


Fig. 2. Linear anamorphoses for EPR signal from a — tissue, b — nuclei, c — mitochondria.

Along the abscissas — $(H-H_0)^2$ in gauss$^2$, along the ordinates — $\frac{H-H_0}{I'}$ (1); lg $\frac{I'}{H-H_0}$ (2) in relative units.

agent — 0.02 M ferricyanide ($K_3Fe(CN)_6$) — led to a decrease of 30-40 percent in the concentration of free radicals.

Two types of centers apparently may be responsible for the free-radical states in the respiratory chain of the mitochondria: the semi-oxidized forms of the flavins and of coenzyme $Q_{10}$. In article (9) it has been shown that some correlation exists between the state of the respiratory chain and the concentration of free radicals in the isolated mitochondria preparation. In the present article we tried to establish a link between the EPR signals in the mitochondria and the oxidation and phosphorylation rates. The same samples for which the oxidation rate and the decrease in inorganic phosphate had been determined previously were measured on the EPR spectrometer.

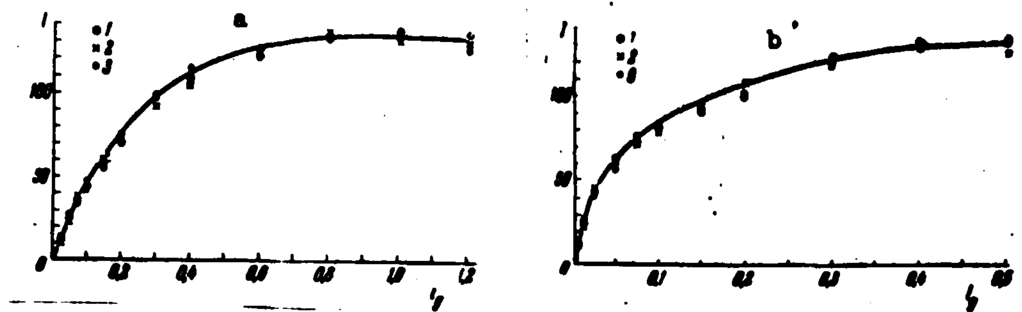


Fig. 3. Saturation curves at 295°K (a) and at 77°K (b).

Along the abscissas — ultra-high frequency detector current in milliamperes, along the ordinates — intensity of EPR signal in relative units. 1 — liver tissue; 2 — nuclei; 3 — mitochondria.

The composition of the incubation mixture: 0.05 M KCl, 0.02 M $KH_2PO_4$, 0.005 M $MgCl_2$, 0.05 M glucose, 0.0015 M ATP, hexokinase (0.3 mg per 1 ml mixture), 0.02 M succinic acid, and mitochondria from 1.5-1.75 g moist liver. Succinic acid was used as the oxidation substrate. A succinic acid concentration of 0.01-0.02 M was enough for creating an excess of substrate. The temperature of the incubation mixture was 18°, the incubation time was one minute (under aerobic conditions). After isolation the mitochondria were rinsed twice. The results of the experiments are given in Table 2.

It is evident from Table 2 that the oxidation and phosphorylation rates also depended on the presence of various cofactors in the incubation mixture of the substrate: hexokinase, ATP, inorganic phosphate, $Mg^{+2}$ ions, and inhibitors. The inhibition of oxidation and phosphorylation in the absence of inorganic phosphate, hexokinase and ATP in the incubation mixture, as well as the interruption of oxidation and phosphorylation by means of DNP (2,4-α-dinitrophenol), had practically no effect on the free-radical states of the respiratory chain. The presence or absence of the oxidation substrate substantially changed the number of free-radical centers. In the samples without an external substrate the concentration of free radicals also depended on the incubation time — in the sample frozen for one minute it is greater than in the sample containing already depleted mitochondria. The onset of anaerobic conditions as a result of the consumption of all the oxygen in the polarographic cell and the inhibition of the transfer of electrons by potassium cyanide (KCN) and azide ($NaN_3$) did not lead to a noticeable change in the free-radical states of the mitochondria. The introduction of ferricyanide into the incubation mixture halved the concentration of free radicals.

TABLE 2

| Composition of Incubation Mixture | Incubation Conditions | Oxidation rate (microamperes O per min.·100 mg mitochondria) | Phosphorylation rate (microamperes P per min.·100 mg mitochondria) | Intensity of EPR signal (in rel. units) |
|---|---|---|---|---|
| Complete | Aerobic | 12.9 | 17.1 | 1.0 |
| Complete | Up to anaerobic conditions | 15.1 | 15.6 | 1.02 |
| Without succinic acid | Aerobic | 1.9 | 3.0 | 0.68 |
| Without succinic acid | Aerobic to depletion | — | — | 0.52 |
| Without $KH_2PO_4$ | Aerobic | 2.8 | 0 | 0.93 |
| Without hexokinase and ATP | " | 3.5 | 0 | 1.03 |
| Without $MgCl_2$ | " | 12.2 | 7.4 | 1.15 |
| Complete | $+ 2\cdot10^{-4}$ M DNP | 15.1 | 0 | 0.89 |
| Complete | $+ 5\cdot10^{-3}$ M KCN | 0 | 0 | 0.91 |
| Complete | $+ 5\cdot10^{-3}$ M $NaN_3$ | 0 | 0 | 0.95 |
| Complete | + 0.0225 M malonate | 0 | 0 | 0.79 |
| Complete | + 0.0225 M $K_3Fe(CN)_6$ | — | — | 0.45 |

If the mitochondria were not rinsed the differences in intensity of the signals would be considerably less. This is apparently caused by the large amount of endogenous substrates in the unrinsed preparation.

Thus the impression is created that the free-radical states of the mitochondria change only in the case of marked effects on specific sections of the respiratory chain. On the one hand the concentration of free radicals decreases with a substantial decrease in the concentration of the oxidation substrates and upon the action of a strong oxidizing agent, ferricyanide. On the other hand the blocking of the oxygen end of the respiratory chain has practically no effect on the signal intensity. This seeming contradiction is resolved if it is assumed that the free-radical centers — the semi-oxidized forms of the flavins and of coenzyme $Q_{10}$ — also take part in the equilibrium between the reduced and oxidized forms of the respiratory chain components. The presence of the signal in the intact preparation shows that even without the occurrence of the enzymatic oxidation process part of the flavins and of coenzyme $Q_{10}$ is in the free radical state. The insensitivity to anaerobic conditions and to inhibition of the oxygen end of the respiratory chain can testify to the presence of a buffer of sufficient capacity between the flavins, coenzyme $Q_{10}$ and cytochrome oxidase. The substance responsible for the recorded EPR signals is apparently much closer to the beginning of the respiratory chain than to the oxygen.

At the same time it should be remembered that the bulk of the free-radical centers observed can be formed on one of the branches of the respiratory chain.

The absence of an effect of the interruption of phosphorylation and oxidation on the free-radical states of the mitochondria attracts attention.

## CONCLUSIONS

1. EPR signals from intact cells and subcellular components differ in shape and width, which testifies to a certain specificity of the free-radical centers responsible for these signals.

2. The contribution of the mitochondria and nucleus to the total number of free-radical centers of the cell is small. It may be assumed that the EPR signals recorded in the tissue are not directly linked with the oxidation process in the mitochondria.

3. Some correlation exists between the free-radical states of the mitochondria and the effects on specific sections of the respiratory chain.

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12 July 1965

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