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# *FREE RADICALS IN SURVIVING TISSUES**

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The first experimental evidence that free radicals occur in animal tissues (and in other biological materials), reported from this laboratory in 1954, was based on electron spin resonance (ESR) studies of frozen-dried samples.[1] ESR signals indicative of low concentrations of free radicals were observed in frozen-dried samples prepared from a variety of animal tissues. The ubiquitous occurrence of the ESR signals and their relative intensities in different tissues suggested that they were due to free radicals associated with metabolic processes.

However, the dry condition of the samples, which was dictated by the relatively low sensitivity of the ESR apparatus then available (the presence of water, being an effective absorber of microwave energy, causes an appreciable loss in the sensitivity of ESR detection), precluded more direct evidence regarding the physiological significance of the observed free radicals.

The development by Jonathan Townsend of more sensitive ESR apparatus has now permitted direct investigation of this question. In the present communication, we report results obtained from a series of ESR investigations of surviving tissues from several laboratory animals and from man. The results show that free radicals occur in living animal tissues and that their concentrations are related to the biological origin of the tissue and vary with certain physiological and pathological conditions. The properties of the free radicals detected in surviving animal tissues are consistent with those previously observed in functional oxidation-reduction enzyme systems, and there is evidence that they are associated with the mitochondria present in the tissue.

*Materials and Methods.*—Tissue samples from laboratory animals are prepared from organs removed whole, as quickly as possible after anesthetization (by ether) of the animal. The entire organ is stored on cracked ice, and tissue slices, about 0.5 mm. thick, are prepared when required. About 50 mg (wet weight) of tissue is suspended in 0.2 ml of 5% glucose solution contained in a flat pyrex ESR cell ($1 \times 5 \times 20$ mm). The tissue is oriented in the cell so that the entire sample occupies the sensitive zone of the ESR spectrometer cavity. The ESR spectrometer employed in these studies, which has been described briefly elsewhere,[2] employs microwave radiation of 9,000 mc/sec, so that resonance absorption due to the spin of the unpaired electron occurs at an external magnetic field of about 3,000 gauss. ESR signals are obtained by scanning the microwave energy absorption as a function of magnetic field strength. About 2 min are required for each run. In the experiments reported below, the instrument was operated with an inherent time-constant of 0.1 sec. The instrument will detect about $10^{-11}$ moles of free radical (of the line width typical of free radicals encountered in biological materials) in a sample containing about 0.1 ml of liquid water.

The ESR signal produced by the spectrometer represents the rate of change in the microwave energy absorbed by the sample with respect to the strength of the external magnetic field. The signal intensity, which is proportional to the number of unpaired electrons sensed by the instrument, is given by the integral of the original signal. To determine the actual free radical content of a tissue sample (on the assumption that each free radical molecule contains one unpaired electron), the integral of the sample signal is compared with the integral of the signal given by a standard solution ($10^{-4}M$) of $MnCl_2$, taking into account that the electron quantum spin number for Mn is $^5/_2$. The free radical concentration of the tissue is then calculated from the wet weight or the nitrogen content (by microKjeldahl) of the sample.

*Results.*—The ESR signals obtained from approximately 50-mg samples of surviving tissue from various guinea pig organs are shown in Figure 1. These observations are typical of results obtained with comparable tissues from rat, mouse, and man. The signals are centered in the region $g = 2.003$ to $g = 2.005$, and line widths (at the point of maximum slope) are 10–20 gauss. In general, the line

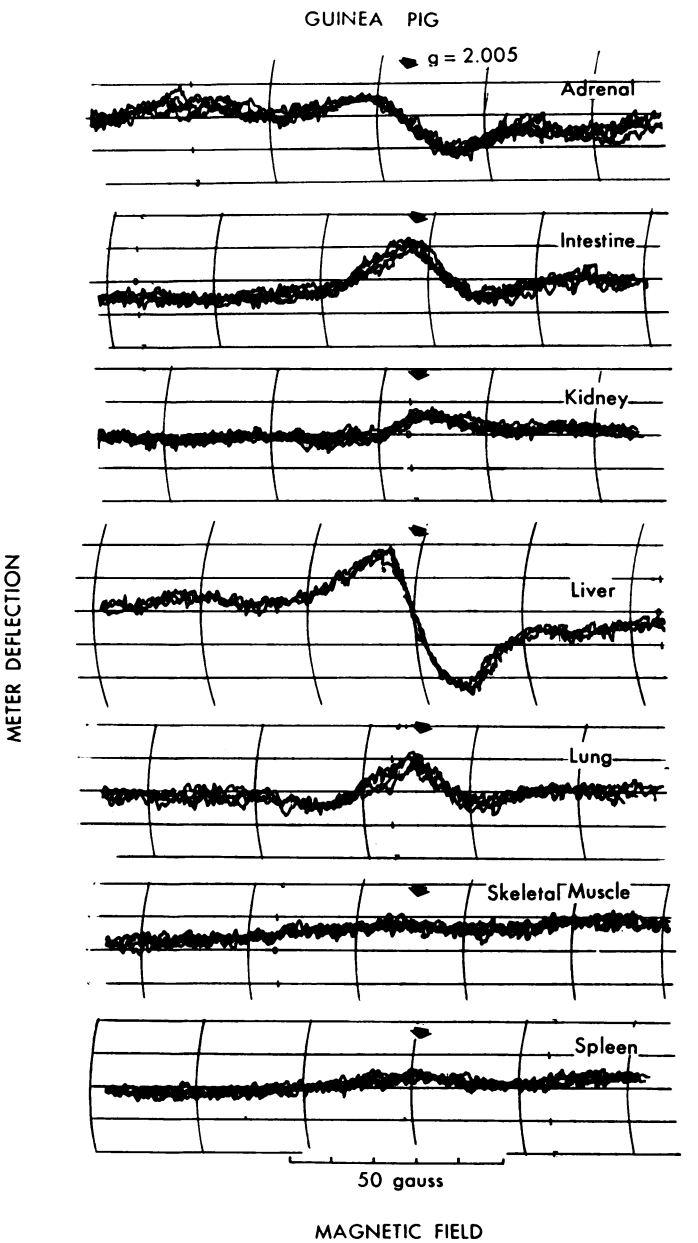


FIG. 1.—ESR signals obtained from surviving samples of guinea pig tissues. Each signal represents superimposed tracings of 3–6 successive runs. The magnetic field increases to the right along the abscissa. The meter deflection (ordinate) represents the rate of change of absorption of microwave energy (9,000 mc/sec) with respect to the field strength. Samples were about 50 mg (wet weight) and the suspending medium was 5 per cent glucose in water. Instrument conditions are described in the text.

shapes are simple and rather similar. The signals are indistinguishable from those exhibited by free radicals formed in redox enzyme systems or in mitochondrial particles which contain such systems.[3–5]

The ESR signal from guinea pig adrenal tissue is unusual and exhibits two subsidiary symmetrical peaks at an interval of about 40 gauss on either side of the main signal at $g = 2.005$. However, adrenal tissue from rat and man exhibit only a simple signal.

By the methods described above, we have determined the total free radical content of a number of single samples of liver, kidney, and heart, removed from a series of adult guinea pigs, rats, and mice (standard laboratory strains). The results illustrated in Figure 2 show that the free radical contents, expressed on either a

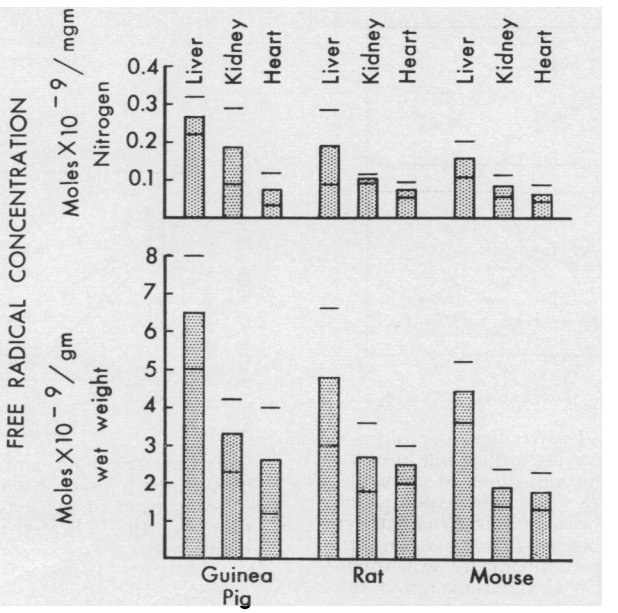


FIG. 2.—Comparison of the free radical concentrations (calculated as described in the text) of surviving liver, kidney, and heart tissue from the guinea pig, rat, and mouse. Bar heights represent average values of 6–8 samples from separate animals. Horizontal lines indicate the standard deviation relevant to each average.

wet-weight or nitrogen basis, while somewhat variable, exhibit mean values which appear to be characteristic both of a given tissue and of a given animal. The free radical content of liver, kidney, and heart declines in the order guinea pig > rat > mouse. In all three animals, the free radical content declines in the order liver > kidney > heart. ESR signals are usually undetectable in samples of skeletal muscle from all three animals; occasionally, it is possible to detect a very weak signal. Comparable relationships among the free radical contents of these tissues have been observed in less complete studies of tissues from man and dog.

The ESR signals observed in surviving animal tissues are characteristically sensitive to ambient temperature. Figure 3(*a*) shows how the free radical content of guinea pig kidney changes as the ambient temperature is varied. The free radical level increases noticeably as the temperature is reduced from 28° to 0°C and persists in the frozen tissue. When the temperature is again elevated, the free radical content falls, but the curves for rising and falling temperature do not coincide. The effects of ambient temperature on free radical content are generally

reversible if the temperature does not exceed about 30°C. However, as shown in Figure 3(*b*) when the temperature of guinea pig kidney tissue is increased from 0° to 38°, an irreversible loss of most of the original signal occurs.

The foregoing results suggest that the free radical contents of surviving tissue samples vary not only with the ambient temperature but also with time. To elucidate this effect, the signal intensity of a series of equivalent samples of rat liver was followed with time at different ambient temperatures. The results, illustrated in Figure 4, show that free radical concentration falls with time, at a constant

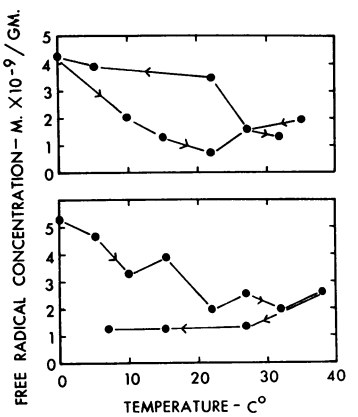


FIG. 3.—Free radical concentration of surviving guinea pig kidney slices during variations in ambient temperature. In the experiment shown in the upper curve (*a*), measurements began with the tissue at 36°C. The temperature was then lowered to 0°C and then raised to 32°C. In the experiment shown in the lower curve (*b*), measurements began at 0°C, and the temperature was increased to 38°C and then reduced again.

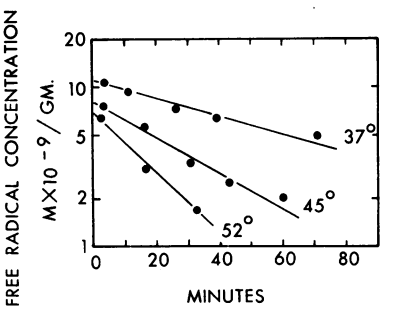


FIG. 4.—Semilog plot showing the change, with time, in the free radical concentration of rat liver slices maintained at the indicated constant temperatures.

temperature, the process being approximately first-order with respect to free radical concentration. As shown in Figure 5, the first-order rate constant rises exponentially with temperature. The heat of activation for the process which limits the loss of free radical, calculated from the data of Figure 5, is about 15–20,000 cal. Temperature effects of this type have been observed in several other tissues; all ESR signals observed in animal tissues (with the exception of those containing melanin, which is a stable free radical[3]) are lost irreversibly when the tissue is boiled for 5 min. This effect, in the case of rat liver, is illustrated in Figure 6.

Oxygen tension has no effect on any of the ESR signals thus far observed in animal tissues. The signals described above were obtained under essentially anaerobic conditions, since the tissue is in a very small volume of medium (about 0.2 ml), which is in turn in contact with air across a minimal area (about .5 $mm^2$). Explicit evidence for this conclusion is given in Figure 7, which shows that neither shape nor intensity of the signal exhibited by a sample of human liver is affected by saturating the fluid medium with $O_2$ or $N_2$ immediately before the ESR determination is made.

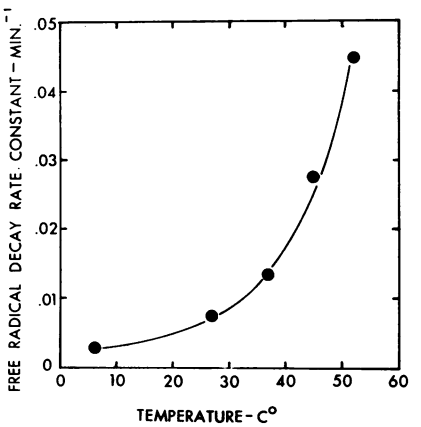


FIG. 5.—The first-order constants for the rate of decline in free radical concentration of rat liver slices at various temperatures, calculated from semilog plots such as those shown in Figure 4.

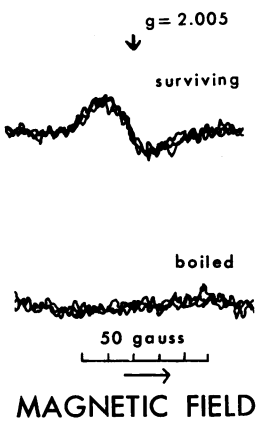


FIG. 6.—ESR signals from surviving rat liver and from the same sample after being boiled for 5 min.

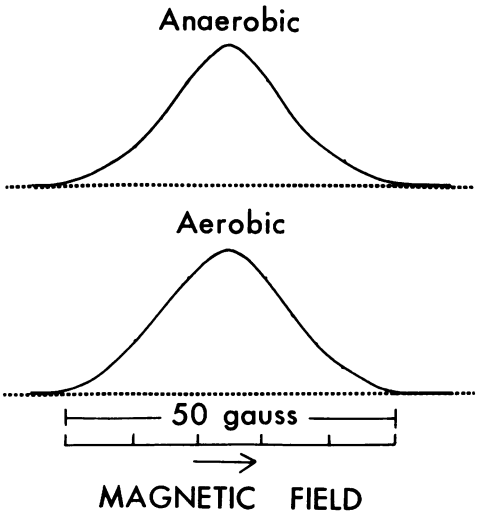


FIG. 7.—Integral signals for 50 mg samples of human liver tissue under aerobic and anaerobic conditions. The free radical concentrations are proportional to the areas under the integral curves.

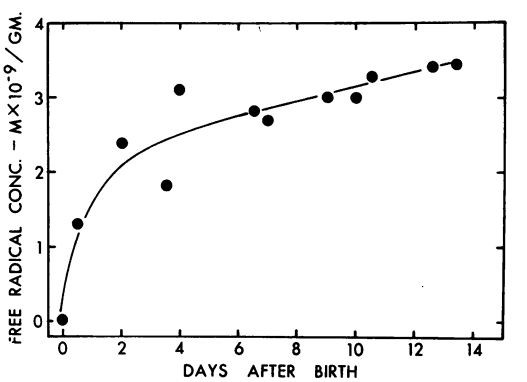


FIG. 8.—Free radical concentrations of samples of liver from litter-mate rats sacrificed at various intervals after birth.

All of the ESR signals described above were observed in tissues from adult animals. We have found, however, that ESR signals are undetectable or noticeably reduced in certain tissues from newborn animals. Figure 8 reports the free radical concentration of liver samples taken from litter-mate rats at intervals after birth. A signal is undetectable in the liver of the newborn rat. Liver from a one-day-old rat exhibits a weak ESR signal, and the free radical concentration increases to a nearly constant value at about the eighth day after birth. Similar effects have been observed in rat kidney and heart tissue.

Additional evidence of a relation between free radical concentration and the physiological status of the tissue has been obtained from some preliminary studies of certain pathological human tissues. A number of human liver samples obtained by biopsy during various surgical procedures have been surveyed for ESR

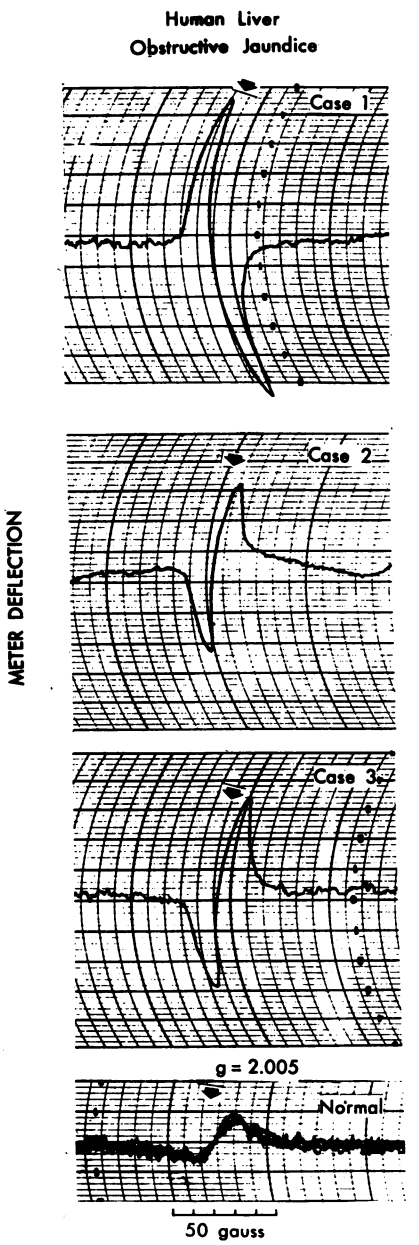


FIG. 9.—ESR signals from 50 mg liver biopsy samples in three cases of extra-hepatic obstructive jaundice compared with a signal from normal human liver. The abscissa and ordinate are as described for Figure 1.

signals. It has been found that liver samples from cases of jaundice due to obstruction of the extra-hepatic bile ducts give a characteristic signal which is about three times as intense as that obtained from normal human liver. Examples of these unusual signals, in comparison with the ESR signal of human liver, are shown in Figure 9. Both signals are irreversibly destroyed by heating at about 40°C; both are enhanced somewhat at temperatures below 20°C and persist in frozen tissue. Liver samples from other types of jaundice (due to cirrhosis and to drugs) do not exhibit an abnormally intense signal. It is possible that this distinction between the ESR signals of liver from cases of obstructive jaundice on the one hand and the signals exhibited by normal liver and by liver from other types of jaundice on the other may provide a basis for a new technique of differential diagnosis. Since present methods for this type of diagnosis are sometimes not subject to unequivocal interpretation, a diagnostic procedure based on the ESR signals may be of considerable practical significance. Clinical trials are now in progress to determine the reliability of differential diagnosis of this and other liver diseases by means of ESR analysis of free radical content.

In our earlier ESR studies of frozen-dried tissues,[1] it was reported that the free radical content of mouse hepatoma was significantly smaller than that of normal mouse liver. We have now re-examined this problem by ESR studies of surviving liver samples. The results, illustrated in Figure 10, show that in contrast with normal liver, free radicals are undetectable in rat and mouse hepatoma. Similarly Figure 11 shows that a biopsy sample of human carcinoma of the colon metastatic to the liver does not exhibit an ESR signal, while tissue from a normal lobe of the same liver exhibits the usual signal. All of the ESR studies of neoplastic tissues up to this time indicate that they are distinguished by relatively

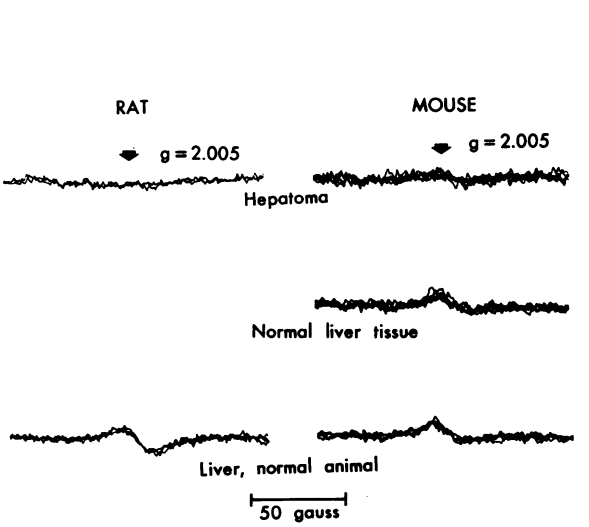


FIG. 10.—ESR signals from 50 mg samples of rat and mouse hepatoma compared with the signals obtained from similar samples of liver tissue from normal animals. In the case of the mouse, the signal from a sample of normal tissue from the hepatoma-bearing liver is also shown. The abscissa and ordinate are as described for Figure 1.

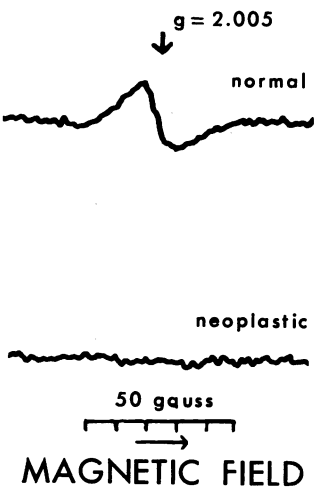


FIG. 11.—ESR signals from 50 mg biopsy samples from a human liver. The upper signal is from a sample obtained from a lobe of the liver containing only normal tissue. The lower signal is from a sample of metastatic carcinoma of the colon occurring in another part of the same liver. The abscissa and ordinate are as described for Figure 1.

low, or wholly undetectable, free radical contents.

*Discussion.*—The foregoing results show that thermally-unstable ESR signals are commonly observed in surviving mammalian tissue, and that the intensities of these signals are related to the biological origin and physiological state of the tissue. The molecular origin of these signals is, of course, of considerable interest.

In this connection, it is instructive to compare the ESR observations on surviving tissues with our earlier ESR studies on the role of free radicals in biological redox systems. If mitochondria are isolated from liver by techniques which preserve their structural integrity (use of hypertonic sucrose solutions, in particular), they exhibit an appreciable ESR signal at $g = 2.004$, which is about 15–20 gauss wide. The signal is clearly associated with enzymatic redox activity. It is lost irreversibly if the preparation is boiled for a few seconds; its intensity is a function of available concentrations of certain substrates (succinate and fumarate, DPNH, and $\beta$-hydroxyburyrate).[6]

Mitochondrial particles that are prepared by somewhat more destructive techniques (especially the use of hypotonic solvents and of extensive washing) do not exhibit an intrinsic ESR signal. However, a strong signal appears when a mixture of succinate and fumarate is added. This signal has a $g$ value of 2.004, a line width of about 15–20 gauss and is irreversibly lost on boiling. It can be shown that the ESR signal exhibited by washed mitochondrial particles in the presence of succinate and fumarate is due to an enzyme-substrate complex of succinic dehydrogenase, which forms an important part of the mitochondrial electron transport chain.[4] These conclusions have been fully confirmed by more recent ESR studies of puri-

fied succinic dehydrogenase systems.[5] Since these free radicals represent forms of an enzyme-substrate complex, they appear in equilibrium systems in which no net oxidation or reduction occurs. This is one reason why the ESR signals exhibited by intact mitochondria, by preparations of washed mitochondrial particles, or by purified succinic dehydrogenase systems are observed when net electron transport (i.e., oxidation) is zero (for example, when oxygen is absent) and may persist for long periods of time.

Less extensive evidence shows that in addition to succinic dehydrogenase several other enzymes commonly present in mitochondria also form free radicals. These include β-hydroxybutyrate dehydrogenase[6] and cytochrome reductase.[3]

In general, the available evidence leads to the expectation that functional mitochondria will exhibit an ESR signal at about $g = 2.004$, about 15–20 gauss wide, which is irreversibly lost on boiling and which is not dependent on net electron transport or the presence of oxygen. It is evident from the experimental evidence reported above that these characteristics are also exhibited by the ESR signals observed in surviving mammalian tissues. In addition, the free radical concentrations characteristic of different tissues generally parallel the known concentrations of mitochondria in these tissues. Thus, the ESR signals from liver and kidney, which are relatively rich in mitochondria, are particularly intense. Heart muscle, which exhibits an appreciable ESR signal, contains a relatively high concentration of mitochondria, while skeletal muscle, which contains relatively few mitochondria, exhibits a barely detectable ESR signal. The increase in free radical concentration observed in rat liver during the first week after birth parallels a similar increase in the concentration of mitochondria and in succinoxidase activity, which has been observed in this tissue by Dawkins.[7] The striking difference between the free radical concentration of normal liver and liver tumor tissue also corresponds to the marked difference in mitochondrial content of normal and neoplastic tissue.

These correlations support the conclusion that the ESR signals observed in surviving mammalian tissues are due to free radicals produced as a result of the enzymatic redox activity of mitochondria. However, in one respect the tissue free radicals appear to differ from those thus far observed in mitochondria and in enzymes isolated from them. Unlike the ESR signals due to mitochondria and to isolated mitochondrial enzymes, the signals exhibited by intact tissues do not become less intense at reduced temperatures but show an opposite effect. This difference is most striking at 0°C. At this temperature, ESR signals are not detectable in mitochondrial particles or in purified succinic dehydrogenase. In contrast, tissue ESR signals rise to a maximum at 0°C and persist in the frozen tissue. There is a similar contrast in the effect of moderately elevated temperatures (i.e., 30–40°C). ESR signals from mitochondrial particles and isolated enzymes are intense and stable over periods of hours at these temperatures. On the other hand, when tissues are maintained at these temperatures, an apparently irreversible loss in signal intensity is observed.

These differences in response to temperature do not necessarily contradict the conclusion that the tissue ESR signals arise in mitochondria. The detailed studies of the succinic dehydrogenase system reported elsewhere[5] show that free radical concentration is dependent not only on enzyme and substrate concentrations but

also on the oxidant/reductant ratio and on the equilibrium constant which governs the interconversion of the normal and free radical forms of the enzyme-substrate complex. It is possible that the effects of temperature on the intensity of tissue ESR signals are due to the influence of temperature on these two parameters. In a complex electron transport system, such as that which is present in intact tissue, the oxidant/reductant ratio relevant to a given enzyme may depend in a quite complicated manner on its own equilibrium constants and on those of other redox enzymes comprising the total chain. In general, the effect of temperature changes on the redox balance of a particular enzyme will reflect *differences* between the temperature effects on the equilibrium constants of individual enzymes that lie above and below it in the electron transport chain. Under these circumstances, decreasing temperature may readily alter the relevant redox balance in such a manner as to increase the free radical concentration.

A possible basis for the effects of moderately elevated temperatures on the free radical concentrations in tissue is suggested by other observations on an isolated enzyme system. It has been found that treatment of purified lactic acid oxidative decarboxylase for 10 minutes at 37°C causes a marked change in the free radical concentration yielded by a given amount of enzyme in the presence of substrate, although specific activity is not affected.[8] The phenomenon is suggestive of a minor effect of temperature on the configuration of the enzyme protein, which could conceivably alter the equilibrium between the free radical and non-free radical forms of the enzyme-substrate complex without appreciably influencing the enzyme's over-all biochemical activity. Such a phenomenon might also account for negative effects of elevated temperature on free radical concentration.

These considerations indicate possible mechanisms which might give rise to the observed effects of temperature on tissue free radical concentrations, and they suggest a line of investigation which may yield the experimental evidence required for an actual explanation of the phenomena.

The wholly empirical observation of decisive differences in free radical concentration related to certain pathological changes in animal and human tissues also suggests certain useful lines of investigation. The distinctive effect of obstructive jaundice on the ESR signal exhibited by liver may on further study provide a clinically useful technique of differential diagnosis. While the remarkable absence of detectable ESR signals in tumor tissue may be only a reflection of the relatively small numbers of mitochondria characteristics of neoplastic tissue, further ESR studies of this phenomenon may provide a useful insight into metabolic differences among tumors and their relation to the effects on antitumor agents. Conceivably, some ESR techniques of diagnostic significance may ultimately result from such studies as well.

In sum, the foregoing ESR observations on surviving mammalian tissues confirm our earlier observations, based on frozen-dried samples, that tissues contain characteristic concentrations of free radicals of metabolic origin. The new observations show also that the ESR signals exhibited by mammalian tissues appear to originate in the redox enzymes associated with mitochondria. The results suggest that ESR determinations of free radical concentrations in tissues may serve to elucidate the metabolic processes characteristic of certain physiological and pathological conditions.

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## *VARIATION IN BASE COMPOSITION OF RIBONUCLEIC ACID IN ESCHERICHIA COLI**

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Santer, Teller, and Andrews[1] have shown that the base ratios of the ribonucleic acid of *Escherichia coli* may be modified by growing this bacterium in different media. These analyses, carried out on the whole RNA of the cell with no attempt to distinguish among various classes of RNA, are supported by a number of experiments dealing with ribosomes and ribosomal-contained RNA of *E. coli*. In these latter experiments modification of RNA has been induced by (1) transferring cells to buffer;[2] (2) suspending cells in a medium containing only an oxidizable carbon source;[3] or(3) growing cells in a phosphate deficient medium until all the phosphate had been exhausted.[4] The change in the RNA has been detected by the release of isotope,[2-4] by observing differences in the ultracentrifuge patterns of the ribosome fraction of cells at various stages in their growth cycle,[5] and by observing the change in ultracentrifuge patterns of cells cultured in different media.[6]

The results presented here show that the base ratio changes induced by changing growth conditions are associated with the ribosome-contained RNA. We have also carried out experiments to determine whether there are changing patterns of RNA synthesis, as measured by $P^{32}$ incorporation into the various nucleotides, during different stages of the growth cycle of *E. coli*. The results of these experiments demonstrate that early log phase cells are producing RNA, associated with the ribosomal fraction, which has a base ratio different from the ratio which is obtained from ribosomal material of late log phase cells.

*Materials and Methods.*—Growth of *E. coli B* and nucleotide isolations and determinations were carried out as described previously.[1] The ribosome and "soluble" RNA fractions were prepared and separated according to Tissières, Watson, Schlessinger, and Hollingworth,[7] using 0.01 *M* Tris (pH 7.4) buffer with $10^{-2}$ *M* $Mg^{++}$ and DNAse (1 $\mu$g/ml) as the suspending medium. Growth experiments were carried out in two kinds of glucose containing medium. The "normal" phosphate medium (NP) is the standard glucose medium used in previous studies.[1] The low phosphate (LP) medium contained $K_2HPO_4$ at a concentration of only 2 $\mu$g/ml