were very thirsty, their limbs became edematous, and blood viscosity was increased. These latter effects were not observed after insulin administration. It is at present not clear whether alteration in the water distribution is due to a direct or an indirect effect of lipogenin or to the presence of mannan. The drop in the blood glucose concentration and the disturbance of the water distribution could be prevented by two intraperitoneal doses of 1.5 ml. of isotonic glucose given together with, and 1 hour after the injection of lipogenin.

While both insulin and lipogenin lower the blood sugar concentration in fasted rats, only the latter increases lipogenesis. This fact suggests that, whereas insulin permits blood glucose to enter the body's cells, lipogenin also causes the conversion of blood glucose into fat. Since this conversion normally proceeds in fed animals, the administration of lipogenin to fed rats does not result in a decrease in the blood glucose, in contrast to the effect of insulin.[3] It is therefore suggested that lipogenin is one of the means of regulation of fatty acid biosynthesis in mammalian organisms.

* Supported by grants from the National Heart Institute, National Institutes of Health, United States Public Health Service.

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[2] G. N. Catravas and H. S. Anker, *J. Biol. Chem.*, **232**, 669, 1958.

[3] S. S. Chernick and I. L. Chaikoff, *J. Biol. Chem.*, **186**, 535, 1950.

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*ELECTRON-SPIN RESONANCE STUDIES OF FREE-RADICAL INTERMEDIATES IN OXIDATION-REDUCTION ENZYME SYSTEMS**

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I. INTRODUCTION

The oxidation-reduction processes that occur in living cells and in systems composed of isolated enzymes involve a succession of reactions in which electron transfer is the common characteristic. This conclusion has been inferred from chemical evidence about the structure and reactivity of the system components, but relatively little is known about the physical processes which govern electron transfer among them. This paper is concerned with one of the unresolved problems: the valence of electron transfer.

Direct chemical evidence indicates that the net effect of many biochemical oxidation-reduction reactions is the transfer of two electrons. In other cases, notably the cytochrome–cytochrome oxidase system, the same type of evidence indicates that the transfer is univalent. The thermodynamic barriers to the necessary coupling of 2-electron and 1-electron processes are formidable, and this problem has been under active consideration for some time.

On the basis of work with model systems, Michaelis proposed a solution which is best summarized in his own statement that "all oxidations of organic molecules, although they are bivalent, proceed in two successive univalent steps, the inter-

mediate state being a free radical."[1] In support of this proposal Michaelis showed that electrometric titration of various organic oxidation-reduction systems exhibits a node which suggests that each of them involves two successive electron transfers. Spectrophotometric studies suggested the presence of intermediates between the fully oxidized state and the fully reduced form of various substances, especially flavins, that participate in biochemical oxidation-reduction processes. Since the fully oxidized and fully reduced forms differ by 2 electrons, the intermediate contains an unpaired electron and is therefore a free radical. The operative conclusion which emerges from Michaelis' work is that free radicals must occur as intermediates in all biochemical oxidation-reduction processes. Following Michaelis, several investigators added to the indirect evidence that favors this hypothesis, but it has also been subjected to a rather critical analysis by Westheimer.[2]

Recently a new experimental approach to the problem has become possible which has the merit of yielding direct physical evidence concerning the occurrence of the unpaired electrons associated with free radicals. The method is based on the phenomenon of electron-spin resonance which may be studied by means of a spectrometer capable of detecting and characterizing the gyromagnetic properties of very small amounts of unpaired electrons (of the order of 10^{-10} moles). The initial efforts to employ this technique to study biological oxidation-reduction processes, reported by Commoner *et al.*,[3] were limited by the fact that the ESR spectrometer then available was not capable of measurements with wet samples. This limitation has now been overcome by the construction (by Professor J. Townsend) of an ESR spectrometer capable of detecting about 10^{-10} moles of unpaired electrons in samples containing about 0.15 ml. of liquid water. With this spectrometer it has been shown that light-induced free radicals occur in chloroplasts and living algae and, in a preliminary way, that free radicals also occur in certain in vitro enzymatic oxidation-reductions.[4] Cursory observations of ESR signals in enzyme systems have also been reported by Ehrenberg and Ludwig and by Beinert.[5]

The present paper reports detailed experiments on a flavoprotein oxidation-reduction enzyme and less detailed studies of a series of common oxidation-reduction enzyme systems. The results confirm Michaelis' proposal that free-radical intermediates are characteristic features of enzymatic electron transport.

II. METHODS

The ESR spectrometer detects the absorption of incident microwave energy (9,000 Mc/sec) which results from energy levels imposed by an external magnetic field on unpaired electrons present in the sample. The gyromagnetic properties of an unpaired electron are such that an external field of about 3,300 gauss will induce absorption of 9,000 Mc/sec incident energy. The spectrometer automatically varies the strength of the external magnetic field over a selected range centered about 3,300 gauss while monitoring the absorption of microwave energy by the sample. The record of a run represents the first derivative of the energy absorbed, plotted against the strength of the external magnetic field. Comparison of the absorption due to a standard preparation of a stable free radical and that yielded by the sample results in a rough approximation of the number of electron spins present in the latter.

A given electron-spin resonance absorption may be characterized in several ways.

(1) The gyromagnetic ratio g is determined from the precise value of the external field at which the center of the ESR absorption occurs, in accordance with the equation: υ = a constant $\times$ g $\times$ H, where υ is the frequency of the incident energy and H is the strength of the external magnetic field. The values of g observed for organic free radicals vary from about 2.000 to 2.007;[6] a given free radical is characterized, but not uniquely, by its g-value. (2) The width of the ESR absorption (usually expressed as the width in gauss, at half the maximum absorption) is indicative of the relaxation processes in which the unpaired electrons are involved. This is a complex, but nevertheless convenient, empirical character. (3) When an unpaired electron is associated with an atomic nucleus that itself possesses a magnetic moment, coupling between the two occurs. This gives rise to a splitting of the energy levels imposed on the electron, so that the ESR absorption then exhibits a characteristic hyperfine structure. In the present work the ESR signals were not sufficiently intense to permit measurements at the high resolving power (and low sensitivity) required to observe hyperfine structure.

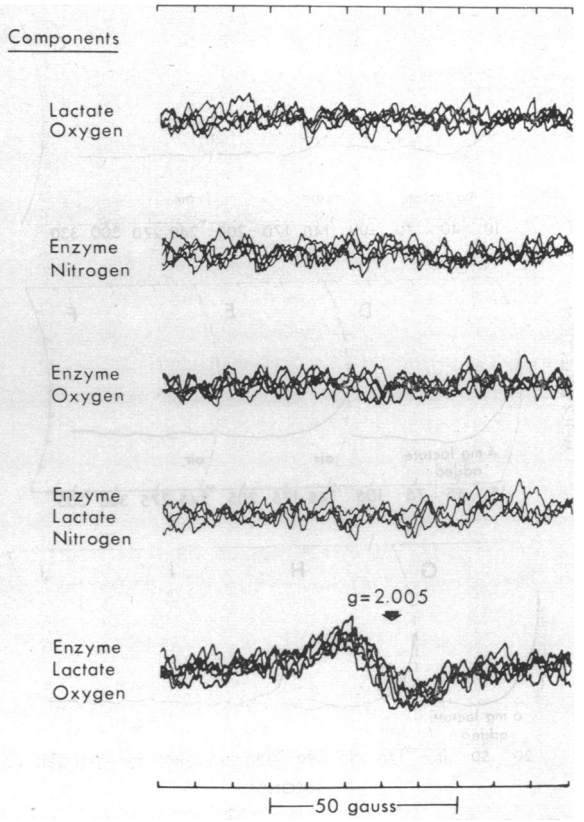

FIG. 1.—Electron spin resonance (ESR) from the separate components of the lactic oxidative decarboxylase system and from the complete system (enzyme plus lactate) in nitrogen and in oxygen: 8 mg. lactic oxidative decarboxylase, 25 mg. *dl*-lactate, and 0.2 ml. PO_4 buffer at pH 5.9. The magnetic field increases toward the right along the abscissa. The ordinate represents the rate of change with respect to field strength of microwave energy (9,000 Mc/sec) absorbed by the sample as the strength of the magnetic field is varied. The records shown are superimposed tracings of four to five sequential runs.

In most experiments 5–50 mg. of the enzyme preparation are placed in an ESR spectrometer cell (a Pyrex cell, 0.5 $\times$ 10 $\times$ 20 mm. internally) and a suitable amount of substrate added in a dry form. About 0.1–0.2 ml. of buffer is then added to the cell, and the contents are mixed rapidly with a stainless-steel wire. In some cases the resulting mixture is a slurry rather than a solution.

The sample cell is inserted into the ESR spectrometer cavity, which is then tuned, and the instrument set to make rapid sweeps across the region of 3,300-gauss external field strength. Each sweep requires a minimum of 90 seconds. The transient character of enzyme intermediates requires that measurements be rapid.

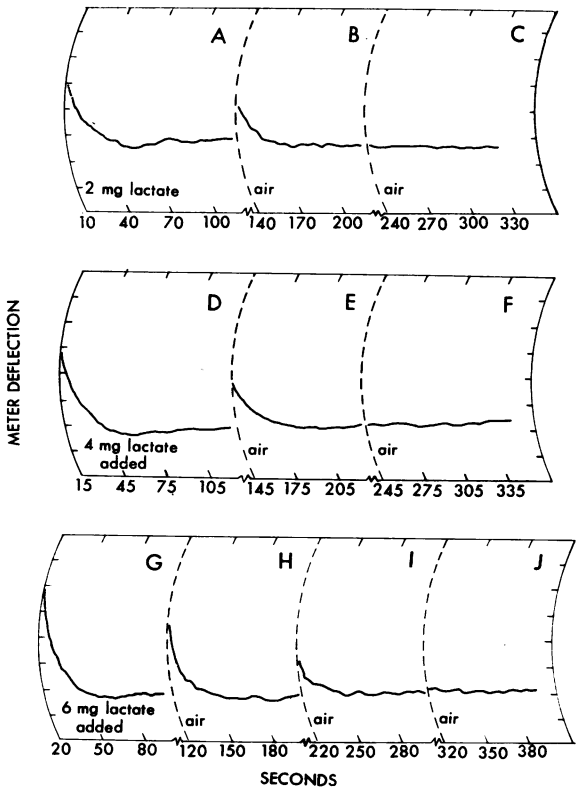

FIG. 2.—Variation in the magnitude of the ESR signal (i.e., the meter deflection at the magnetic field at which this deflection is maximum) with time after the addition of varying amounts of lactate to a sample of 8 mg. of lactic oxidative decarboxylase in 0.2 ml. PO_4 buffer at pH 5.9. The system was saturated with air at the beginning of each run. Curves *A*, *B*, and *C* show the effects of adding 2 mg. of lactate to the enzyme at time zero, and of aeration at 130 and 230 seconds. Curves *D*, *E*, and *F* show the effects of adding 4 mg. of lactate at time zero to the enzyme system after the completion of run *C*. The system was aerated at 135 and 235 seconds. Curves *G*, *H*, *I*, and *J* show the effects of adding 6 mg. of lactate to the system at time zero, after the completion of curve *F*, with aeration at 110, 210, and 310 seconds.

Under these conditions inherent noise is a significant factor, and it becomes necessary to distinguish between meter deflections due to noise and those due to an ESR signal. This may be accomplished by superimposing, by tracing, the records of successive runs; random deflections due to noise cancel out, and the deflections due to the signal coincide.

During the course of the experiments reported in this paper it was discovered that exposure of solutions of flavoprotein enzymes to moderate intensities of visible light induces the formation of free radicals. Accordingly, in most cases, the data reported below refer to systems which were protected from direct exposure to light. This problem is discussed in detail in another paper.[7]

III. MATERIALS

Enzyme preparations used in the experiments described below were derived from the following sources. Cytochrome C reductase was isolated and purified according to the method of Mahler *et al.*[8] Cytochrome oxidase was isolated and purified according to the method of Wainio *et al.*[9] The SC factor was prepared according to the method of Clark *et al.*[10] Lactic oxidative decarboxylase, a crystalline preparation, was isolated from *Mycobacterium phlei* and was kindly supplied by Dr. William B. Sutton. D-α-amino acid oxidase, purified, and old yellow enzyme, purified, were obtained from Mann Research Laboratories. Aldolase, crystalline, and glyceraldehyde phosphate dehydrogenase, crystalline, were obtained from Worthington Biochemical Company. Alcohol dehydrogenase (yeast), purified; lactic acid dehydrogenase, purified; glucose-6-phosphate dehydrogenase, purified; and cytochrome C, crystalline, were obtained from Sigma Chemical Company. Succinic acid dehydrogenase, purified, was obtained from Delta Chemical Company.

IV. RESULTS

1. *Lactic Oxidative Decarboxylase.*—This enzyme was selected for detailed study. It has been isolated, purified, and studied with respect to biochemistry by Sutton.[11] We are indebted to Dr. Sutton for very generous supplies of extensively purified and recrystallized enzyme. Sutton's investigations show that this enzyme will rapidly dehydrogenate and decarboxylate lactic acid in the presence of molecular O_2, the products being carbon dioxide, acetic acid, and water. The enzyme is a flavoprotein and contains 2 molecules of FMN per enzyme molecule. Sutton has suggested that the catalytic activity of the enzyme involves a series of electron transfers, with a free radical occurring as an intermediate.[12]

Figure 1 shows the results of a series of ESR runs obtained from the enzyme alone, from lactate alone, and from the complete system. Neither the enzyme itself nor the lactate exhibits an ESR signal. If the enzyme and substrate are present together but under anaerobic conditions, no signal is detected. However, if oxygen is also present, a distinct ESR signal is observed during the first few minutes after the components are mixed. The ESR has a g-value of 2.005 and a half-width of about 13 gauss.

The effects of variation in lactate concentration and in oxygen tension on the size of the ESR are shown in Figure 2. In this experiment the spectrometer's magnetic field is set at the position of the resonance, so that the ESR signal causes only a simple deflection of the indicating meter. The recording chart is employed to register the changes, with time, in the meter deflection. By this means rapid changes in ESR magnitude can be followed during the course of the enzyme reaction.

When 2 mg. of lactate are added to 8 mg. of previously aerated enzyme, the magnitude of the ESR signal declines rapidly as soon as measurements begin, and the signal disappears within 40 seconds (curve A). After the signal declines to zero, aeration of the sample restores the signal to a degree (curve B). The meter deflection again falls to zero in 20 seconds. Curve C shows that an additional aeration now fails to restore the signal. These results show that first oxygen and then lactate are rapidly depleted in the sample cell and limit the size of even the initial signal that is observed.

Curve D shows that enzyme activity and the ESR signal can be restored in a system depleted of lactate by the addition of this substrate. Again the signal declines with time. Curve E shows that this effect is due in part to depletion of oxygen, but finally lactate is also lacking (curve F). Curve G and those following show the effects of a second addition of 6 mg. of lactate and of subsequent aeration. As expected, the addition of the larger amount of lactate prolongs the time during which the system continues to respond to the presence of oxygen.

This experiment shows that the magnitude of the ESR signal is a function of the lactate and oxygen concentrations and that a single sample of enzyme exhibits an ESR in a succession of reactions. It will be noted that the earliest observed signals were obtained about 10–12 seconds after the system components are mixed—i.e., the time required to tune the spectrometer. However, it is possible to estimate the size of the true initial signal and its relation to the amount of lactate added to the system. In Figure 3, curves A and D and G of Figure 2 are plotted semilogarithmically. The signal size declines exponentially, so that extrapolation to

zero time (the time at which the lactate is added to the sample) is possible. This intercept is a measure of the true initial signal value.

From a series of such experiments it is possible to describe the relationship between lactate concentration and the true initial size of the ESR signal. The results are shown in Figure 4. Up to a concentration of 6 mg. of lactate per 0.2 ml. of solution (containing 8 mg. of crystalline lactic oxidative decarboxylase) the size of the ESR signal is proportional to the lactate concentration. Beyond this point

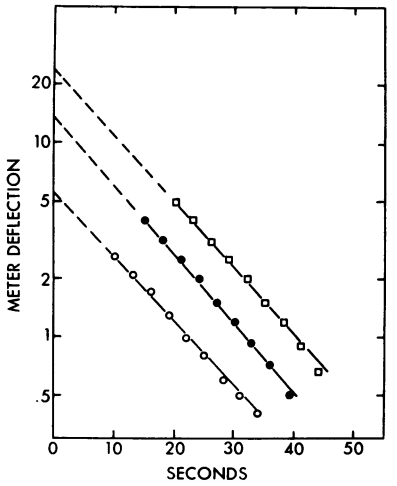

FIG. 3.—Semilogarithmic plots of the meter deflections from Fig. 2 against time. Open circles represent curve *A* of Fig. 2; solid circles, curve *D;* and open squares, curve *G*. The broken lines are extrapolations of the curves to zero time (the time at which the lactate is added to the sample). This intercept represents the initial meter deflection.

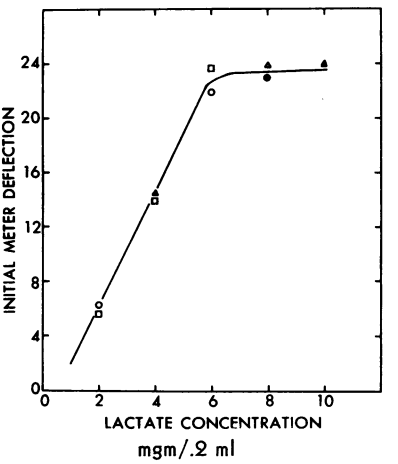

FIG. 4.—The relation of lactate concentration to the initial meter deflection (obtained by extrapolating data similar to that in Fig. 2 to zero time). Circles, squares, and triangles represent experiments carried out on different days using different preparations of enzyme. Open symbols represent sequential additions of lactate to a single sample of enzyme. Closed symbols represent a single addition of lactate to a sample of enzyme.

the initial ESR signal remains constant despite increasing lactate concentration The experimental values extrapolate to zero ESR signal near zero lactate concentration. Data derived from three separate experiments, carried out at different times, show an excellent quantitative agreement.

A series of similar experiments in which the enzyme concentration is varied in the presence of 6 mg. of lactate gives the results shown in Figure 5. With considerable precision, the magnitude of the initial ESR signal at the time of mixing is proportional to the enzyme concentration and extrapolates to zero near zero enzyme concentration.

Figure 6 shows the effects of two inhibitors of the lactic oxidative decarboxylase—pyruvate (which competes with lactate for the enzyme's active sites) and *p*-chloromercuribenzoate (PCMB), which appears to inactivate essential -SH groups.[13] The control curve of Figure 6 shows that if excess lactate is added to the enzyme in the absence of inhibitor, the ESR signal appears and is maintained for a period of 10 minutes. If the same amount of lactate is added to the enzyme after a previous

addition of pyruvate (30 mg. in 0.15 ml.), no ESR signal appears. Thus, in the presence of a competitive concentration of pyruvate, the enzyme fails to act on lactate and shows no ESR signal. PCMB has a similar effect. In this case the inhibitor itself, on addition to the enzyme, generates a free radical, possibly because it brings the -SH group into its half-oxidized state. However, when lactate is added, the signal fails to increase in size.

It can be shown that the failure of the enzyme to respond to lactate when these inhibitors are present is not due to inactivation of the enzyme's prosthetic group. We have found that flavins, either free or in the form of enzyme prosthetic groups, will respond to visible light by forming a free radical apparently identical with that which occurs as an oxidation-reduction intermediate.[7] If lactic oxidative decarboxylase is illuminated in the presence of either PCMB or pyruvate after the addition of lactate has failed to elicit an ESR signal, light has its expected effect, and a substantial new ESR is observed (Fig. 6). This shows that, despite the presence of the inhibitor, which prevents the enzyme's response to lactate, the FMN prosthetic group is still capable of forming a free radical. This result is expected from the fact that these inhibitors act only on the protein moiety of the enzyme.

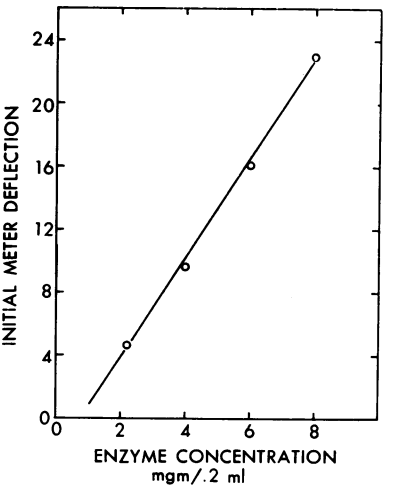

FIG. 5.—The relation between concentration of lactic oxidative decarboxylase and initial meter deflection (obtained by extrapolation to zero time of data similar to that in Fig. 2). In all cases 6 mg. of lactate was introduced at time zero. The point at 8-mg. enzyme is the average of two experiments, while the other three points represent single experiments.

The foregoing results confirm Sutton's proposal that the activity of the lactic oxidative decarboxylase proceeds through a free-radical intermediate. Qualitatively, the experiments demonstrate an obligate relationship between enzyme activity and the presence of the free radical. The quantitative results also conform with the expectation that the free radical is an intermediate which achieves a steady-state concentration that depends on the rate of enzyme activity. The turnover number of lactic oxidative decarboxylase, calculated by Sutton from the rate of enzymatic activity, is about 2,000. On this basis the enzyme system described in Figure 3 under optimum conditions can convert 2 mg. lactate and return to a zero rate of electron transport in 20 seconds; 4 mg. can be converted in 40 seconds and 6 mg. in 60 seconds. Figure 3 shows that in the three cases the times during which an ESR is detectable are 50, 65, and 80 seconds, respectively. This agreement shows that the free radical is present only during the course of enzymatic electron transfer.

The quantitative relationships between the size of the ESR and the enzyme and lactate concentration also support Sutton's proposal. Like the rate of enzyme activity, the size of the ESR is proportional to the enzyme concentration (Fig. 5). The curve relating lactate concentration to size of initial ESR (Fig. 4) resembles the Michaelis-Menten function, which relates the rate of enzyme activity to substrate concentration.

So long as an inhibitor prevents lactate from being bound by the enzyme or from transmitting electrons to FMN, the free radical is absent. Substitution of another source of electrons, through the effect of light, induces an ESR which is not distinguishable from that found during ordinary enzyme activity. This suggests that the ESR observed when the enzyme is active is probably due chiefly to an FMN free radical.

In sum, all the foregoing results support the conclusion that electron transfer mediated by lactic oxidative decarboxylase gives rise to a free-radical intermediate probably derived from the enzyme's prosthetic group and that the steady-state concentration of free radical varies with the rate of electron transport.

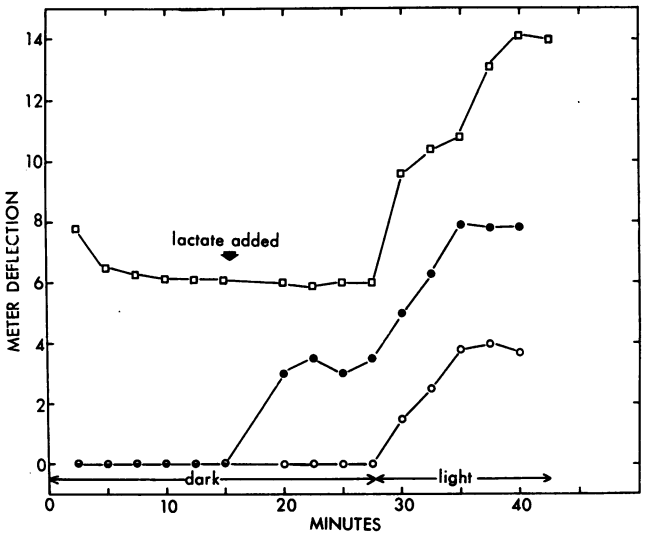

FIG. 6.—The effect of inhibitors and light on the electron-spin resonance obtained from lactic oxidative decarboxylase. The maximum meter deflection is plotted against time. In all cases the system contained 8 mg. of enzyme and 0.15 ml. of phosphate buffer at pH 5.9. The closed circles represent a control experiment in the absence of inhibitor. In the experiment represented by the open circles 30 mg. of pyruvate were present. In the experiment represented by the open squares 5 mg. of *p*-chloromercuribenzoate were present. The arrow indicates the time at which 30 mg. of lactate were added to all three samples.

2. *Other Enzymes.*—ESR results obtained from a number of enzyme systems are summarized in Table 1. The enzymes studied include 5 dehydrogenases, 5 flavoprotein enzymes, and the cytochrome C–cytochrome oxidase system.

All the experiments with dehydrogenases (alcohol, lactic acid, glucose-6-phosphate, 3-phosphoglyceric acid, and succinic) show that an ESR appears during the activity of the complete system consisting of the substrate, the enzyme, and the appropriate coenzyme. No resonances were observed in any of the separate components. The 3-phosphoglyceraldehyde dehydrogenase was also studied in a more complex system, in which aldolase and fructose 1,6-diphosphate was present to generate the necessary 3-phosphoglyceraldehyde. Again an ESR was observed only when the system included the enzymatically formed substrate, dehydrogenase, and

DPN^+. This result shows, incidentally, that the activity of aldolase, an enzyme which does not appear to mediate electron transfer, is not accompanied by free-radical formation. Results obtained with alcohol dehydrogenase and with glyceraldehyde phosphate dehydrogenase show that the activity of this enzyme leads to an ESR signal whether the process is in the "forward" direction (i.e., coupled oxidation-reduction of alcohol or glyceraldehyde phosphate and DPN^+) or in the "reverse" direction (coupled oxidation-reduction of acetaldehyde or phosphoglyceric acid and DPNH).

The experiments with lactic oxidative decarboxylase that have already been described offer the most detailed evidence regarding the occurrence of free radicals during the activity of a flavoprotein enzyme. Three other flavoproteins have been studied in a preliminary way: D-amino acid oxidase, Warburg's old yellow enzyme, and cytochrome reductase. Unlike the decarboxylase, none of these preparations was crystalline. Commercial preparations of D-amino acid oxidase and of Warburg's old yellow enzyme in the absence of their substrates exhibited a small ESR, which showed a significant transitory increase when substrate was added. These are the only instances in which an ESR was observed in other than the complete enzyme system. As shown elsewhere, when flavoproteins are exposed to ordinary room light, free radicals which may be quite stable in some conditions are readily produced.[7] It is possible that the signals observed in these enzyme preparations were due to exposure to light at some stage during their preparation.

Results of experiments with cytochrome reductase have been described elsewhere.[14] This enzyme is a flavoprotein which is reversibly oxidizable, DPNH serving as electron donor and cytochrome C as electron acceptor. The reduction of the reductase by DPNH is accompanied by a transitory ESR signal. The ESR is enhanced if cytochrome C is then added. The reduced form of the reductase is then reoxidized, and the cytochrome C is reduced. When this signal itself becomes diminished with time, the addition of cytochrome oxidase (which reoxidizes reduced cytochrome C) regenerates an intensified ESR signal. The presence of atabrine, an inhibitor of cytochrome reductase, causes the ESR exhibited by the DPNH–reductase–cytochrome C system to disappear. These results show that the enzymatic activity of the several flavoproteins studied is uniformly associated with an ESR.

The electron transfers mediated by the cytochrome–cytochrome oxidase system are necessarily univalent, since they involve a univalent change in the state of an iron atom. Results summarized in Table 1 show that this transfer is associated with an ESR. Oxidized cytochrome C does not exhibit an ESR. When cytochrome C is reduced with ascorbate, a small signal is observed. This may be due to some ascorbate free radical which is easily formed and stable in neutral solution. When cytochrome oxidase, which itself lacks an ESR, is added to the reduced cytochrome C and O_2 is present, an intensified signal appears. The presence of cyanide, which inhibits cytochrome oxidase specifically, prevents this effect.

The ESR signals described in Table 1 are not sufficiently intense to permit a search for hyperfine structure. However, the observed g-values and line widths fall within the range of corresponding values observed in a number of organic free radicals.[6]

In the case of the flavoprotein enzymes, it can be concluded that the observed ESR is due, at least in part, to a free-radical form of the flavin prosthetic group.

TABLE 1

ENZYME	SYSTEM COMPONENTS	ELECTRON-SPIN RESONANCE g-Value	ELECTRON-SPIN RESONANCE Half-width (Gauss)	APPROXIMATE MOLES OF FREE RADICAL DETECTED × 10^{-10}
Lactic oxidative decarboxylase	Complete: enzyme (8 mg.), *dl*-lactate (10 mg.), O_2	2.005	13	4
	Enzyme only	...	...	...
	dl-Lactate only	...	...	...
	Enzyme, lactate in N_2	...	...	...
D-α-amino acid oxidase	Complete: enzyme (50 mg.), alanine (20 mg.), O_2	2.002	15	3
	Enzyme only	2.002	15	2
	Alanine only	...	...	...
Warburg's old yellow enzyme	Complete: enzyme (50 mg.), TPNH (5 mg.), O_2	2.003	14	5
	Enzyme only	2.003	14	2
	TPNH only	...	...	...
Glyceraldehyde phosphate dehydrogenase	Complete: enzyme (10 mg.), 3-phosphoglyceric acid (30 mg.), DPNH (10 mg.)	2.001	13	1
	Enzyme only	...	...	...
	Phosphoglyceric acid only	...	...	...
	DPNH only	...	...	...
	Complete: enzyme (10 mg.), aldolase (10 mg.), fructose-1,6-diphosphate (30 mg.), DPN^+ (10 mg.)	2.001	13	1
	Aldolase only	...	...	...
	Fructose-1,6-diphosphate only	...	...	...
	DPN^+ only	...	...	...
	Aldolase, fructose-1,6-diphosphate, DPN^+	...	...	...
Alcohol dehydrogenase	Complete: enzyme (50 mg.), ethyl alcohol (50 per cent, 0.06 ml.), DPN^+ (30 mg.)	2.006	13	1
	Enzyme, DPN^+	...	...	...
	Enzyme only	...	...	...
	Alcohol only	...	...	...
	DPN^+ only	...	...	...
	Complete: enzyme (50 mg.), acetaldehyde (0.1 ml.), DPNH (30 mg.)	2.006	13	1
	Enzyme, DPNH	...	...	...
	DPNH only	...	...	...
	Acetaldehyde only	...	...	...
Glucose-6-phosphate dehydrogenase	Complete: enzyme (30 mg.), glucose-6-phosphate (10 mg.), TPN^+ (10 mg.)	2.007	18	1
	Enzyme only	...	...	...
	Glucose-6-phosphate only	...	...	...
	TPN^+ only	...	...	...
	Enzyme, TPN^+	...	...	...
DPNH–cytochrome C reductase	Enzyme (30 mg.), DPNH (8 mg.)	2.006	18	2
	DPNH only	...	...	...
	Complete: enzyme (30 mg.), DPNH (8 mg.), cytochrome C (30 mg.)	2.006	18	2
	Cytochrome C only	...	...	...
	Complete + inhibitor: enzyme, DPNH, cytochrome C, atabrine (30 mg.)	...	...	...
	Complete + oxidase: enzyme (30 mg.), DPNH (8 mg.), cytochrome C (30 mg.), cytochrome C oxidase (30 mg.)	2.006*–2.004	18*–11	3

TABLE 1—*Continued*

Enzyme	System Components	Electron-Spin Resonance: g-Value	Electron-Spin Resonance: Half-width (Gauss)	Approximate Moles of Free Radical Detected × 10^{-10}
Cytochrome C oxidase	Complete: enzyme (50 mg.), reduced cytochrome C (40 mg.), O_2	2.004	11	4
	Enzyme only	...	...	...
	Reduced cytochrome C only	2.004	11	2
	Complete + inhibitor: enzyme, reduced cytochrome C, KCN (5 mg.), O_2	2.004	11	2
	Enzyme, KCN	...	...	...
Succinic dehydrogenase	Complete: enzyme (10 mg.) succinate (10 mg.), cytochrome C (10 mg.), SC factor (0.03 ml.)	2.007	16	3
	SC factor only	2.007	16	<1†
	SC factor, succinate	2.007	16	2
	Enzyme, SC factor, succinate	2.007	16	2.5
	Enzyme only	...	...	...
	Succinate only	...	...	...
	Complete + oxidase: SC factor, succinate, enzyme, cytochrome C, cytochrome C oxidase	2.007	16	4

* Upon the addition of cytochrome oxidase to the complete cytochrome reductase system, the g-value of the resonance begins a systematic shift from $g = 2.006$ to $g = 2.004$ and the half-width shifts gradually from 18 to 11 gauss. The changes are those expected in a shift from a resonance due to the cytochrome reductase system to a resonance due to the cytochrome oxidase system.

† The resonance due to the SC factor only is extremely small compared to the noise present and may not represent a significant ESR.

When flavoprotein enzymes are illuminated in the absence of both electron donor and acceptor, an ESR is observed which appears to be similar to that produced during enzymatic activity. In the case of dehydrogenases it is not known at present to what degree DPN and TPN free radicals and possible free-radical forms of the substrates contribute to the observed ESR's. Similarly, the precise molecular origin of the ESR observed in the cytochrome–cytochrome oxidase system cannot be specified as yet. However, in all cases the observed ESR's are due to free radicals which are present only during the course of enzyme activity.

V. CONCLUSIONS

The foregoing experiments confirm both the specific and the generic aspects of Michaelis' hypothesis.

Detailed study of lactic oxidative decarboxylase shows that enzyme activity is invariably associated with a free radical, probably due to the FMN prosthetic group. When the rate of enzyme activity is varied by regulating the substrate and enzyme concentrations, the steady-state concentration of free radical varies proportionally. This behavior is expected of an oxidation-reduction intermediate. The effects of inhibitors on the free-radical level are equally characteristic of such an intermediate. The data confirm Sutton's specific proposal that the reactions mediated by this enzyme include at least one intermediate which has a free-radical form.

The survey of a series of oxidation-reduction enzymes shows that in all cases free radicals occur during the course of activity. The enzymes studied are of sufficient variety and occur so widely among living cells as to give substantial support to Michaelis' proposal that all biological transport is mediated by free-radical intermediates.

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*LIGHT-INDUCED FREE RADICALS IN FMN AND FLAVOPROTEIN ENZYMES**

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I. *Introduction.*—There is some evidence that visible light may influence the rate of cellular respiration[1] and of isolated oxidation-reduction enzyme systems.[2, 3] The evidence is not sufficiently extensive to indicate whether the effect is a general one, nor is it sufficiently detailed to suggest a possible mechanism.

The experiments reported below provide evidence on these questions. They show that flavin prosthetic groups and flavoprotein oxidation-reduction enzymes readily form free radicals when exposed to ordinary intensities of visible light. The light-induced free radicals appear to be identical with those ordinarily formed as intermediates in the oxidation-reduction process carried out by these components. This effect provides a mechanism which can mediate the influence of light on cellular oxidation-reduction. Since flavoprotein enzymes participate in a wide range of metabolic systems, the evidence also indicates that the observed effects of light on cellular processes is probably of quite general significance.

II. *Methods.*—In the experiments reported below, free radicals were detected and characterized by the electron-spin resonance technique. The electron-spin resonance spectrometer employed and the general features of the technique have been described in previous papers.[4, 5] The properties of the spectrometer that are of particular interest in the present work are (*a*) that the sample may contain as much