Flavin and Pyridine Nucleotide Oxidation-Reduction Changes in Perfused Rat Liver

I. ANOXIA AND SUBCELLULAR LOCALIZATION OF FLUORESCENT FLAVOPROTEINS

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SUMMARY

Flavin and pyridine nucleotide fluorescence was continuously monitored from the surface of the hemoglobin-free, perfused rat liver with the use of a new double fluorometer. In response to anoxia or after addition of inhibitors of the respiratory chain, the fluorescence intensity excited at 436 µm decreased, while that excited at 366 µm increased, representing reduction of flavin and pyridine nucleotides, respectively. Subsequent reoxidation of pyridine nucleotides in the cytosol by the addition of pyruvate was not accompanied by a change in flavin fluorescence. Maximal flavin reduction could be achieved by complete inhibition of the respiratory chain in the presence of oxygen. Titration with acetoacetate showed that mitochondrial pyridine nucleotides were in equilibrium with the fluorescent flavoproteins. Microsomal oxidation-reduction reactions appeared to be reflected by small changes in pyridine nucleotide fluorescence, but not by changes of flavin fluorescence. It is concluded, therefore, that the flavin signal is predominantly due to mitochondrial flavoproteins, with approximately equal contributions from flavin pools located on both the substrate and the oxygen side of the rotenone block. The continuous monitoring of flavin and pyridine nucleotide fluorescence as an experimental approach for studies of compartmentation of oxidation-reduction systems is discussed.

The study of metabolic control mechanisms in highly complex systems such as intact tissues has been limited in the past by the lack of suitable methods permitting both continuous and direct measurements. Usually, insight into cellular events is obtained by static analyses of tissue contents as well as by dynamic analyses of the extracellular fluid. Optical methods have the distinct advantage of monitoring cellular parameters without disruption of the tissue (1). Their application is, however, limited in the presence of hemoglobin because of its strong absorption band in the range of visible light (2-4). One useful optical parameter is the tissue fluorescence excited at distinct wavelengths, introduced in 1959 by Chance and Jobis (5). Subsequently, a close correlation between a blue fluorescence excited at 366 µm and the content of reduced pyridine nucleotides of various tissues has been clearly established (6-8). Furthermore, studies of absorbance and fluorescence changes in isolated mitochondria indicate that a yellow fluorescence excited at 436 µm is due to certain oxidized flavoproteins (9).

Recently, Chance, Mayer, and Legallais² developed a double fluorometer for the simultaneous measurement of flavin and pyridine nucleotide fluorescence in suspensions of isolated mitochondria (10). The purpose of the present paper is to describe the application of this new apparatus to the isolated, hemoglobin-free, perfused rat liver. It was previously shown that changes in fluorescence intensity excited on the liver surface by 366 µm light represent oxidation-reduction changes in both mitochondrial and extramitochondrial NAD systems (8, 11). In this study, however, we will present evidence that a change in fluorescence intensity excited at 436 µm is predominantly due to mitochondrial flavoproteins. Simultaneous measurement of the fluorescence changes due to flavin and pyridine nucleotides, therefore, allows the study of compartmentation of oxidation-reduction systems. Preliminary results have been reported previously (12, 13).

EXPERIMENTAL PROCEDURE

Methods

Hemoglobin-free Perfusion of Isolated Rat Liver—The perfusion system described by Scholz and Bücher (7) and by Scholz (8) obtained by static analyses of tissue contents as well as by dynamic analyses of the extracellular fluid. Optical methods have the distinct advantage of monitoring cellular parameters without disruption of the tissue (1). Their application is, however, limited in the presence of hemoglobin because of its strong absorption band in the range of visible light (2-4). One useful optical parameter is the tissue fluorescence excited at distinct wavelengths, introduced in 1959 by Chance and Jobis (5). Subsequently, a close correlation between a blue fluorescence excited at 366 µm and the content of reduced pyridine nucleotides of various tissues has been clearly established (6-8). Furthermore, studies of absorbance and fluorescence changes in isolated mitochondria indicate that a yellow fluorescence excited at 436 µm is due to certain oxidized flavoproteins (9).

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EXPERIMENTAL PROCEDURE

Methods

Hemoglobin-free Perfusion of Isolated Rat Liver—The perfusion system described by Scholz and Bücher (7) and by Scholz (8)
is used with slight modifications. The perfusion fluid consists of 100 ml of Krebs-Henseleit buffer, pH 7.4 (14), containing 4 g of bovine serum albumin per 100 ml (Fraction V, Sigma; twice dialyzed). This solution is saturated in a temperature regulated (37°) disc oxygenator, usually with mixtures of oxygen and carbon dioxide (95:5), or with mixtures of nitrogen and carbon dioxide (95:5). After the gas supply is changed, 90% saturation occurs in less than 1 min, indicating the high efficiency of the oxygenator. After passing through a gauze filter placed in a bubble trap, the saturated perfusion fluid is pumped through the liver via a cannula inserted in the portal vein. It leaves the liver via a cannula inserted in the vena cava, and passes an oxygenator. After washing with pulses of 366 and 436 nm light, the rotation of the disc being used as a time sharing device. The emitted fluorescence is detected by photomultipliers after passing through secondary filters having transmission maxima at 450 and 570 nm, respectively.

Male albino rats, Holtzman strain, 200 to 220 g, are deprived of food for 24 to 30 hours prior to anesthesia with pentobarbital (50 mg per kg). Details of the surgical procedure are described elsewhere (15). The advantage of the method is that an ischæmia or even hypoxia is totally avoided, since the perfusion is started in situ by inserting a polyethylene cannula into the portal vein with perfusion fluid running.

Oxygen concentration in the effluent perfusion fluid is monitored polaroagraphically with a modified Clark type platinum electrode.

Surface Fluorometry—The mechanical and electronic details of the double fluorometer are described elsewhere. The apparatus (Fig. 1) consists of a high intensity mercury arc lamp, reference and measure photomultipliers, combinations of primary and secondary filters mounted on a rapidly rotating disc, and necessary electronic components. The liver is alternately illuminated with pulses of 366 and 436 nm light, the rotation of the disc being used as a time sharing device. The emitted fluorescence is detected by photomultipliers after passing through secondary filters having transmission maxima at 450 and 570 nm, respectively.

Solutions of reduced pyridine nucleotides have fluorescence maxima at about 470 nm when excited with 366 nm light (16, 17). Moreover, this maximum is shifted to shorter wave lengths after binding to certain enzymes (18). We detect a fluorescence maximum between 440 and 450 nm when the liver surface is illuminated with 366 nm light (Fig. 2), suggesting that pyridine nucleotides in the intact cell are predominantly bound to protein. Although oxidized flavin nucleotides emit a yellow fluorescence when excited with 436 nm light (19), most isolated flavoproteins are not fluorescent (20). In suspensions of isolated mitochondria, however, changes of fluorescence with a maximum at about 570 nm are closely correlated with absorption changes of flavoproteins (9, 21). This fluorescence maximum is also observed under similar conditions in the perfused liver.

Materials

Reagent grade chemicals were obtained from Sigma, Calbiochem, Aldrich, or K and K Chemicals. Acetoacetate was prepared from the methyl ester (22). Rotenone was dissolved in dimethylsulfoxide (100 mg per ml), antimycin A in acetone (50 mg per ml).

RESULTS

Cycle of Anoxia—Distinct and reversible intracellular oxidation-reduction changes are obtained when liver is subjected to a cycle of anoxia (7). After oxygen withdrawal, the intensity of the fluorescence excited at 436 nm decreases, while that excited at 366 nm increases, representing a reduction of both flavin and pyridine nucleotides. The changes roughly index the maximal oxidation-reduction shift possible from the aerobic steady state level. They are reversible after restoration of the oxygen supply, indicating a reoxidation of the coenzymes. From a comparison of the kinetic changes in the fluorescence excited at 366 nm with tissue analyses of reduced pyridine nucleotides, it was concluded that these changes predominately represent changes in tissue NADH content with only a minor contribution of NADPH (7, 8).

So far, such a direct correlation between fluorescence changes and tissue content is not available for flavoproteins. However,

The perfusion apparatus and oxygen electrodes were constructed in the workshop of the Institute for Physiological Chemistry, University of Munich. The authors thank Dr. B. Brauser for the kind gift of miniature oxygen electrodes.
excitation and emission spectra of liver slices clearly identify this component as flavoprotein, particularly at low temperatures. In the following, we will describe a fluorescence increase excited at 366 mp as pyridine nucleotide reduction, and a fluorescence decrease excited at 436 mp as flavin reduction.

In the initial phase of the anoxic cycle, a small increase of the pyridine nucleotide fluorescence is observed, while no change in flavin fluorescence is recorded (Figs. 3 and 4, Phase I). Twenty seconds after the onset of anoxia, a rapid flavin reduction occurs, which is approximately paralleled by the pyridine nucleotide reduction (Phase II). Thereafter, the pyridine nucleotide reduction continues at a slower rate, while considerably less change is observed in the flavin trace (Phase III).

The reduction of cytochromes a and c reaches about 50% before the onset of the rapid pyridine nucleotide reduction occurs (23). Furthermore, a decrease in phosphate potential precedes the rapid increase in tissue NADH (7). It is concluded, therefore, that cytochromes, phosphate potential, and flavoproteins react in anoxia prior to the major pool of pyridine nucleotides. However, a small component of the pyridine nucleotide pool, detectable by tissue analysis (8) and fluorescence (Phase I), becomes reduced before an apparent reaction of the respiratory chain.

When oxygen is restored to the perfusion fluid, a rapid oxidation of flavoproteins is followed by an oxidation of pyridine nucleotides (Phase IV). The original oxidation-reduction state of pyridine nucleotides is slowly re-established. The kinetics of flavoprotein reoxidation shows an "overshoot" phenomenon. It is suggested that immediately after oxygen restoration, electron flux through the respiratory chain is maximal, owing to the excess ADP produced during anoxia, i.e. State 5 → 3 transition (24). Thus, the highly oxidized state of the oxidation-reduction carriers which is observed in State 3 of isolated mitochondria could explain this overshoot. A similar reaction of the mitochondrial pyridine nucleotides is apparently masked by the extramitochondrial pool. Concomitant with the completion of ADP phosphorylation, a State 3 → 4 transition occurs, which is accompanied by a partial reduction of the electron carriers.

Subcellular Localization of Fluorescent Flavoproteins—In previous studies with perfused liver, it was shown that both mitochondrial and extramitochondrial NADH pools are detectable by surface fluorometry (8, 11). Since the present paper is the first detailed report of flavin fluorescence continuously monitored in intact tissue, we must resolve which flavins are being measured. It is known that flavoproteins of isolated mitochondria have different quantum efficiencies (21). Also, fluorescent flavins have recently been detected in the microsomal fraction of rat liver. However, a quantitative comparison of flavin fluorescence in isolated subcellular fractions with that of intact tissue is not possible, owing to their different fluorescence enhancement and quenching properties. The subcellular localization of fluorescent flavoproteins in perfused liver, therefore, must be studied indirectly by comparing multiple oxidation-reduction changes under different metabolic conditions.

This problem can be approached by using inhibitors known to interact at specific points in the respiratory chain (Fig. 5). Rotenone and Amytal inhibit between two flavoproteins, $F_p D_1$ and $F_p D_2$.

$^4$ B. Chance and G. Van Rossum, unpublished observations.
$^6$ See the legend to Fig. 5 for an explanation of the abbreviations $F_p D_1$ and $F_p D_2$. 

![Fig. 3. Flavin and pyridine nucleotide oxidation-reduction changes during a cycle of anoxia in the perfused rat liver. An upward deflection reflects a reduction of pyridine nucleotides (upper trace, increase of fluorescence intensity excited at 366 mp) and flavoproteins (middle trace, decrease of fluorescence intensity excited at 436 mp). The lower trace represents the oxygen concentration in the effluent perfusion fluid, measured continuously with a platinum electrode. At the points indicated, the gas supply to the oxygenator was changed from O$_2$:CO$_2$ (95:5) to N$_2$:CO$_2$ (95:5), and vice versa. A time lag of 15 to 20 sec is due to both gas exchange and dead space in the perfusion system before anoxia or oxygen restoration occurs in the liver tissue. Different phases (I to V) of the anoxic cycle (7) are indicated by dotted lines.](https://example.com/fig3)

![Fig. 4. Oxidation-reduction changes during a normoxia-anoxia transition (7) in the perfused liver: oscilloscope recording of flavin (lower trace) and pyridine nucleotide (upper trace) fluorescence. An upward deflection represents reduction of these coenzymes. One vertical scale division represents a 2% change in fluorescence intensity.](https://example.com/fig4)

$F_p D_3$ (NADH dehydrogenase (EC 1.6.99.3)), which are characterized by different fluorescence yields and oxidation-reduction potentials (21, 26). $F_p D_4$ is thought to be coupled with lipoamide dehydrogenase, and is reduced by NADH, $\alpha$-keto-
glutarate, or pyruvate, whereas FpD is transferred electrons to the cytochrome chain (10, 27). FpD is coupled with succinate dehydrogenase, and is reduced in the rotenone-blocked system only by succinate, α-glycerophosphate, or fatty acids. Although antimycin A and sulfide inhibit at different points, they both block the transfer of electrons from flavoproteins to oxygen. Thus, anoxia and inhibitors of electron flux in the cytochrome chain should cause the same degree of reduction of mitochondrial flavoproteins. Furthermore, the problem of subcellular localization can be approached by compensating oxidation-reduction changes by the addition of pyruvate or acetoacetate, substrates known to react with dehydrogenases in different compartments.

**Rotenone**—Shortly after the addition of rotenone, a rapid flavin reduction is observed, followed after a time lag of about 2 sec by a reduction of pyridine nucleotides (Fig. 6). Maximal responses are reached in 2 min. The half-times of both reactions are about 30 sec. Occasionally, a slight reoxidation of pyridine nucleotides occurs, reaching a constant level after about 5 min (cf. Fig. 10, below). Concomitantly with the oxidation-reduction changes, the oxygen consumption decreases about 50%.

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![Diagram](image_url)

**Fig. 5.** Scheme for the mitochondrial flavin chain (25). FpD, NADH dehydrogenase (EC 1.6.99.3); FpL, lipoamide dehydrogenase (EC 1.6.4.3); FpS, succinate dehydrogenase (EC 1.3.99.1). Rotenone or Amytal separates FpD from FpD.

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![Diagram](image_url)

**Fig. 6.** Fluorescence changes following the addition of rotenone to the perfusion fluid: oscilloscope recording of flavin (upper trace) and pyridine nucleotide (lower trace) fluorescence. An upward deflection represents reduction. Rotenone (1 mg) was dissolved in dimethylsulfoxide (10 μl) and added to a sample of perfusion fluid with rapid stirring. Since precipitation of rotenone could not be avoided, the final concentration should be less than 25 μM. One vertical scale division represents 4% change in fluorescence intensity.

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![Diagram](image_url)

**Fig. 7.** Effect of pyruvate on flavin and pyridine nucleotide reduction in the perfused liver following inhibition by rotenone. Eight minutes after the addition of rotenone, 0.2 μmole of sodium pyruvate was slowly added to the perfusion fluid. Pyruvate, which gives maximal effects with a concentration of 2 mM, reverses 40% of the increment of pyridine nucleotide fluorescence observed after rotenone addition, if the base-line drift (see left hand side of the trace) is considered. We cannot explain why the flavin reduction in this experiment is slower than that usually observed (see Figs. 6, 9, and 10).

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Based on the known site of rotenone inhibition, the fluorescence changes are expected to represent primarily the reduction of respiratory chain components located on the substrate side of the rotenone block, i.e. FpD-lipoamide dehydrogenase complex and mitochondrial NAD. Extramitochondrial NAD, however, is also expected to be involved, owing to the stimulation of glycolysis following a decreased phosphate potential.

**Compensation of Oxidation-Reduction Changes**—It can be concluded that in anoxia or with rotenone or Amytal, pyruvate reacts predominantly with extramitochondrial NADH, whereas mitochondrial reactions, i.e. pyruvate carboxylation and decarboxylation, are prevented because of the lack of energy and NAD. Furthermore, the first step in pyruvate decarboxylation appears to be unlikely since lipoamide dehydrogenase would already be reduced, owing to its rapid equilibration with NADH dehydrogenase (25). The reoxidation of pyridine nucleotides reduced by rotenone following the addition of pyruvate (Fig. 7, about 40%) and the diminished reduction during anoxia in the presence of high pyruvate concentrations (Fig. 8) are, therefore, primarily due to an extramitochondrial NADH pool which is in connection with lactate dehydrogenase, as shown previously.

Flavin reduction is not diminished during anoxia in the presence of high pyruvate concentrations compared to a control in the absence of a hydrogen acceptor for extramitochondrial NADH (Fig. 8). Likewise, less than 10% of the flavoproteins reduced by rotenone can be reoxidized by the addition of pyruvate (Fig. 7). Thus, the reoxidation of extramitochondrial NADH does not affect the oxidation-reduction state of fluorescent flavoproteins.

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1 R. Schols, J. Grunst, F. Schwarz, and T. Bücher, manuscript in preparation.
Fig. 8. Flavin and pyridine nucleotide oxidation-reduction changes of the perfused liver during a cycle of anoxia in the presence and in the absence of high pyruvate concentrations. One minute after the gas supply to the oxygenator was switched from \( \text{O}_2-\text{CO}_2 \) to \( \text{N}_2-\text{CO}_2 \), pyruvate was infused into the perfusion fluid before it entered the liver, maintaining a concentration of approximately 2 mM. An accumulation of pyruvate could occur after 2 min of infusion, owing to the turnover in the perfusion system (volume, 100 ml; flow rate, 40 ml per min). After 4 min of anoxia, the gas supply to the oxygenator was switched to \( \text{O}_2-\text{CO}_2 \); the pyruvate infusion was stopped.

Further support is obtained from similar experiments in which acetooacetate is added after rotenone inhibition (Fig. 9). High concentrations of acetooacetate in the perfusion fluid only partially reoxidized pyridine nucleotides (about 70%), but reoxidize flavoproteins completely. In contrast with pyruvate, acetooacetate is a hydrogen acceptor for mitochondrial NADH, its main interaction in liver being with \( \beta \)-hydroxybutyrate dehydrogenase. This experiment indicates that part of the NAD reduced by rotenone is not in equilibrium with this dehydrogenase, i.e. NAD in the cytosol. Conversely, the entire flavoprotein pool reduced by rotenone appears to be in equilibrium with mitochondrial NAD linked to \( \beta \)-hydroxybutyrate dehydrogenase.

Antimycin A and Sulfide—Another flavin pool containing \( \text{FAD}, \text{succinate dehydrogenase}, \) and the flavoproteins, involved in fatty acid oxidation remains oxidized in the rotenone-inhibited state. This pool becomes reduced when the electron flux to oxygen is completely stopped by anoxia or by addition of antimycin A (Fig. 10). The flavin reduction observed shortly after the addition of antimycin A exceeds that reached in 4 min of anoxia; however, nearly the same level of reduction is reached if the anoxia is continued for a longer time period (not shown in this experiment). The changes in flavin fluorescence caused by rotenone and subsequently by antimycin A are almost equal.

In the presence of rotenone, the reoxidation of pyridine nucleotides after anoxia is incomplete. However, the same fluorescence increase is reached with antimycin A in comparable time intervals as with anoxia. Furthermore, this pyridine nucleotide reduction continues, while the flavin reduction maintains a maximal level. Assuming that the oxidation-reduction state of flavoproteins reflects that of mitochondrial NAD, the prolonged pyridine nucleotide reduction following antimycin A inhibition indicates the absence of a rapid equilibrium between extra- and intramitochondrial pools.

The fact that anoxia does not cause a larger flavin reduction than inhibition with antimycin A is confirmed in a similar experiment with sulfide (Fig. 11). Oxygen withdrawal after maximal inhibition with sodium sulfide does not further reduce fluorescent.
flavoproteins, indicating that in the absence of sulfide or antimycin A the flavin reduction observed in anoxia is due to mitochondrial flavoproteins. Unexpectedly, pyridine nucleotide reduction increases rapidly after oxygen withdrawal in the presence of sulfide. Since the egress of reducing equivalents from the mitochondria as well as the generation of NADH in the cytosol should not be accelerated under these conditions, the interaction of reduced pyridine nucleotides with extramitochondrial oxidases should be considered.

Microsomal Oxidation-Reduction Reactions—It might be expected that extramitochondrial flavoproteins should also be reduced in anoxia. Cyanide-insensitive respiration was found to be 10 to 20% of the total oxygen uptake of the perfused liver (7, 15), indicating the activity of extramitochondrial oxidases. The negligible contribution of these flavoproteins to the fluorescence changes in anoxia could be due, first, to a lower quantum efficiency of their fluorescence, or second, to smaller oxidation-reduction changes to that of flavins linked to the respiratory chain. However, if microsomal oxidation-reduction changes are detectable, they should become apparent when substrates for mixed function oxidation, such as aminopyrine, are metabolized. Shortly after the addition of aminopyrine, an increase in oxygen consumption of about 5% and an oxidation of pyridine nucleotides occur (Fig. 12). These responses indicate that microsomal flavin-enzymes, such as NADH-NADPH diaphorase (EC 1.6.1.1) and NADPH-cytochrome c reductase, are reacting; but no change in flavin fluorescence is observed. Furthermore, it was recently suggested that ethanol is a substrate for the mixed function oxidase system (28). Usually, ethanol metabolism is reflected by a reduction of pyridine nucleotides and flavoproteins (12). However, in the presence of pyrazole, a potent inhibitor of alcohol dehydrogenase (29), addition of ethanol has no effect on flavin fluorescence, indicating that microsomal ethanol oxidation does not cause detectable fluorescence changes, if the assumption is made that pyrazole is without effect on this system. Thus, no evidence is obtained that changes in flavin fluorescence must be attributed to flavoproteins located outside mitochondrial membranes.

DISCUSSION

Determination of Oxidation-Reduction States in Different Subcellular Compartments of Intact Tissue—Studies of oxidation-reduction compartmentation are confronted with the experimental problem that tissue analyses do not allow insight into the oxidation reduction states of different subcellular compartments. Furthermore, these different oxidation-reduction states cannot be maintained during the process of subcellular fractionation at the present time. Dissection of subcellular components from tissues followed by microanalysis has been accomplished in a variety of cells (30, 31). Although this laborious technique has not been applied to problems of oxidation-reduction compartmentation, it seems to be possible. A completely different approach, developed mainly by Bücher and Klingenberg (32), makes the assumption that thermodynamic equilibrium is obtained in different oxidation-reduction systems. Subcellular oxidation-reduction states, therefore, are calculated from the tissue content or extracellular concentrations of substrate couples, the dehydrogenases of which are localized exclusively in one compartment. Much attention has been devoted to the continuous read-out of metabolic signals from intact tissues (1). With respect to oxidation-reduction studies, such signals may be derived from the fluorescence of coenzymes, particularly reduced pyridine nucleotides, as shown by Chance and Jobsis for intact sartorius muscle (5). Two general principles are followed. First, the fluorescence of different cellular organelles can be observed by microfluorometry. This method has been successfully applied to the direct read-out of pyridine nucleotide reduction, mainly in perinuclear mitochondria and the clear cytoplasm of single cells.
(33). A second approach is the use of fluorescence signals derived exclusively from one subcellular compartment. While pyridine nucleotide fluorescence is derived almost equally from both mitochondria and cytosol, flavin fluorescence of liver tissue, as shown in these experiments, is derived predominantly from mitochondrial flavoproteins. Since these flavoproteins are thought to be closely equilibrated with mitochondrial NADH, the overall pyridine nucleotide signal of the tissue may be corrected for the mitochondrial component.

**Oxidation-Reduction Changes of Mitochondrial Flavoproteins**—In this study, several indirect approaches have been used to show that flavin fluorescence in the perfused liver is an indicator for the mitochondrial space. First, the addition of a hydrogen acceptor for extramitochondrial NADH, such as pyruvate, in the rotenone-inhibited or anoxic state causes a considerable reoxidation of pyridine nucleotides, but only a negligible reoxidation of flavoproteins. However, a complete reoxidation of flavoproteins reduced by rotenone occurs after the addition of acetoclastic, an acceptor for mitochondrial NADH. This flavin pool, therefore, represents the oxidation-reduction state of mitochondrial, rather than extramitochondrial, NADH.

Moreover, a complete reoxidation of reduced pyridine nucleotides by high concentrations of either pyruvate or acetoadetate was not possible under these conditions. This observation agrees with similar experiments, where the reoxidation in anoxia (8) and in the Amytal-inhibited state was also titrated. Thus, the withdrawal of reducing equivalents from one compartment does not effect a rapid efflux from the other compartment. Since rotenone, Amylans, and anoxia all decrease the energetic state of the liver cell, it is possible that these two compartments fail to interact rapidly because of a lack of energy.

Second, anoxia did not cause a greater flavin reduction than inhibition of the respiratory chain between oxygen and the flavin region. A possible reduction of microsomal flavoproteins in anoxia, therefore, can give only a minor contribution to the fluorescence signal. This is also shown under conditions of increased microsomal activity, in which no changes of flavin fluorescence are detectable. Ultimately, a more accurate analysis may lead to direct measurements of flavoproteins associated with extramitochondrial processes as well. However, at present, they constitute such a small portion of the total signal in the types of metabolic transitions studied here that they may be neglected.

All of the available experimental evidence, therefore, confirms the conclusion that more than 90% of the flavin fluorescence changes observed from the liver surface is due to mitochondrial flavoproteins. In a transition from the aerobic steady state to a completely reduced state, the flavin pools on either side of the rotenone block give almost equal contributions to the fluorescence changes. However, less than 30% of the total flavin extractable from isolated liver mitochondria can account for a flavin pool on the substrate side of the rotenone block. This confirms the observation from mitochondrial experiments that this pool has a higher fluorescence yield than the one on the oxygen side (20).

**Kinetics of Intracellular Oxidation-Reduction Reactions in Tissues**—Of importance to the interpretation of experimental data on intracellular reactions and on the modelling of intracellular reactions by computer techniques is information on the speed with which these reactions occur. Kinetics of reactions in subcellular fractions and single cells can be determined by the use of techniques of rapid mixing. But these techniques, such as the flow apparatus (34), are not applicable to intact tissues. In a perfused organ, in which cellular reactions are initiated via the transport of substances by the perfusion fluid, the time difference between the onset in different cells can reach several seconds. The initial phase of fluorescence changes, therefore, is influenced by the statistical distribution of a population of 10^9 to 10^10 liver cells. Thus, the observed kinetics of the "on" reaction in anoxia or rotenone inhibition with half-times of 15 and 30 sec, respectively, is much slower (at least one order of magnitude) than in experiments with isolated mitochondria (27).

In the "on" reaction of anoxia, the reduction of cytochromes a and c (23) and of flavoproteins occurs before the reduction of the major portion of pyridine nucleotides. A minor portion, however, becomes reduced before any reaction of the respiratory chain is detectable. This phenomenon is not observed in an aerobic-anoxic transition of isolated mitochondria or with rotenone inhibition in perfused liver. Thus, the reduction in Phase I of anoxia appears to be independent of the respiratory chain. It may be due to a pool of pyridine nucleotides coupled to an oxygen acceptor with a K_m for oxygen greater than that of cytochrome oxidase, such as oxidases located in peroxisomes (35) or in the smooth endoplasmic reticulum.

**Studies of Compartmentation of Oxidation-Reduction Systems**—The subcellular distribution of fluorescent flavoproteins, as shown in this paper, has unique applications to the study of compartmentation of oxidation-reduction systems. Thus, flavin or pyridine nucleotide fluorescence can be used as a continuous monitor for mitochondrial or for mitochondrial plus cytosolic oxidation-reduction states, respectively.

A decrease of the electron flux in the respiratory chain, initiated either by oxygen withdrawal or by the addition of specific inhibitors, causes primarily a reduction of mitochondrial pyridine nucleotides and a fall in the phosphate potential. A consequent reduction of extramitochondrial pyridine nucleotides could be due to the following possibilities: (a) an egress of reducing equivalents from the mitochondria, (b) a decreased rate of extramitochondrial oxidation of NADH and NADPH, and (c) an increased generation of NADH in the glycolytic chain stimulated by the adenosine phosphate system. The experiments described in this paper give no evidence that a flux of reducing equivalents across mitochondrial membranes is involved. Since the oxidation-reduction changes in both cytosol and mitochondria can be separately compensated by specific hydrogen acceptors, it was previously concluded that a rapid bidirectional hydrogen flux does not occur when the respiratory chain is inhibited, i.e. in the absence of energy. Furthermore, evidence exists that, in anoxia, a decreased extramitochondrial oxidation of pyridine nucleotides is, in part, involved. The reduction in Phase I and the increased pyridine nucleotide reduction in anoxia after prior inhibition with sulfide may be ascribed to an inhibition of the extramitochondrial oxidases. Coupling of extra- and intramitochondrial oxidation-reduction reactions via the adenosine phosphate system appears to be the important mechanism of cytosolic pyridine nucleotide reduction following a decreased electron flux in the respiratory chain.

Under more nearly physiological conditions, i.e. in the presence of energy sources, a bidirectional flux of reducing equivalents...
across the mitochondrial membrane is observed to occur. During ethanol or lactate oxidation, this flux is directed into the mitochondria, whereas during gluconeogenesis or fatty acid oxidation the flux is in the opposite direction. The simultaneous monitoring of flavin and pyridine nucleotide fluorescence and oxygen consumption allows the study of speed and extent in the intracellular transfer of reducing equivalents, as well as the relative involvement of different transport shuttle mechanisms.

REFERENCES