Kinetics of mitochondrial flavoprotein and pyridine nucleotide in perfused heart

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The increasing fund of knowledge on the properties of respiratory pigments of isolated mitochondria coupled with new techniques for their study in cells and tissues affords new approaches to the study of four fundamental biochemical and physiological relationships: a) the nature and function of the electron transport system in the intact tissue, b) the interaction between energy sources and energy sinks in a functional tissue such as cardiac muscle, c) the effectiveness with which oxygen is transported to the intracellular mitochondrial spaces from the extracellular capillary space, and d) the nature of the specific activator of mitochondrial function in situ, ADP + Pi and/or Ca2+.

Particularly important to the problem of cell function is the identification of metabolic states in mitochondria controlled by the substrate, oxygen, and energy levels. A transition between such states that is significant in muscular activity is the resting-active transition in which the energy demand on mitochondrial energy conservation vastly alters the steady state and kinetic properties of the electron carriers (12, 24). In previous studies repetitive stimulation of the isolated sartorius muscle activated the resting (state 4) to active (state 3) transition and increased the oxidation of reduced PN (16, 23, 38). Such transitions are observed in the steady-state level of reduced PN by spectrophotometry (28) and fluorometry (16) and of cytochrome b by spectrophotometry (22, 38, 40). More recent studies of isolated mitochondria (26) show that the energy state of the mitochondria can be deduced from the kinetics of respiratory enzymes in the anaerobic-aerobic transition of cytochromes b, c, and a3 caused by O2 pulses (11). These experiments identify accelerated responses of the flavoproteins and pyridine nucleotides when ADP and Pi are present and even faster rates of these transitions when the energy is needed for transporting calcium as well (unpublished observations). In essence, the mitochondrial respiratory chain contains sensitive indicators of the extent and nature of the energy demand. Since one of the principal problems of tissue bioenergetics in normal and abnormal states is the functionality of mitochondria in situ (an example is the irreversible damage in stroke and shock (43)), it seems desirable to be able to apply the techniques developed for isolated mitochondria directly to the intact tissue.

The metabolic transition employed in our mitochondrial experiment was from anoxia to normoxia in the rapid flow apparatus; oxygen pulses are delivered to the anaerobic suspension of isolated mitochondria. The oxidation time for Fp and PN was as short as 100 msec (unpublished observations). When oxygen pulses are delivered to perfused organs the time from anoxia to normoxia may be 20–30 sec (25, 66) and even with the intact organ circulated in vivo, recovery from ischemia in the liver requires 2–3 sec (20) due to diffusion limitation in the tissue.

Spontaneous or evoked contractility affords fast perturbation of the metabolic state of skeletal and cardiac muscle (16, 23, 38) and causes a fast oxidation of PN due to the arrival of ADP (or Ca2+) at the mitochondria (17). This perturbation has the great advantage that diffusion time from the myofibrils to the mitochondria is short (<100 μsec) and in particular cases mitochondrial responses 200 msec after a single muscle twitch are observed (see ref. 17, Fig. 1).

The diffusion limitation in the anaerobic-aerobic transition can be avoided by methods that perturb the biological system after diffusion equilibrium of the relevant metabolites has been established. Specific perturbation of single enzymes is not possible with available relaxation methods
which alter only the fundamental variables of temperature, pressure, volume, etc. For example, a temperature perturbation alters the enzyme activities and metabolic flux rates on a wide range of components rather than a specific component as we desire. An example of a temperature perturbation is afforded by a laser-induced temperature jump (≈10 °C) applied to a toad sartorius muscle (21). A biphasic decrease of the fluorescence of NADH was observed. The fast decrease was due to a decrease in quantum efficiency and the slow decrease was due to a temperature-induced change of metabolism that reached a peak at 200 msec. This type of perturbation was exploited further in cell suspensions (47) in which xenon flash and joule heating were employed. The 5°C temperature jump reached equilibrium in about 400 msec, causing an activation of the enzymes of glycolysis as evidenced by an increased NADH oxidation. However, the response could not be identified with a single enzyme or chemical species. One way to obtain a more specific perturbation is the electrophoretic injection of substrate into the cytosol of a single cell which gives a fluorescence increase due to NAD or NADP reduction with a half-time of a few hundred milliseconds (42).

While the temperature jump perturbations caused extensive transients in the glycolytic metabolism because the temperature coefficients of the steps are large and nonidentical, the successive steps of electron transport in mitochondria have nearly identical temperature coefficients and thus little effect can be observed with temperature perturbation. However, a large and specific activation of mitochondrial electron flow is caused by flash photolysis of cytochrome as-CO in the presence of oxygen. This technique has previously been employed in the study of isolated cytochrome oxidase (34) and can now be effectively applied to suspensions of isolated mitochondria (11, 13). In brief, the technique involves carbon monoxide inhibition of the anaerobic mitochondria, their rapid mixture with oxygen in the regenerative flow apparatus, illumination with a photolyzing light of sufficient intensity to break the cytochrome as-CO bond (6), and a sufficiently rapid optical readout system to follow the rapid oxidation of cytochrome as + a (11) and cytochrome e (6). Under these conditions, the observed reaction rates are limited neither by the speed of photolysis of the CO compound nor by the mixing of oxygen with the cytochrome oxidase; the intrinsic rates of the electron transport reactions are directly measured. In addition to electron transport activation, ATP formation, ion pumping, and substrate transport are rapidly initiated and thus this technique can be of great value in relating the response time for activation of these functions with overall physiological activities. The kinetics of activation of electron transport can be read out not only in terms of cytochromes of types e, a, and a3 but also from the energy-dependent cytochrome b5 (26, 69) and the fluorescent electron transport component, oxidized Fp and reduced NAD (unpublished observations). The rate of the response can be sensitive indicators of the mitochondrial phosphate potential (ADP/ADP + P1) (15) or energy charge (1).

With flash photolysis techniques in general, it is essential that the mixing time for the as-CO compound with oxygen be shorter than the time for spontaneous dissociation of the as-CO compound. While this is easy to achieve in the regenerative flow apparatus, it can become of critical importance in tissues where the mixing of oxygen with the CO-inhibited oxidase depends on the effectiveness of perfusion and the speed of diffusion from the capillaries to the tissue mitochondria. From our previous experimental results on the relatively slow recovery of tissues from anoxia, it is clear that the rate of dissociation of carbon monoxide from cytochrome a3 at 20°C (6) is fast enough so that some difficulties might be encountered. This paper describes the resolution of these problems, the design of a satisfactory experimental approach to flash photolysis of cytochrome a3-CO in the presence of oxygen in cardiac tissue with the readout of the kinetics of electron transport and tissue bioenergetics in terms of the fluorescent Fp and reduced PN components of the mitochondria.

The rat heart has been selected because of its physiological and biochemical characteristics. The high volume ratio of mitocondria (0.34) (56) together with the relatively small contribution of tubular memranae to the total sarcolemna area make an experimental material suitable for evaluation of mitochondrial function in contraction-relaxation processes. Thus, a further investigation of the role of mitochondria in muscular function (37, 41, 49, 51) and a more critical evaluation of our previous conjectures may be possible (8). In addition, this work may provide an essential first step on the way to the study of kinetics of enzyme reactions in tissues.

**METHODS**

An illustration of the apparatus as employed for recording fast fluorescence changes in the perfused heart is provided by Fig. 1. The general characteristics of the time-sharing fluorometer for Fp and PN have been described previously (14, 18). The apparatus consists of two sets of filters for excitation and emission measurements of PN (366 and 480 nm, respectively) and Fp (460 and 580 nm, respectively) which are rotated at 1,800 rpm before the excitation lamp (H6 Hg lamp) and measuring photomultiplier PMM. The timing drive connects the rotating disk to a synchronous motor. The rotation is synchronous with the alternating light pulses from the a-c driven lamp. The light from the mercury arc is passed through local absorbing filters that diminish the intensity of the light as well. An image of a limiting aperture is projected as a 1–3 mm spot upon the surface of the perfused heart. The emitted fluorescence is imaged upon PMM via a lens and an appropriate filter. The waveform of pulses corresponding to the fluorescent signals are balanced against a similar series of pulses obtained from a compensating photomultiplier PMO so that for the normoxic state of the heart, the pulses are exactly canceled out and the effect of light intensity fluctuations is negligible. In the anoxic state of the heart the pulses are unbalanced and a gated detector circuit identifies this difference and reads out the resulting traces for the Fp and PN signals on a strip chart and storage oscilloscope (Tektronix 564). Photolysis of the cytochrome a3-CO compound in the presence of O2 is ob-
KINETICS OF FLAVOPROTEIN AND PYRIDINE NUCLEOTIDE IN HEART

209

Perfused Heart - 28 J Xenon Flash

FIG. 1. A diagram of perfused heart illustrating relationship of fluorometer and photolysis by liquid dye laser and xenon flash lamp.

tained by flash illuminating it just after perfusion with oxygen is begun. The flash may be derived from a typical photoflash unit (28-joule (J) xenon flash, supplemented with a 430-nm filter) or alternatively from a liquid dye laser emitting monochromatic light at 585 nm at an intensity of 100 mJ and a pulse width of 0.4 μsec. Tension is measured with a strain gauge and in some experiments left ventricular pressure is measured. Fluorescence excitation is applied normally to the heart so that the specular reflection travels back along the incident path and not into the fluorescence detector, and the scattered light does not change appreciably with the motion of the heart. In some cases, the heart rests upon a glass surface; for example, a small funnel which collects the perfusate which helps thereby to damp the gross movement of the heart. Fluorescence emission is observed at an angle of 30° through the secondary filter. Each 180° turn of the disk holding the excitation and emission filters brings them into alignment for reduced PN (366 nm excitation, 460 nm emission) and for oxidized Fp (460 nm and 580 nm, respectively).

Depth of penetration and size and location of field of view. The fluorometer observes a portion of the surface of the heart between 2 and 4 mm in diameter and on the left to right ventricle as may be desired. The depth of penetration has been estimated in experiments in which successive layers of tissue are piled one upon the other and the increase of the fluorescence signal is observed. The “end point” of the fluorescence increase is identified with the end point of the penetration of the excitation and emission wavelengths. For PN (366 nm excitation, 460 nm emission) the value is 0.36 mm. For Fp (excitation 460 nm, 570 nm emission) the value is 0.84 mm. Thus the fluorescence recording is from about the first third of the thickness of the wall of the heart (3 mm).

Fluorescence change in response to a normoxic-anoxic cycle were recorded from the left and right ventricles with satisfactory results although somewhat larger signals were obtained from the left ventricle (50%) which was employed in most experiments (experiments in collaboration with Mr. Guy Salama). No more detailed survey of the heart surface has been made.

Photolysis. Two light sources afford photolysis for the carbon monoxide-inhibited cytochrome system. A xenon flash lamp (2-msec flash, 28-J white light) (Braun F 270) is filtered through a 430 ± 10-nm filter so that interference with the PN readout at 480 nm is diminished. A reflector in the flash lamp plus a Lucite light cone increased the...
efficiency of illumination. Two xenon flash lamps and cones may be employed (see below).

Photolysis with the liquid dye laser (LDL) (General Laser GL-1,000) (585 nm, 100 mJ intensity) is via a small mirror. The laser affords this power output at an operating voltage of approximately 14 kv when employing a Rhodamine 6G dye. The laser beam is highly parallel and of roughly 1 cm diam. Thus, the heart is uniformly illuminated by the laser beam.

The heating of the heart from the 0.1-mJ laser flash is readily calculated since 4 J are required to raise 1 ml of H2O by 1 C. Two calculations can be made. The first one assumes that all the laser light is uniformly absorbed in the appropriately 1 ml volume of the heart. The temperature rise under these conditions is 0.1/4 or 0.025 C. A second calculation assumes that the laser light is all absorbed in a square 5 mm in a side and 3 mm thick, the thickness of the heart wall. The temperature rise would then be 0.3 C. A third calculation assumes that only the mitochondria are heated within this volume of tissue since the mitochondria comprise about one-third of the total tissue volume (56), the temperature rise would be 1 C. The possibility that the laser damages the mitochondria as suggested by experiments at higher powers (3, 33) is unlikely; repeated flashes cause no decrease of mitochondrial signals. A response to increased temperature to be expected is the decrease of PN fluorescence at about 1%/°C due to the decrease of quantum yield; no significant effect is seen. The jump responses to flash photolysis do not depend upon which ventricle is illuminated and thus a specific effect upon the pacemaker tissue with oxygen and another flash occurs several seconds later. The two xenon flash lamp is trigger the appropriate synchronizing circuit. Two Lucite cones are employed to direct the laser beam is short compared to the 75- to 100-msec rise time of the phosphorescence of Cytochrome oxidase.

If some flash artifact occurs, the recovery of the PM output is short compared to the 75- to 100-msec rise time of the metabolic responses of Fp and PN.

A trigger for the synchronizing circuit can conveniently be derived from either one of the two pulses from the compensating photomultiplier (PMc). Such a pulse triggers the delay circuit which provides a relay closure for firing the flash lamp at an appropriate phase angle. A Tektronix storage scope 564 is provided with a suitable pretrigger adjustable from 0.5 to 3 sec with respect to the photolysis flash. Usually the oscilloscope trace is triggered at the time the oxygen-saturated perfusate is turned on. The operation thereafter is automatic.

Characteristics of preparation. Male albino rats of Holtzman strain, weighing 220-289 g, are fed Purina laboratory chow and water ad lib. The experiment is started with no special reference to the rat feeding cycle.

The rats were decapitated, the heart rapidly excised and securely attached to a Y glass cannula (15 gauge equivalent) via the aortic root. As explained below, it is essential to avoid prolonged anoxia between the interval of excision of the heart and the perfusion with oxygen. The estimated interval for most preparations is ~45 sec. A Langendorf perfusion apparatus was employed and the perfusate was not recirculated. The perfusion pressure was 50 ± 10 mm Hg and the temperature was 24 C. The flow rate was 12-17 ml/min. (For further details see ref. (32).) The perfusate was Krebs-Ringer bicarbonate (45) containing half the usual calcium concentration. When perfused with lanthanum, a HEPES buffer solution containing isotonic sucrose (no PO4) was used (59).

Sodium nitrite, 0.1 mM, was usually added to the perfusate in order to oxidize the tissue myoglobin and any residual hemoglobin (66) and thereby avoid absorbancy changes due to these oxygen carriers in the aerobic anoxic cycles. We obtained no evidence for a significant effect of the nitrite at this concentration on the metabolism of the heart.

In order to alternate aerobic and CO anaerobic perfusates, 95% O2 + 5% CO2, or 67% CO + 28% N2 + 5% CO2, was equilibrated with the perfusate in duplicate vessels each at the same height (Fig. 1 illustrates stopcocks for changing the perfusion media). However, pinch clamps prior to the Y connection were found to be more rapid. The dead volume between the pinch clamps and the heart is 0.1 ml. Thus the new perfusate arrives at the heart in 0.1 sec at the flow rates of 10 ml/min. The duration of carbon monoxide exposure was 1 min and a 5 min aerobic recovery interval was afforded. When prolonged intervals of anoxia were employed the heart was supplemented with 1-10 mM dextrose in order to maintain a strong heart beat.

Heart contractility. Ventricular pressure was measured directly with a 20-gauge needle in the left ventricle and a Sanborn pressure transducer and d-c amplifier. The tension was measured from a hook at the apex of the heart combined with a strain gauge. The measurements were not localized to the exact region of fluorometric observation.
but did afford a control of the generalized physiologic function of the heart.

Reproducibility of preparation. The experiments described here are based upon the studies of over 300 rat hearts and the charts included here are representative of the vast majority of these experiments. As described below under Experimental design, a successful experiment requires rapid reoxygenation of the tissue following an anoxic episode. In hearts from the Holtzman strain, this was regularly observed and responses from Fp and PN are obtained as indicated in the diagrams of this paper. In some rats from other strains, the reoxidation of PN and particularly Fp was incomplete after the first cycle of anoxia. Furthermore, the ventricular pressure which fell sharply following this cycle failed to recover for an extended interval (ca. 10 min). No detailed studies have been made of this phenomenon but it is assumed that the tissue circulation in the area under observation is somehow impaired by a brief episode of anoxia. Thus the first response of the heart to anoxia was carefully monitored. In addition, special precautions were taken to avoid an anoxic episode in the interval between excision of the heart and transfer to the perfusion apparatus.

Calibration procedure. The calibration of the fluorometer is: 1 scale division = 12.5% change of fluorescence. The aerobic heart itself is used as the fluorescence standard, the amplifier gain and PM voltage is set to give l-v signal (2 scale divisions) from the aerobic heart. The amplifier gain is then increased 4 times so that each scale division represents 12.5%. The time base is usually 1 cm/sec, but occasionally 0.5 cm/sec. The response speeds employed range from approximately 1 sec or 40 msec (for 10-90% response).

In actual operation the heart is initially put in the anoxic condition with 67% CO + 28% N₂ + 5% CO₂. The perfusate is then changed to 95% O₂ + 5% CO₂ by altering the fluid connections and at the same time the sweep is triggered. The photolysis flash occurs 0.5–1.0 sec after the recovery from anoxia is detectable. The photolysis flash then activates the respiratory carriers and the metabolic state of the tissue can be read out in terms of their rate of oxidation. The system is allowed to reestablish its normoxic state and an interval of 5 min elapses before the next episode of toxic anoxia.

Measurement of cytochrome a₃-CO. The apparatus described in Fig. 1 can be employed for spectroscopic measurements as well (14, 18). Interference filters of 445 and 455 nm are inserted into additional holes in the disk. A PM views the light transmitted through a single thickness of the heart wall via a 1-mm diam light pipe.

Experimental design. Optimal results for this experiment require a careful evaluation of the parameters. Ideally 100% saturated cytochrome a₃-CO is completely photolyzed by the light flash to give 100% reduced a₃ which combines with excess O₂ to give 100% oxidized a₃. This ideal is only imperfectly realized; the amount of photolysis depends on the interaction of four factors: 1) the CO concentration of the perfusion, 2) the extent of preflash photolysis, 3) the extent of flash photolysis, and 4) the oxygen concentration. Whereas in the dark a few tenths of a percent of CO will satisfy the requirements for initial CO saturation in view of the high affinity for CO (10⁻⁴ M) (13), the photolysis caused by the fluorescence excitation shifts the CO affinity to lower values due to an increase of k' of equation 6 of ref. 1 and causes "preflash photolysis." The extent of preflash photolysis is diminished by high CO (kₚ is increased (see equation 5 of ref. 6)). A high fluorescence excitation intensity is required for the fast readout of fluorometric signals; thus, a signal to noise ratio in excess of 10 may result in a preflash photolysis of up to 25% of the total oxidation reduction change. Flash photolysis of cytochrome a₃ was over 95% complete with the laser source or the xenon flash source, taking, in this case, 100% to be that obtained with the double-flash technique (see above and below). The filling of the capillaries and the diffusion velocity of oxygen in the tissue should be fast enough so that the oxygen gradient moves through most of the a₃-CO molecules in the field of observation in the interval between initiating the oxygen perfusion and the flash photolysis. Under these conditions the oxidation rates of PN and Fp will be indicative of the ADP + P₁ and Ca²⁺ levels in the cytosol.

The interaction of these factors above leads to four categories of cytochrome a₃ molecules at the time of photolysis. First, cytochrome a₃-CO molecules that are in the presence of a sufficiency of oxygen, i.e., an oxygen concentration which upon photolysis will give a significant oxidation of Fp and PN. Second, cytochrome a₃ molecules that are in a deficiency of oxygen such that no significant response of Fp and PN is observed on photolysis. Third, reduced cytochrome a₃ molecules from which CO has dissociated but which oxygen has yet not reached. Fourth, oxidized cytochrome a₃ molecules from which CO has already dissociated and with which oxygen has already reacted prior to the time of the photolysis flash.

The populations of molecules in these categories will vary with time. For example, the fourth category is increased rapidly upon photolysis, those in the third category will recombine with CO if oxygen does not arrive in a short time. The molecules in category 2 are of considerable interest because these can acquire category 1 status as the oxygen diffusion gradient moves through the tissue which is in turn related to the effectiveness of tissue oxygenation. The category 1 molecules will respond rapidly to photolysis if both oxygen and high Ca²⁺ are present (unpublished data).

Categories 1 and 2 are identified respectively with a sufficiency and a deficiency of oxygen. This can be quantitated as follows: molecules are in category 2 if on photolysis they produce a cytochrome oxidation rate slower than the observed oxidation of Fp (tₛ = 60 msec). Since the second-order velocity constant for cytochrome a₃ with oxygen is 3 × 10⁶ M⁻¹ sec⁻¹ (11), a half-time of 60 msec would be obtained at <1 μm tissue oxygen; cytochrome a₃-CO molecules photolyzed in <1 μm oxygen are therefore in category 2.

The partitioning of molecules between category 1 and category 4 depends on the rate at which cytochrome a₃-CO molecules dissociate in the time interval between the entry of oxygen into the tissue and the flash photolysis. It is for this reason that the coronary perfusion and the oxygen diffusability in the tissue are important variables. In addition, the photolysis of the cytochrome a₃-CO compound by the measuring light now becomes of critical importance and appropriate control experiments are described below.
the presence of 20% CO (Fig. ZC), the cytochrome a3-CO (Fig. ZB), the xenon flash causes no absorbance change. In anoxia cytochrome a3-CO is afforded by Fig. 2, B and C. In anoxia absorption is indicated by the downward deflection of the trace to the original baseline. Evidence of flash photolysis of cytochrome a3-CO is converted to the oxidized form as indicated by the upward deflection while in B and C, it is in a downward deflection as indicated by appropriate arrows.

where the rate of this preflash photolysis is decreased by a decrease of the fluorescence excitation intensity.

**EXPERIMENTAL RESULTS**

Representative charts of the experimental results illustrate typical responses of the perfused heart. Usually a statistical analysis is unnecessary since each experiment contains a prior control or allows a recovery of the heart to its initial state, after which the experiment is repeated. Thus, the illustrative charts represent one of a series of repetitions of the particular test.

**Cytochrome a3-CO reaction in perfused heart.** In order to identify the a3-CO compound in the cardiac tissue and to afford a basis for the application to cardiac tissue of studies on the a3-CO compound in isolated mitochondria, we illustrate in Fig. 2 dual-wavelength absorbance measurements at 445-455 nm made through the wall of the heart into a light pipe and then to the measuring photomultiplier. The heart is initially under normoxic conditions and is perfused with nitrogen-saturated medium. The consequent anoxia causes an upward deflection of the trace corresponding to increased absorbance at 445 nm. Following complete reduction of cytochrome a3, perfusion with 0.6% CO is begun and at this low CO concentration approximately 6 min are required to reach the saturation value of cytochrome a3-CO. The absorbance change with CO is about half of that in the presence of CO (B) and in the presence of CO (C). Absorbance changes are measured at 445 nm with respect to 455 nm. Time proceeds from left to right in this and in succeeding diagrams. Other details are described in text.

**METHODS.** The cardiac tissue is perfused with 95% oxygen and after the dead volume is cleared out a significant oxygen concentration has accumulated in the tissue, increased fluorescence of oxidized Fp and decreased fluorescence of reduced PN are observed.

The experiment is repeated in Fig. 3B with CO replacing 67% of the nitrogen; both traces exhibit greater amplitude. After perfusion with the oxygen saturated medium, rapid oxidation of reduced PN and Fp occurs as in the previous experiment. The half-times (1.5 sec) for the normoxic-anaoxic transitions are approximately the same in Fig. 3, A and B. The amplitudes and hence the initial slopes are, however, different. In nitrogen anoxia there is partial oxygenation of the surface of the heart and a diminished amplitude of oxygen concentration has accumulated in the tissue, increased fluorescence of oxidized Fp and decreased fluorescence of reduced PN are observed.

The experiment is repeated in Fig. 3B with CO replacing 67% of the nitrogen; both traces exhibit greater amplitude. After perfusion with the oxygen saturated medium, rapid oxidation of reduced PN and Fp occurs as in the previous experiment.
extent of flash photolysis may be evaluated in two ways: as shown by experiments on isolated mitochondria. The diffusion described below. However, the rate of PN and Fp oxidation does not depend upon the degree of photolysis intensity is needed to photolyze and thus to assay the molecules in category 1, particularly for studies of oxygen diffusion gradient reached the mitochondria very rapidly.

**Fig. 5.** A graph of effect of number of filters (abscissa) on photolysis response of PN and Fp. See Figs. 3 and 4 for details.

The intensity profile for this phenomenon is indicated in Fig. 5 where four filter densities are employed. It is apparent that fluorescence excitation obtained through three to four filters is sufficiently small so that a maximal response of PN and Fp is obtained, corresponding respectively to 62 and 50% of the total normoxic-anoxic change. At present the difference between the category I cytochrome a2-CO molecules assayed by Fp and PN responses is not regarded to be significant. The fact that these responses do not rise to 100% as the fluorescence excitation intensity decreases is due to the dissociation of cytochrome a2-CO (k0 of equation 1 in ref. 6) which is thermally activated and occurs without illumination. Thus, 100% response could only occur if the oxygen diffusion gradient reached the mitochondria very rapidly.

**Effect of photolysis flash intensity.** An adequate flash photolysis intensity is needed to photolyze and thus to assay the molecules in category I, particularly for studies of oxygen diffusion described below. However, the rate of PN and Fp oxidation does not depend upon the degree of photolysis as shown by experiments on isolated mitochondria. The extent of flash photolysis may be evaluated in two ways: 1) by a stepwise variation of the intensity of a single flash lamp, and 2) by a sequence of two flashes. The first method is simple to employ but does not identify 100% photolysis. A pair of flashes spaced at a small time interval identifies 100% photolysis when the second flash causes no response.

In Fig. 6, two 28-J flash lamps are employed. Each is equipped with blue filters giving maximal transmission near 430 nm. The increment of photolysis afforded by the second flash following the first (Fig. 6B) by 0.6 sec is small (<5%). When the second flash follows the first by 1.3 sec a measurable increment of photolysis is observed on the PN trace (~30%). Thus, photolysis is practically complete with the first flash of the 28-J lamp. In many cases, a single laser flash verified these results. The increment of photolysis observed with the second lamp is due to a further progress of the oxygen diffusion outward from the capillaries.

**Identification of components involved.** In previous studies with the perfused heart, fluorescence signals have been identified with the mitochondrial Fp and with mitochondrial and cytosolic PN. Based on studies of perfused liver, the equilibration of mitochondria and cytosolic (10, 12) PN requires a few seconds (61) due to the H shuttle between the two spaces. The shuttles which equilibrate the mitochondrial and cytosolic spaces do not operate rapidly enough to afford a cytosolic PN signal that correlated in time with Fp signal, and the kinetics of oxidation of the two components are indistinguishable as shown in Fig. 3 (cf. 61).

**Effect of timing of flash.** As an essential control, laser flashes in the normoxic and anoxic states give no photolysis jump. The jump response does vary with the time after admitting oxygen to the heart as shown in Fig. 7, essentially varying the number of category I molecules. Figure 7A is a control without flashes. In Fig. 7, B and C, the time increment between the flashes is 1 sec. The response to the early flash (Fig. 7B) (0.5 sec after the start of oxidation) is typical of the previous charts; its extent is bounded by the slope discontinuities. However, in Fig. 7C, the delay is 1.5 sec and the prephotolysis oxidation reaction has proceeded about 50% to the normoxic steady state. A flash at this time completes the oxidation of Fp and essentially completes the oxidation of PN. Thus, about 1.5 sec after the start of oxidation no cytochrome oxidase molecules remain in categories 2 and 3, all are in 1 or 4 and negligible further oxidation occurs after the flash photolysis has substantially depleted the category I molecules.

**Rate of photolysis response.** Most of the recordings here are indicated on a sufficiently compressed time scale that the kinetics of the Fp and PN response are not detailed. Where the xenon flash is employed, the amplitude of the jump is...
measured as a slope discontinuity in the traces. In other cases, for example, in Fig. 7, where the liquid dye laser is employed, negligible artifact appears on that trace. This is generally the case with the Fp trace; for example, see Fig. 7C. A series of measurements have been made of the time between the laser flash and the attainment of 90% of the fast response. The end point is identified by the slope discontinuity between the fast and the slow portions of the trace as, for example, in Fig. 7B. Since the response is exponential in nature, the time to reach 90% is measured rather than the time to reach 100%. Ten experiments show values of 100 ± 20 msec for Fp and PN. Recordings were taken on a variety of time scales including 200 msec per division.

Response to physiological parameters. The foregoing has outlined the kinetic aspects of the mitochondrial response under a variety of conditions. Selected biochemical and physiological parameters are varied in the following experiments.

Since the fractional occupancy of mitochondria by CO and oxygen just prior to the photolysis activation is a measure of the effectiveness of oxygen diffusion from the capillaries, the extent of response in the nitrite-perfused organ has been compared with that of the nitrite-free organ in Fig. 6. In Fig. 8A, nitrite is absent and ferromyoglobin would be expected to be assisting in the tissue oxygenation (60, 71). In Fig. 8B, nitrite is present, and not only would the myoglobin be oxidized in the met-form but also vasodilation of the heart would be expected (36). The jump of PN and Fp on photolysis in the presence of nitrite is larger than in the absence of nitrite (increased by 50% for Fp and 100% for PN in Fig. 8B as compared to Fig. 8A). A second flash follows the first flash in both these cases to ensure that photolysis is substantially complete. Apparently the vasodilation is significant but no contribution of heart myoglobin to tissue oxygenation is identified under these conditions.

The relationship between external calcium and the respiratory activity of the perfused heart suggests that higher calcium would cause an increased mitochondrial activity (64). An example of a fourfold increase of calcium (0.9–3.6 mM) is indicated in Fig. 9. On the left is a control experiment which shows a small photolysis jump due to the category I cytochrome a2 molecules (0.7 division for Fp and 0.9 for PN). Following perfusion with the higher level of calcium (Fig. 9B), the Fp and PN responses increased, the former by over twofold. Thus, higher Ca2+ in the perfusate enhances the photolysis jump, which we interpret to indicate a higher proportion of category I a2-CO molecules which, as explained above, depends on the presence of both O2 and Ca2+ at the mitochondria.

Previous work (50–53) shows that lanthanum is a specific inhibitor of mitochondrial calcium uptake (6). Its effect on the sarcoplasmic reticulum is significantly different (27, 31). Direct applications of lanthanides to the study of excitation-contraction processes in cardiac tissue have been carried out (59) and more recently in perfused hearts (R. A. Floyd, in preparation). It should be noted that phosphate was omitted over the whole interval of the heart perfusion in order to avoid a precipitation of lanthanum phosphate.

This chart is of great value since it indicates that the photolysis jump can be substantially eliminated in the La3+-perfused heart and thereby adds strong evidence that the photolysis jump is not an artifact but is related to the energy metabolism of the heart.

As the normoxic heart is perfused with 10 μM La3+ (Fig. 10A), an increasing reduction of PN and Fp is observed over an interval of 30 sec, corresponding to a decrease of energy demand on the mitochondria. If now the heart is perfused with CO, and then with O2, the xenon flash at the appropriate time during the reoxidation of Fp and PN gives no photolysis jump. Thus, the La3+ interferes with the Ca2+ transport in the muscle and the fast response characteristic of category I is not observed.
DISCUSSION

**Principle of method.** A fundamental limitation to the measurement of enzyme kinetics in solid tissue is afforded by the structure of the particular organ. Thus, the basic approach to enzyme kinetics is, so far, based on destruction of the cell structure, and isolation and purification of the enzyme. While these studies have been of inestimable value for the identification of enzyme intermediates (5) and their reaction mechanisms, a better understanding of metabolic control phenomena (7) has led to the realization that enzymes cannot be regarded simply as isolated catalytic units in the tissue but have the capability to interact with one another in linear and nonlinear fashions (19, 54). An appreciation of the high concentrations of enzymes in situ also questions the relevance of the study of highly diluted isolated systems (35, 62). Any feasible approach to the problem of enzyme kinetics in solid tissues is of greatest interest from the standpoint of physical chemistry, metabolic regulation, and, as we shall see, the physiological aspects as well. The parameters which rapidly perturb the steady-state levels of biochemical components of the living cell are temperature, pressure, and volume. In the case of single enzymes, perturbations of these fundamental variables have yielded remarkable results (30). But the perturbations are not “enzyme” specific and it is usually not possible to predict or to interpret in a multienzyme sequence the consequences of such perturbations. An exception was afforded by Kreezer and Kreezer (46) where the luminous intensity of a light-emitting reaction was read out from the bacterial cell. In another case, the temperature of a small region on the surface of the perfused heart was rapidly raised by the absorption of the light from a 25-J ruby laser (694 nm) (21). In this case, the NADH change as read out by surface fluorometry shows a metabolic response reaching a peak at 200 msec, largely attributed to a thermal activation of enzymes, mostly of glycolysis. That interpretation was supported by subsequent temperature jump studies on cell suspensions (47).

Photoactivation has offered unique opportunities for specific activation of particular enzyme systems involved in electron transport in green leaves and in purple bacteria (9, 29, 33, 70). The technique is applicable to plant tissues; it represents a unique opportunity for observing rapid perturbations of electron transport systems in vivo. Recent experiments have employed chlorophyll absorption, not only for the flash activation of the electron flow but for a photodestruction of the membrane system as well, as a “catastrophic” perturbation (33).

As easy as these perturbations of the living plant tissue may be, because of the chlorophyll pigment similar specific perturbations of tissue enzymes are possible only where specific “photolabels” are available. The system on which most of the photolysis studies of enzyme systems have been based is the iron-carbonyl bond recognized to be photolyzable at the respiratory enzyme by Warburg (67) in 1929 and based is the iron-carbonyl bond recognized to be photolyzable at the respiratory enzyme by Warburg (67) in 1929 and identified with Keilin and Hartree’s (41) cytochrome a3 (6). The CO compound of cytochrome a-CO is photolyzable at sufficiently high quantum yields to be useful experimentally with xenon flash techniques (42). Recently, the use of liquid-dye and solid-state lasers has made possible photolysis of cytochrome a3-CO in suspensions of mitochondria. The photolysis of this compound enables the abrupt activation of electron transport in the respiratory chain and can be applied to solid tissue that has been perfused with oxygen. The ideal perturbation, namely, that by oxygen directly, i.e., rapidly to generate oxygen in the tissues may be possible since we have demonstrated the flash photolysis of the Fe2+ compound of catalase in the presence of H2O2 in accordance with the steady-state photolysis studies (63) and unpublished observations). The photolysis of the cytochrome a3-CO compound is employed here. In vitro, the rate of dissociation of carbon monoxide from cytochrome oxidase is very slow in the dark (~0.02 sec⁻¹) (6) but background illumination may increase the rate several fold decreasing the half-time to that approximately required for the oxygen to come into equilibrium with the tissue space. Thus, one of the essential design parameters is the rate of photolysis of the a3 CO compound by the measuring light in relation to the rate of diffusion of oxygen from the capillary bed to the mitochondrial cytochrome a3-CO.

Photoysis of cytochrome a3-CO in the presence of O2 provides a pulsed activation of electron transport that rapidly oxidizes the components of the respiratory chain ranging from the very rapidly responding components, a3, a, c, c1, to the more slowly responding components, cytochrome b, the flavoproteins, quinones, and pyridine nucleotides. Depending on the information desired, different components of the chain afford a suitable readout. The fast reactions of electron transfer of cytochromes a, c, and c1 are slightly affected by the presence of extramitochondrial calcium and the slower kinetics of cytochrome b, flavoprotein, quinone and pyridine nucleotides are more sensitive (unpublished observations). The readout of flavoprotein and NADH can be made by fluorometry which is directly applicable to tissue surfaces, as opposed to transmission measurements which are required for cytochrome b and quinone and for which both sides of a tissue of appropriate thickness must be available. In the case of the perfused heart, the total thickness is too great for satisfactory absorbancy measurements of the cytochromes and thus we have punctured the heart wall with the light pipe to obtain the single thickness of ~3 mm for absorbancy measurements. Since this puncture is not necessary for surface fluorometry, a better biochemical and physiological condition is possible.

**Experimental results.** The principal result from these experiments is related to mitochondrial function in cardiac biocnertics. The result is read out from the Fp and PN kinetics in response to carbon monoxide-oxygen transitions followed by the flash photolysis, in the presence or absence of lanthanum.

While ADP and phosphate increase the rate of PN and Fp responses by fourfold, calcium at concentrations of about 100 μM causes a further fivefold increase to half-times of ~100 msec (unpublished observations). Thus, Fp and PN afford a dual readout: the rate is related to the “energy environment” and distinguishes ADP + P i from calcium as the energy donor to the mitochondria. The result of these flash-photolysis studies is that the response of Fp and PN is in the 100-msec range and that Ca2+ is a significant contributor to the energy environment of cardiac mitochondria in the beating perfused heart.
Prior to drawing general conclusions it is appropriate to evaluate various interpretations of the experimental data. The simplest is that mitochondria in situ respond more rapidly to energy demands than do those in vivo. This hypothesis is contrary to several experimental data. First, oxygen pulse or photolysis experiments on intact cells and organelles may fail to show the characteristic rapid response of Fp and PN of the beating cardiac tissue. Ascites cells, liver cells, and kidney tubules, which remain adequately suspended to permit their use in flow or flow-flash apparatuses, exhibit half-times for PN oxidation of about 1 sec (unpublished observations). This result suggests that the mitochondria in such cells are responding to ADP + Pi and low levels of Ca\(^{2+}\) (\(\sim 10^{-3} \text{M}\)).

The second possibility is that the cardiac tissue mitochondria are uncoupled and that PN and Fp respond rapidly for this reason. Here again objections can be raised, this time from the physiological standpoint. Anoxia greatly reduces the ventricular pressure indicating that the functionality of the mitochondria is responsible for a significant portion of the work on the heart.

The third possibility is that cardiac mitochondria acquire kinetic properties in vivo that they do not exhibit in vitro, namely, a characteristic rapid response of Fp and PN on flash photolysis of the beating heart regardless of the ADP + Pi or Ca\(^{2+}\) levels. This possibility has been substantially eliminated by the lanthanum experiments which show that the mitochondria respond slowly to nitrogen oxygen transition as is the case with the ascites tumor cells and kidney tubules mentioned above.

Thus, the fourth possibility is that the isolated and in situ mitochondria show comparable responses and that the cardiac mitochondria have an environment of high calcium or high calcium and ADP + Pi in the beating tissue. Essentially, the active metabolic state 3 of cardiac tissue mitochondria is due to both ADP + Pi and Ca\(^{2+}\).

The last alternative could be a consequence of the biochemical condition at the time of flash photolysis since glucose is present in the perfusate. Indeed the glycolytically produced ATP seems adequate for the cardiac contraction and activates the calcium pump of the endoplasmic reticulum and possibly that of the mitochondria as well. Thus, anoxia limits the total energy resource available to the beating heart rather than eliminating a particular pathway of ion transport. Yet the mitochondria in the transition from anoxia to normoxia appear to be in a high Ca\(^{2+}\) and ADP + Pi environment characteristic of the beating heart.

Lanthanum is chiefly useful to study the Fp and PN responses to an active → resting state transition (state 3 to 4 (21)) and to flash photolysis in a heart in which the energy load due to Ca\(^{2+}\) transport is negligible. While direct inhibition of Ca\(^{2+}\) uptake by mitochondria in the sarcoplasm by La\(^{3+}\) is possible, based on its great effectiveness in vitro (48, 52, 55), the possibility of high phosphate levels in the sarcoplasm may restrict its initial effects to Ca\(^{2+}\) transport through the plasma membrane causing a lack of Ca\(^{2+}\) in the sarcoplasm. However, the ruthenium red (55, 56) which forms soluble phosphate compounds gives similar results (unpublished observation).

**Mitochondrial calcium accumulation in cardiac muscle.** The purpose of this paper is twofold: first, to demonstrate that mitochondrial kinetics can be recorded in solid tissues, and second, to make some preliminary interpretations of the mitochondrial kinetics based on a comparison of their properties in vivo and in vitro. Observations of a fast Fp and PN response of the mitochondrion in situ in the rat heart suggest a high Ca\(^{2+}\) concentration external to the mitochondria. This result seems to be inconsistent with the current views for the calcium cycle in muscle (8, 58). Nevertheless, it is appropriate to comment on the suitability of the mitochondrial calcium pump as a factor in cardiac contractility (2, 4, 8) and to emphasize that more recent studies show their capabilities in reducing the extramitochondrial calcium concentration to extremely low values (52). The use of mitochondria as intracellular calcium indicators is obviously a topic that extends well beyond the scope of this paper and a comparative study of a wide range of perfused organs over the gamut of their physiological conditions is feasible with this approach and may lead to an appropriate explanation of the results.

**Tissue oxygen diffusion.** The progress of an oxygen diffusion gradient from the capillary to the mitochondria is at present qualitatively indicated by these results. If the gradient were to move very rapidly compared to the photolysis of cytochrome a\(_3\)CO by the measuring light and by the spontaneous dark reaction, all the mitochondria would be in category 1 and the extent of photolysis would correspond to 100% in Fig. 5 for both Fp and PN instead of to the observed 62 and 50%, respectively. Our data indicate that the effect of the measuring light can substantially be eliminated by decreasing its intensity progressively (see Fig. 5). There remains, however, a spontaneous dissociation (\(k_0\) of equation 1 of ref. 6). Thus, in the simplest case of interpretation, the photolysis jump is essentially a count of the number of mitochondria in category 1 at the time of the flash, a value established by an oxygen diffusion gradient in the tissue that is just able to balance the spontaneous dissociation of CO from cytochrome a\(_3\) (\(k \sim 0.02 \text{sec}^{-1}\) at 25°C).

At this point it seems appropriate only to point to a new method of determining oxygen diffusion from the capillary bed to the mitochondria and to suggest that a suitable criterion is the percentage of the total Fp or PN oxidation which can be observed a) under conditions where the photolysis flash is of sufficient intensity to afford complete breakdown of the \textit{category 1} molecules, b) where the measuring light is so diminished that the photolysis jump is maximal, and c) assuming that the O\(_2\) concentration gradient reaches the capillaries serving the tissue under observation at the same time. The calculation of the actual diffusion rates depends on computer simulation of the geometries, reaction kinetics, and stoichiometries involved. At present, however, it seems highly appropriate to collect data and to compare values of percentage photolysis from various perfused organs with normal and abnormal circulatory and tissue diffusion patterns.

**Limitations of method.** The method as described here gives acceptable signal-to-noise ratios for readout of fast Fp and PN signals from perfused cardiac tissue. The coronary
perfusion is sufficiently effective that the oxygen diffusion wave exceeds the rate of breakdown of cytochrome a$_2$-CO induced by the measuring light. In other tissues conditions may be less favorable. In the perfused liver preliminary experiments indicate that the maximum photoysis jump may be no greater than 10% identifying the less effective transfer of oxygen from the blood vessels to the tissue in that organ. Studies on the brain in situ are feasible and preliminary experiments indicate photoysis jumps of about 25% (unpublished observations). Interference from hemoglobin may not be formidible in intact tissues. When carbon monoxide-saturated erythrocytes are replaced by oxygen-saturated erythrocytes the mixing of the two species in capillaries and the photoysis of HbCO in the presence of O$_2$ will occur much more rapidly than O$_2$ diffusion through the tissues. Thus, while all physical methods have limitations in their applications to physiological systems, we can visualize the kinetic method for studying mitochondrial responses in tissues as having a somewhat wider range of applicability than the steady state approaches.

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REFERENCES

30. Gibson, Q. H., and C. Greenwold. Reactions of cytochrome oxi-


