

[54] **METHOD AND APPARATUS FOR OBSERVING RATES OF REACTION OF OXYGEN IN LIVING TISSUES**

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[58] Field of Search **128/2 A, 2 R, 2.1 R, 2.1 Z; 424/9; 356/39**

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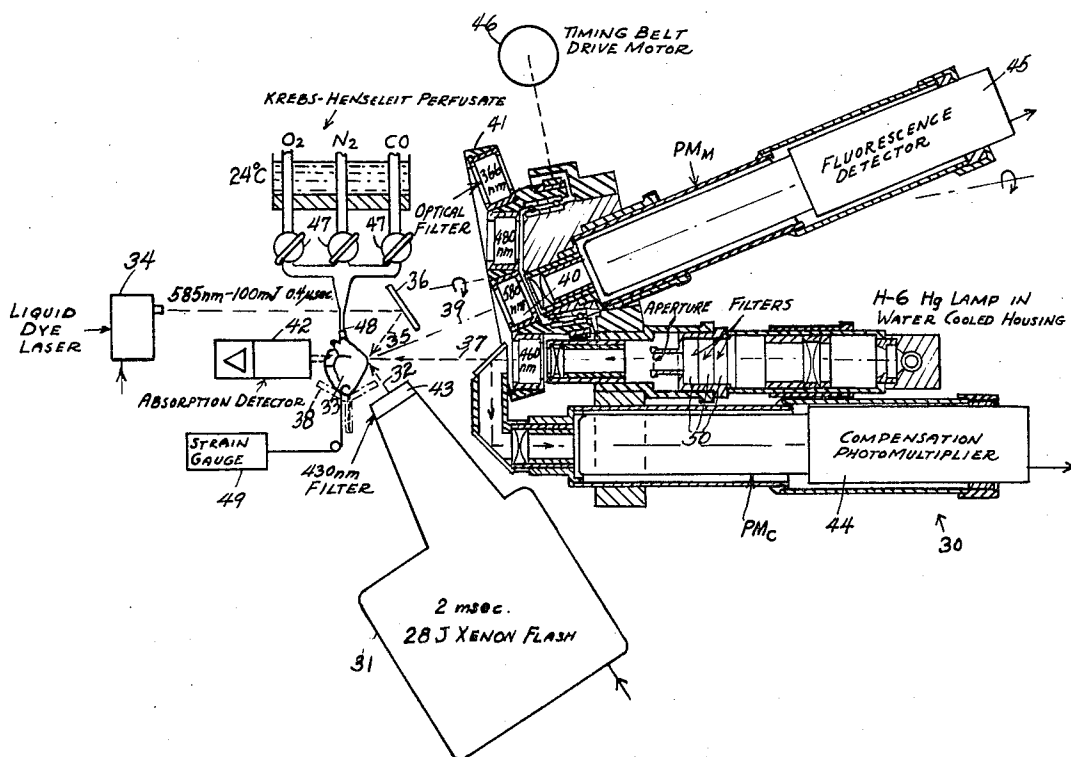
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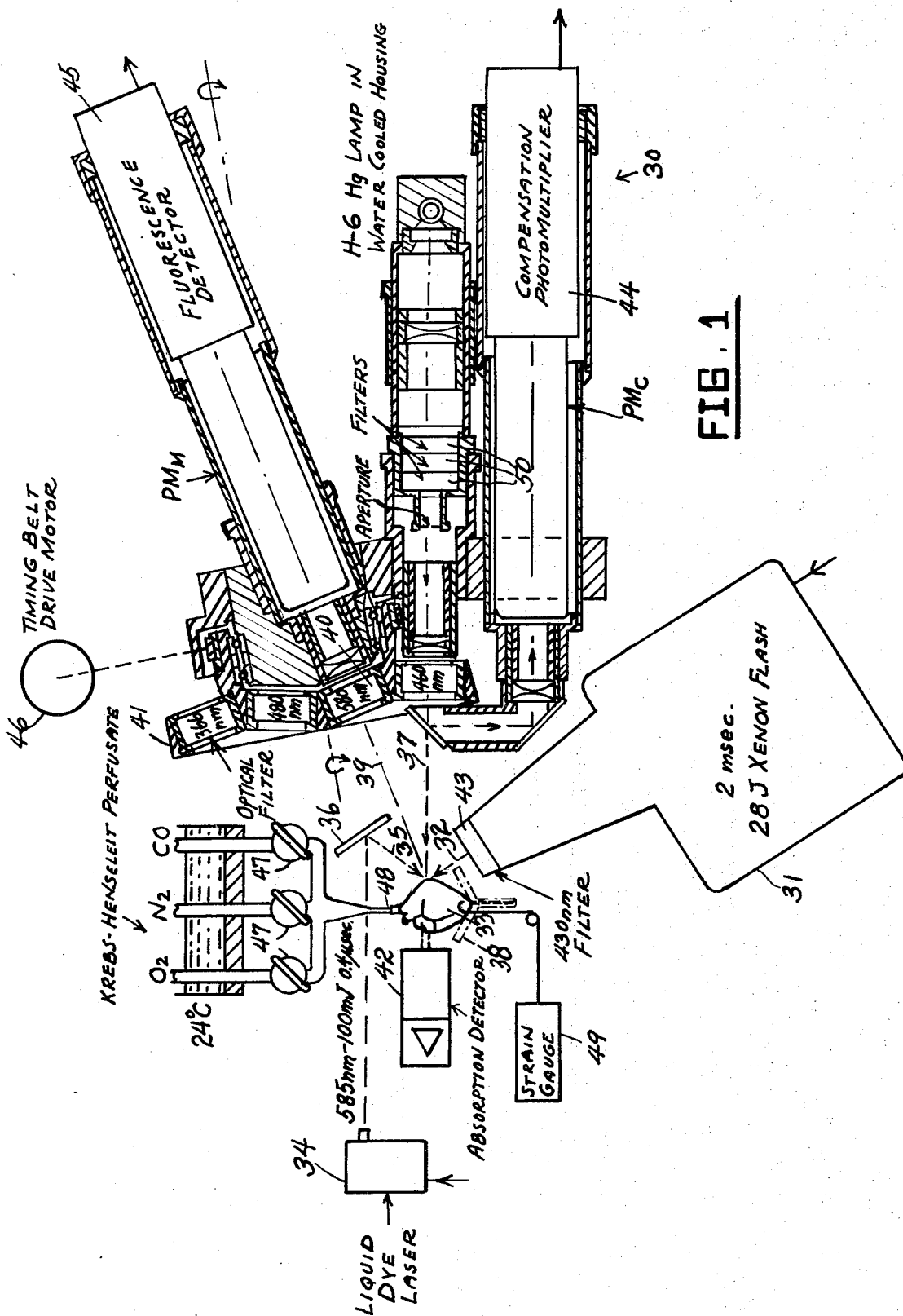
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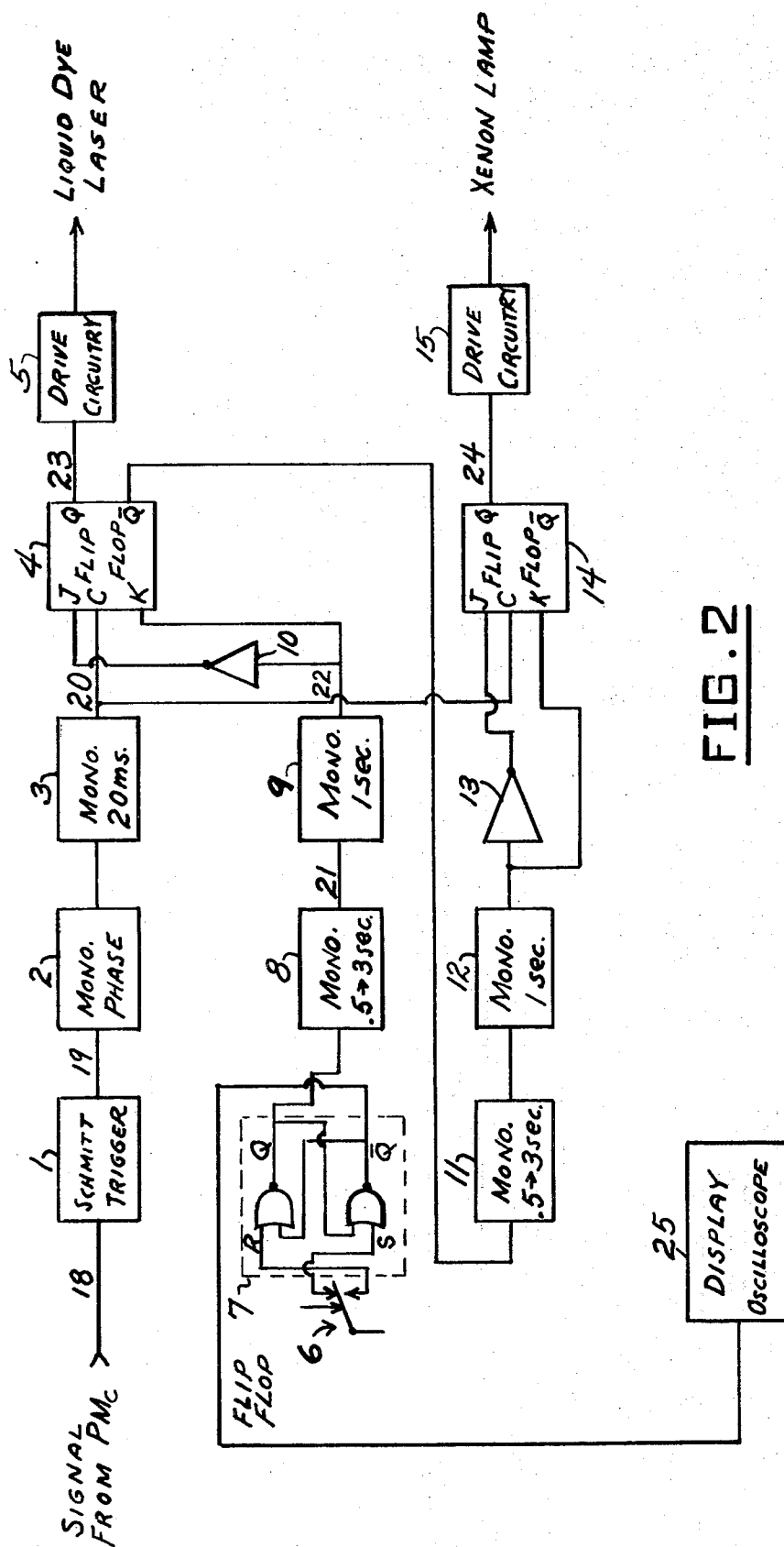
[57] **ABSTRACT**

A system for providing flash photolysis activation of CO-inhibited cytochrome oxidase in living tissue in the presence of oxygen. In a typical procedure employing cardiac tissue this initiates oxidation of reduced pyridine nucleotide (PN) and flavoprotein (Fp), with a high rate of response. The fractional extent of the photolysis response of PN and Fp indicates the fraction of the total mitochondrial population containing cytochrome a_3 CO to which oxygen has diffused at the time of the photolysis flash, thereby providing an indication of the effectiveness of oxygen diffusion in the tissue without destruction of the tissue. A double flash is used to evaluate the extent of photolysis, one flash occurring a few seconds after perfusing the tissue with oxygen, followed by another flash a few seconds later. The readout is obtained on a storage oscilloscope, using a double beam, time-shared photometer assembly with a compensating photomultiplier. The flash lamps are triggered by a pulse from the compensating photomultiplier, with a delay to fire the flash lamps at an appropriate phase angle.

14 Claims, 6 Drawing Figures







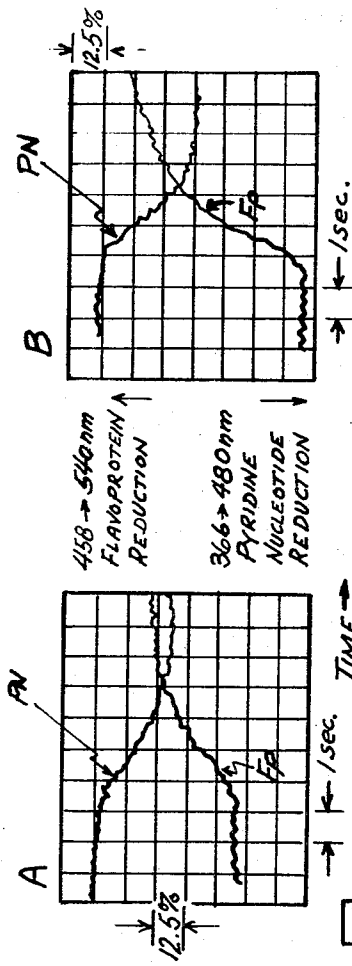
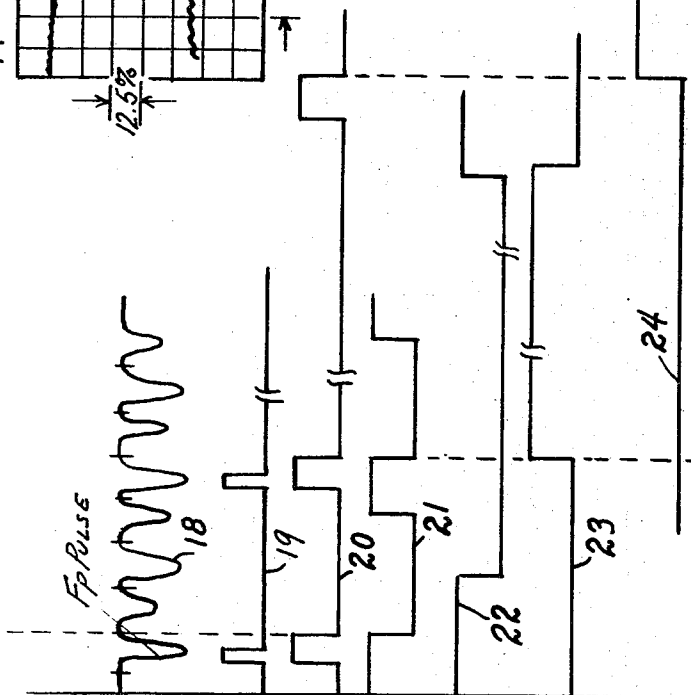
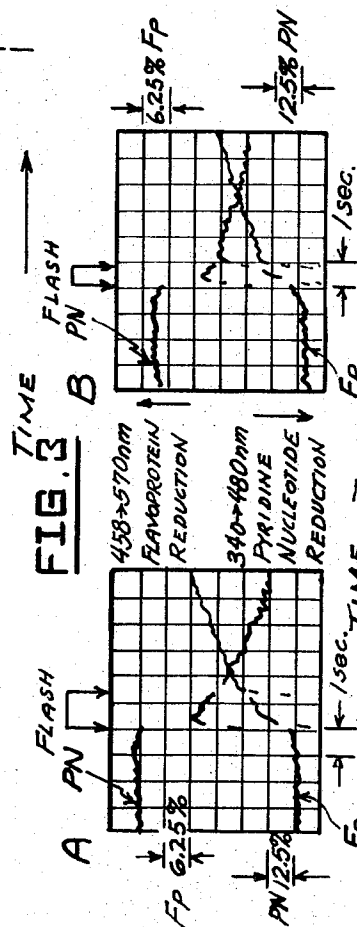
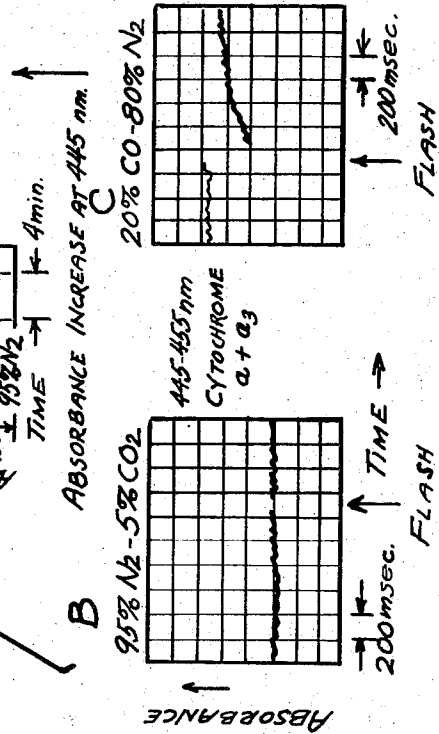
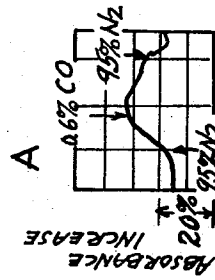


FIG. 5



SIGNAL
COMPENSATION
P.M.C.

SCHMITT
TRIGGER 1

MONO. 3

MONO. 8

MONO. 9

FLIP FLOP 4

FLIP FLOP 14

METHOD AND APPARATUS FOR OBSERVING RATES OF REACTION OF OXYGEN IN LIVING TISSUES

This invention relates to methods and means to study mitochondrial electron transport, and more particularly to systems for non-destructively determining the effectiveness of oxygen diffusion in tissue.

A main object of the invention is to provide a novel and improved system for obtaining fast activation of mitochondrial electron transport in intact tissue perfused with oxygen, whereby to identify the nature and magnitude of the metabolic energy load of the mitochondria.

A further object of the invention is to provide an improved system for studying the nature and magnitude of the metabolic energy load of mitochondria, employing flash photolysis activation of CO-inhibited cytochrome oxidase in the presence of oxygen so as to initiate the oxidation of reduced pyridine nucleotide (PN) and flavoprotein (Fp), with a high rate of response, the system including means to provide an indication of the fraction of the total mitochondrial population containing cytochrome a_3 CO to which oxygen has diffused at the time of the photolysis flash.

A still further object of the invention is to provide an improved system as above described wherein a double flash is employed to evaluate the extent of photolysis, one flash occurring a short time after perfusing the tissue under study with oxygen and the second flash occurring shortly thereafter.

A still further object of the invention is to provide a novel and improved apparatus for the flash photolysis of intact tissue perfused with oxygen to obtain information as to the extent to which oxygen has diffused to the mitochondrial population.

Further objects and advantages of the invention will become apparent from the following description and claims, and from the accompanying drawings, wherein:

FIG. 1 is a diagram showing a system in accordance with the present invention for measuring the effectiveness of oxygen diffusion in a perfused heart.

FIG. 2 is a block diagram of the double flash circuitry employed with the system of FIG. 1.

FIG. 3 is a diagram showing typical wave forms present at various points of the circuit shown in FIG. 2.

FIG. 4 comprises graphs showing transitions from anoxia to normoxia as indicated by flavoprotein (Fp) and pyridine nucleotide (PN), graph A representing the condition wherein carbon monoxide is absent and graph B representing the condition wherein carbon monoxide is present.

FIG. 5 comprises graphs respectively illustrating the reaction of cytochrome a_3 with carbon monoxide in the anaerobic heart (graph A), and the effects of flash photolysis upon cytochrome a_3 of the heart mitochondria in the substantial absence of CO (graph B) and in the presence of substantial CO (graph C).

FIG. 6 comprises graphs providing an evaluation of the extent of photolysis by the double flash technique, graph A representing the results obtained when the interval between flashes is about 1.3 second and graph B representing the results when the interval between flashes is about 0.6 second.

The increasing fund of knowledge regarding the properties of respiratory pigments of isolated mito-

chondria 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; (d) the nature of the specific activator of mitochondrial function in situ, ADP + Pi and/or Ca^{2+} .

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 upon the mitochondrial energy conservation vastly alters the steady state and kinetic properties of the electron carriers. (See B. Chance and G. R. Williams, "The Respiratory Chain and Oxidative Phosphorylation" in "Advances in Enzymology," F. F. Nord, ed., Interscience Publishers, New York, 1965, vol. XVII: pp. 65-134). 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. (See B. Chance and F. F. Jobsis, "Changes in Fluorescence in a Frog Sartorius Muscle Following a Twitch," "Nature," 184: 195-196, 1959; B. Chance and A. M. Weber, "A Steady State of Cytochrome b During the Rest and After Contraction in Frog Sartorius," J. Physiol., 169:263-277, 1963; C. M. Connelly and B. Chance, "Kinetics of Reduced Pyridine Nucleotides in Stimulated Frog Muscle and Nerve," Fed. Proc. 13:29, 1954). Such transitions are observed in the steady state level of reduced PN by spectrophotometry (see C. M. Connelly and B. Chance, above cited) and fluorometry (see B. Chance and F. F. Jobsis, above cited) and of cytochrome b by spectrophotometry (see B. Chance and A. M. Weber, "Steady State Changes of the Cytochromes Following Isometric Twitches," Fed. Proc., 16:146, 1958; "Early Kinetics of the Cytochrome b Response to Muscular Contraction," Ann. N.Y. Acad. Sci., 81:505-509, 1959; F. F. Jobsis, "Spectrophotometric Studies on Intact Muscle, I. Components of the Respiratory Chain," J. Gen. Physiol., 46:905-928, 1963). More recent studies of isolated mitochondria (see B. Chance, D. F. Wilson, P. L. Dutton and M. Erecinska, "Energy-coupling Mechanisms in Mitochondria: Kinetics, Spectroscopic and Thermodynamic Properties of an Energy-transducing Form of Cytochrome b," Proc. Natl. Acad. Sci. 66:1175-1182, 1970) show that the energy state of the mitochondria can be deduced from the kinetics of respiratory enzymes in the anaerobic-aerobic transition of cytochrome b, c and a_3 caused by O_2 pulses. (See B. Chance and M. Erecinska, "Flow Flash Kinetics of the Cytochrome a_3 - oxygen Reaction in Coupled and Uncoupled Mitochondria Using the Liquid Dye Laser," Arch. Biochem. Biophys., 143:675-687, 1971). 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. 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 (examples are irreversible damage in stroke and shock, see A. G. B. Kovach, "The Function of the Central Nervous System After Hemorrhage," *J. Clin. Path.*, 23:Suppl. (Royal Coll. Path.) 4:202-215, 1970), it seems desirable to be able to apply the techniques developed for isolated mitochondria directly to the intact tissue.

In mitochondrial experiments conducted with the system of the present invention the metabolic transition employed was from anoxia to normoxia in the rapid flow apparatus; oxygen pulses were delivered to the anaerobic suspension of isolated mitochondria. The oxidation time for Fp and PN was as short as 100 msec. When oxygen pulses are delivered to perfused organs the time from anoxia to normoxia may be 20-30 sec. (see B. Chance, J. R. Williamson, D. Jamieson and B. Schoener, "Properties and Kinetics of Reduced Pyridine Nucleotide Fluorescence of the Isolated and In Vivo Rat Heart," *Biochem. Z.*, 341:357-377, 1965; J. R. Williamson and D. Jamieson, "Metabolic Effects of Epinephrine in the Perfused Rat Heart. I. Comparison of Intracellular Redox States," *Tissue pO₂ and Force of Contraction*, *Mol. Pharmacol.*, 2:191, 1966) and even with the intact organ circulated in vivo, recovery from ischemia in the liver requires 2 to 3 sec. (see B. Chance and B. Schoener, "A Correlation of Absorption and Fluorescence Changes in Ischemia of the Rat Liver In Vivo," *Biochem. Z.*, 341:340-345, 1965) due to diffusion limitation in the tissue.

Spontaneous or evoked contractility affords fast perturbation of the metabolic state of skeletal and cardiac muscle and causes a fast oxidation of PN due to the arrival of ADP (or Ca²⁺) at the mitochondria. (See B. Chance, G. Mauriello and M. Aubert, "ADP Arrival at Muscle Mitochondria Following a Twitch In Muscle as a Tissue," K. Rodahl and S. Horvath, eds., McGraw Hill, New York, 1962, pp. 128-145). This perturbation has the great advantage that diffusion time from the myofibrils to the mitochondria is short (<100 μ sec.) and in particular cases mitochondria responses 200 msec. after a single muscle twitch are observed.

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 desired component. An example of a temperature perturbation is afforded by a laser induced temperature jump (about 10°) applied to a toad sartorius muscle. (See B. Chance, B. Schoener and D. DeVault, "An Attempt to Apply the Temperature Jump Technique to Enzyme Reactions in Tissues," *Science*, 144: 561, 1964). 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 (see I-Y. Lee and B. Chance, "Method for Creating Rapid Cellular Temperature Perturbation," *Anal. Biochem.*, 29: 331-338, 1969) in

which xenon flash and joule heating were employed. The 5° temperature jump reached equilibrium in about 400 msec., and caused 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 msec. (See E. Kohen, C. Kohen and B. Thorell, "Rapid Microfluorimetry of Enzyme Reactions in Single Living Cells," *Biochim. Biophys. Acta*, 234:531-536, 1971).

While the temperature jump perturbations caused extensive transients in the glycolytic metabolism because the temperature coefficients of the steps are large and non-identical, 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 *a₃* in the presence of oxygen.

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 *a₃*-CO bond (see B. Chance, "The Carbon Monoxide Compounds of the Cytochrome Oxidase," *J. Biol. Chem.*, 202:407-416, 1953), and the use of a sufficiently rapid optical read-out system to follow the rapid oxidation of cytochrome *a₃* + *a* and cytochrome *c*. 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 *c*, *a* and *a₃* but also from the energy-dependent cytochrome *b_T* (see B. Chance, D. F. Wilson, P. L. Dutton and M. Erecinska, "Energy-coupling Mechanisms in Mitochondria: Kinetics, Spectroscopic and Thermodynamic Properties of an Energy-transducing Form of Cytochrome *b*," *Proc. Natl. Acad. Sci. U.S.A.*, 66:1175-1182, 1970; D. F. Wilson and P. L. Dutton, "Energy Dependent Changes in the Oxidation-reduction Potential of Cytochrome *b*," *Biophys. Res. Comm.*, 39:59-64, 1970) and the fluorescent electron transport components, oxidized Fp and reduced NAD. The rate of the response can be a sensitive indicator of the mitochondrial phosphate potential (see B. Chance and G. Hollunger, "The Interaction of Energy and Electron Transfer Reactions in Mitochondria. III. Substrate Requirements for Pyridine Nucleotide Reduction in Mitochondria," *J. Biol. Chem.*, 236:1555-1561, 1961) or energy charge (see D. E. Atkinson and G. M. Walton, "Adenosine Triphosphate Conservation in Metabolic Regulation," *J. Biol. Chem.*, 242:3239-3241, 1967).

With flash photolytic techniques in general, it is essential that the mixing time for the a_3 -CO compound with oxygen be shorter than the time for spontaneous dissociation of the a_3 -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 upon the effectiveness of perfusion and the speed of diffusion from the capillaries to the tissue mitochondria. From 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 a_3 at 20° (see B. Chance, "The Carbon Monoxide Compounds of the Cytochrome Oxidase," *J. Biol. Chem.*, 202:407-416, 1953) is fast enough so that some difficulties might be encountered. The system of the present invention provides for the resolution of these problems and provides a satisfactory experimental approach to flash photolysis of cytochrome a_3 -CO in the presence of oxygen in cardiac tissue, with the readout of the kinetics of electron transfer and tissue bioenergetics in terms of the fluorescent Fp and reduced PN components of the mitochondria.

In typical studies employing the system of the present invention the rat heart was selected because of its physiological and biochemical characteristics. The high volume ratio of mitochondria (0.34) together with the relatively small contribution of tubular membrane to the total sarcolemma also make it an experimental material suitable for evaluation of mitochondrial function in contraction-relaxation processes. Thus, the system is useful for further investigation of the role of mitochondria in muscular function and for the further study of the kinetics of enzyme reactions in tissues.

A typical illustration of the apparatus of the present invention 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, generally indicated at 30, have been described previously (see B. Chance, D. Mayer and L. Rossini, "A Time Sharing Instrument for Direct Readout of Oxidation-reduction States in Intracellular Compartments of Cardiac Tissue," *IEEE Transactions on Bio-Medical Engineering*, 2:118-121, 1970, and B. Chance, N. Graham and D. Mayer, "A Time Sharing Fluorometer for the Readout of Intracellular Oxidation-reduction States of NADH and Flavoprotein," *Rev. Sci. Instr.*, 42:951-957, 1971). Modifications required for flash photolysis in the perfused heart are indicated by the diagram. These modifications comprise the provision of a suitably mounted first flash device 31, for example, a Xenon flash assembly arranged to deliver a flash beam 32 to the surface of the heart 33 under study and the provision of a suitably mounted second flash device 34, for example, a liquid dye laser assembly, arranged to deliver a flash beam 35 via a suitably angled mirror 36 to the heart surface area under study. The heart can be perfused with either oxygen or nitrogen plus carbon monoxide by shifting between reservoirs equilibrated with the appropriate gas mixture. Fluorescence excitation is applied normally to the heart by an intermittent beam 37 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 may be supported upon a glass surface, for example, a suitably supported

small funnel 38 which collects the perfusate which helps thereby to damp the gross movement of the heart. Fluorescence emission is observed at a beam 39 at an angle of 30° through the secondary filter 40. Each 180° turn of the disk 41 holding the excitation and emission filters brings them into alignment for reduced PN (366 nm and 480 nm, respectively) and for oxidized Fp (460 nm and 580 nm, respectively).

The fluorometer observes a portion of the surface of the heart 33 between 2 and 4 mm in diameter and on the left or 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 "endpoint" 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 (about 3 mm.).

Absorption measurements may be obtained by employing an absorption detector 42 in the manner disclosed in B. Chance, D. Mayer and L. Rossini, "A Time Sharing Instrument for Direct Readout of Oxidation-reduction States in Intracellular Compartments of Cardiac Tissue," *IEEE Transactions on Bio-Medical Engineering*, 2:118-121, 1970.

Fluorescence change in response to a normoxic-anoxic cycle may be recorded from the left and right ventricles with satisfactory results, although somewhat larger signals are obtained from the left ventricle (about 50 percent), which technique has been employed in most experiments.

The two light sources 31 and 34 afford photolysis for the carbon monoxide-inhibited cytochrome system. The xenon flash lamp 31 (2 msec flash, 28 J white light) (Braun F 270) is filtered through a 430 ± 10 nm filter 43 so that interference with the PN readout at 480 nm is diminished. A reflector in the flash lamp plus a lucite light cone increases the efficiency of illumination. Two xenon flash lamps and cones may be employed.

Photolysis with the liquid dye laser 34 (General Laser GL-1,000) (585 nm, 100 mJ intensity) is via the small mirror 36. 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 diameter. Thus, the heart is uniformly illuminated by the laser beam 35.

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 H_2O by 1°C. Two calculations can be made. The first one assumes that all of the laser light is uniformly absorbed in the approximately 1 cc volume of the heart. The temperature rise under these conditions is 0.1/4 or 0.025°. A second calculation assumes that the laser light is all absorbed in a square 5 mm on a side and 3 mm thick, the thickness of the heart wall. The temperature rise would then be 0.3°. A third calculation assumes that only the mitochondria are heated within this volume of tissue since the mitochondria comprise about 1/3 of the total tissue volume, and the temperature rise would be 1°. The possibility that the laser damages the mitochondria as suggested by experiments at higher powers 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 percent per degree due to the decrease of quantum yield; no significant effect was seen. The kinetic responses do not depend upon which ventricle is illuminated and thus a specific effect upon the pacemaker cells is unlikely. This viewpoint is supported by studies with electrically driven hearts.

The xenon flash lamp with the 430 nm filter 43 causes about the same photolysis as the liquid dye laser; the extinction coefficient of cytochrome oxidase is approximately 10 times greater at 430 nm than at 585 nm.

A double flash is useful to evaluate the extent of photolysis. One flash occurs a few seconds after perfusing the tissue with oxygen and another flash may be applied several seconds later. The two flash lamps 31 and 34 are triggered by an appropriate synchronizing circuit such as shown in FIG. 2, presently to be described. Two lucite cones can be employed to direct the light from these lamps upon the same area of the heart.

In addition to the circuitry for the time sharing fluorometer, the photolysis lamps 31 and 34 are also synchronized with the rotating disk 41 so that their flashes are triggered during an interval when the photomultiplier 45 is not illuminated. The xenon flash occurs following the Fp measurement since the 580 nm emission filter 40 protects the photomultiplier 45 from the blue flash which decays substantially in the 8 msec interval between fluorescence measurements.

In the case of the liquid dye laser 34, the 0.4 μ sec flash is terminated in the 8 msec interval between measurements. The 585 nm light does not interfere with the 450 nm PN measurement but does interfere with Fp measurement at 580 nm. The laser is flashed just after the aperture for Fp measurement is closed and thus about 14 msec is available for recovery of this channel prior to the first post-flash Fp measurement. In this way the time sharing system not only alternates the filters for the fluorescence measurement but also acts as a phosphoroscope to guard the measuring photomultiplier 45 against overload during the flash photolysis of the cytochrome oxidase-CO compound.

If some flash artifact occurs, the recovery of the photomultiplier 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, shown at 44. Such a pulse triggers the delay circuit which provides a relay closure for firing the flash lamps at an appropriate phase angle. A Tektronix Storage Scope, Model 564 is provided with a suitable pre-trigger adjustable from 0.5 to 3 sec. with respect to the photolysis flash. In usual operation, the oscilloscope trace may be triggered at the time the oxygen saturated perfusate is turned on. The operation thereafter is automatic.

FIG. 2 is the block diagram of the typical double flash circuitry which may be employed, and FIG. 3 shows the key waveforms for the circuit.

The signal 18 from the compensation photomultiplier 44 is squared up by Schmitt trigger 1, resulting in waveform 19. Monostable 2 allows phase adjustment so that both flash lamps 31 and 34 can be flashed at a given point with respect to waveform 18. Monostable 3 generates a 20 ms gate shown as waveform 20 and is ap-

plied to the toggle inputs of flip-flops 4 and 14 as the clock or synchronous input. Pushbutton switch 6 initiates the flashing sequence. Flip-flop 7 provides a suitable trigger to monostable 8 and the storage oscilloscope 25. Thus, a sweep is started. Monostable 8 delays the flashing sequence from 0.5 to 3 sec. to permit storage of baseline on oscilloscope 25. After said delay monostable 9 is triggered, which results in waveform 22. The negative going edge of waveform 21 is phased to occur approximately 1 ms after the Fp pulse signal of waveform 18. The first negative transition of waveform 21 after waveform 22 has gone negative causes waveform 23 at the output of flip-flop 4, which is suitably shaped by drive circuit 5 to trigger xenon lamp 31 or liquid dye laser 34. Inverter 10 keeps the J and K inputs of flip-flop 4 in 180° phase opposition. Monostables 11 and 12 delay the flashing of liquid dye laser 34 from 0.5 to 3 sec. Inverter 13 and flip-flop 14 act to trigger liquid dye laser 34 coincidentally with the negative transition of waveform 20, resulting in waveform 24, which is shaped by drive circuit 15 to trigger lamp 34, thus completing the double flash cycle.

A 1800 RPM synchronous motor 46 is employed. The rotating disk 41 is mounted directly on the shaft of the motor.

The display oscilloscope 25 is a Tektronix, Model 564, using a four channel vertical amplifier and a time base used in the external trigger input mode.

In typical experiments, after suitable preparation rats were decapitated, the heart rapidly excised and securely attached to a "Y" glass cannula (15 gauge equivalent) at 48 via the aortic root. It is essential to avoid a prolonged anoxia between the interval of excision of the heart and the perfusion with oxygen. The estimated interval for most preparations is about 45 seconds. 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°. The flow rate was 12–17 ml/min. (For further details see R. B. Fisher and J. R. Williamson, "The Oxygen Uptake of the Perfused Rat Heart," *J. Physiol. (London)* 158:86–101, 1961). The perfusate was Krebs Ringer bicarbonate (see H. A. Krebs and K. Henseleit, "Untersuchungen Über Die Harnstoffbildung Im Tierkörper," *Hoppe-Schler's Z. für Physio-Chemie*, 210:33–37, 1932) containing half the usual calcium concentration.

0.1 mM NaNO_2 was added to the perfusate in order to oxidize the tissue myoglobin and any residual hemoglobin and thereby avoid absorbancy changes due to these oxygen carriers in the aerobic anoxic cycles.

In order to alternate aerobic and CO anaerobic perfusates, 95% O_2 , 5% CO_2 or 67% CO , 28% N_2 , 5% CO_2 was equilibrated with the perfusate in duplicate vessels each at the same height. FIG. 1 illustrates the use of stopcocks 47 for changing the perfusion media. The dead volume between the stopcocks and the heart was about 0.1 ml. Thus the new perfusate arrived 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 to maintain a strong heart beat.

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 49. 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.

The experiments described here are based upon studies of many rat hearts and the graphs shown in FIGS. 4, 5 and 6 are representative of the results obtained in the vast majority of these experiments.

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 were obtained as indicated in FIGS. 4 and 6. It appears 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.

In actual operation, after suitable calibration of the fluorometer, the heart is initially put in 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 to 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 re-establish its normoxic state and an interval of 5 min. elapses before the next episode of toxic anoxia.

The apparatus illustrated in FIG. 1 can be employed for spectroscopic measurements as well, as above mentioned. 445 and 455 nm interference filters are inserted into additional holes in disk 41. A photomultiplier in unit 42 views the light transmitted through a single thickness of the heart wall via a 1 mm diameter light pipe.

Optimal results for experiments such as above described require a careful evaluation of the parameters. Ideally 100 percent saturated cytochrome *a*₃-CO is completely photolyzed by the light flash to give 100 percent reduced *a*₃ which combines with excess O₂ to give 100 percent oxidized *a*₃. 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 pre-flash 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 (10⁻⁶M), the photolysis caused by the fluorescence excitation shifts the CO affinity to lower values due to an increase of *k*₆' in equation 6 mentioned in B. Chance, *Jour. Biol. Chem.*, 202: 407, 1952, and causes "pre-flash photolysis." The extent of pre-flash photolysis is diminished by high CO (*k*₅ · *i* is increased; see equation 5 of the last-named reference). 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 pre-flash photolysis of up to 25 percent of the total oxidation reduction change. Flash photolysis of cytochrome *a*₃ was over 95 percent complete with the laser source or the xenon flash source, taking in this case 100 percent to be that obtained with the double flash technique above described and to be fur-

ther discussed below. The oxygen concentration in the perfusate and the diffusion velocity of oxygen in the tissue should be high enough so that the oxygen gradient moves through most of the *a*₃-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 + Pi and Ca²⁺ levels in the cytosol.

The interaction of these factors above mentioned leads to four categories of cytochrome *a*₃ molecules at the time of photolysis. First, cytochrome *a*₃-CO molecules which are in the presence of a sufficiency of oxygen, that is, an oxygen concentration which upon photolysis will give a significant oxidation of Fp and PN. Second, cytochrome *a*₃ molecules that are in a deficiency of oxygen such that no significant response of Fp and PN is observed on photolysis. Thirdly, reduced cytochrome *a*₃ molecules from which CO has dissociated but which oxygen has not yet reached. Fourth, oxidized cytochrome *a*₃ 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, and 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 the mitochondria are in a region of high Ca²⁺ (about 10⁻⁴M).

Categories 1 and 2 are identified with a sufficiency and 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 (half-time, *t*_{1/2} = 60 msec.). Since the second order velocity constant for cytochrome *a*₃ with oxygen is 3 × 10⁷M⁻¹sec⁻¹ (see B. Chance and M. Erecinska, "Flow Flash Kinetics of the Cytochrome *a*₃ — oxygen Reaction in Coupled and Uncoupled Mitochondria Using the Liquid Dye Laser," *Arch. Biochem. Biophys.*, 143:675-687, 1971) a half-time of 60 msec. would be obtained at less than 1 μM tissue oxygen; cytochrome *a*₃-CO molecules photolyzed in less than 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 *a*₃-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 *a*₃-Co compound by the measuring light becomes of critical importance and the rate of this pre-flash photolysis is suitably decreased by a decrease of the fluorescence excitation intensity.

The fluorescence excitation intensity is decreased in the typical arrangement of FIG. 1 by the use of a number of suitable filters 50 in the beam from the excitation source. The filters may comprise blue Corning glass filters, such as Corning Model CS-5-60 (5543) which transmit 10 percent of the incident light at 366 nm and 45 percent at 460 nm, corresponding to absorbancy in-

crements of 0.10 and 0.26 per filter. Three or four filters may be employed. The signal-to-noise ratio is lower at the lower excitation intensity. Such a low signal-to-noise ratio is satisfactory in many experiments, but higher signal-to-noise ratio and more rapid speed of response can be obtained with fewer filters to attenuate the source light. In such cases the number of category 1 molecules is less but the speed of the Fp and PN oxidation is more clearly recorded.

It has been found that fluorescence excitation obtained through 3 to 4 filters is sufficiently small that a maximal response of PN and Fp is obtained, corresponding to 62 and 50 percent of the total normoxic-anoxic change. At present the difference between the category 1 cytochrome a_3 -CO molecules assayed by the Fp and PN responses is not regarded to be significant. The fact that these responses do not rise to 100 percent as the fluorescence excitation intensity decreases is due to the dissociation of cytochrome a_3 -CO which is thermally activated and occurs without illumination. Thus, 100 percent response could only occur if the oxygen diffusion gradient reached the mitochondria very rapidly.

Representative graphs shown in FIGS. 4, 5 and 6 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, following which the experiment is repeated. Thus, the illustrative graphs each represent one of a series of repetitions of the particular test.

In order to identify the a_3 -CO compound in the cardiac tissue and to afford a basis for the application to cardiac tissue studies on the a_3 -CO compound in isolated mitochondria, FIG. 5 illustrates dual wavelength absorbancy measurements at 445–455 nm made through the wall of the heart into a light pipe and then to the measuring photomultiplier of unit 42. In graph A 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 absorbancy at 445 nm. Following complete reduction of cytochrome a_3 , perfusion with 0.6 percent CO is begun and at this low CO concentration approximately 6 minutes are required to reach the saturation value of cytochrome a_3 -CO. The absorbancy change with CO is about half of the oxygen-nitrogen change identifying the formation of cytochrome a_3 -CO. On addition of oxygen, the a_3 -CO compound is converted to the oxidized form as indicated by the downward deflection of the trace to the original base line. Evidence of flash photolysis of cytochrome a_3 -CO is afforded by graphs B and C of FIG. 5. In anoxia (FIG. 5B) the xenon flash causes no absorbance change. In the presence of 20% CO (FIG. 5C) the cytochrome a_3 -CO compound rapidly photolyzes and gives an abrupt absorbancy decrease at 445 nm. After the flash, CO rapidly recombines with cytochrome a_3 . If however oxygen were present as in FIG. 6, oxidation of cytochrome a_3 and the mitochondrial components would follow. In these experiments residual hemoglobin and tissue myoglobin are converted from the ferrous to the ferric forms by nitrite perfusion and thus, interference with their CO and oxygen compounds is negligible.

The kinetics of recovery from anoxia are indicated in FIG. 4A for nitrogen anoxia and CO anoxia. The

oxidation-reduction changes are read out from the fluorescence of reduced PN and oxidized Fp. The cardiac tissue is rendered anoxic by perfusion with nitrogen saturated medium. At the beginning of the trace the heart is perfused with 95 percent oxygen and after the dead volume is cleared out and 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. 4B with CO replacing 67 percent of the nitrogen; both traces exhibit greater amplitude. After perfusion with oxygen saturated medium, rapid oxidation of reduced PN and Fp occurs as in the previous experiment. The half-time (1.5 sec.) for the normoxic-anoxic transitions are approximately the same in FIGS. 4A and 4B. The amplitude 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 the oxidized-reduced changes. In the presence of carbon monoxide, Fp and PN are more highly reduced. Nevertheless, the anoxic-normoxic transition proceeds rapidly; illumination of the heart by the fluorescence excitation causes photolysis of the cytochrome a_3 -CO compound, and consequently rapid oxidation of Fp and PN.

An adequate flash photolysis intensity is needed to photolyze and thus to assay the molecules in category 1, particularly for studies of oxygen diffusion. 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 step-wise 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 percent photolysis. A pair of flashes, such as by laser 34 followed by xenon unit 31, spaced at a small time interval identifies 100 percent photolysis when the second flash causes no response.

In FIG. 6, two 28 J flash lamps were employed. Each was 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 (less than 5 percent). When the second flash followed by the first by 1.3 sec. (FIG. 6A) a measurable increment of photolysis is observed on the PN trace (about 30 percent). Thus, photolysis is practically complete with the first flash of the first 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 outwards from the capillaries.

Experiments have shown different responses to early and late flashes. With an early flash, for example, 0.5 sec. after the start of oxidation, only a portion of the total amount of Fp and PN is oxidized. However, in an experiment wherein the delay was 1.5 sec. and the pre-photolysis oxidation reaction had proceeded about 50 percent to the normoxic steady state, the flash completed the oxidation of Fp and nearly completed 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 categories 1 or 4, and negligible further oxidation occurs after the flash photolysis has substantially depleted the category 1 molecules.

Recordings on varying time scales (1.0, 0.5 and 0.2 sec/div.) indicate the time for the photolysis response to reach 90 percent for PN and Fp was similar, and a value of 100 msec $\pm$ 20 msec was obtained in a sample of 10 experiments.

In order to further test for factors which influence tissue oxygenation at the cellular level, the effect of nitrite on the nature of the response was studied. When nitrite is absent ferromyoglobin would be expected to be assisting in the tissue oxygenation. When nitrite is present not only would the myoglobin be oxidized in the met-form but also vasodilation of the heart would be expected. The jump of PN and Fp on photolysis in the presence of nitrite was larger than in the absence of nitrite. A second flash followed the first flash in both these cases to ensure that photolysis was substantially complete. Apparently the vasodilation is significant but no significant contribution of heart myoglobin to tissue oxygenation was 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. An example of a 4-fold increase of calcium (0.9–3.6 mM) was studied. Following perfusion with the higher level of calcium not only was the Fp response approximately doubled, but the further oxidation of Fp and PN proceeded more rapidly. Calcium addition increased the rate of oxidation of PN and Fp and increased the size of the photolysis jump.

Usually the perfusate was supplemented with 10–20 mM dextrose in order to maintain glycolytic activity at a maximum rate, and thereby afford a minimal depletion of energy reserves in the anoxic interval preceeding the flash photolysis. Such supplements of substrate may be unnecessary for the initial anoxic cycles but are of considerable importance after half a dozen anoxic episodes. The striking effects of substrate supplementation are demonstrated in experiments where the beat rate of the heart in the anoxic interval is slow. Under these conditions, substrate supplement increases cytoplasmic ATP so that sufficient energy for mitochondrial calcium uptake is available and photolysis response is observed in the anoxic-normoxic transition.

It can be concluded that photolysis of cytochrome a_3 -CO in the presence of O_2 provides a pulsed activation of electron transport that rapidly oxidizes the components of the respiratory chain ranging from the very rapid responding components, a_3 , a , c , c_1 , 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 c_1 are slightly affected by the presence of extra-mitochondrial calcium and the slower kinetics of cytochrome b , flavoprotein, quinone and pyridine nucleotide are more sensitive. The readout of flavoprotein and NADH can be made by fluorometry which is directly applicable to tissue surface as opposed to transmission measurements which are required for cytochrome b and quinone, and for which both sides of a tissue 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 it is necessary to penetrate the heart wall with the light pipe to obtain the single thickness of about 3 mm for absorbancy measurements.

Since this puncture is not necessary for surface fluorometry, a better biological and physiological condition is possible.

The principal result from the experiments conducted is related to mitochondrial function in cardiac bioenergetics. The result is read out from the Fp and PN kinetics in response to carbon monoxide/oxygen transitions followed by the flash photolysis.

The above points to a new method of determining oxygen diffusion from the capillary bed to the mitochondria and suggests 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 category 1 molecules and (b) where the measuring light is so diminished that the photolysis jump is maximal. The calculation of the actual diffusion rates depends upon computer simulation of the geometries, reaction kinetics and stoichiometries involved.

While a specific embodiment of a system for providing flash photolysis activation of CO-inhibited cytochrome oxidase in tissue in the presence of oxygen has been disclosed in the foregoing description, it will be understood that various modifications within the spirit of the invention may occur to those skilled in the art. Therefore it is intended that no limitations be placed on the invention except as defined by the scope of the appended claims.

What is claimed is:

1. Apparatus for determining the effectiveness of oxygen diffusion in animal tissue comprising a photometer device, means to support a sample of intact animal tissue in the field of observation of said photometer device, a source of anoxygenating material including oxidation-inhibiting material, means to perfuse the sample with said anoxygenating material from said source, whereby to place the sample in anoxic condition, a source of oxygenating material, means to perfuse the anoxic sample with oxygenating material from said second-named source, whereby oxidation of the anoxic sample will then take place at a relatively inhibited rate, and means to excite the sample with a pulse of photolyzing radiant energy shortly after oxygenation of the anoxic sample commences, said pulse being of sufficient strength to destroy the inhibiting effect of said anoxygenating material, said photometer device including means to continuously read out the degree of oxidation of the anoxic tissue with time, whereby the destruction of said inhibiting effect will provide a read-out indicating the effectiveness of oxygen diffusion in the tissue sample.

2. The apparatus of claim 1, and wherein the photometer device comprises a fluorometer including means to excite the tissue sample with radiant energy and means to measure the fluorescence of the tissue caused by said last-named radiant energy.

3. The apparatus of claim 1, and wherein the photometer device comprises a time-sharing system delivering time-spaced excitation beams of different wavelength to the sample, generating corresponding time-spaced optical responses of the tissue sample, whereby responses to photolysis of a plurality of components in the tissue sample may be sequentially measured.

4. The apparatus of claim 1, and wherein the photometer device comprises a time-sharing cyclic fluorometer including means to excite the tissue sample with time-spaced excitation beams of different wavelength

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and means to measure corresponding time-spaced fluorescence responses of the tissue sample, whereby the responses of a plurality of components in the tissue sample may be simultaneously measured.

5. The apparatus of claim 1, and means to excite the tissue sample with a second pulse of photolyzing radiant energy subsequent to the first pulse for evaluating the extent of photolysis.

6. The apparatus of claim 1, wherein the anoxxygenating material includes carbon monoxide.

7. The apparatus of claim 1, and wherein the photometer device comprises a time-sharing system delivering time-spaced excitation beams to the sample, generating corresponding time-spaced optical responses of the tissue sample, said means to excite the sample with the pulse of photolyzing radiant energy comprising a flash lamp means adapted to be located adjacent the sample, and circuit means to momentarily energize the flash lamp means in the interval between a pair of said time-spaced excitation beams.

8. The apparatus of claim 7, and wherein the photometer device includes means to generate a noise-compensating electrical pulse coincidentally with each excitation beam, said circuit means including means to energize said flash lamp means after a predetermined time interval following a noise-compensating electrical pulse.

9. The apparatus of claim 29, and a second photolysis flash lamp means adapted to be located adjacent the sample, and wherein said circuit means includes means to momentarily energize the second flash lamp means after a second predetermined time interval following the momentary energization of the first-named flash

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lamp means, for evaluating the extent of photolysis produced by the first-named flash lamp means.

10. A method of determining the effectiveness of oxygen diffusion in intact animal tissue comprising the steps of perfusing a sample of the intact tissue with an anoxxygenating material which inhibits oxidation of the tissue sample, then perfusing the sample with oxygen to initiate oxygenation thereof, continuously measuring the progress of oxidation with time, and then rapidly terminating the inhibiting effect shortly after oxygenation has commenced by illuminating the sample with a photolyzing flash of radiant energy of sufficient intensity to destroy the inhibiting effect to thereby allow the sample to rapidly react with the oxygen, whereby to obtain a readout in accordance with the extent to which the oxygen has diffused in the tissue sample at the time of the photolyzing flash.

11. The method of claim 10, and wherein the oxidation-inhibiting material is carbon monoxide.

12. The method of claim 10, and illuminating the sample with a second photolyzing radiant energy flash shortly after the first flash, for evaluating the extent of photolysis.

13. The method of claim 12, and wherein the first photolyzing flash is applied approximately 1.5 seconds after the start of the perfusion of the sample with oxygen and the second photolyzing flash is applied between 1 and 2 seconds after the first flash.

14. The method of claim 10, and wherein the photolyzing flash is applied approximately 1.5 seconds after the start of the perfusion of the sample with oxygen.

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