17 August 1962, Volume 137, Number 3529 SCIENCE

Intracellular Oxidation-Reduction States in Vivo

The microfluorometry of pyridine nucleotide gives a continuous measurement of the oxidation state.

Britton Chance, Peter Cohen, Frans Jobsis, Brigitte Schoener

Direct observation of intracellular biochemical events and their relationship to physiological function presents a challenging and long-standing problem (*1*). The determination of intracellular oxygen levels in tissues has already been the subject of much discussion (*2*), but even the most refined method for measuring oxygen tension in blood and tissue fails to indicate the oxidation-reduction states of the respiratory carriers (*2*). Observations of the latter are especially important because they reflect the intracellular levels of phosphate and phosphate acceptor which control the intensity of cell metabolism (*3, 4*).

Keilin used microspectroscopy to observe changes in the oxidation-reduction level of cytochrome *c* of the thoracic muscles of the wax moth during wing motion (*5*). Millikan developed sensitive methods for following changes in oxymyoglobin concentration caused by contractions in cat soleus muscle (*1, 6*). Arvanitaki and Chalazonitis made early and significant observations of changes of pigments in nerve (*7*), and Lundegårdh has studied cytochromes in wheat roots for some years (*8*).

The reduced pyridine nucleotide component of cells (*9*) and of isolated mitochondria (*3, 4*) has a larger absorption band than other components of the respiratory chain. In tissues that can be perfused and excised, spectrophotometric measurements can be made of pyridine nucleotide (*10–12*) and cytochrome components (*13*). Oxidation of these components after contractions indicates increased concentrations of adenosine diphosphate at the muscle mitochondria; cytochrome *b* and pyridine nucleotide are more highly oxidized in the active muscle than they are in the resting muscle. This method was first used to detect the formation of adenosine diphosphate in a single contraction of muscle (*12, 12a*), a result that now appears to find confirmation in biochemical analysis (*14*).

The reduced pyridine nucleotide component of mitochondria has a fluorescence emission (*15, 16*) characteristic of the bound form (*16*), as observed in studies of binary (*17*) and ternary (*18*) complexes of liver alcohol dehydrogenase. Measurement of the fluorescence emission appears to be especially suitable for studies of mitochondrial pyridine nucleotide in excised tissues, since light transmission through the tissue is unnecessary; excitation of fluorescence and measurement of the emission require only one free surface. In preliminary experiments with excised muscles hemoglobin did not appear materially to reduce the sensitivity (*19, 20*). Also, microfluorometry has sufficient sensitivity that apertures of 5 microns can be used and less than 10^5 molecules of reduced pyridine nucleotide can be detected (*21–23*). Other components of the respiratory chain cause little interference with the measurements of the intense fluorescence emission band of mitochondrial pyridine nucleotide (*16*); thiamine and flavin fluorescence does not interfere (*24*) with measurements of the changes of oxidation-reduction state of pyridine nucleotide.

In this article we report the application of two new instruments to the study of fluorescence emission of rat kidney and rat brain, and to the study of the localized metabolic response of portions of these tissues to variation in oxygen concentration and to the addition of respiratory inhibitors. The results give a preliminary indication of the relationship between intracellular oxidation-reduction levels of mitochondrial pyridine nucleotide and some elementary physiological functions. A preliminary report has appeared (*25*).

Experimental Methods

In the identification of the material under observation, fluorescence emission spectra are considered more definitive than fluorescence excitation spectra. Absorption (and excitation) maxima for free and bound pyridine nucleotide are 335 millimicrons ($m\mu$) for bound and 340 $m\mu$ for free pyridine nucleotide, and in mitochondria they are indistinguishable from each other (*11, 16*). But bound and free pyridine nucleotide have different fluorescence emission peaks—463 and 480 $m\mu$, respectively, on the relative energy basis used here. Thus, the fluorescence emission spectra identify qualitatively the contribution of bound and free material. Under the conditions of our experiment, it is not possible to distinguish between reduced diphosphopyridine nucleotide (DPNH) and reduced triphosphopyridine nucleotide (TPNH), but in the case of the brain (and kidney) TPNH can be neglected (*26*).

The authors are affiliated with the School of Medicine, University of Pennsylvania, Philadelphia. The experiments were carried out in the Johnson Research Foundation.

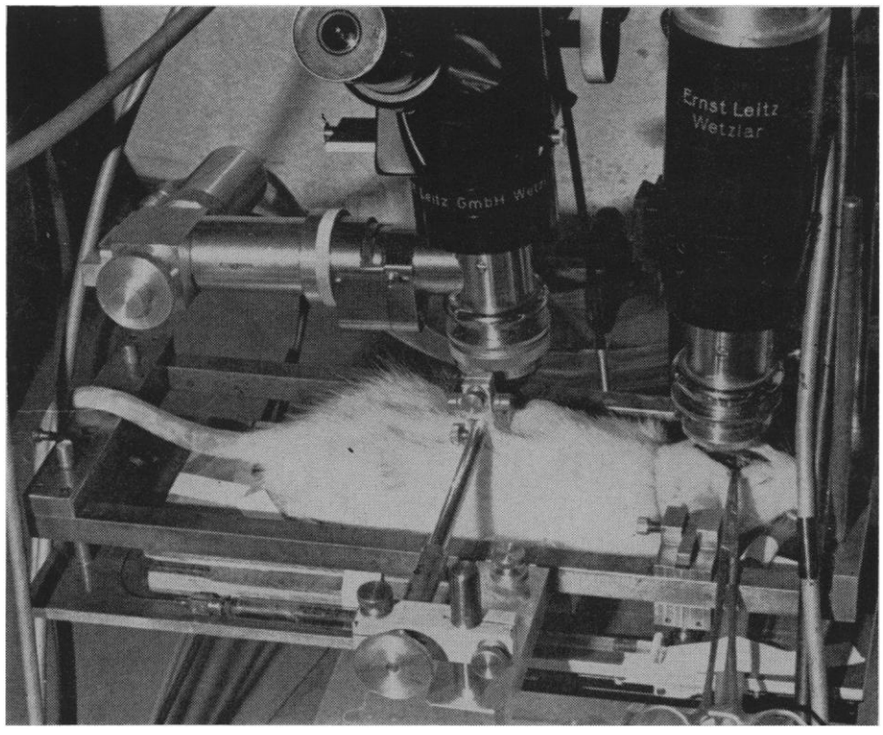

Fig. 1. Experimental arrangement for simultaneous microfluorometry of brain and kidney cortex in the rat. Two microfluorometers are focused on the exposed surfaces of the brain and kidney. By means of a tracheal cannula the oxidation-reduction level of the intracellular pyridine nucleotide can be altered and the corresponding fluorescence changes can be recorded by the two microfluorometers. For the kidney fluorometer, the water-cooled lamp housing attached to the Leitz "Ultrapak" illumination system is shown. Light choppers and photomultipliers are not shown.

Differential spectrofluorometer. In studies of mitochondria and cell suspensions, spectra representing the differences in absorption of the oxidized and reduced forms of the respiratory carriers have provided the most incisive information (*27*). We have devised a spectrofluorometer that plots a spectrum representing the difference in fluorescence emission of two portions of excised tissue which differ in the oxidation-reduction state of pyridine nucleotide (*28*). The excitation energy is selected by a grating monochromator from a high-intensity ultraviolet source (General Electric type AH6). An excitation wavelength of 366 mμ is usually employed. The excitation falls upon a vibrating sample holder which exposes either one of the samples to the excitation beam for 1/20 second. The fluorescence emission passes through a secondary filter (Wratten 2C) and thence to a second monochromator for analysis, and to a photomultiplier for intensity recording. The emission spectra are not corrected for the sensitivity curve of the photosurface but indicate exactly the energy distribution observed by the microfluorometer, described in the next paragraph. Corrected emission spectra of isolated mitochondria have already been published (*16*). Electronic circuits are arranged in such a way that the recorder plots the difference in emission intensity between the two samples. The two specimens are separately bathed on both sides by flowing Ringer's solution. Thus, one may be aerobic and the other anaerobic, or suitable chemicals may be added to one and not to the other. When both samples are aerobic, the recorder plots a very nearly flat base line over the wavelengths desired (390 to 600 mμ) and the instrument detects exceedingly small changes in the fluorescence emission of one of the samples that are due to changes in chemical environment.

Microfluorometer. In order to observe oxidation-reduction changes of pyridine nucleotide near the surface of intact organs of the anesthetized animal, we have modified the microfluorometer described previously (*21, 23, 29*) so that excitation and emission of fluorescence require only the free surface of the tissue sample. The magnification has been decreased, since localization of metabolic activity in a circle 20 microns in diameter is sufficient. Sufficient sensitivity is available for measuring the emitted intensity to within less than 1 percent, even at the low levels available from such tissues as the electric organ of *Electrophorus* (*30*). Thus, the cardioid condenser (*29*) is replaced by Leitz "Ultrapak" illumination for excitation. The apparatus has the following features. Excitation energy from a 1-kilowatt water-cooled lamp (type AH-6) is filtered by a multi-element glass filter (Eppendorf) so that only the 366-mμ region is transmitted. At this low intensity tissues can be observed over a period of hours without measurable decrease in fluorescence. The Ultrapak focuses the excitation energy on the surface of the tissue, occasionally through a water immersion dipping cone.

The fluorescence emission is viewed through an 11× objective, a secondary filter (Wratten type 2C), and a 3× ocular, with provision for simultaneous visual observation (this has been found to be especially useful for controlling mechanical artifacts in observation of inhomogenous tissues). A 0.6-mm aperture is vibrated at 60 cycles per second in the image plane and alternately shields and illuminates a photomultiplier with the emitted fluorescence. The output of the photomultiplier is connected to a cathode follower, a switch demodulator, an adjustable resistance-capacitance filter, a millivolt amplifier, and a chart recorder. The sensitivity of the apparatus is periodically calibrated with a fluorescent glass (Corning type 360).

The magnification of 33 is suitable for observing areas between larger vessels on the brain cortex and for observing small glands such as the adrenals. Without much difficulty quartz light pipes could be employed for recording from less accessible organs.

Biological preparations. Excised tissues used in studies with the differential spectrofluorometer were cut in the Stadie-Riggs tissue slicer in sections about 0.75 mm thick. Two identical slices were used in the differential spectrofluorometer. When desired, perfusion through the vena cava and aorta was continued until the kidney was completely blanched (approximately 200 ml of perfusion fluid was used).

Rats were prepared for in vivo observation under ether anesthesia followed by administration of urethane. The kidney was cleared of fat and placed in a cup-shaped holder just above the abdominal cavity. Thus it rested free from contact with the intestine; in this way mechanical disturbance due to

respiratory movements was avoided. The objective was sharply focused on the capillaries lying immediately below the tissue surface. The kidney capsule was left intact.

Reagents were added to the circulation by cannulation of the vena cava or, in some cases, the renal artery. Changes in the oxygen supply to the kidney were accomplished by one of three methods. (i) A simple mechanical clamp was used to shut off the blood flow through the vessels supplying the kidney. (ii) For a 300-gram rat, 10 micrograms of norepinephrine in the vena cava or 1 microgram of norepinephrine in the renal artery caused a 2- to 4-minute occlusion of circulation, sufficient to give a high degree of pyridine nucleotide reduction (*31*). (iii) The animal was provided with a tracheal cannula, and inspired gases were controlled as desired and monitored by the Pauling-Wood oximeter. This method was the most satisfactory. In studies of the kidney it was found desirable to treat the kidney beforehand with phentolamine methane sulfonate (Regitine) (about 1 mg for a 300-gram rat) prior to anoxia in order to avoid irregular reoxygenations of the kidney, possibly due to norepinephrine release in anoxia.

In studies of the brain cortex a section of the skull was removed carefully in order to minimize bleeding into the field of observation; about 25 square millimeters of the cortex was exposed. The dura was left intact. Observations were made with or without a dipping cone on the objective. The head was held by an instrument similar to that used in stereotaxic studies of the brain and was kept sufficiently motionless for continued observation of a small area between major blood vessels. In a few experiments, observations were made simultaneously on the cortex of the brain and on the kidney by means of two independent microfluorometers. In such cases the kidney was held in a clamp on the back of the animal. Cannulation of the vena cava or the carotid artery was provided. The apparatus is shown in Fig. 1.

Selection of field of view. Areas of the brain and kidney were selected which showed a bright and uniform distribution of fluorescent material. Areas containing large blood vessels were avoided. On the brain cortex a field was selected in which the number of visible capillaries was minimal. Since continuous viewing is possible during photoelectric measurement of the fluorescence, these positions were monitored to make sure that mechanical artifacts were avoided.

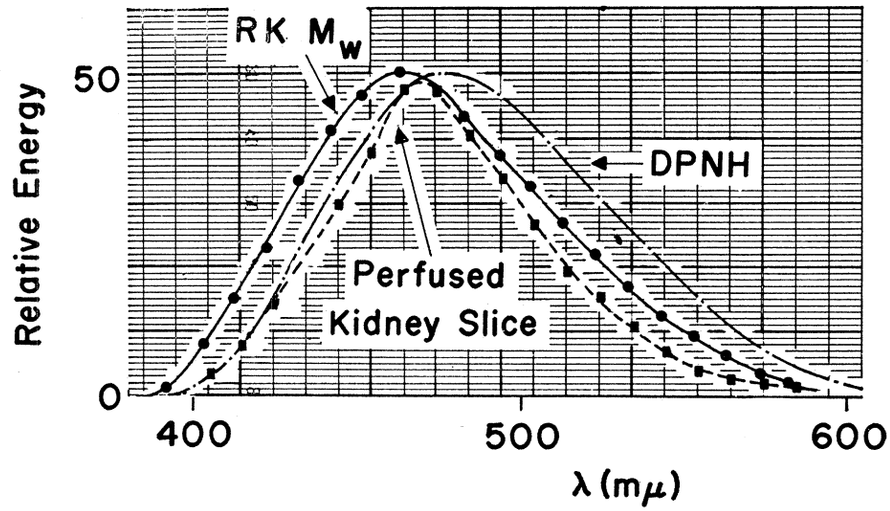

Fig. 2. Fluorescence emission spectra. (Dashed-dotted curve) spectrum due to a solution of DPNH; (solid curve) spectrum due to DPN reduction in a suspension of rat kidney mitochondria; (dashed curve) spectrum due to an aerobic-anoxic transition in a slice of kidney cortex obtained from a perfused rat. The peaks of the emission bands, uncorrected for photocell sensitivity, are at 480, 463, and 472 mμ, respectively.

Depth of penetration of fluorescence excitation. Since the 366-mμ excitation energy is highly absorbed by the tissues, it is to be expected that the field of view will not extend far below the surface of the tissue. This is, of course, highly desirable for localizing metabolic responses in well-defined functional areas such as the cortex of the brain and kidney. In order to obtain quantitative information on the depth of the field of observation, we compared the intensity of fluorescence emission of slices of perfused kidney cortex of thicknesses in the range 1.1 down to 0.52 mm. The intensity of fluorescence emission decreased only 14 percent in this range; this suggests that localization is mainly in layers less than 0.5 mm thick. The gray matter of the brain cortex probably gave the major response. A higher degree of localization near the tissue surface could be obtained by using excitation of shorter wavelength than the 366 mμ employed; that is, an extremely high degree of localization at the surface would be obtained by exciting reduced pyridine nucleotide fluorescence at 334 mμ or even at the absorption peak of the adenine band (260 mμ).

Correlation with the physiological function. In these preliminary experiments, where attention has been focused upon the successful operation of the microfluorometer and the exercise of suitable controls to avoid artifacts, no instrumentation for observation of physiological function has been included. Instead, we have resorted to the simplest of physiological criteria in the experiments with anoxia—namely, the moment when breathing ceased (*32*). This provided a convenient index of the integrated function of the central nervous system and is here correlated with the degree of intensification of fluorescence in hypoxia. Obviously, more refined procedures can be used in the future; it is highly desirable to correlate active transport in the kidney and electrical activity in the brain with the level of reduction of pyridine nucleotide in the tissues.

Experimental Results

Changes in fluorescence emission spectra of kidney and brain cortex caused by aerobic-anoxic transition. The component of fluorescence emission of the intact tissues which is of interest here is that which changes with the degree of oxygenation of the tissue or the degree of metabolic activity—fluorescence changes due to changes in the extent of reduction of pyridine nucleotide. A considerable increase in fluorescence occurs when nitrogen replaces oxygen in the fluid bathing one of the two slices of kidney examined in the differential spectrofluorometer. The blood was removed from the slices by perfusion. The spectrum of such an increase in fluorescence is indicated in Fig. 2 (dashed curve); for our experimental conditions this spectrum has a peak at 472 mμ for an excitation wavelength of 366 mμ.

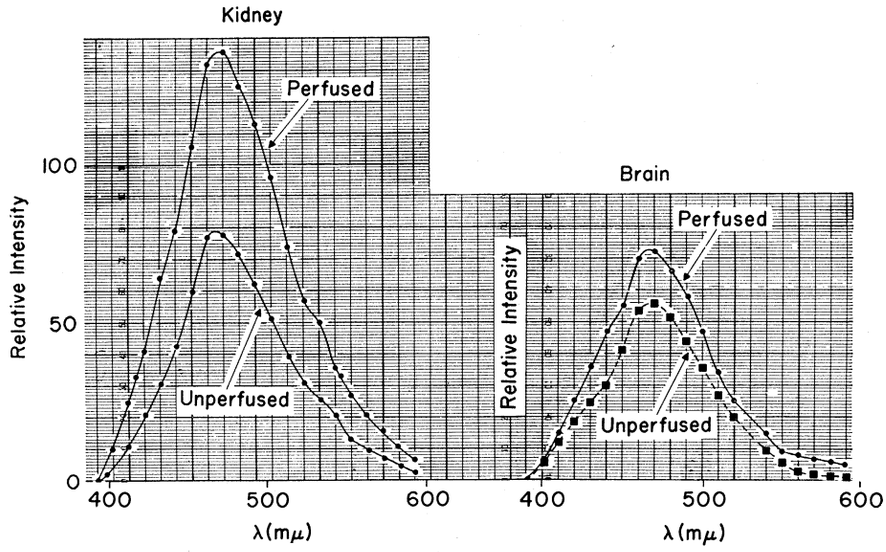

Fig. 3. Effect of perfusion on aerobic-anoxic fluorescence emission changes in slices of kidney and brain cortex. The uncorrected emission maxima are at 472 mμ. The same energy sensitivity was used in the four experiments, and thus the heights of the peaks indicate the relative intensities of emission from kidney and cortex slices.

In order to identify this increase in fluorescence we have included the spectrum for a solution of DPNH (Fig. 2, dashed-dotted curve) and that for a suspension of mitochondria from rat kidney (Fig. 2, solid curve). The binding of DPNH within the mitochondria shifts the emission to a shorter wavelength (463 mμ) than that of a solution of DPNH (480 mμ). The increase in fluorescence of the tissue slice in nitrogen is sufficiently similar to that due to DPN reduction in mitochondria to allow us to tentatively identify the one with the other. The contribution due to cytoplasmic pyridine nucleotide is best indicated by Amytal treatment, as described later.

The emission spectra of the fluorescing components of the kidney and brain cortex slices do not change in that the aerobic-anoxic transition consists of a broad band covering the range 460 to 540 mμ. This spectrum is due in part to a steady-state reduction of pyridine nucleotide, but it also contains a band near 520 mμ which may be due to flavin. However, the spectra of Fig. 2 lack distinctive fluorescence emission in this region. Thus, if there is a flavin component present, it does not change its oxidation-reduction state in oxygen-nitrogen transitions in these tissue slices (see Fig. 3).

Effect of hemoglobin on fluorescence emission spectra. In Fig. 3 the emission spectra of slices of kidney and brain cortex from perfused and unperfused animals are compared in order to determine whether the fluorescence emission can be satisfactorily measured in the presence of hemoglobin. The peaks of the emission spectra show no shift (472 mμ), but the intensities are less in the presence of blood. This diminution is attributed to an absorption of the exciting wavelength by erythrocytes. From the graphs we compute that 43 percent of the excitation is absorbed in the kidney and 23 percent is absorbed in the brain slices. Thus, changes in fluorescence emission can readily be measured in the presence of erythrocytes with surprisingly little loss of sensitivity.

The shape of the emission spectra of Fig. 3 is not significantly altered by the concomitant deoxygenation of hemoglobin in the unperfused tissue. This result indicates that the spectral changes of hemoglobin contribute too little to the absorption of fluorescence emission in the unperfused tissue to be measurable.

It is apparent from these studies of perfused and unperfused slices of kidney and brain cortex that the increase in emission that occurs in the aerobic-anoxic transition can be attributed to the reduction of pyridine nucleotide. We have not yet identified any other fluorescing component that changes with the oxidation-reduction state of the tissue when 366-mμ excitation is used.

Observations of changes in fluorescence emission in intact organs. Fig. 4*A* shows a recording of the time course of intensity changes as observed with the microfluorometer focused on the left kidney of a urethane-anesthetized rat. The time scale runs from left to right, and an increase in fluorescence intensity is indicated by a downward deflection of the trace. Regitine (phentolamine, 0.4 mg) had been introduced into the vena cava prior to the time represented in the record, and the animal was breathing oxygen through a tracheal cannula. At the junction of the arrows above the left upper portion of the trace, the inspired gas is changed to nitrogen. Thirty-two seconds later the fluorescence abruptly increases,

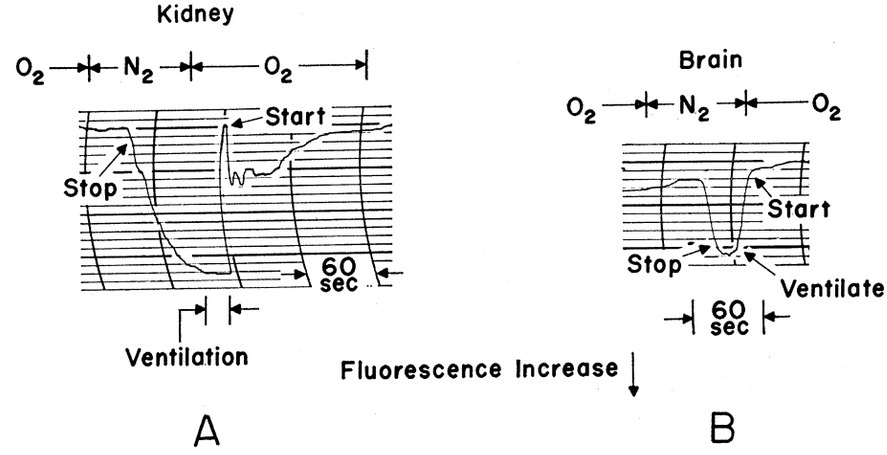

Fig. 4. Microfluorometer recordings of the kinetics of increases in fluorescence observed in oxygen-nitrogen transition for kidney (*A*) and brain (*B*) cortex of urethane-anesthetized rats. The time scale proceeds from left to right, and increases in fluorescence are recorded as a downward deflection. The times when gas in the tracheal cannula was changed from oxygen to nitrogen and the times when breathing stopped and started again are indicated. The photoelectric sensitivity in recordings of the brain cortex is 2½ times that for recordings of the kidney. (Experiment 425, I and II.)

and it follows a roughly exponential course to a plateau in 1 minute. The increase in fluorescence observed at the time respiration ceases is 11 percent of the total increase during anoxia. As soon as a plateau is reached, the lungs are ventilated with oxygen three times. Three seconds after ventilation is ended an abrupt decrease in fluorescence is observed, and breathing starts as the decrease reaches its peak. Irregularities in the extent of oxidation in the fluorescence response are observed for the next 30 seconds, but the fluorescence remains above the initial value for 2½ minutes. Then the value observed prior to the start of the experiment is reestablished.

Figure 4*B* illustrates a similar response in the brain cortex. No fluorescence changes are observed for 30 seconds after the inspired gas is changed from oxygen to nitrogen. Then a small but consistently observed diminution of fluorescence occurs, and 10 seconds thereafter hyperventilation is observed. About 6 seconds thereafter, an abrupt increase in fluorescence is recorded, which proceeds rapidly toward its plateau value and reaches 80 percent of this value when breathing stops. After a plateau is established, ventilation with oxygen is commenced, and 3 seconds thereafter an abrupt decrease in fluorescence occurs, which extends slightly beyond the level observed prior to the administration of nitrogen. Six seconds after the decrease in fluorescence reaches its plateau, breathing commences.

These records with brain and kidney indicate that anoxia causes similar fluorescence changes in vivo, in excised slices, and in isolated mitochondria. We will now describe a number of controls to make sure that these changes in fluorescence correspond to reduction and oxidation of intracellular pyridine nucleotide.

Effects of terminal respiratory inhibitors. Hydrogen sulfide, like hydrogen cyanide, causes inhibition of cell respiration to such an extent that hemoglobin is not deoxygenated and histotoxic anoxia [the classic "death with red blood" of Claude Bernard (*33*)] is obtained. Thus, the change in the hemoglobin would be opposite to that caused by nitrogen; hemoglobin becomes more oxygenated when the animal is given oxygen manually after injection of hydrogen sulfide or hydrogen cyanide. Thus, this type of experiment provides an excellent control with respect to spectroscopic changes of oxyhemoglobin-hemoglobin transformation affecting the fluorescence measurements.

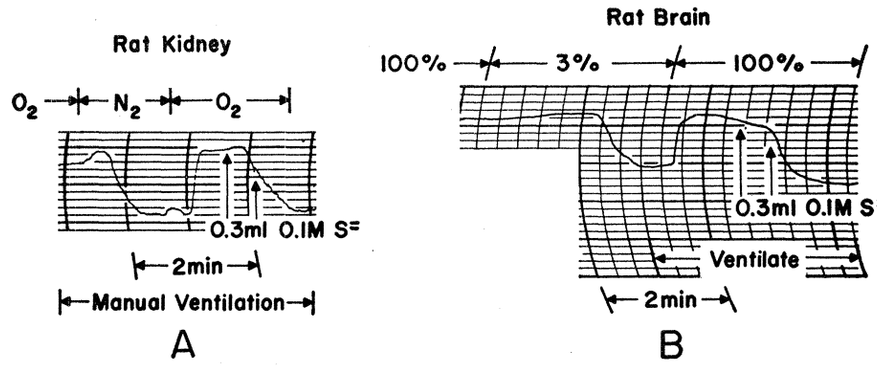

Fig. 5. Microfluorometric recording of fluorescence increases caused by oxygen-nitrogen transition and by sulfide infusion into the vena cava, for kidney (*A*) and brain cortex (*B*) of a urethane-anesthetized rat. At points marked above the record the inspired gas was changed from oxygen to nitrogen for the kidney, and from oxygen concentrations of 100 percent to concentrations of 3 percent for the brain. The time scale proceeds from left to right, and increase in fluorescence is indicated as a downward deflection. In both experiments the oxygen-nitrogen-oxygen transition is followed by slow infusion of a solution of 0.1*M* sulfide. The records indicate that the increase in fluorescence caused by sulfide inhibition of cytochrome oxidase is about the same as, or greater than, that observed in the oxygen-nitrogen transition, where hemoglobin is deoxygenated as well. The sensitivity in recordings on the brain is 2.5 times that in recordings on the kidney. (Experiments 422 and 425, II.)

In Fig. 5*A* the fluorescence increase caused by anoxia is recorded, the animal being under manual ventilation. Thirty seconds after administration of nitrogen, an increase in fluorescence is obtained which reaches a plateau in about a minute. The inspired gas is changed to oxygen, and 30 seconds later the abrupt rise of the trace to a plateau value occurs. Thirty seconds after this, a slow infusion into the vena cava of 0.1*M* sodium sulfide is begun. Fifteen seconds later an increase in fluorescence begins, which continues for 45 seconds until a plateau is reached. This plateau value is only 5 percent less than that obtained with nitrogen. A series of experiments shows consistently that over 80 percent of the fluorescence increase caused by anoxia can be achieved by injecting sulfide into the aerobic rat.

Fig. 5*B* illustrates a similar experiment on the brain cortex. The rat is initially equilibrated with 100 percent oxygen, and then transferred to 3 percent oxygen. As the concentration approaches 3 percent, the fluorescence diminishes slightly and remains constant for a minute. Thereafter, the fluorescence increases and breathing ceases. The fluorescence intensity reaches a plateau about 20 seconds later. Seven seconds after the changeover from 3 percent to 100-percent oxygen, the fluorescence abruptly diminishes, and it overshoots in a characteristic manner. Infusion of 0. 1*M* sulfide is begun, and after 0.3 ml has been added, the fluorescence increases to a level somewhat in excess of that observed with 3-percent oxygen. Again, there appears to be no evidence of hemoglobin interference.

As a further control on the possible effects of oxyhemoglobin-hemoglobin changes, we have compared the fluorescence increase in the kidney caused by the transition from oxygen to nitrogen with that caused by the transition from oxygen to carbon monoxide. It is found that the fluorescence increase with carbon monoxide is 96 percent of that obtained with nitrogen. Since the absorption bands of carboxyhemoglobin and reduced hemoglobin differ considerably, this result seems to be consistent with the conclusion that oxyhemoglobin-hemoglobin changes interfere to a negligible extent with the measurement of fluorescence changes.

Effect of barbiturates. Addition of amobarbital (Amytal) to mitochondria causes an increase in the reduction of mitochondrial pyridine nucleotide to its maximum value (*34*) and thereby affords a specific reaction of mitochondrial pyridine nucleotide. Figure 6 (left) shows the slow infusion (over an interval of 1 minute) of 7.5 mg of Amytal (0.3 ml of a 25-mg/ml mixture) into the vena cava of a rat whose kidney is under microfluorometric observation. About 20 seconds after the

beginning of the infusion the fluorescence increases, and it approaches a plateau value at the end of 2 minutes, a response suggesting the reduction of mitochondrial diphosphopyridine nucleotide. (The animal is then given oxygen manually.) Due to the distribution of the Amytal into the body spaces, the effect diminishes somewhat after it reaches its plateau value. In order to discover whether any pyridine nucleotide remains oxidized under these conditions, the gas is changed to nitrogen; after approximately 1 minute a further 40-percent increase in fluorescence is observed. This experiment suggests that at least 60 percent of the increase in fluorescence emission observed in anoxia is due to an Amytal-sensitive reaction.

The effect of Amytal is observed in the kidney at lower blood concentrations than in the brain. A demonstration of this has been obtained with the simultaneous recordings on the two organs, and the result is illustrated in Fig. 6 (right). With the animal breathing oxygen, an infusion of Amytal (25 mg/ml) is begun. By the time 0.1 ml (2.5 mg) has been added, an increase in fluorescence is observed on the kidney; this increase continues in an almost linear fashion while the infusion is continued to a total volume of 0.4 ml. Then there is a slight decrease, probably due to dilution of Amytal infused into the body spaces. On the brain, a scarcely measurable downward deflection of the trace is observed; this is followed in the course of the next 2 minutes by a slight upward rise. In order to calibrate the amount of fluorescence increase that can be obtained with nitrogen, the inspired gas is changed to nitrogen, and after 1 minute an increase in fluorescence intensity is observed on both traces. These calibrations indicate that the same blood level of Amytal causes a fluorescence increase of 5 percent on the brain and of 30-percent on the kidney. High concentrations of Amytal have a greater effect on the brain; direct infusion of 7.5 mg of Amytal into the carotid artery causes a fluorescence increase which is 24 percent of that obtained with nitrogen.

Effects of perfusion. In order to control further the effects of hemoglobin on the fluorescence emission from these tissues, we compared the fluorescence levels obtained in a rat, breathing oxygen, when blood was circulating through the kidney and when a large part of the blood volume had been replaced with Ringer solution. These tests indicate that 60 percent of the fluorescence excitation is absorbed by the circulating blood, as compared with 43 percent absorbed by the noncirculating blood of the unperfused tissues (Fig. 3). The noteworthy result of the experiment was, however, that both the aerobic and anoxic levels of fluorescence increased proportionately, suggesting that the oxyhemoglobin-hemoglobin change does not appreciably affect the nature of the fluorescence change.

Oxygen sensitivity of the brain cortex. In tests of the oxygen sensitivity of the brain cortex we measured the increment of pyridine nucleotide reduction as a percentage of the change from aerobiosis to anoxia. Oxygen sensitivity of the cortex can be evaluated relative to that of the kidney, or with reference to a fixed level of hypoxia; here the point when breathing ceases after inspired oxygen has been replaced by nitrogen is used (Fig. 4). An examination of four rat kidneys indicates that a fluorescence increase of 28 percent had been reached at the time breathing ceased (these data were obtained from animals which had been anesthetized for less than 1 hour). Thirteen tests (experiment No. 425) on the brain of one rat indicated that the fluorescence increase had reached 87 percent of the plateau value at the time breathing ceased. Tests of other rats gave similar values. These data indicate a difference in the degree of pyridine nucleotide reduction within two organs associated with a given partial pressure of inspired oxygen in the artery.

A second approach to the question of the oxygen sensitivity of the cortex is afforded by experiments in which the oxygen concentration in the inspired air is held at low values that give various percentages of fluorescence increase. Such an experiment is illustrated in Fig. 7. The first measurable increase in fluorescence is observed when the percentage of oxygen falls to 8. A partial recovery of the initial fluorescence is obtained by returning to an oxygen concentration of 20 percent. In a second interval of anoxia the fluorescence increase fluctuated between 30 and 60 percent with an oxygen percentage of about 4. It is difficult to stabilize the values at a 50-percent increment of reduction.

Response to vasoconstrictors. The measurement of intracellular oxidation-reduction levels should provide information on the effects of vasoconstrictor drugs similar to these obtained with microelectrode or oximeter records (see *35*). Noradrenaline (30 μg/kg) causes vasoconstriction to the point of anoxia in the kidney for periods of several minutes (*31*). Simultaneous observations of the effects upon the kidney and upon the brain show large and rapid increases in fluorescence on the kidney and a decrease on the brain that is smaller and longer-lasting. Presumably the noradrenaline affects the circulation of the kidney much more markedly than that of the brain. Obviously, these are matters which may well be investigated in greater detail by supplementing fluorometry with oxygen microelectrode studies.

Discussion

In addition to the considerable advantages of studying physiological reactions of organs on a biochemical basis, the microfluorometric method has real value for the study of integrated biochemical processes themselves. Among the advantages of using whole organs rather than tissue slices for biochemical study are the following.

1) The tissue can readily be kept oxygenated by its own circulation, and at any level of blood-oxygen concentration desired. Localized intracellular oxygen concentrations can be measured.

2) When inhibitors, substrates, and so on, are added to the local circulation of the organ, not only the onset of the effects but also, in the case of reversible reactions, dissociation phenomena can readily be observed. For example, the reversibility of Amytal or sulfide inhibition of electron transfer can readily be observed because of the transient nature of the increase in fluorescence (see Fig. 6).

3) It is now possible to compare the responses of intact tissues and tissue homogenates with more accuracy, particularly with respect to factors affecting energy-linked reactions (*36*).

4) Metabolic states as observed in isolated mitochondria (*3*, *4*) can be correlated with those of the intact organs.

Accuracy of results. It is appropriate first to discuss the accuracy with which the oxidation-reduction state of pyridine nucleotide may be measured in vivo. The experimental results obtained so far suggest that hemoglobin causes a diminution of fluorescence excitation but does not appreciably affect the shape of the spectrum of fluorescence

emission (see Fig. 3). In a separate series of experiments on the unperfused rat diaphragm, the absorption of the fluorescence change is found to be greater below 470 mμ than is indicated in Fig. 3, presumably because the mitochondrial fluorescence is smaller in relation to the hemoglobin content (*37*).

It is possible to interpret these effects of fluorescence emission in terms of the absorption changes occurring in the conversion of oxyhemoglobin to hemoglobin. There is considerable increase of absorption in the region 430 to 450 mμ and a smaller decrease in absorption in the region 450 to 500 mμ (*38*). Since the energy sensitivity of the photocell-filter combination is nearly uniform from 420 to 490 mμ, and since the emission spectrum is distributed on both sides of the isobestic point for the oxyhemoglobin-hemoglobin change, a considerable cancellation of the changes takes place. The net effect should be a small increase in fluorescence in the region of 470 mμ due to the oxyhemoglobin-hemoglobin change. Apparently this increase is so small that we have not yet been able to detect it in comparisons of fluorescence increases observed on ventilation with nitrogen with those observed on the addition of sulfide.

Carbon monoxide provides a valuable control. Here the transformation is from oxyhemoglobin to carboxyhemoglobin. There is a very little change in the region of 430 mμ. At 470 mμ the decrease in absorption is only 20 percent of that due to hemoglobin formation. Thus, carboxyhemoglobin formation should decrease the fluorescence emission while hemoglobin formation should increase it. Our experimental data indicate that the increase in fluorescence on ventilation with carbon monoxide is 96 percent of the value obtained with nitrogen. The 4 percent decrease in the case of carbon monoxide could be explained on this basis, but more accurate data are needed.

At 500 mμ there is very little change in the oxyhemoglobin-carboxyhemoglobin spectra. By using an interference filter at 500 mμ in addition to a Wratten 2C filter, we find results that are the same as those already presented—very nearly equal fluorescence intensity that increases on ventilation with nitrogen, carbon monoxide, and sulfide.

Thus, our explanation of the currently observed insensitivity to the oxygenation and deoxygenation of hemoglobin

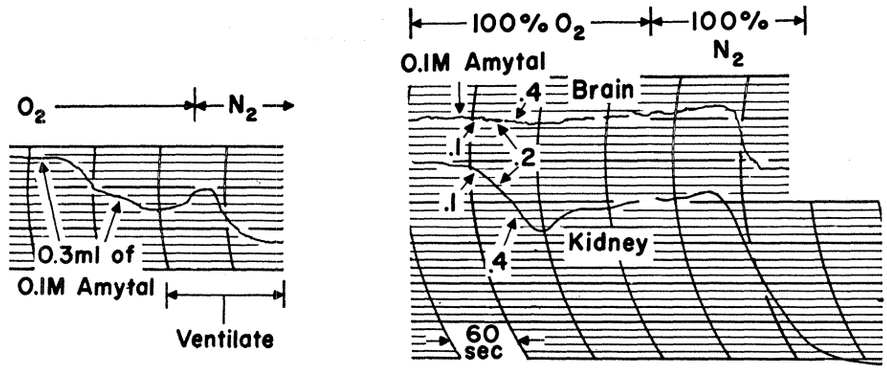

Fig. 6. (Left) Microfluorometric recordings, for the kidney, of increases in fluorescence intensity caused by infusion of Amytal into the vena cava of a urethane-anesthetized rat as compared with the increases caused by the changeover from oxygen to nitrogen. The time scale proceeds from left to right. Increases in fluorescence intensity are indicated as a downward deflection. (Right) Simultaneous microfluorometric recordings, for kidney and brain, of changes in fluorescence intensity caused by an infusion of Amytal. The Amytal sensitivities of the two organs are comparable to their sensitivities with respect to the changeover from oxygen to nitrogen. The amplifier sensitivity used in recording from the brain was 4.6 times higher than that used in recording from the kidney.

is that the change in the absorption of excitation light by the capillaries is too small to be detected, while the effects on the fluorescence emission largely cancel out. (More recently a recording microspectrofluorometer has been perfected and it is found that the shape of the fluorescence emission spectrum in the region 400–600 mμ is the same in aerobiosis and anoxia; the intensity is of course altered.)

The calibration of the fluorometer is readily repeatable to within 3 percent; fluctuations in the arc intensity are less than this. Also, the sensitivity is repeatedly calibrated with a fluorescence standard. The intensity of fluorescence emission from a series of rat kidneys is most consistent with respect to the anoxic level of fluorescence intensity. The aerobic level may fluctuate somewhat, possibly as a result of different levels of biochemical activity, as has been noted in the case of muscle (*19*). For a particular animal, measurements of the increment between the aerobic and the anoxic levels are reproducible to within at least 10 percent.

The very large changes in fluorescence on pyridine nucleotide reduction, that are associated with different metabolic states of isolated mitochondria (*3*, *4*) indicate that the currently available accuracy is adequate for a correlation of the oxidation-reduction state of pyridine nucleotide with physiological and biochemical function.

Identification with mitochondrial pyridine nucleotide. In metabolically active tissues such as kidney and brain, a considerable amount of mitochondrial pyridine nucleotide may be oxidized in the aerobic state (state 3) (*3*, *4*). The aerobic-anoxic transition should show an abrupt increase in pyridine nucleotide reduction, correspond-

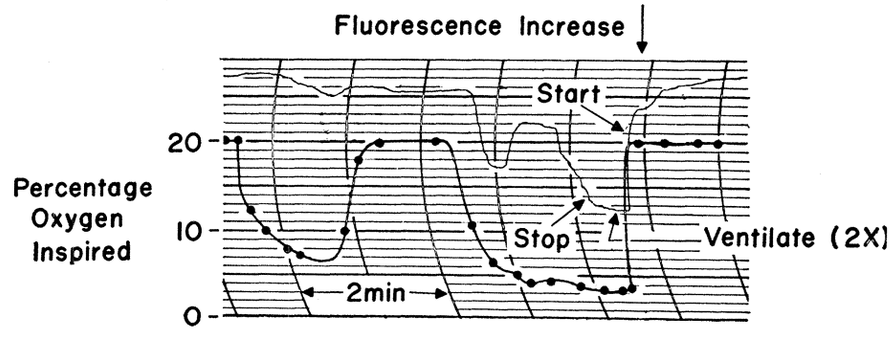

Fig. 7. Correlation of the percentage of oxygen in inspired air with the increase in fluorescence intensity, as measured on the brain cortex of a rat under urethane anesthesia. The oxygen percentages were measured with a Beckman oximeter, and the increases in fluorescence, with the microfluorometer. The recording sensitivity is the same as that of Fig. 4*B*.

ing to the abrupt increases in fluorescence observed experimentally in the study under discussion (Fig. 4) and in a suspension of mitochondria (*3*). In these tissues, cytoplasmic pyridine nucleotide may become more reduced as well. This is a slow process in excised frog muscle and may require several hours at 25°C (*10*, *12*, *39*).

In the only cell type in which the kinetics of reduction of localized fluorescence of the cytoplasm have been compared with those of a mitochondial aggregate (*22*), no change in cytoplasmic fluorescence was observed in the time required for complete reduction of the mitochondrial pyridine nucleotide in the aerobic-anoxic transition at 26°C. If these results on grasshopper spermatid are applicable to the mammalian tissues, the aerobic-anoxic transitions recorded in this article largely involve pyridine nucleotide reduction in the mitochondria.

In studies of ascites tumor cell suspensions, Amytal is found to cause reduction of about half the total pyridine nucleotide, and the addition of glucose doubles the pyridine nucleotide reduction. This indicates that at least half of the total change is due to mitochondrial pyridine nucleotide.

We may further consider whether di- or triphosphopyridine nucleotide contributes to the observed fluorescence. Chemical assays of pyridine nucleotide of rat kidney and brain indicate that diphosphopyridine nucleotide is the major component of these mitochondria (*26*, *40*, *41*).

Klingenberg has made an attempt to obtain in vivo chemical assays of the DPNH and DPN contents of the brain and other organs of the rat (*26*). Unfortunately, data are not available for the kidney, but the heart shows a threefold increase of DPNH in the in vivo to post mortem change (presumably anoxic). In this case the post mortem reduction level reaches the level for the mitochondrial portion (this is true for liver and skeletal muscle, as well). In the assays of the brain there is no post mortem increase; the in vivo level indicates that all the mitochondrial pyridine nucleotide is reduced. Klingenberg (*42*) indicates the great difficulty of this technique as applied to the brain, and further results are awaited.

In summary, there appears to be reasonable experimental evidence that the reduction of the diphosphopyridine nucleotide component of mitochondria of the cells under observation is responsible for over half of the fluorescence increase observed in anoxia.

Metabolic state of kidney and brain. In excised muscle the transition from rest to activity caused by contractions is marked by an oxidation of mitochondrial pyridine nucleotide, which may be measured either spectrophotometrically (*12*) or fluorometrically (*19*, *20*). In kidney and brain, transitions to various levels of functional activity require more subtle physical or chemical control than has as yet been exercised. Thus, we do not have information as to which oxidation-reduction levels of the pyridine nucleotide in vivo correspond to a metabolically active state (state 3) or to a resting state (state 4). However, by making some simple assumptions we can obtain a preliminary estimate. In kidney, the ratio of the anoxic to the aerobic fluorescence has been observed to exceed 2. In brain, the ratio is about 1.8. Such large ratios of aerobic to anoxic DPN reduction are consistent with the idea that the cells under observation are in a highly active metabolic state (state 3) (*3*, *4*). (More recent data obtained with the resting gastrocnemius muscle of the rat show this ratio to be only 1.1.)

Experiments with uncoupling agents should confirm the view that the metabolism of the kidney and brain cells is in a highly activated state, for if it were not, injection of uncoupling agents should cause a diminution of fluorescence corresponding to a further oxidation of the aerobic steady-state levels. So far, our inability to obtain decreases in fluorescence with uncoupling agents supports the idea that kidney and brain cortex are in state 3, but such studies must await a variety of more detailed controls, particularly with respect to permeability factors.

It is also possible that the oxidation-reduction level lies between that of the active (state 3) and the resting state (state 4). In this case an expected response to physiological function is the change observed during the oxidative recovery from anoxia in brain cortex, where a higher oxidation level of pyridine nucleotide is observed for about 2 minutes after an interval of anoxia.

Difference in Amytal sensitivity of brain and kidney. At first sight, the result of Fig. 6 suggests that the brain is much less sensitive to the same blood levels of Amytal that cause large pyridine nucleotide reduction in the kidney. Possibly the striking effects of Amytal on electron transport are only incidentally related to its anesthetic properties (*42a*). However, more detailed consideration of the effects of barbiturates on intact tissues is required before conclusions can be drawn, since permeability factors, number of units, and localization of the anesthetic effect must be considered. Nevertheless, the rapid, sensitive, and reversible responses of the kidney to Amytal clearly demonstrate the in vivo response of an intact organ to this interesting inhibitor.

Critical oxygen tension in the brain cortex. On the basis of these preliminary results it is possible to make some estimates of the oxygen tension in the cortical cells that are relevant to integrated function. We find that an increment of pyridine nucleotide reduction of 80 percent of the range from the aerobic steady-state level to the anoxic level is associated with cessation of breathing. (Subsequent experiments associate this level with the diminution of the monopolar electroencephalogram on the area of fluorometric observation.) While this value stands independently of other approaches, it is useful to convert these increments of pyridine nucleotide reduction observed in vivo to localized tissue oxygen concentrations.

We have recently evaluated the oxygen concentrations that give varying increments of pyridine nucleotide reduction in cell suspensions, particularly rapidly respiring cells such as bakers' yeast (*43*). Although such experiments are not yet feasible with brain mitochondria, we have no reason to believe that the data on the respiratory chain of pyridine nucleotide reduction of 80 percent as defined above correspond to the extremely small oxygen concentration of 0.1 micromole. This then appears to be the oxygen concentration that is critical for intracellular function.

A criterion of critical oxygen concentration used previously (*43a*) is that at which a decrease of oxygen utilization is noted. For resting frog sartorius muscle at 10–20°C a value of 0.5–2.0 mm Hg (1–3 micromoles) is obtained. In the rat cortex, the inspired oxygen concentration at which an increment of pyridine nucleotide reduction (1.0 percent) is observed is about 8 percent. Based on the studies of yeast cells described above, this corresponds to an intracellular oxygen concentration of about 1 micromole, which is in reasonable agreement with Hill's value for

resting frog muscle. It is possible to compare these values with determinations of oxygen concentrations in the brain cortex by means of the platinum microelectrode.

According to Davies and Bronk (*41*), oxygen tension against a pial vessel of the cat brain is 33 mm with a 7.8-percent concentration of inspired oxygen, and oxygen tension in the venule is 15 mm (*44*, *45*). If the data on the cat brain are assumed to apply to the rat, the gradient of oxygen tension from the pial vessel to an averaged intracellular concentration is from 33 to 1.0 mm-Hg. Although Davies's data are for cat brain, the results of Cater (*35*) on the rat brain suggest no large differences between the two animals. Thus a decline of oxygen tension from that of the capillaries to the values observed by these methods must be seriously considered (*44*, *45*).

At a concentration of inspired oxygen of 4 percent (30 mm), respiration is just sustained and the increment of pyridine nucleotide reduction is 30 to 60 percent (roughly 50 percent). The corresponding intracellular oxygen concentration is 0.3 micromole, or 0.2 mm-Hg. These results emphasize the extremely efficient use of intracellular oxygen by the respiratory chain and suggest that the intracellular measurements may be more readily correlated with physiological function than the extracellular values.

We may also compare the oxygen tensions in hypoxic brain and kidney on the basis of the data of Fig. 4 and the section on oxygen sensitivity of the brain cortex, which give correlations between the increment of pyridine nucleotide reduction and the moment of cessation of breathing in a rapid transition from aerobiosis to anoxia. We may assume that the oxygen affinities of kidney and brain mitochondria are the same, since we have as yet no data to suggest large differences in the oxygen affinity of the mitochondria in different tissues. On the basis of the results described, we find that the 28-percent increment of reduction in the kidney corresponds to 1 micromole, or 0.6 mm of O_2, and that the 87 percent increment in the brain corresponds to 0.1 micromole or 0.06 mm of O_2—a tenfold difference in the intracellular oxygen tension in these two organs at the moment of cessation of breathing.

Other applications of microfluorometry of pyridine nucleotide in tissue. In addition to the applications of microfluorometry to rat kidney and rat brain described in this article, studies on liver are in progress, although at present the controls are not clear-cut. Studies on the skeletal muscle have already been carried out on a large scale (*19*), and a number of preliminary experiments on the intact frog and on the toad sartorius muscle are in progress. Preliminary studies of the rat diaphragm and the brown fat body have been carried out by Serlupi-Crescenti and are being continued by him elsewhere. R. P. Davis has applied the Aminco-Bowman fluorometer to the study of large areas of the excised toad bladder (*46*).

We have used the microfluorometer for observation and localization of fluorescence changes consequent to electrical activity in the electric organ of *Electrophorus,* in collaboration with R. D. Keynes. Correlations of active transport and energy metabolism in the frog skin and toad bladder are being studied in Copenhagen with an identical microfluorometer by Hans Rasmussen and Ulla Fugmann.

Summary

It now appears to be possible to continuously record changes in intracellular oxidation-reduction levels in terms of the fluorescence of reduced pyridine nucleotide in mitochondria of various tissues and organs *in situ*. Studies of kidney and brain cortex in the rat show that changes in fluorescence are not measurably affected by the presence of oxyhemoglobin. Nitrogen, sulfide, cyanide, and carbon monoxide cause increases in fluorescence to very nearly the same levels, and the increases are attributed to larger reduction of mitochondrial diphosphopyridine nucleotide. Amytal at a low blood concentration causes increased reduction in the kidney cortex, and at a high blood concentration, in the brain cortex. The qualitative response of the pyridine nucleotide to low oxygen concentrations shows the brain to be more sensitive than the kidney. The first measurable increase in pyridine nucleotide reduction observed on the brain occurs at a concentration of inspired oxygen of 8 percent. Breathing stops when the percentage increase of pyridine nucleotide reduction on the brain reaches about 90; at this point the percentage increase for the kidney is only about 30. This difference corresponds roughly to a tenfold difference in oxygen tension. Half-maximal increase in pyridine nucleotide reduction on the brain occurs at a concentration of inspired oxygen of about 4 percent and corresponds to an intracellular oxygen tension of about 0.2 mm (*47*).

References and Notes

1. G. A. Millikan, *Proc. Roy. Soc. London* **B123**, 218 (1937).
2. B. Chance, *Federation Proc.* **16**, 665 (1957).
3. ——— and G. R. Williams, *J. Biol. Chem.* **217**, 409 (1955).
4. ———, *Nature* **176**, 250 (1955); *Advan. Enzymol.* **17**, 65 (1956).
5. D. Keilin, *Proc. Roy. Soc. London* **B98**, 312 (1925).
6. F. Urban and H. B. Peugnet [*Proc. Roy. Soc. London* **B125** (1938)] early claimed to have observed oxidation-reduction changes in flavin and cytochrome pigments of excised gastrocnemius. Later studies [B. Chance and C. M. Connelly, *Proc. Philadelphia Physiol. Soc.* **29**, 12 (1953–54); *Am. J. Med. Sci.* **227**, 710 (1934); F. Buchtal, G. G. Knappeis, T. Sjostrand, *Skand. Arch. Physiol.* **82**, 249 (1939)] have indicated that the tissue was probably anaerobic at the time of the experiments, and the use of unperfused tissue involves hemoglobin interference with the observation of the oxidation-reduction change by colorimetry.
7. A. Arvanitaki and N. Chalazonitis, *Arch. Intern. Physiol.* **54**, 406 (1947).
8. H. Lundegårdh, *Arkiv. Kemi* **3**, 69 (1951).
9. B. Chance, *Nature* **169**, 215 (1952); *Federation Proc.* **11**, 196 (1952).
10. C. M. Connelly and B. Chance, *Federation Proc.* **13**, 29 (1954).
11. B. Chance, *Harvey Lectures Ser.* **49 (1953–54)**, 145 (1955).
12. ——— and C. M. Connelly, *Nature* **179**, 1235 (1957).

12a. B. Chance, G. Mauriello, X. Aubert, in *Muscle as a Tissue*, K. Rodahl, Ed. (McGraw-Hill, New York, 1962).

13. A. M. Weber, *Federation Proc.* **16**, 267 (1957); J. R. Ramirez, *J. Physiol.* (*London*) **147**, 14 (1959; F. Jobsis, *Ann. N.Y. Acad. Sci.* **81**, 505 (1959).
14. D. F. Cain and R. E. Davies, in *Muscle as a Tissue*, K. Rodahl, Ed. (McGraw-Hill, New York, 1962), p. 84. See also W. F. H. M. Mommaerts, *ibid.*, p. 146; D. F. Cain and R. E. Davies, *Biochem. Biophys. Res. Commun.*, in press.
15. O. Warburg, *Wasserstoffubertragende Fermente* (Saenger, Berlin, 1948), p. 21.
16. B. Chance and H. Baltscheffsky, *J. Biol. Chem.* **233**, 736 (1956).
17. P. D. Boyer and H. Theorell, *Acta Chem. Scand.* **10**, 447 (1956); L. N. M. Duysens and J. Amesz, *Biochim. Biophys. Acta* **24**, 19, (1957).
18. A. D. Winer and H. Theorell, *Acta Chem. Scand.* **13**, 1038 (1959).
19. B. Chance and F. Jobsis, *Nature* **184**, 195 (1959).

20. B. Chance, G. Mauriello, X. Aubert, in *Muscle as a Tissue*, K. Rodahl, Ed. (McGraw-Hill, New York, 1962).

21. B. Chance and B. Thorell, *Nature* **184**, 931 (1959).
22. ———, *J. Biol. Chem.*, **234**, 3044 (1959).
23. B. Chance and V. Legallais, *Rev. Sci. Instr.* **30**, 732 (1959).
24. F. S. Sjostrand, *Acta Anat. Suppl.* **1**, 1 (1944).
25. B. Chance, P. Cohen, F. Jobsis, B. Schoener, *Science* **136**, 325 (1962).
26. M. Klingenberg, *Mosbach Colloq.* (Springer, Berlin, 1960), p. 82.
27. B. Chance, *Science* **120**, 767 (1954); ———, in *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo-Kyoto, 1957* (Maruzen, Tokyo, 1958), p. 295.
28. ———, in preparation.
29. ———, *Ann. N.Y. Acad. Sci.* **97**, 431 (1962).
30. ——— and R. D. Keynes, in preparation.
31. D. M. Aviado, *Anesthesiology* **20**, 71 (1959).
32. W. E. Spoerel, *Anesthesia Analgesia, Current Res.* **40**, 94 (1961).
33. C. Bernard, *Leçons sur les Substances Toxiques* (Paris, 1857).
34. B. Chance in *Enzymes: Units of Biological Structure and Function*, O. H. Gaebler, Ed.

(Academic Press, New York, 1956), p. 447.
35. D. B. Cater, *Proc. Roy. Soc. London* **B155**, 136 (1961).
36. H. A. Krebs and L. V. Eggleston, *Biochem. J.* **82**, 134 (1962).
37. G. Serlupi-Crescenti, unpublished data.
38. R. Lemberg and J. W. Legge, *Hematin Compounds and Bile Pigments* (Interscience, New York, 1949).
39. B. Chance and C. M. Connelly, unpublished data.
40. M. Klingenberg, W. Slenczka, E. Ritt, *Biochem. Z.* **332**, 47 (1959).
41. G. E. Glock and P. McLean, *Biochem. J.* **61**, 388 (1955).
42. M. Klingenberg, personal communication.
42a. W. N. Aldridge, *Ciba Foundation on Enzymes and Drug Action* (Churchill, London, 1962), p. 155.
43. Unpublished data.
43a. D. K. Hill, *J. Physiol.* **107**, 479 (1948).
44. P. W. Davies and D. W. Bronk, *Federation Proc.* **16**, 689 (1957).
45. P. W. Davies, personal communication.
46. R. P. Davis and M. Canessa-Fischer, *Abstracta* (53rd Annual Meeting of the American Society for Clinical Investigation, Atlantic City, May 1961), p. 15; M. Canessa-Fischer, C. M. Edelman, Jr., R. P. Davis, *Federation Proc.* **21**, 148 (1962).
47. Dr. Blake Reeves participated in early phases of the development of the microfluorometer and Dr. J. Ramirez applied it to a study of excised heart strips. Dr. Hans Rasmussen and Dr. Ulla Fugmann applied the method to excised frog skin and toad bladder. Dr. Richard Keynes participated in applications of microfluorometry to the excised electric organ of *Electrophorus*. Dr. Serlupi-Crescenti applied the spectrofluorometer to excised liver diaphragm and the brown fat body. Dr. Frans Jobsis participated in experiments on renal artery perfusions of rat kidney. Dr. William Masland carried out the brain operations. Victor Legallais contributed the excellent designs of the spectrophotometer and microfluorometer. We are grateful to our colleagues for thoughtful criticism of this manuscript and for many useful suggestions. This work has been supported in part through grants from the National Science Foundation, the U.S. Public Health Service, and the Army Chemical Center. Dr. Peter Cohen is a member of the department of anaesthesiology and is supported under training grant 2G-215C4 from the U.S. Public Health Service.

Cesium-137 in Man

Fallout was decreasing at a rate equivalent to a half-life of 1 year before testing was resumed.

Charles O. Onstead, Erich Oberhausen, Frank V. Keary

The results of our measurements of the cesium-137 level in 1000 individuals living in Germany were reported in an earlier publication (*1*). We made these measurements in Landstuhl, Germany, during the period June through December 1959, with the aid of the two-π liquid scintillation whole-body counter designed by Anderson and his coworkers (*2*). The program was continued, and by 1 September 1961, levels in more than 6000 individuals living in Germany, whose average diet is shown in Table 1, had been measured. The large number of measurements makes possible a thorough study of the influence of age and sex, as well as diet, on the cesium-137 content in the human body. Because cesium and potassium are chemically similar, and because earlier work (*1, 3*) had revealed that the concentration of potassium in the human body is dependent on age and sex, we expected to find that levels of cesium-137 are age- and sex-dependent. Our studies also enabled us to follow the cesium-137 content of the general population up to the time that atmospheric testing of nuclear weapons was resumed by the Soviet Union. Various investigators (*4–6*) have reported a relatively steady increase in the cesium-137 levels during the period 1956 through 1959. This was to have been expected, since testing of fallout-producing nuclear weapons continued until the latter half of 1958. It was realized that, pending the resumption of testing, fallout, and consequently the cesium-137 content in the human being, would eventually begin to decrease. The factor primarily responsible for determining the rate of decrease is the length of time the fission products remain in the stratosphere. In a recent article, Rundo (*6*) reported that the cesium-137 content in the human being has been decreasing since December 1959. His study was based on regular measurement levels in 11 individuals. No conclusions were reached regarding the rate of the decrease.

With our more voluminous material, an attempt was made to follow the changing concentration of cesium-137 in man by studying the average findings in the general population. It was necessary to establish correction factors for sex and age, since the average cesium-137 values were found to be dependent, to a certain extent, on sex and age as well as on time. Figures 1 and 2 demonstrate the relationship of levels of cesium-137 to age for males and females of our study, as indicated by measurements made between 1 July 1959 and 1 September 1961. These results are not corrected for variations in the cesium-137 content in man which occurred over this long period. This may account for the distance of some of the points from the curves.

It is evident from Fig. 1 that the cesium-137 content per kilogram of body weight is age-dependent. Since the ratio is much lower for children than for adults, the dose rate from this isotope is correspondingly lower for children.

Assimilation, by the body, of cesium and of potassium differs with sex and age, as indicated in Fig. 2. The fact that the ratio of cesium-137 to potassium is much lower in children than in adults indicates that children discriminate against cesium in favor of potassium, whereas, as other workers have found (*5*), adults discriminate against potassium in favor of cesium. At an age of about 22 years, the ratio of cesium-137 to potassium reaches a constant figure; thereafter it is independent of age. However, the ratio remains lower in females than in males. We investigated the possibility that this variation could have been caused by a systematic error in our measurements or calculations but found no discrepancies. Since the findings are statistically valid, it must be concluded that the ratio of cesium-137 to potassium varies with sex and varies between child and adult. Since potassium, and presumably cesium, are primarily intracellular constituents of the human body, it may be said that certain groups of cells keep changing their discrimination patterns with respect to these two elements until the individual is 22. This conclusion,

Major Onstead is with the U.S. Army Medical Research Unit, Europe. Dr. Oberhausen is a physicist at the Institute of Biophysics, University of the Saarland, Homburg, Germany. Mr. Keary, who was formerly with the Army Medical Research Unit, Europe, is now at the University of Ottawa, Ottawa, Ont.