

Oxygen Tension: Direct Observations on the Critical Level for the Brain*†

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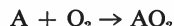
In this lecture I want to address myself to the questions: What is the lowest oxygen tension compatible with adequate function, and what are the factors that set this limit? Much of the background to the material presented here has been reviewed extensively in the not too distant past (Jöbbsis '64), and I will take the liberty of letting that article provide most of the detailed justification of my remarks today.

I. Mechanisms of Oxygen Utilization

In mammals there are three mechanisms for the utilization of dissolved oxygen. First, I will list these, and then I will emphasize the last one of these, since it accounts for the major part, by far, of the oxygen uptake in the body. These three are:

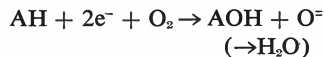
A. Oxygen transferases

for which the generalized reaction can be written



in which A is a substrate that accepts two separate oxygen atoms;

B. Mixed function oxidases with the reaction



where the last notation signifies the rapid final step of water production from $O^=$ and $2H^+$ ions;

* Supported in part by Grant NB 06729 from the National Institutes of Health.

† Presented at the *Symposium on Intracellular pH, PCO₂, and PO₂*, September 29, 1967, Medical College of Virginia, Richmond.

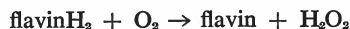
C. Electron transfer oxidases catalyzing the reaction



Numbers one and two are mainly active in the catalytic oxidation of amino acids and of some steroids and account at most for 1% of the total O_2 consumption in mammals. They are, therefore, of very little importance to us in this symposium.

The enzyme systems catalyzing the last category can be subdivided into, firstly, autoxidative flavoproteins and, secondly, the respiratory chain.

1. The oxidation of autoxidative flavoproteins can be written



The hydrogen peroxide formed is removed by the action of catalase. These reactions may account for about 10% of the total oxygen uptake in the body. We are not too sure where this uptake takes place or even what subdivision of the cell is the site of this oxygen consumption. One of the few known variables affecting this reaction is acclimation. Russian workers have shown that, during hibernation, the fraction mediated by the autoxidative flavoproteins goes from 10% to approximately 25% (Skulachev, 1962). Another important fact is that no mechanism has been found by which energy conservation takes place in this reaction. More specifically, no high energy phosphate production has been found associated with this reaction.

2. The respiratory chain accounts for all the rest of the oxygen taken up by the tissues. This is the mitochondrial contribution to overall oxidative metabolism. A system

of redox couples catalyzes the transfer of reducing equivalents from the substrates to oxygen and results in the formation of water. This is shown in Figure 1 together with some of the inhibitors and side reactions of the respiratory chain.

In the respiratory chain three locations, identified by asterisks in the above scheme, contribute to the overall process by being loci for the conservation of energy in the form of high-energy phosphate bonds ($\sim P$) that are transferred to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP). The reaction, which is referred to as oxidative phosphorylation, is obligatorily coupled to the electron transfer steps in the chain. This coupling produces an effective control mechanism for the rate of oxygen uptake. If no ADP is available, no oxidative phosphorylation can take place, and electron transport is slowed down dramatically. This gives rise to the difference in rates of O_2 consumption during rest and during strenuous exercise. Failure of oxidative phosphorylation to take place is the main harm associated with anoxia. The respiratory chain plus its side reaction, oxidative phosphorylation, is, therefore, the reaction system that must be understood for any discussion of critical O_2 levels. I would like to discuss these processes now.

Starting at the substrate side of the respiratory chain, we enter with certain substrates and their dehydrogenases which will reduce NAD^+ to form NADH (nicotinamide-adenine dinucleotide). [The old-fashioned name was DNP, and many people still refer to it as DPN^+ and $DPNH$]. NADH is able

to reduce flavoproteins and, then, from there, follow a set of redox couples, each one oxidizing its predecessor by electron transfer and being oxidized by the next one until O_2 is reached. This is a set of cytochromes identified by letters, i.e., cytochrome *b*, cytochrome *c*₁, cytochrome *c*, cytochrome *a* and cytochrome *a*₃. Potential differences between the numbers of the respiratory chain are neatly arranged along the line so that electron transfer proceeds naturally toward O_2 .

At three locations, the potential energy of the overall reaction—which in the end is really the reaction of hydrogen in the substrate with oxygen to form H_2O —this potential energy of burning up the substrate is saved. In these three spots, reactions take place resulting in the formation of ATP from ADP and inorganic phosphate. There are three of these sites, and three P moieties are formed per oxygen atom. However, there are certain substrates, such as succinate, that are oxidized without benefit of NAD, and their oxidation only yields 2~P, since the site between NAD and flavoprotein is lost.

These two functions, electron transfer and high energy phosphate production, are very tightly coupled. They are obligatorily linked. Without the one the other does not take

place, unless something is amiss. This brings with it some very important corollaries. For instance, if there is not enough ADP near the mitochondria, oxygen uptake comes to a halt, because partial blocks occur at three steps. This is referred to as respiratory control. In the same vein, the absence of electron acceptor (O_2) results in stoppage of electron transfer and, therefore, no further oxidative phosphorylation takes place. This is the fatal outcome of asphyxiation.

II. Main Phases of Foodstuff Degradation

After this very rapid introduction, I would like to discuss some of these points more quantitatively and emphasize the methods that are available for the study of the respiratory chain. I think this is very important, and I am glad that Professor Patterson emphasized your interest in methods. Without an understanding of the methods, we really don't have a thorough understanding of the reliability of a scientific assertion, especially at the level of complexity that we meet in biology. Therefore, I will emphasize the methods as we go along.

Starting then on the substrate side, Table 1 summarizes the re-

actions between the ingestion of food and the form in which it enters the Krebs cycle. The table is adapted from Krebs and Kornberg (1957) and speaks further for itself. What occurs is a rather amazing simplification of a great variety of compounds. The cell modifies and simplifies these complex substances into a rather small number of intermediates. Thus, we end up with acetyl coenzyme A and a few other things such as α -ketoglutaryl and oxaloacetic acid. In the third phase these three feed into the citric acid cycle, and there decarboxylation and dehydrogenation take place. This, of course, is the important step; this is where NADH is being formed. I always like to think of decarboxylation as just getting rid of the carbons. No energetically advantageous steps are involved in it. The carbons can be looked upon as nothing more than vehicles for carrying hydrogens around the citric acid cycle for the reduction of NAD and flavoproteins.

In Table 2 I have taken these same four phases and emphasized the high energy yield per carbon atom. It is very straightforward for carbohydrates: 6.4~P per carbon. It is a bit arbitrary for fats, because they differ so much in chain length. I have chosen palmitate as

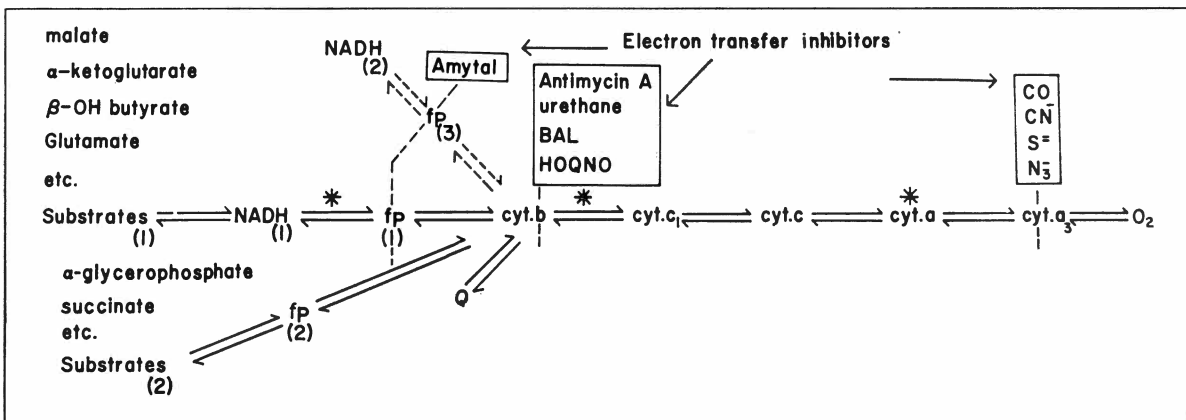


Fig. 1—The respiratory chain. The asterisks indicate the probable locations of the phosphorylative steps. The dashed lines illustrate the point of action of inhibitors blocking the transfer of electrons to oxygen. (BAL: British Anti-Lewisite; HOQNO: δ -hydroxy quinoline N-oxime.) (Reprinted with permission from *Handbook of Physiology*, Jöbsis, 1964.)

TABLE 1
The four main phases of foodstuff degradation

Phase	Outline of Chemical Change						Primary Reactions	
I	carbohydrates ↓ hexoses		proteins ↓ about 20 amino acids		fats ↓ glycerol; fatty acids		depolymerization	
II	hexoses ↓ lactic acid	glycerol ↓ pyruvic acid	several amino acids (alanine, serine, cysteine) ↓ acetyl coenzyme A	fatty acids ↓ acetyl coenzyme A	several amino acids (3 leucines, tyrosine, phenylalanine) ↓ acetyl coenzyme A	several amino acids (glutamic acid, histidine, prolines, arginine) ↓ α-ketoglutaric acid	several amino acids (aspartic acid, tyrosine, phenylalanine) ↓ oxaloacetic acid	dehydrogenation, decarboxylation, deamination and shortening of the carbon chain
III	acetyl coenzyme A		α-ketoglutaric acid		oxaloacetic acid		decarboxylation and dehydrogenation	
IV	NADH		flavin-H ₂		respiratory chain		H ₂ O production	

TABLE 2
Location, products and profits of the four main phases of foodstuff degradation

Phase	Primary Chemical Change	Location	Waste Products	Reducing Equivalents		~P Yield Per Carbon Atom	
				Yield	Destination	Carbohydrates	Fats
I	Depolymerization	Extracellular and cytoplasmic	None	None		None	None
II	Dehydrogenation, decarboxylation, deamination, and shortening of the carbon chain	Cytoplasmic and intramitochondrial	NH ₃ and urea; small amounts of CO ₂ and H ₂ O	Small amounts of NADH and flavin-H ₂	Used in IV	0.33	-0.06
III	Decarboxylation and dehydrogenation	Intra-mitochondrial fluid phase	CO ₂	Large increase in NADH and flavin-H ₂ ; small increase in NADPH	Used in IV	0.33	0.50
IV	H ₂ O production	Mitochondrial membranes	H ₂ O	Large decrease in NADH and flavin-H ₂		5.70	8.00

an example. The longer the chain, the higher your net yield, since you have to pay a standard price in activation no matter how long the chain. I have not even attempted to calculate a similar number for amino acids; they are too dissimilar.

Note that, in the first stage, there is no yield of high energy phosphates. In the second stage there is a very small yield, and actually there is utilization of high energy phosphates in fatty acid metabolism. There is an extremely small provision by so-called substrate level phosphorylations in the third phase, the citric acid cycle level. In the fourth or final phase, however, the great bulk of high energy phosphates arises. In other words, the most important yield derives from the last phase, and it is mediated by the respiratory chain.

It is simple enough to measure these reactions in a vessel, a manometric one if you need to know the amount O_2 consumed. It is more meaningful, however, to study the system in the intact, circulated tissue rather than with slices or iso-

lated mitochondria. It is here that special instrumental methods are needed. Almost without exception such methods have been pioneered since 1946 by Dr. Britton Chance at the Johnson Foundation, Departments of Biophysics and Physical Biochemistry of the University of Pennsylvania.

III. Split-Beam Spectrophotometric Method

The great boon in the study of the respiratory chain, and actually the factor upon which the discovery of the cytochromes hinged, is the fact that each of the members has optical activity: they absorb light at specific wavelengths. In the 1920's, Professor Keilin first described the series of components that is now known as the cytochrome chain (Keilin, 1925). Although he drew some strong inferences regarding its function, he did not know, at that time, that energy was being conserved in conjunction with the cytochrome activity. That had to wait until the

late 1930's. All of his conclusions were the result of visual spectroscopy. Nowadays we use spectrophotometry to do the same thing. In this approach one first needs to determine the identity and the concentration of different components. For this purpose a special type of spectrophotometer, the so-called "split-beam," is used. It is especially designed for highly light-scattering preparations such as mitochondria suspended in a solution or intact tissue. The instrument is shown in Figure 2. Starting from the light source, the beam is diffracted in a monochromator and is alternately deflected by means of an oscillating mirror through two cuvettes placed side by side. One of these is the sample; the other serves as the reference. The light emerging from these two cuvettes is measured by a photomultiplier. There is a bit of electronic circuitry concerned with unscrambling the pulses so that one can keep track of the light absorption of the sample as compared with the reference cell. After subtraction, the difference is recorded logarithmically. This renders the data directly in OD units which are proportional to concentrations. The monochromator sweeps through the wavelengths from about $650 m\mu$, which is the dark red, to about $300 m\mu$ in the very near UV. The differences in absorption are recorded on a wavelength scale. Starting with two suspensions of isolated mitochondria, for example, the first run provides a base line, since one started out with equal samples. After an experimental manipulation, for instance the removal of oxygen, one sweeps through again, and the difference between the oxidized and the reduced materials is now recorded. Figure 3 shows two absolute spectra and their difference spectrum, using hemoglobin as an example. The absorption spectrum of hemoglobin in the oxygenated form is shown by the solid curve, in the disoxygenated form by the broken curve. These are absolute spectra;

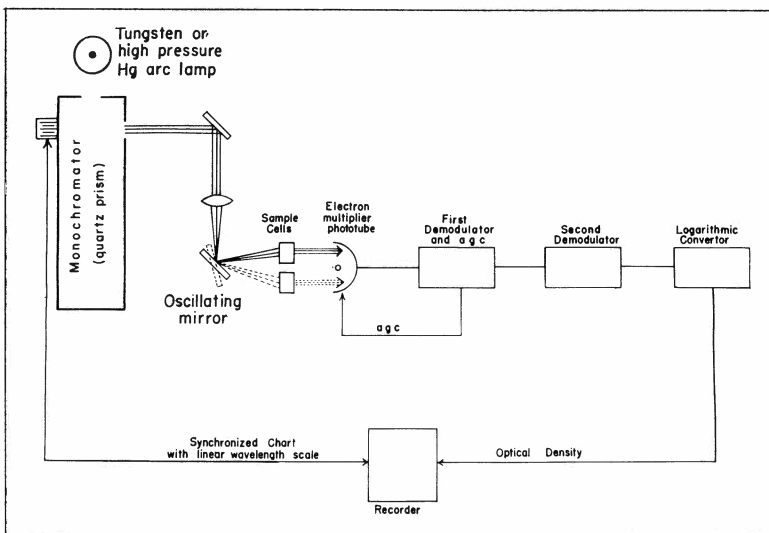


Fig. 2.—The split-beam, difference spectrophotometer. The difference in light intensity emerging from the two sample cells is logarithmically recorded on a chart directly coupled with the wavelength drive of the monochromator. The resulting graph is a recording of the difference in optical density at the various wavelengths. (Reprinted with permission from *Methods in Enzymology*, Chance, 1957.)

but if one puts a disoxygenated sample of hemoglobin in the sample cuvette and oxygenated hemoglobin in the reference cuvette, the resulting curve is a difference spectrum, as shown in the upper part of Figure 3.

Studies on Mitochondria

The same thing can be done for the respiratory chain in the mitochondria. Mitochondria are obtained from the cells by grinding and differential centrifugation. They are suspended in an appropriate medium and aliquots are pipetted into the two cuvettes. The spectrum in the top of Figure 4 shows the differences in OD between oxygenated and anoxic mitochondria. The latter were on the sample side; the former served as reference. The location of the peaks identifies the components; the height of the peaks, the concentration. Reduced cytochromes and NADH have absorption bands that are sharper and more intense than those of the oxidized form. Therefore, the peaks are in the upward direction. The cytochromes produce a complex of beta bands which are rather indefinite and are grouped together. Note that the large trough at 465 $m\mu$ is related to flavoprotein reduction; in contrast with the cytochromes, the flavoproteins absorb more strongly in the oxidized form.

Studies on Muscle

Since I am a physiologist, I consider it important to show that this is not a biochemist's artifact; that it is possible to measure the cytochrome absorption peaks in whole cells also. One does this by taking two pieces of tissue, for instance, two muscles carefully dissected, one from the left leg and one from the right leg, and measuring the difference spectrum between the anoxic sample and the oxygenated reference. The result is the lower

spectrum of Figure 4. With some minor differences it is much the same as the spectrum of the isolated mitochondria. So it is possible to make these observations and measure the concentrations in intact tissues. For a physiologist that is reality. Now we can use the level of reduction as an indicator system. For Professor Keilin it was the beginning. After his visual observations of the cytochrome bands in intact cells, he was obliged to isolate and identify the compounds giving rise to them. Some thirty

years later Dr. Chance was able to quantitate the observation in tissues with his new instrumentation. Nowadays it is not too difficult to make identifications and measure concentrations in most any tissue that interests you.

Reduction Level of Metabolic States

Since we now have a method for following changes in oxidation and reduction of the members of the respiratory chain, the ques-

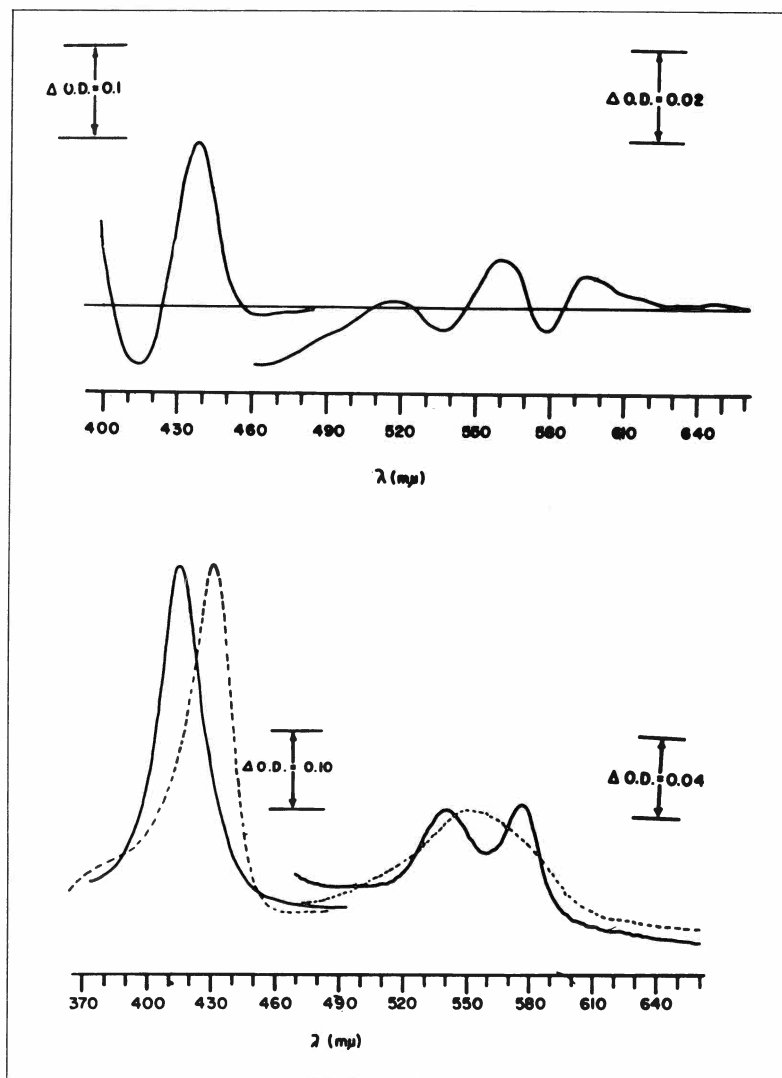


Fig. 3—Absolute spectra of oxygenated (solid line, bottom) and disoxygenated hemoglobin (broken line) and their difference spectrum (top).

tion of their reduction level in different metabolic states can be tackled. Not that we are so very interested in the cytochromes per se, but they serve as indicators of changes in the rate of oxygen uptake. For example, O_2 uptake will be slow when ADP is lacking in a suspension of mitochondria otherwise supplied with all the needs for oxidative phosphorylation (i.e., O_2 , inorganic phosphate and oxidizable substrates). Addition of ADP will then produce a rapid uptake of oxygen, which will slow down again when all the ADP has been turned into ATP. This can be followed very easily with an oxygen polarograph, an "oxygen electrode." This is the ideal experiment for determining what happens to the reduction states of the members of the respiratory chain when oxidative metabolism changes, as I will show in just one minute. Concomitantly with the O_2 , substrate, ADP and inorganic phosphate will disappear. A measure of the efficiency of phosphorylation is the well-known P to O ratio, i.e., the ratio of the amount of inorganic phosphate that disappears to the number of oxygen atoms utilized. For an NADH-linked substrate it is 3; for a flavo-protein substrate it is 2; when the entire Krebs cycle is operating, we get an intermediate value, say, 2.5 to 2.7.

Such measurements are possible with mitochondria in a cuvette, but not in intact cells. What is possible, however, is the monitoring of the optical density changes related to reduction level changes of the cytochromes. These absorption effects were first measured in isolated mitochondria and were found to be very specific as we do the ADP addition experiment. For this we use a slightly different spectrophotometer. The first one I described can only be used for steady states when we are identifying components or looking for concentrations. Now we need to follow changes in absorption over a limited time span.

IV. Double-Beam Spectrophotometric Method

For this purpose Dr. Chance developed the so-called "double-beam" spectrophotometer (Fig. 5). A single light source is used for two monochromators. One is set for the wavelength of an absorption peak, the other at an indifferent wavelength to serve as a reference beam. The two beams are alternately switched by a vibrating mirror, proceed through the sample, and the emerging light is measured by an end-window photomultiplier. This

system is needed, because the suspensions (and the tissues as well) are turbid. What is more, the light scattering is greatly affected by all sorts of metabolic reactions. When light-scattering changes take place, they are indistinguishable from absorption changes. Both result in changes in photomultiplier current, because light is lost. However, it is lost equally, at first approximation, from the reference and measuring wavelengths. Therefore, changes in the difference between the two beams are significant, not the total signal. Total signal changes 20

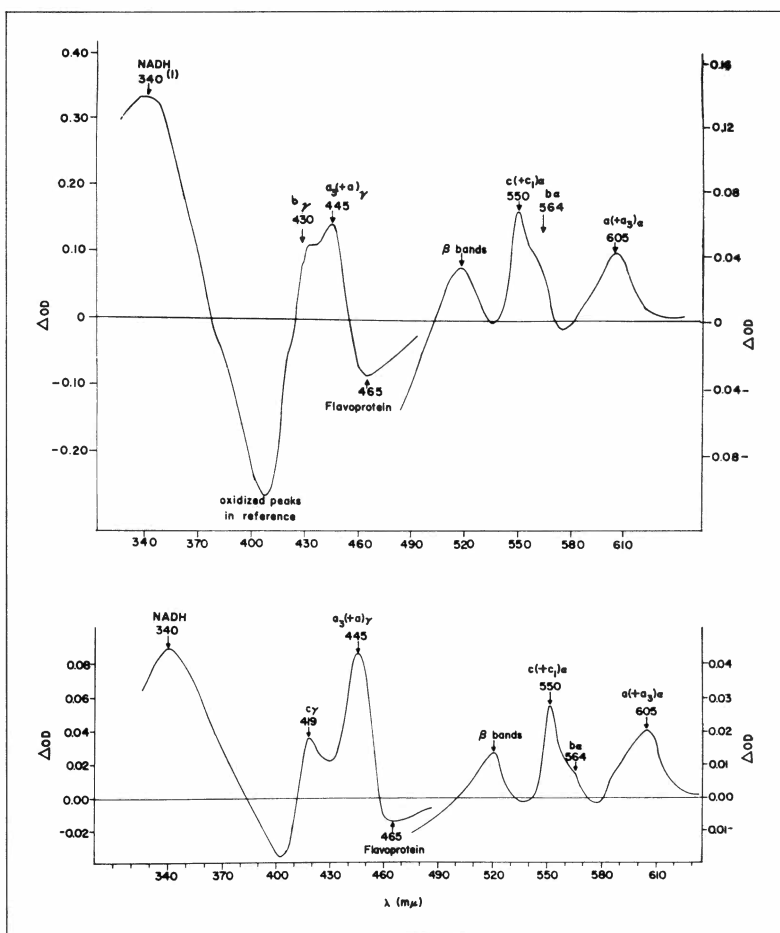


Fig. 4—Difference spectra of the respiratory chain. Sample: reduced by anoxia; reference: in the presence of oxygen. Upper curve: two aliquots of suspended mitochondria isolated from rat liver [anoxic (state 5) minus starved (state 2)]. Lower curve: two excised toad sartorius muscles [anoxic (state 5) minus resting with endogenous substrates (state 1)]. (Reprinted with permission from *Handbook of Physiology and J. Gen. Physiol.*, Jöbsis, 1963 and 1964.)

times greater than the true absorption changes can thus be tolerated.

Studies on Mitochondria

If we follow the absorption of one of these cytochromes as we go through some of the more obvious metabolic conditions, we will see a characteristic pattern emerge (Fig. 6). At 430 – 400 m μ , cytochrome *b* is monitored. As the mitochondria come out of the centrifuge tube and are put in a cuvette, we start the trace. Now we add ADP, and a decreasing absorption at 430 m μ compared to 400 m μ , signifies that an oxidation of cytochrome *b* takes place. This is inevitable. Substrates have not been added. The ADP-stimulated, high rate of O₂ uptake soon exhausts the endogenous substrate supply of the mitochondria, and cytochrome *b* goes fully oxidized. At this point β -hydroxybutyrate is added, NAD is reduced: we see a change towards a higher level of reduction. A plateau is maintained for a short while; then another change occurs. Concomitant biochemical determinations will show that at this point the ADP that was added has now been phosphorylated to ATP. Electron transfer slows down, and a more reduced state results because of the blocking of the respiratory chain further down, close to oxygen. Reducing equivalents accumulate, therefore, in cytochrome *b*. When oxygen uptake is stimulated again by a new addition of ADP, cytochrome *b* returns to the previous, more oxidized state while ATP is being formed. After a number of such cycles, all the oxygen is used up, and an extremely large change will indicate that *b* goes completely reduced. Very much the same thing occurs for NADH, as is shown in the right-hand record of Figure 6.

These steady-state changes are the important ones for our attempts to measure metabolic rates in the whole cell—especially this last transition to full reduction when the PO₂ is inadequate. It provides the

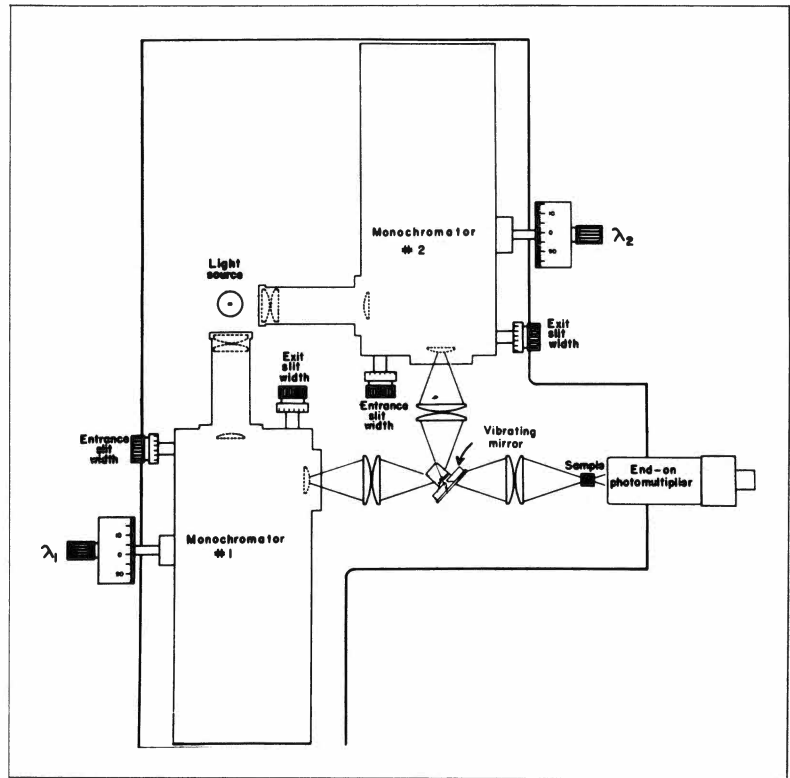


Fig. 5—The double-beam, difference spectrophotometer. The difference in light absorption of two beams of different wavelengths are recorded as a function of time. The resulting graph shows the kinetics of the wavelength-specific absorption changes; for instance, after the addition of a reactant. (Reprinted with permission from *Methods in Enzymology*, Chance, 1957.)

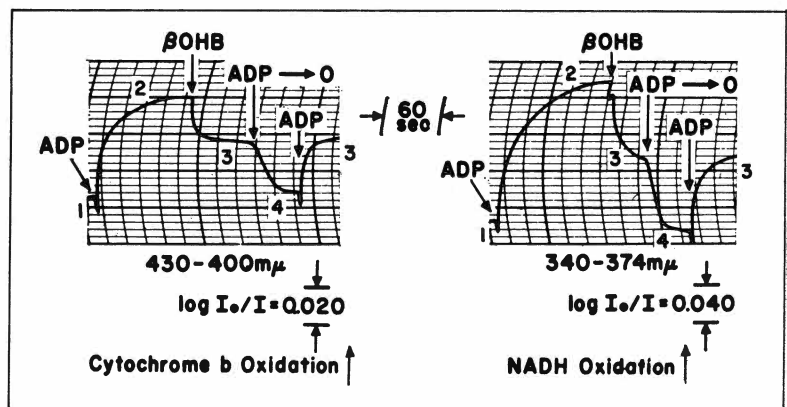


Fig. 6—Oxidation-reduction levels of cytochrome *b* and NADH, in suspensions of rat liver mitochondria in states 1 to 4. Note the reduction that occurs after addition of NAD-dependent substrate (β -hydroxybutyrate) and the return from the state -4 to the state -3 level upon the last ADP addition. (Reprinted with permission from *Handbook of Physiology*, Jöbsis, 1964, adapted from *J. Biol. Chem.*, Chance and Williams, 1955.)

OXYGEN TENSION

signal to determine the critical level of oxygen needed for the cell. In Table 3 the metabolic states are listed as they were defined in the original article of Chance and Williams in 1955. The numbers of the states correspond to those along the traces in Figure 6.

Studies on Muscle

Showing again my bias as a physiologist, I do want to emphasize that these changes do not only occur in mitochondria in a cuvette, but can also be seen in muscles. The same double-beam system can be used. Light of two wavelengths shines alternately through a muscle in the resting metabolic state. This provides the base line for the experiment (Fig. 7). A number of rapid twitches are induced, ATP is hydrolyzed to ADP and recovery metabolism starts. All the optical changes of Figure 7 are in complete accord with the notion that we are observing the switch to rapid metabolism (state 4 to state 3). When the ATP has been rephosphorylated, metabolism slows down and the optical density returns to that of the resting state. NADH and cytochrome *b* went through a cycle of increased oxidation; cytochrome *c* went more reduced. The difference between *c* in the muscle and the effect listed in Table 3 is not significant. Since 1955 it has been shown that *c* can be more reduced

in state 3 than in state 4. It depends on the tissue from which the mitochondria were prepared and on the type of substrate furnished. The important conclusion is that steady-state changes in the respiratory chain components can be demonstrated in intact tissues when the rate of O₂ consumption changes.

V. Fluorometric Method

Studies on Toad Sartorius Muscle

In addition to spectrophotometry, fluorometric observation of NADH is also possible and has great advantages, especially in tissues with intact blood supply. This method rests on the fact that reduced NAD fluoresces when illuminated with light near its absorption peak at 340 m μ . It then emits light with a broad maximum at about 450 m μ : a bluish cast comes from the tissue. Not all of it is derived from NADH, but a large part is. Since the oxidized form (NAD⁺) does not fluoresce, we now have another optical measure of the reduction state of NAD.

This method is actually simpler. The intensity of the fluorescent light is measured with a photomultiplier. The same surface that is exposed to the excitation light (usually the 366 m μ line of a high pressure mercury arc) is used for observation. An appropriate secondary

filter between the photomultiplier and the tissue screens out the reflected 366 m μ light. In addition to being simpler, the method is also more sensitive than the spectrophotometric one. This is shown in Figure 1, which shows the response to a single twitch. Note that the spectrophotometric cycles of Figure 8 were the responses to 30 twitches. Since this figure was published, further improvements have been made, so that now a response of the size shown in Figure 8 shows practically no noise. I have actually performed experiments with a reproducibility within 2% for a dozen single twitches over a period of three or four hours.

Studies on Tissue In Vivo

However, we need to know about the whole tissue, the intact tissue in vivo. Excised tissue is only one step in that direction. Now that there is a method available for looking at the surface of tissues and saying something about the reduction of NAD, we can apply it to tissues with intact circulation. For observation we can use any exposed tissue, in our case, most significantly, the cerebral cortex. The apparatus is relatively simple. A microscope with an epi-illumination system looks down on the cerebral cortex. Light from an arc lamp is made to emerge as a cone around the objective of the microscope and is

TABLE 3
Metabolic states of mitochondria and associated oxidation-reduction levels of respiratory enzymes

Characteristics						Steady-State Percentage Reduction of Components				
State	[O ₂]	ADP level	Substrate level	Respiration rate	Rate-limiting substance	a	c	b	Flavo-protein*	NADH ₁
1	>0	Low	Low	Slow	ADP	0	7	17	21	~90
2	>0	High	~0	Slow	Substrate	0	0	0	0	0
3	>0	High	High	Fast	Respiratory chain	4	6	16	20	53
4	>0	Low	High	Slow	ADP	0	14	35	40	99
5	0	High	High	0	Oxygen	~100	~100	~100	~100	~100

These values are based on the amount of flavoprotein of the respiratory chain. Other fractions, reduced by anoxia, are present but disregarded here. [After Chance & Williams]

focussed on the observed spot. Emitted fluorescence, as well as reflected excitation light, enters the objective lens and goes up the tube. The latter is filtered out so that only the fluorescence light remains. Much of it, but certainly not all, is that derived from NADH. A photomultiplier measures the light intensity at the level of the ocular.

Minimal O_2 Requirement of the Brain

This instrumentation enables us to evaluate the minimal O_2 requirements of the brain. The first type of experiment to do is the administration to the animal of gas mixtures low in O_2 . The type of effect we look for is an increase in fluorescence, signalling that in some parts of the tissue the respiratory chain is not furnished with O_2 and NAD^+ goes to NADH. Such an experiment on the kidney and brain of the rat is shown in Figure 9. The animal is switched from air to 100% N_2 or to 3% O_2 in N_2 . This results in a rise in the fluorescence, indicated by the downward excursion of the trace in Figure 9. I apologize sincerely for the change in direction. Dr. Chance and I have always been opposed in our views about how to display this, so I am afraid you will have to switch back and forth between the experiments that we did together and the ones that I did myself. In his case, an increase in fluorescence is downward, and in my case an increase in fluorescence is upward.

As expected, an increase in fluorescence accompanies anoxia. Addition of sulfide also increases fluorescence since it blocks the chain at the level of cytochrome a_3 . All other components of the chain, including NAD, become reduced. The same thing can be shown for cyanide and CO; they all block at the level of a_3 . Amytal will also produce increased fluorescence by blocking the chain, this time, however, by inhibiting the flavoproteins oxidizing NADH. These maneuvers with anoxia (or

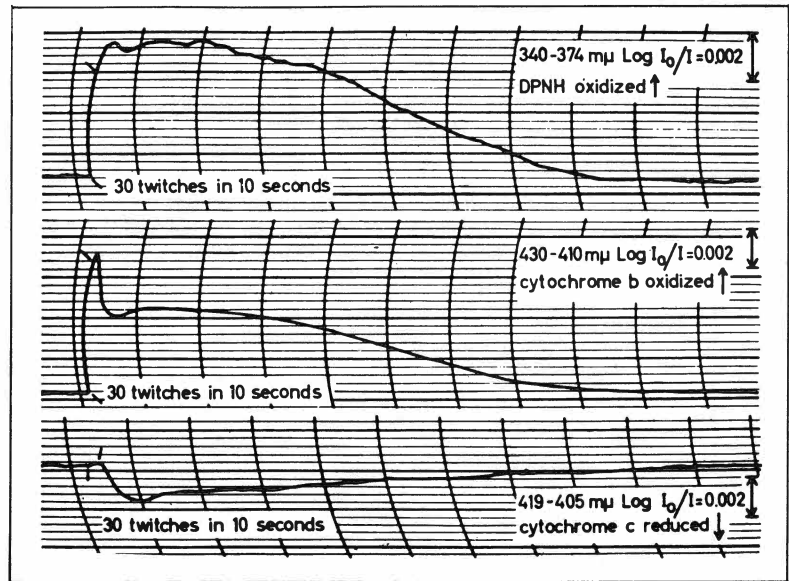


Fig. 7—Steady-state changes of DPNH (NADH), cytochrome b and cytochrome c in toad sartorius muscles during oxidative recovery from contractile activity. The vertical ordinates are one minute apart. (Reprinted with permission from *J. Gen. Physiol.*, Jöbbsis, 1963.)

hypoxia) and various inhibitors show that the instrumentation does perform in the intact tissue as hoped for.

Limiting Factors in O_2 Uptake

Now we should stop for a moment and reflect on the exact question of the day. We are asking: What are the parameters that

determine the level of O_2 necessary for the tissue? It has been my experience that most of us tend to look immediately toward the parameters of circulation, the source of the O_2 , such as hemoglobin, PO_2 , blood flow, A-V differences, etc. However, although terribly relevant, there is a more basic aspect. That is the question: What limits the rate of O_2 uptake when PO_2 does not?

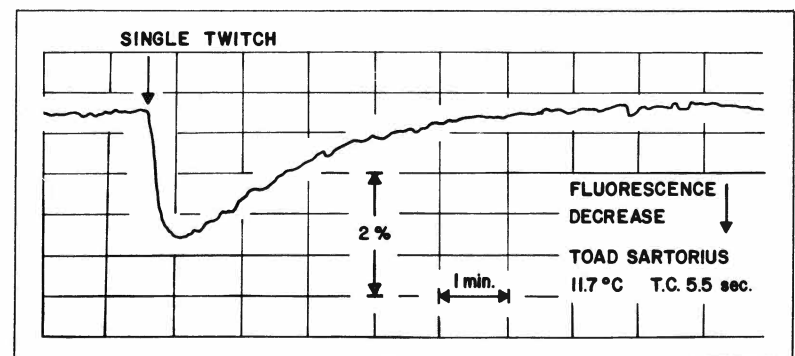


Fig. 8—Fluorescence cycle induced by an isometric twitch. Toad sartorius muscle. A decrease in NADH concentration would be registered in a downward direction. (Reprinted with permission from *J. Gen. Physiol.*, Jöbbsis and Duffield, 1967.)

Studies on Cells

First we should discuss what happens in intact cells. We can measure the rate of oxygen uptake as a function of the partial pressure of oxygen in the cuvette. Diffusion problems from the blood vessels to the cells are sidestepped. In Figure 10 we see such an experiment for yeast cells. The same thing has been done with other cells. It is im-

portant to obtain a good measurement of the PO_2 at which the rate of oxygen uptake is decreased. It is immediately obvious from the two sets of experiments that the presence of glucose makes an enormous difference on both the rate of oxygen uptake and the PO_2 at which the plateau rate starts to fall. The important point is that in the absence of glucose, the availability of substrate was the limiting factor long

before the PO_2 . Succinate, although metabolized rapidly, penetrates slowly and thus provides only inadequate substrate levels in the cells. Those experiments were, therefore, run in a semi-starved condition. The PO_2 at which the two systems begin to show signs of being limited by O_2 availability are 2.5 and 0.21 mm Hg. This tenfold difference is the result of the difference in rate of metabolism due to substrate limitations. When you ask me what the limiting level of O_2 is, I will have to ask you, first, what the rate of respiration is in terms of total capacity and, then, what is limiting the rate? Because it could also quite well be that the limit is not the substrate but the blocks accompanying a decreased demand for oxidative phosphorylation. The effects of these two causes of a less than maximal rate will be slightly different, but, for the moment, that is of secondary importance.

One can make a rather complete mathematical description of the respiratory chain system, if that is necessary and useful. Then one can calculate a number of constants, such as the apparent K_m for O_2 ; extrapolate the data to infinite concentrations of O_2 , substrate, ADP and inorganic phosphate; and finally come out with a limiting value for PO_2 near the mitochondria. I have done my homework on this and the calculated, "corrected" curves are shown as broken lines in Figure 11. In this way I obtained values of approximately 0.5 mm Hg ($1\mu MO_2$) for 50% inhibition and 5 mm Hg ($9\mu MO_2$) for a 10% loss in O_2 uptake. However, the numbers have little meaning except as upper limits. Any time the rate of O_2 uptake is not maximal, these minimal levels will be considerably lower; in the case of nearly starved yeast, perhaps 25 times.

The figures above are for conditions in which the respiratory chain is the only limiting factor. However, as I said, that is hardly ever the case. Usually the system is

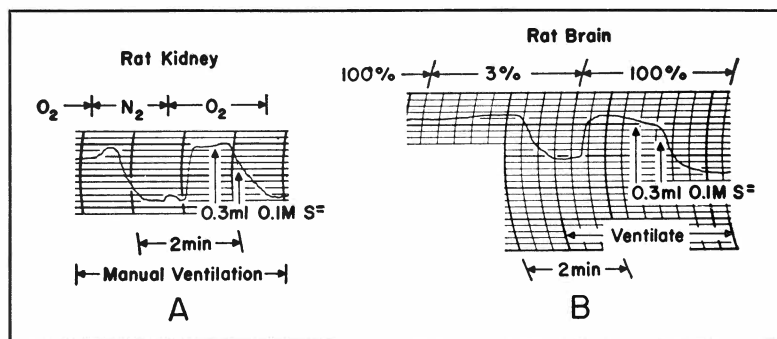


Fig. 9—Fluorescence changes induced by anoxia or severe hypoxia and by sulfide (an electron flow inhibitor) in the kidney and brain of rats. The top line shows the periods during which different gases were respired. An increase in NADH concentration would be registered in a downward direction. (Reprinted with permission from *Science*, Chance et al., 1962.)

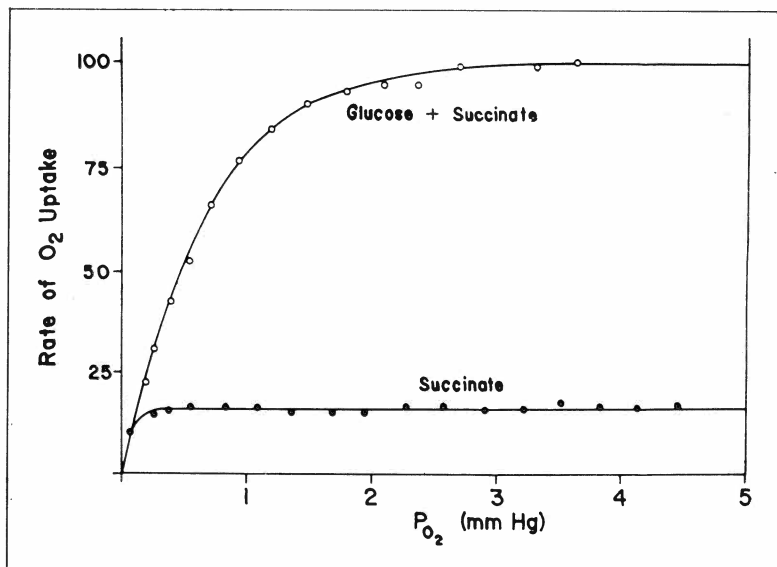


Fig. 10—The rate of oxygen consumption of yeast cells in succinate buffer as a function of PO_2 . Top curve: glucose added as substrate. (Reprinted with permission from *Handbook of Physiology*, Jöbsis, 1964, adapted from *J. Cellular Com. Physiol.*, Winzler, 1941.)

limited either by the rate of oxidative phosphorylation or by the substrate supply. This is difficult to distinguish. It turns out that the resting metabolism of most cells—at least the ones that we have looked at—is not limited by a lack of ADP only. During resting respiration they do not have fully reduced levels of mitochondrial NAD such as isolated mitochondria have. There is also a subtle but very effective control of the substrate level. In resting metabolism the substrates are not present in excess. So there must be at least one other control step, but most likely there are several. We do not really know where they are located except that they are not in the respiratory chain. This type of control operates only in the intact cell, not in isolated mitochondria. In all tissues that we have studied there is this very definite limit of substrate provision. This limitation is overcome immediately upon initiation of more active oxygen uptake. In other words, there is a multi-pronged control system of overall oxidative metabolism. Physiological activity produces an increase in oxygen uptake and oxidative phosphorylation by an increase in ADP, but at the same time it spurs substrate provision. It certainly can do so by increased glycolysis. This results in an increased availability of pyruvate for the citric acid cycle. Apparently there are similar controls in the other pathways feeding into the Krebs cycle. Of course, there is an absolute necessity for meeting the increased needs. Happily, in an experiment it is often possible to get around this problem by increasing the substrates from the outside.

Dr. Patterson: Could you define for some of us the $K_{m_{O_2}}$?

Dr. Jöbss: My apologies. The shorthand stands for the Michaelis constant for oxygen.

Let me define it very pragmatically. If one does a plot of the rate of oxygen uptake as a function of the PO_2 , one comes up, as I showed

with a curve which looks something like those in Figure 10. It is useful to talk about that level of oxygen where the rate is 50% of the maximal rate. It is often referred to as the apparent K_m . When I was quoting numbers for the $K_{m_{O_2}}$, I was talking about the more theoretical system, i.e., when one would have provided all conditions so that only the respiratory chain was limiting. The higher the rate of O_2 uptake in the experiment, the closer one is approaching the true K_m —the real point of reference for the

respiratory chain as an enzyme system.

Question: Is this the Michaelis formulation applied to your system?

Dr. Jöbss: Yes, but, of course, it is only a pragmatic constant. A midpoint or 50% point is usually better defined than a “perceptible decrease.” Therefore, I often like to use the experimental, the *apparent* $K_{m_{O_2}}$.

Question: Wouldn't it be a PO_2 , then, corresponding to that $5 \mu M$?

Dr. Jöbss: No. The K_m number is 0.5 mm Hg or $1 \mu MO_2$. One can

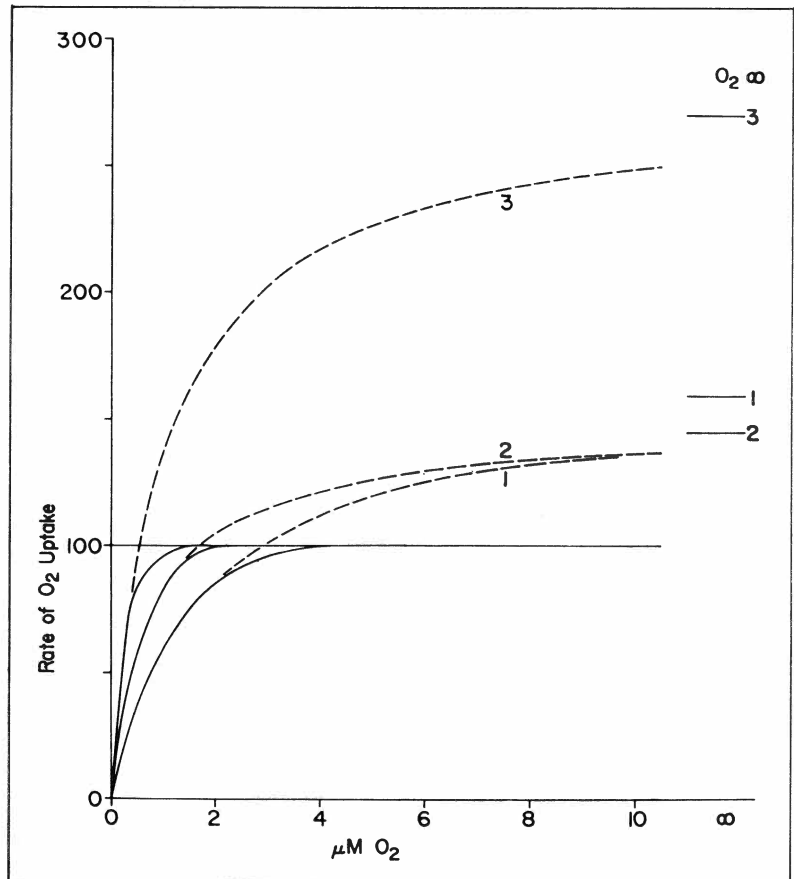


Fig. 11—The rate of oxygen consumption as a function of O_2 concentration for ascites tumor cells (curve 1, $37^\circ C$), liver cells (curve 2, $40^\circ C$), and isolated liver mitochondria (curve 3, $20^\circ C$). The maximal rate observed experimentally (solid lines) is given the value 100. The upper curves (dashed lines) were calculated after extrapolation of the experimental data in the hypoxic range to infinite O_2 concentration by a Lineweaver-Burk plot. The lines on the right-hand margin indicate the theoretically achievable maximal rate unlimited by the supply of reducing equivalents. (Reprinted with permission from *Handbook of Physiology*, Jöbss, 1964.)

use about 10 times that level as a lower limit for plentiful O_2 , i.e., 5 mm Hg or about $9\mu M$. That is an average for 10% decrease in O_2 uptake rate, as shown in Figure 11. I'm not very interested in that number, however, because it's a non-physiological one. It is calculated for the system when the respiratory chain is the only limiting situation, and that is hardly ever the case, if ever.

Question: Micromoles per what?

Dr. Jöbsis: Micromolar—i.e., micromoles per liter of fluid.

Studies on Animals

In the physiological case the number of interest is, of course, the one for that undefined condition when substrate is partially limiting. We would have to make some really awful generalizations to define it. In addition we would have to tackle the job of calculating the PO_2 midway between capillaries from blood flow and blood content data. There are too many badly known parameters to inspire real confidence. Instead we can use the experimental approach that is made possible by our ability to determine the adequacy of the PO_2 by observing the respiratory chain directly. We will find that PO_2 in the inspired air at which we first see NAD going reduced.

What Chance and his co-workers did was to combine a double-beam reflection spectrophotometer with a fluorometer, both of them looking at the same spot on the cerebral cortex (Chance, Schoener and Schindler, 1964). The spectrophotometer shows the changes in the color of the hemoglobin as it goes from the oxygenated to the disoxygenated form, and the fluorometer gives a measure of the adequacy of the oxygen for the respiratory chain. What is really being measured in this tissue is the behavior of the source, which is hemoglobin, in relation to that of the sink, which is the respiratory chain. In addition, we would like to

have something physically located in between—myoglobin might be just right—but the brain does not contain that, of course.

In the experiment an animal is respired on different gas mixtures. This is the independent variable and is given on the ordinate of Figure 12. It is, of course, a much different scale of PO_2 than in the previous figure. Starting out at 150 mm Hg, we find a situation where we have certain values of the currents of the photomultipliers associated with the normal tissue. The difference between these and the currents recorded when the animal is completely anoxic, we give the value of 100 for both the spectrophotometer and the fluorometer. These values are set out on the ordinate. Now we let the animal respire on a mixture low in O_2 , and we will look at the hemoglobin to see how much of it is in the oxygenated form and at the NADH to see if it goes up, i.e., shows signs of hypoxia. For each gas mixture you get two points: an average tissue PO_2 from the hemoglobin and an index of the adequacy of O_2 provision to the mitochondria from the flu-

orescence. At about 40 mm Hg inspired, the method did not see anything but disoxygenated hemoglobin. Actually that means less than about 10% oxygenated Hb, because that is the level of confidence of these measurements. At the same time the fluorometer recorded that the level of fluorescence had gone up only 35%. In other words, most of the cells were not yet anoxic. A further decrease in the inspired PO_2 finally resulted in complete anoxia as defined by a stable, high intensity of fluorescence.

Before we decide upon a limiting PO_2 value, we should discuss one other property of the chain. It concerns the interpretation of the increased NADH level in terms of effects of O_2 lack, and it arises from some kinetic considerations. The real O_2 sink is, of course, reduced cytochrome a_3 , because that is the one that reacts with oxygen. It turns out, again from mitochondrial experiments in cuvettes, that when one follows the rate of oxygen uptake as a function of the PO_2 one will notice many members of the chain at a more reduced level be-

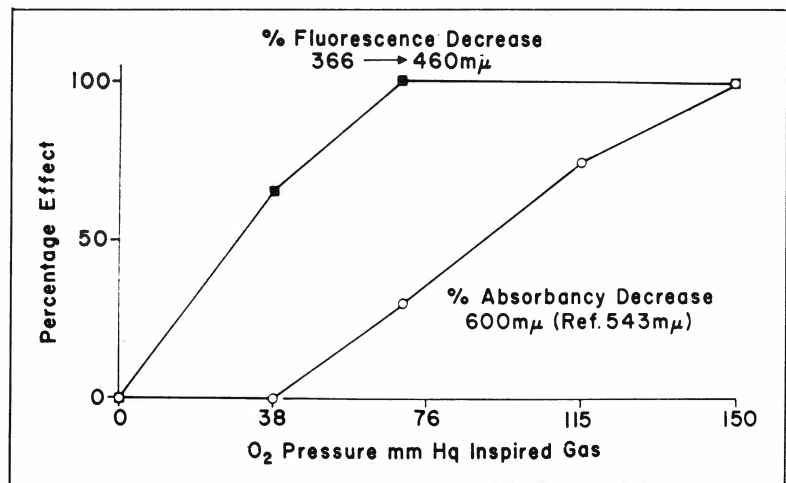


Fig. 12—Blood oxygenation level and NADH fluorescence in the intact cerebral cortex of the rat. Abscissa: PO_2 of inspired gas; ordinate: change in the signals as a percentage of the total change produced by complete anoxia. (Reprinted with permission from *Handbook of Physiology*, Jöbsis, 1964, adapted from *Oxygen in the animal organism*, Chance et al., 1964.)

fore a measurable change in the rate of oxygen uptake occurs. The reason is a perfectly straightforward one, arising from chemical kinetics. Since reduced a_3 reacts with oxygen, the reaction rate is proportional to the reduced a_3 concentration times the O_2 concentration. Under conditions of plentiful O_2 , cytochrome a_3 is only 1% reduced or less. There is, therefore, an enormous capacity for cushioning the effect of low PO_2 on the reaction rate. All that is needed is an increased level of reduced a_3 . This is what occurs automatically when O_2 starts getting scarce, since it will take longer before a reduced a_3 molecule and an O_2 molecule will collide. Over the range of hypoxia the reduction of a_3 is reflected further and further down the chain by increased levels of reduction of the other components. The last one to be affected significantly will be NAD. Roughly speaking one can use a value of a measurably increased NADH level (10%) as an end point. Here the cell reaches the detrimental range of hypoxia.

Question: This is an intracellular oxygen tension?

Dr. Jöbssis: No. In Figure 12 we are speaking of the PO_2 in the gas mixtures. The numbers on the ordinate are oxygen tensions in the respired air. Practically speaking this is, of course, the crucial number. From another point of view, however, we might want to know the average PO_2 in the tissue at which 10% reduction of NAD occurs in brain mitochondria. This turns out to be, again, about 5 mm Hg; in this respect the brain mitochondria are not greatly different from other mitochondria. However, this number is of limited value only. The tissue level of O_2 varies, of course, depending on the distance from capillaries. This is the best we can say at the moment.

It is also possible to approximate from the oxygen dissociation curve of rat hemoglobin the average capillary PO_2 . When NAD reduction due to hypoxia is about

10% complete, the spectrophotometric signal shows that hemoglobin is still 20% in the oxygenated form. Assuming for the brain tissue an average venous PO_2 midway between arterial and venous blood (i.e., 70 mm during the air-breathing part of the experiment), then 1/5 the difference in signal between that level and the anoxic one gives us, from the O_2 saturation curve for rat hemoglobin, a PO_2 of 25 mm Hg. This is again a gross average, this time between venous and arterial fractions. Perhaps the only usable numbers will come out of fluorometric experiments such as those shown in Figure 12, with or without the differential spectrophotometer trace.

Limitations of Fluorometric Method

I believe that these, then, are the results and the present state of the art. There are a number of limitations to the method as it stands at the moment. The main one is a limitation in resolving power. We are limited by the penetration of the excitation light into the tissue. So, at the moment there is not much sense in decreasing the lateral dimensions, i.e., decreasing the diameter of the optical field. The problem is that fluorescence from a depth of a millimeter still contributes to the signal. That is, of course, terribly much. One possibility is the use of a different line of the mercury arc, say, the 330 $m\mu$ line. In that region there is a sizeable change in the depth of penetration of light with shorter wavelengths, but while 330 $m\mu$ penetrates less deeply, it is also more harmful. In other words, there are problems, but perhaps they can be overcome step by step.

This is where I want to stop. Discussion of this paper will be after the coffee break, and I would like to start the discussion by covering two slightly different points.

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DISCUSSION

Dr. Patterson: Our discussion session now will concern this marvelous and beautifully delivered material that Dr. Jöbsis has given us. I do want to introduce a gentleman who has come at the suggestion of Dr. Doolan, our associate dean, to help us discuss some material that is so basic and so difficult for a number of us: Dr. Lutz Kiesow, who is head of the division of molecular energetics at the Naval Medical Research Institute. I think Dr. Jöbsis would like to show two slides before the quizzing begins.

Dr. Jöbsis: Thank you for indulging me once more. I would like to bring up two more things, and since they are a little distant from the previous focus, I thought it would be best to wait until after the coffee break.

I have looked at the fluorescence of the cerebral cortex from a functional point of view, trying to relate physiological activity with levels of fluorescence. The activity I have studied is paroxysmal firing induced by strychnine or Metrazol. The experimental system was the cat with an exposed cerebral cortex, electrodes on the pia mater and microscopic observation of fluorescence. With this arrangement it is possible to show that a decrease in fluorescence accompanies each burst of epileptiform activity. A considerable amount of instrumental development has shown that this is not an artefact caused by increased blood vessel diameter. Monitoring of that, as well as the administration of 10% CO₂, which maximizes vessel diameter even in rest, has shown the reliability of this observation.

These results show that it is possible to distinguish between less active and more active physiological conditions by the relative intensity of the fluorescence, i.e., the relative intensity of the metabolism. In terms of the scheme of Chance and Williams (Table 3), the extra physiological activity tends to force

the respiratory chain further away from the resting level (state 4) to the active level (state 3). The fact that it is possible to show this in the intact tissue is the first point I wanted to make.

The second point concerns, perhaps, a minor issue that can be settled with the fluorescence method. For quite a while it has been known that epileptiform discharges are accompanied by increased metabolism and an expansion of the cerebral vascular bed. Because of this knowledge it has been speculated that the end of a paroxysmal burst of high voltage EEG activity might well be brought about by the occurrence of anoxia in the cortex. This I am now able to deny. Under normal respiratory conditions, oxidations are invariably noted without a trace of a tendency toward the increased fluorescence that is characteristic of anoxia or hypoxia. It is possible to produce signs of hypoxia during an attack, but either very low gas mixtures must be administered during the experiment or a very low rate of artificial respiration must be maintained. In addition, under such conditions signs of hypoxia may occur during an epileptiform burst without much effect on the further progress of the burst. Apparently the discharges can continue for some time without the need of continuous oxidative restoration of the ~P energy stores required for recouping the loss of ions.

Dr. Worsham: Are we to understand that the length of these episodes would be the same as without anoxia? Have you terminated the episode by the anoxia?

Dr. Jöbsis: Your understanding is correct; the burst is *not* terminated by anoxia.

Dr. Patterson: Is your burst the equivalent of a convulsive seizure?

Dr. Jöbsis: Right. Usually they are produced by strychnine and Metrazol together. It is a horrible mixture, but it's very effective.

Dr. Patterson: The floor is open for quizzing. I think we'd like to hear

from the members of our panel first.

Dr. Kiesow: I think what has to be done here is to correct a few things. First of all, there is your extremely modest presentation of your contribution to a rather important field of modern cell physiology. You mentioned Britton Chance and his almost 20-year-old efforts of analyzing and characterizing the cellular respiratory chain and its elements. But I think your contributions are almost as important as his, since you made the step back to the physiological system. You freed yourself from this dangling sword of Damocles of being a mitochondriac, and you went back to as intact and as physiological a system as you could find. In this context I would like to ask a question or two. One has to realize, of course, that the mitochondriac working with either mitochondria, or even subunits thereof, quite often has the advantage of less interferences caused by cell constituents such as substrates, coenzymes and enzymes. These are present in intact cells but are frequently absent in mitochondria and their subunits. The question, therefore, concerns your studies on the fluorescence of either tissue in situ or of muscle when excited with light of a wavelength of 340 or 366 m μ . To what extent does this fluorescence really reflect changes of the NADH concentration alone, and to what extent does it reflect changes of total of intracellular pyridine nucleotides—NADPH as well as NADH? I would also be interested in data which would break down the intracellular pyridine nucleotides of your biological materials into the NAD and the NADP system. Furthermore, as you subdivide the total, what are the ratios of the reduced over the oxidized forms of both systems?

Dr. Jöbsis: This is such an invitation to expand! Dr. Kiesow has put his finger right on a very sore spot. I've thought a lot about it and done some experiments. I can gladly give some answers, although I'm sure

they will not be as complete as he would like to have them. This is a very important point, and I did not go into it only because one has to limit a lecture in some way.

Next to NAD (nicotinamide adenine dinucleotide) there is NADP (nicotinamide adenine dinucleotide phosphate), which is the same molecule with an extra phosphate on the ribose of the adenosine. This is also contained within the mitochondria, and when it is reduced, it has very much the same absorption and fluorescence properties as NADH. It is also involved in certain mitochondrial reactions. This is not the only possible source of confusion, since, in addition to mitochondrial NAD and NADP, there is NAD, which is not in the mitochondria but in the cytoplasm. It is involved in glycolytic metabolism, which is, of course, highly active. I am sure this will be brought up later on. Finally, there is another fraction of NAD in the mitochondria which is, as far as we know at the moment, not too directly in equilibrium with the chain. It is not reduced directly by substrates but by a so-called reversed flow of electrons. For this reducing equivalents flow in the opposite direction, as usual; that means uphill in an energy diagram. This costs energy, of course, and high-energy phosphates or high-energy intermediates are needed to do this. I don't want to go too far into it. It does not add to the present discussion. I just wanted to bring it up to show that the picture is complex.

Let us go from the easier part of the question to the more difficult. The NAD in the cytoplasm is glycolytically active. This fraction does not interchange freely with that which is in the mitochondria. In fact, NADH added to isolated mitochondria will not be oxidized. There is a separate pathway for intercommunication of reducing equivalents, but it doesn't concern us here because the problem is: Since it is NADH and becomes alternately oxidized and reduced, how do you

know that you're not partially measuring it? The only indications I have are some studies on muscle, excised muscle, where we looked very hard for possible cytoplasmic effects in the presence and absence of iodoacetate. There was no contribution that we could see. This appears puzzling, because the concentrations are very similar. Happily for us, the fact is that NADH has a tremendously increased fluorescence efficiency when in mitochondria. It fluoresces up to 20 times more intensely per concentration unit than it does in solution or, apparently, in the cytoplasm. This is the reason why our signal is derived, as far as I can see, from mitochondrial NADH.

NADPH is a different problem. It is involved in various other biochemical steps, and it is also oxidizable and reducible and should contribute absolutely the same type of signal. There are two points that save the day. First, there happens to be very little NADP present compared to NAD. Second, both Dr. Chance and I, independently—Dr. Chance for liver and I for brain—have analyzed biochemically for NADP. In my case, the experiments were closely related to the fluorescence experiments. We measured the fluorescence in one spot. We produced a maximal decrease in fluorescence with strychnine administered *i. v.* and took a sample from the other hemisphere. In other words, in the middle of a strychnine attack we froze a piece of tissue and removed it. Next we let the brain go fully anoxic by apnea and took a second sample right in and around the point of optical observation. Then we analyzed with enzymatic methods and compared left side against right side, anoxic against oxidized. It turns out that the NADPH concentration doesn't change in such an experiment, while the NADH does and, incidentally, shows a good correlation with the increase in fluorescence. So, we're pretty sure that NADPH does not interfere. Dr. Chance has

been able to pick up some signs of an effect in the liver but with slow kinetics. In other words, he has seen NADPH changes, but they were at a different rate than the fluorescence and the NADH changes.

In conclusion, then, we appear to be rather safe. However, there remains the cytoplasmic fraction of NADH. We cannot analyze for it differentially. It is probably also affected by some of the same factors that affect electron transport. The only reassurance is the fact that, in various other preparations such as muscle, it has been shown that its quantum efficiency of fluorescence is some 10 to 20 times less than that of NADH associated with the oxidative phosphorylation function of the respiratory chain. That is the answer, I think.

Dr. Kiesow: You were referring to the problem of energy-linked reversal of the electron transport. If I remember correctly, Chance recently published a paper where he demonstrates that this step is affected by hyperbaric oxygen. He claims that this effect is not due to an increased rate of autoxidation of reduced flavoprotein, but is due to a direct effect of hyperbaric oxygen on this reversal of the electron transfer. What would you consider to be hyperbaric conditions here; hyperbaric in regard to oxygen, that is?

Dr. Jöbbsis: If you want a hard number, I'm sure I cannot give it to you. I think anything that produces a level higher than about 30 to 40 mm Hg-averaged tissue PO_2 is probably hyperbaric. That's the range for which you need a hyperbaric chamber. But I'm not too well acquainted with hyperbaric work. The experiment that you refer to was the following. When mitochondria are exposed to high oxygen pressure (5 to 12 atmospheres) it appears that one inhibits, the reduction of the NAD fraction which receives its reducing equivalents by reversed electron flow (the pathway that goes from succinate

via flavoproteins to NAD). Exposure to hyperbaric oxygen prevents NAD reduction upon the addition of succinate. This is suggestively linked to some measurements of Sanders, Woodhall and their group in which they have shown that very high levels of succinate in the blood protect against cerebral damage by hyperbaric oxygen. (A. P. Sanders, I. H. Hall, P. J. Cavanaugh and B. Woodhall. In *Proceedings 3rd International Conference on Hyperbaric Medicine*. I. W. Brown, Jr. and B. G. Cox (eds.). Nat. Acad. Sci.—Nat. Res. Council, Publ. No. 1404, pp. 73-82, 1966). The suggestive implication is that a tremendous amount of succinate—and these are really enormous levels they are working with—produces a protective effect by partially overcoming this inhibition. However, I don't believe that Chance has been able to verify this by direct observation. Until now, regardless of the dose of administered succinate, he just hasn't seen it.

Dr. Robin: Dr. Jöbsis, I would like to join my colleagues in thanking you for this superb discussion, and I won't end that compliment by using a harpoon. I should like to ask you a question. It's a self-serving one, because after you answer it, I would like to make a comment, regardless of how you answer the question. I think that one of the purposes of a conference like this is to highlight matters that are of potentially great interest for the future in the areas that are being discussed. The question that I would like to ask is: Are there data available concerning whether the peroxidase pathways, the non-cytochrome pathways involving energy generation, are linked, on one hand, with ATP generation and, on the other hand, with acetyl Co-A as an intermediary? In other words, how closely are they linked to the usual kind of oxidative phosphorylation?

Dr. Jöbsis: Well, I'm very glad that you gave me a choice of any

answer, because I am not too well acquainted with these systems. I have looked into this matter a bit, since I worried that these reactions might affect the mitochondrial reaction. Being a rather pragmatic experimentalist, I immediately stopped going into further details when I found that they did not seem to hinder my system of measurement. Hence, I would really like to hear your comments.

Dr. Robin: What I should like to indicate is that the area of non-cytochrome-linked energy generation is, in my opinion, a potentially very important one and that there are at least two physiological circumstances that I know of in which the possibility of non-cytochrome-linked energy generation has been raised. With your permission, Dr. Jöbsis, I might indicate these.

One system is the alveolar macrophage, which is being extensively investigated by a number of workers, including my colleague, Dr. Bernard Gee. This fits in with this type of audience, because there are lung types and heart types, and one of the important functions of the alveolar macrophage, of course, is to phagocytize inert, dead bacteria, and living bacteria. Almost certainly this is an endergonic, an energy-requiring process, and one can establish this by several lines of evidence, but one of the most striking lines of evidence is that, if you measure the oxygen consumption of the alveolar macrophage in its resting state and then let it gobble up dead bacteria, there is a brisk increase in oxygen consumption. Now, if you manipulate this system by partially inhibiting cytochrome b by using antimycin, or you use cyanide and partially inhibit a_3 so that the cells are still living, there is obviously a profound decrease in oxygen consumption, but there is no effect on phagocytosis. There is evidence which indicates that, although the general level of oxygen consumption is decreased in these cells, there is still, at this reduced oxygen consumption, an increase under

circumstances of phagocytosis. Obviously, the easiest way to reconcile these data would be to say that there must be another energy link, another oxygen-requiring process, which does not involve the respiratory chain.

The second system is the nucleated red cell of the shark, and the findings to be discussed are probably characteristic of most nucleated red cells. Unlike mammalian red cells, nucleated red cells are brisk oxygen consumers. One can measure oxygen consumption in these cells by standard methods. One of the important energy-requiring processes in red cells generally is the process by which sodium is transported from the inside of the red cell to the plasma against an electrochemical gradient. If one is interested in energetics, one is, of course, anxious to quantitate the metabolic costs of performing this transport work. (Dr. Jöbsis's colleague, Dr. Tosteson has spent a good part of his professional life in this area.) Now, if you take this cell and inhibit anaerobic glycolysis by using one of the standard inhibitors, there will be a marked decrease in sodium transport. So, presumably, the major energy source for this transport process is anaerobic glycolysis. If you take large doses of cyanide and you inhibit the respiratory chain, you get no change in transport. This raises an important question, because presumably both respiratory chain activity and anaerobic glycolysis result in ATP generation. How can the cell distinguish between the ATP which is being generated oxidatively, which is not available for transport, and the ATP that is being generated by anaerobic glycolysis? Even more interesting than this is the finding that exposure of cells to nitrogen leads to a decrease in transport rate. I can summarize these results as follows. Let us assume the normal sodium transport rate is 15 mEq Na^+ /kg red cell H_2O per hour, transported from the inside of the

red cell to the outside. If one stops anaerobic glycolysis, the transport rate falls to 5 mEq Na⁺/kg red cell H₂O. If one cuts out the respiratory transport chain by either antimycin or cyanide, the transport rate remains at 15 mEq Na⁺/kg red cell H₂O. On the other hand, if one decreases oxygen availability by exposing the cells to 100% nitrogen, the transport rate falls to, say, 8 mEq Na⁺/kg red cell H₂O. This suggests that some aliquot of energy cost for this process is being mediated by non-cytochrome oxygen consumption, and the sum total of this is, then, as I said at the beginning, that non-cytochrome-mediated energy generation is probably a much more important field than has been appreciated in the past and may be a "hot" area of work in the future. Would you comment on this?

Dr. Jöbbsis: I am very glad to hear these data. For the last half year a graduate student, Mr. Hersey, and I have been looking for these kinds of systems. I guess we will just have to go to the Caribbean and catch a shark or two.

I would like to point out one thing. Earlier in my presentation I talked about autoxidative flavoproteins, and I mentioned that there is no known energy-saving step. High energy phosphate is definitely not being formed. This could really say something else again, namely that energy utilization, although not mediated by ATP, might perhaps be a direct function of the membrane components without the intervention of ATP. It is a most important point. Altogether, I'm very glad indeed that this gives us a good excuse to go to the Caribbean around Christmas.

Dr. Carter: I have just three short questions: 1) Does the fluorescence generation result in any significant temperature change of the tissue? 2) Have you thought of the possibility of using a fiber optic to come beneath to limit your depth? We've had some success in using fiber optics in kidney, in other words, pok-

ing in and coming up. It sounds a little drastic, but remember that these penetrations are very small, and it's conceivable that you would have a known depth of un-damaged kidney and brain under these circumstances. And finally, 3) Could fluorescence possibly be used as a measurement of blood flow? I should certainly like to hear a little more about why fluorescence is related to blood flow. Specifically, we would be very much interested in measuring blood flow on the surface of the kidney.

Dr. Patterson: If I remember correctly, you actually said you corrected the fluorescence for changes in blood flow, and I was fascinated by the possibility that this technique might be used to measure blood flow.

Dr. Jöbbsis: I didn't measure it; I just got rid of it.

Your first point, Dr. Carter, was about the temperature. We have a highly filtered light beam. Practically all infrared is eliminated. In addition, we have measured the temperature by using a small thermistor, enclosing it in a drop of saline and putting that on top of the cortex. The rate of heating is insignificant as long as the blood supply is intact. This carries heat away very effectively. However, if you stop the circulation, in a dead cat, for example, you have a slight rise in temperature; but there is greater danger in direct radiation damage of the tissue. We don't know the mechanism, but it is signified by pinprick hematomas appearing in the optical field. This is much worse for the shorter wavelengths. The 366 m μ line is a good compromise between effectiveness of NADH excitation and relative lack of damage.

The second point was about the fiber optics. First of all, I didn't know one could go down to that small dimension. I'd love to talk instrumentation with you for a while after the meeting. This certainly sounds very exciting. There is a problem which I'm sure will be solved in the near future, and

that is that the last time I looked into fiber optics, the index of refraction of available materials was such that the wavelengths of light between 300 and 400 m μ were not transmitted well by the fiber optics. I'm sure that something can be done about it. I'm not sure that I'm the right person to try to do it, though.

Comment from audience: There are plastic ones now available.

Dr. Jöbbsis: They are very bad at the moment, because plastics have some absorption in that region. For small thicknesses it is no problem. In fact, all my cuvettes and muscle chambers are made of lucite, but they are limited to about a millimeter of thickness in the light path. If you're going through fiber optics, however, you are working with centimeters.

Comment from Audience: But you've got too much light, anyway, you said.

Dr. Jöbbsis: Well, that's relative. An additional problem is a faint blue fluorescence excited by 366 m μ .

Now, the final point about blood flow. This was very much a headache, especially changes of blood content in the field. Hemoglobin will diminish the fluorescence in two ways. First, it will absorb excitation light, and then it will absorb the emitted fluorescent light. When light penetrates a red cell, practically all the 366 m μ light is absorbed, and almost the same occurs for the fluorescence. Therefore, the problem that you face is the following. You are looking down on an optical field on the surface of the tissue. Through the field run capillaries, venules, and arterioles. By eye, it looks very much like a network of black threads running through a blue background. Lateral and depth resolution are not sufficient to avoid anything but the very largest vessels. When something increases the metabolic activity, blood flow increases either by an opening up of more capillaries or by distention of vessels. The effective field from

which you are recording decreases, therefore, every time you have an increased distention of the vessels.

Excitation light is scattered in the tissue, and some of it is picked up by the objective. This fraction is affected by changes in blood volume in much the same way as the fluorescence. With appropriate optical beam-splitters and filters, it can be measured and provides a control for the light absorption changes affecting the fluorescence. I have often wondered whether these changes in the scattered 366 $m\mu$ light might not provide the basis for a useful means of monitoring blood flow parameters.

Dr. Patterson: You are actually not correcting the change of blood flow, but the changes in blood volume.

Dr. Jöbsis: Correct.

Dr. Worsham: It is really farther removed than that. What you are really correcting is the amount of hemoglobin in the field.

Dr. Jöbsis: Yes. You see more hemoglobin in the field due to distention of the vessels. Incidentally, the response time is quite good. The time resolution is in the neighborhood of two seconds.

Dr. Patterson: We would now like to throw this program open to general questioning of our distinguished speakers.

Dr. Huf: I have two questions. The first one is related to the problem which the first discussant raised about the importance of specific factors located outside of the mitochondria. I am now raising the question of the importance of unspecific factors. Take the muscle, for instance. When you throw the muscle into activity, you are bound to get all kinds of unspecific changes, membrane changes, protein contraction, pH changes, osmotic changes. Hence, you don't really look at a specific system, let's say the B system, in the same physicochemical environment. To what extent do unspecific factors, the kind mentioned, affect at least the

transient changes and, perhaps, steady-state changes as well?

The second question deals with the point which you made about the significance of the rate at which metabolites enter into cells. When you come into the region of very low oxygen tension, would it be useful to consider as a limiting factor the rate of oxygen supply? You do have a high oxygen diffusion coefficient, but you have reduced the oxygen gradient to practically nothing; so it comes, as it were, to a competition between limiting factors. You may have a high concentration of substrate of low mobility, or you may have a low concentration of oxygen which has a high diffusion coefficient. So, what is limiting?

Dr. Jöbsis: Let us start with the second question first. We are pretty sure that oxygen is limiting at very low PO_2 levels; say, less than 0.5 mm Hg. Above that there is a large region in which we can not decide what the limiting factor is. With tissues, and especially tissue slices, it probably would be the low PO_2 due to diffusion path lengths. There is no telling when this is the case unless you do an experiment with varying PO_2 levels. The uncertainty about the diffusion is, of course, part of all considerations of this nature. Fortunately, most cell membranes do not exclude either O_2 or CO_2 , so no significant new barriers are erected.

There is one aspect of the control of a sequence of reactions that escapes the uninitiated. I think Dr. Worsham has some very significant things to say about this. The problem is that most of our kinetic thinking is derived from enzymes in a cuvette or a test-tube. We can throw a substrate in and measure how fast it is converted. When you have added an insufficient amount of substrate and you see a decrease in the rate, you know immediately and quite correctly that your substrate concentration has limited the velocity. However, in a mitochondrial system and even

much more so in cells, there is a very complex situation with many control points. Take, for instance, the glycolytic chain. There is usually plenty of glycogen, yet lactate formation does not proceed maximally. Here there may be as many as six different control points. In the mitochondrial system itself there are three already in oxidative phosphorylation. There is not one primary control point; each one of the sites of oxidative phosphorylation provides control, and the rate becomes different if you take just one away. There is, therefore, a very complex interaction, complex enough to boggle the mind really. One needs to use computers to represent all the individual rates and their influences on each other. However, at very low oxygen levels, I think it is warranted to say oxygen is limiting and, of course, in a tissue, oxygen diffusion is one of the limiting factors.

As to your first point, yes, there is a tremendous amount of difficulty with non-specific changes. Just from an instrumental point of view, it is difficult, because when a muscle contracts, it scatters light quite differently than when it is in the resting condition. The double-beam method is necessary to overcome this. For a first approximation this is all right. However, light of different wavelengths is not scattered equally. Low wavelengths are scattered more intensely than high wavelengths. When your expected absorption is very small, this will get in the way. Then you must use two reference wavelengths, straddling the wavelength that you want to measure. In other words, if I want to measure 550 $m\mu$, I use as reference 540 and 560 $m\mu$, thereby getting rid of most of the wavelength specific effect. However, the increase in scatter is not linear with the wavelength; it is a power function, and one has to correct also for that.

Dr. Worsham: It depends on the direction of scattering, too, unless you have a spherical molecule.

Dr. Jöbbsis: That doesn't bother us too much, because that effect is not too wavelength specific. It's a very correct point, but pragmatically we don't worry about it much.

Now, your final point. There are not only instrumental difficulties, but physiochemically a contracted muscle is a different system. We can only rely at this point on changes we can observe in mitochondria in cuvettes and compare them to those in the tissue. It turns out that we don't get too many differences by changing any extracellular parameters. The functions we are studying are rather insensitive to pH. Only large pH changes show anything, and I have some data on changes in intracellular pH relevant to this.

Dr. Huf: What are large changes of pH?

Dr. Jöbbsis: .2 of pH unit, or something close to that.

Dr. Huf: How much does the pH change when the muscle contracts?

Dr. Jöbbsis: I have looked at the pH changes after small amounts of contractile activity and before glycolysis is stimulated. During glycolysis really large changes occur in the internal pH. But keeping away from lactic acid formation as best you can, pH changes occur as a result of a single twitch, which are in the neighborhood of .05 pH unit. Other people have looked at this long ago and found the same.

Then there may be osmotic changes. They are very unpleasant when they are large. It looks, however, as if that does not occur in the muscle cell, with the possible exception of some increased osmotic pressure due to long term anoxia. In general, we have covered the difficulties pragmatically, but we certainly have not stopped trying to study each for its own worth.

Question from the Audience: At what level of hypoxia does this reaction of strychnine disappear completely?

Dr. Jöbbsis: You mean an increased EEG? I have not tried that. The spontaneous EEG disappears some-

where in the neighborhood of breathing 3% to 5% oxygen, at least in our blissfully anesthetized animals. I really don't know at what level of PO_2 the paroxysmal, strychnine-induced EEG stops.

Dr. Kontos: I was wondering whether your method is fast enough to observe any rapid spontaneous changes in living muscle, for instance, in response to changes in blood flow. The records you have shown before you interfered with some particular intervention looked pretty close to straight lines. Is this true?

Dr. Jöbbsis: These muscle records were from excised muscle, so we avoided that problem. Dr. Stainsby, Dr. Renkin and I have planned to investigate that a bit later this fall. Dr. Renkin is specifically interested because of possible changes in capillary distribution, distribution of open and closed capillaries. Dr. Stainsby is interested in the relation between lactate production during exercise and possible hypoxia in muscles doing different kinds of work. I am just interested.

Dr. Worsham: I want to make a comment, if I may, but first I want to ask a question. I'd like to ask how well we know the characteristics of each step along this cytochrome chain. Do we know the rate constants and the Michaelis enzyme constants all the way along for each step?

Dr. Jöbbsis: We do not know enough, but we know approximately. You can observe each component of the respiratory chain with the exception of cytochrome c_1 . Many of the rate constants are well determined, although one always wishes for better.

Dr. Worsham: I'd like to suggest that perhaps the time has come for you to join forces with the theorists, if you classify yourself as a pragmatist, and go ahead and try to simulate your system from the known in vitro characteristics of these various steps. The best thing you could hope for is that your computer simulation, a model built

up from the known isolated characteristics, would then agree with what you see in some of your experiments. I am impressed with how well you have cut out a lot of complications. This is always the place to begin for a good theoretical approach; in fact, you think like a theorist and work like an experimentalist, but you have done the best experimental job of getting a real simple system to study. Perhaps you have a chance now of building a model, putting it on to a fairly modest computer and trying to see if your model does agree with what you have observed. One needs theory to program an analog computer to simulate the reactions that occur in a metabolic pathway. Dr. Jöbbsis has well indicated the nature of the problem. He wiggles the oxygen concentration at one end of the chain, and he wants to know what happens to the NADH rate turnover at the other end of the chain. This problem is not easy to solve.

Dr. Patterson: Well, you are teaching kinetics of reversible processes, and a lot of these are irreversible.

Dr. Worsham: That's not true. The difference is that the metabolic systems in the animals we have studied have no beginning. That's really the difference. There's no C_0 in the processes that you study. There's a steady state you can think of, a metabolic steady state; you perturb this, and it goes to another steady state. What I'm saying is that the kinetics you think of as pot kinetics are quite different from the kinetics of steady-state metabolic path rates to additional steady states.

Dr. Kiesow: I completely agree that the problem here is the flow kinetics—rather the pot kinetics—and I was just wondering, since it obviously is a question of measuring the intracellular oxygen concentration with an indicator as close as possible to oxygen, whether you have ever tossed around the thought of using the photodissociation of the carbon monoxide complex of a_3 as an indicator for intracellular

oxygen? The second question: Although the autoxidation contributes to only 10% of the total oxygen consumption, it should have, and it does have, the characteristic of being much more proportional to the partial pressure of oxygen than this a_s , which is very low saturation pressure. Isn't it possible, perhaps, also to use the state of the redox system at the level of flavoprotein as an indicator for intracellular oxygen pressure based on the autoxidation where the rest of the chain is hooked off by either an inhibitor, antimycin A, or cyanide or carbon monoxide?

Dr. Jöbbsis: Actually I'd like to go in reverse order, which is not too pleasant, but I don't think that would be an insurmountable problem. From the specific to the more general, I have never thought of using the autoxidative flavoproteins as indicators. However, it sounds like a very, very good suggestion indeed if we can be sure we have blocked the other flavoproteins. We need cyanide. That can be solved. A bit of difficulty lies in trying to get an index to flavoproteins. They have rather weakly differentiated spectra. However, a colleague of all of us in this field, Dr. José Ramirez of Mexico City, who unfortunately died in a car accident, had, at the time of his death, completed a system of fluorescence measurements of flavoprotein that he thought was very promising. I am sorry to say that progress on this ceased with his death.

As for the remark of Dr. Worsham, I really wanted him to come up with this very important point that we are not working in the same type of chemical kinetics as that so often found in textbooks.

Dr. Worsham: You mean they taught you the wrong thing?

Dr. Jöbbsis: Yes, for this purpose it is wrong. Recently, however, the physical chemists have been getting more interested in such systems. I am glad that they are correcting themselves. I should be more humble at this point, because

I am a rank amateur in computer work, but I love to play around with computers and sometimes have to tear myself away from the analog because it's so wonderfully neat. You put in the values, the curves come out beautifully, and you think, "Now I'm doing science!" Of course, you're not doing science at all, because a dirty experiment is really what science is made of. To interpret a dirty experiment, however, you need an analog computer. Although I've done a bit of this sort of work, this is being done very intensively at the Johnson Foundation by a whole group of computer people. In fact, they have made a system, starting out with glucose and some other substrates in glycolysis, taking in the whole respiratory chain and taking ATP consumer reactions into account, also.

Dr. Worsham: Have you tried their model to simulate your experiments?

Dr. Jöbbsis: No. I, myself, have not played with the analog computer in this system. I have played with it in the interpretation of energy turnover in muscle contraction.

Dr. Worsham: Why don't you go to their computer and say, "Let me . . ."

Dr. Jöbbsis: Well, they are doing this, so I am just waiting for them to do it. Of course, sometimes they don't ask the right questions.

Dr. Worsham: That's where they need you.

Dr. Jöbbsis: Yes, but you'll have to convince them of that. There are two fellows, each with his own group; one working with an analog computer group, the other with a digital computer group. By the way, I think they have purposely been put next to each other to produce some healthy competition.

Dr. Worsham: By the way, your comment was excellent. Thomas Huxley said, "The great tragedy of modern science is the slaying of a beautiful theory by an ugly fact."

Dr. Robin: Two comments. Dr. Jöbbsis, maybe this afternoon I can provide you with an animal in

which you can completely knock out a_s and have both the animal and the brain survive for at least one to two weeks. Secondly, since we're being philosophical and comparing the differences between how things work in a complicated cell and a complicated animal, macromolecules and ions being put in a test-tube with some water and shaken up together, I think the problem is even deeper than that. We have no C_0 . In many kinetic processes the kinetic changes that take place depend on the previous point along the curve, and, therefore, if we are describing any of these multicomplexed steps, we have to describe them each in terms of a family, at best. Consequently, I am not sure you've been teaching the wrong kind of physical chemistry. I am not sure that the right kind of physical chemistry yet exists which can accurately define these kinds of systems, and, therefore, what we try to do is try to make the best approximation we can with the method and thinking that is available until, hopefully, somebody comes along with a physical-chemical-theoretical structure which can deal with variables which change so rapidly.

Dr. Patterson: Haven't people like Dr. Prigogine, working with thermodynamics of irreversible processes, and others studying the irreversible processes helped somewhat in this area?

Dr. Robin: Yes, although I think you are referring to something that is a little bit different. The difference between reversible and irreversible thermodynamics is involved in your question. The basic assumption that is made in classical thermodynamics is that you deal with equilibria, and, of course, in living systems there are no equilibria. The consequences of not having equilibria are that the best you can deal with are steady states or quasi-steady states, and the general equations that apply to each of these two different approaches are quite different.