the "spasm reactor" in your practice
The Machine Age man still possesses a Stone Age stomach; sometimes the job of merely coping with today's environmental stress may prove too much. For some (the "spasm reactors" in your practice), tension, anxiety and worry may find expression through the voice of gastrointestinal or other smooth muscle spasm. To treat these patients with antispasmodics alone is often to miss the point of origin of their disturbance; to rely solely on tranquilizers often proves discouragingly slow or ineffective in relieving spasm and pain.

To quiet and quell Donnatal can promptly and effectively quell the spasm and quiet the tensions that trigger it. Prescribed by more physicians than any other antispasmodic-sedative, Donnatal continues to provide the classic answer.

The "Donnatal Effect" The characteristic, over-all effect of Donnatal has been observed in many thousands of children and adults, clearly establishing its value as a versatile sedative-antispasmodic. Outstanding in effectiveness, safety, economy, uniformity of composition and dosage convenience, Donnatal continues to be desired and prescribed by a majority of physicians.

In a multiplicity of indications Particularly useful when anxiety and tension accompany, aggravate or account for smooth muscle spasm, Donnatal is indicated for the symptomatic relief of recurring, persistent or chronic visceral spasm. More than two dozen distinct and separate indications for Donnatal are listed on page 974 in the current PDR.

Brief summary Blurring of vision, dry mouth, difficult urination, and flushing or dryness of the skin may occur on higher dosage levels, rarely on usual dosage. Administer with caution to patients with incipient glaucoma or urinary bladder neck obstruction. Contraindicated in acute glaucoma, advanced renal or hepatic disease or a hypersensitivity to any of the ingredients.

Each tablet, capsule or 5cc. of elixir (23% alcohol) Extental®

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>hyoscyamine sulfate</td>
<td>0.1037 mg.</td>
</tr>
<tr>
<td>atropine sulfate</td>
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</tr>
<tr>
<td>hyoscine hydrobromide</td>
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<tr>
<td>phenobarbital (1/4 gr.)</td>
<td>0.0195 mg.</td>
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<tr>
<td>(warning: may be habit forming)</td>
<td></td>
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</tbody>
</table>

QUIETS THE STRESS/QUELLS THE SPASM
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COVER: Structure of hydrated hydronium ions superimposed on two tissue cells.
Design based on Figure 43, Page 151 (E. Wicke) from THEORETISCHE BIOCHEMIE by Hans Netter. Berlin: Springer-Verlag, 1959.

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INTRODUCTORY REMARKS

JOHN L. PATTERSON, JR., M.D., Chairman

It is a pleasure to welcome the three authorities who will carry the major load of presentations in our symposium today. They are Dr. Frans Jöbsis of Duke University School of Medicine, Dr. Eugene Robin of The University of Pittsburgh School of Medicine, and Dr. Norman Carter of The University of Texas Southwestern Medical School. We are also grateful for the presence of Dr. Lutz Kiesow of the Naval Medical Research Institute, Bethesda, Maryland. Dr. Kiesow will assist in the discussions to be held after each major presentation. Each of these men is an international authority in his field, and we are more than fortunate to have this group with us today.

The topics for this symposium were chosen almost as a matter of necessity. In recent years, some of the best basic and applied research in the physiology of the circulation and respiration has been concerned with the physiological and biochemical roles in the body economy of the tensions of oxygen and carbon dioxide, and of the hydrogen ion concentration or activity. In our own group, we find ourselves more and more frequently forced into consideration or speculation of the probable behavior of the “tissue” or intracellular tensions of these gases and of the pH in our attempts to explain experimental phenomena. We feel safe in predicting increasing emphasis on the intracellular values of these three major variables in the next few years, and we have felt the urgent need to take stock now of the best present information. Our speakers will define the variables under consideration and discuss methods for their measurement and their physiologic and biochemical roles in the cellular economy. They have been told that they are free to emphasize those aspects of the subject of greatest interest to them.

Again, I wish to welcome each of the speakers and present the first of them, Dr. Frans Jöbsis, whose topic will be “Oxygen Tension: Direct Observations on the Critical Level for the Brain.”
Oxygen Tension: Direct Observations on the Critical Level for the Brain*†

FRANS F. JÖBSIS

Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina 27706

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 (Jöbsis '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
   \[ A + O_2 \rightarrow AO_2 \]
   in which A is a substrate that accepts two separate oxygen atoms;

B. Mixed function oxidases
   with the reaction
   \[ AH + 2e^- + O_2 \rightarrow AOH + O^+ \quad (\rightarrow H_2O) \]
   where the last notation signifies the rapid final step of water production from \( O^+ \) and \( 2H^+ \) ions;

C. Electron transfer oxidases
   catalyzing the reaction
   \[ 4e^- + O_2 \rightarrow 2O^+ \quad (\rightarrow 2H_2O) \]
   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, autooxidative flavoproteins and, secondly, the respiratory chain.

1. The oxidation of autooxidative flavoproteins can be written
   \[ \text{flavinH}_2 + O_2 \rightarrow \text{flavin} + H_2O_2 \]
   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 autooxidative 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 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 (~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 \( \text{NAD}^+ \) to form \( \text{NADH} \) (nicotinamide-adenine dinucleotide). [The old-fashioned name was DNP, and many people still refer to it as DPN'] 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 $c$, cytochrome $c_1$, 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 $P$ potentials, 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 $2\sim P$, 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 asphyxiatiation.

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 reactions 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 $\alpha$-ketoglutaral 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: $6.4\sim P$ per carbon. It is a bit arbitrary for fats, because they differ so much in chain length. I have chosen palmitate as
TABLE 1
The four main phases of foodstuff degradation

<table>
<thead>
<tr>
<th>Phase</th>
<th>Outline of Chemical Change</th>
<th>Primary Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>carbohydrates → hexoses; proteins → about 20 amino acids; fats → glycerol; fatty acids</td>
<td>depolymerization</td>
</tr>
<tr>
<td>II</td>
<td>hexoses → glycerol; several fatty acids; several amino acids; several amino acids</td>
<td>dehydrogenation, decarboxylation, deamination and shortening of the carbon chain</td>
</tr>
<tr>
<td>III</td>
<td>acetyl coenzyme A → α-ketoglutaric acid → oxaloacetic acid</td>
<td>decarboxylation and dehydrogenation</td>
</tr>
<tr>
<td>IV</td>
<td>NADH → flavin-H₂ → H₂O production</td>
<td>H₂O production</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Phase</th>
<th>Primary Chemical Change</th>
<th>Location</th>
<th>Waste Products</th>
<th>Reducing Equivalents</th>
<th>~P Yield Per Carbon Atom</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Depolymerization</td>
<td>Extracellular and cytoplasmic</td>
<td>None</td>
<td>None</td>
<td>None None</td>
</tr>
<tr>
<td>II</td>
<td>Dehydrogenation, decarboxylation, deamination, and shortening of the carbon chain</td>
<td>Cytoplasmic and intramitochondrial</td>
<td>NH₃ and urea; small amounts of CO₂ and H₂O</td>
<td>Small amounts of NADH and flavin-H₂</td>
<td>Used in IV 0.33 -0.06</td>
</tr>
<tr>
<td>III</td>
<td>Decarboxylation and dehydrogenation</td>
<td>Intra-mitochondrial fluid phase</td>
<td>CO₂</td>
<td>Large increase in NADH and flavin-H₂; small increase in NADPH</td>
<td>Used in IV 0.33 0.50</td>
</tr>
<tr>
<td>IV</td>
<td>H₂O production</td>
<td>Mitochondrial membranes</td>
<td>H₂O</td>
<td>Large decrease in NADH and flavin-H₂</td>
<td>5.70 8.00</td>
</tr>
</tbody>
</table>
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. 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 consumed. It is more meaningful, however, to study the system in the intact, circulated tissue rather than with slices or isolated 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 mµ, which is the dark red, to about 300 mµ 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;
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 indefinable and are grouped together. Note that the large trough at 465 mµ 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-
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 flavoprotein 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., Jóbsis, 1963 and 1964.)](image-url)
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 mμ, 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 mμ compared to 400 mμ, 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. 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.)
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 \( \text{O}_2 \) 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 \( \text{m} \mu \). It then emits light with a broad maximum at about 450 \( \text{m} \mu \): 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 \( \text{m} \mu \) 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 \( \text{m} \mu \) 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**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Steady-State Percentage Reduction of Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>State</td>
<td>([\text{O}_2]) level</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>&gt;0</td>
</tr>
<tr>
<td>2</td>
<td>&gt;0</td>
</tr>
<tr>
<td>3</td>
<td>&gt;0</td>
</tr>
<tr>
<td>4</td>
<td>&gt;0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

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 $a_d$. 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 $a_d$. Amytal will also produce increased fluorescence by blocking the chain, this time, however, by inhibiting the flavoproteins oxidizing NADH. These maneuvers with anoxia (or 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, $P_O_2$, 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 $P_O_2$ does not?
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 important 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 lJ.MO$_2$) for 50% inhibition and 5 mm Hg (9 lJ.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
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 \( K_m \) ?

Dr. Jöbsis: 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 \( P_{O_2} \), 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 \( K_m \), 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. Jöbsis:** 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 \( P_{O_2} \), then, corresponding to that 5 \( \mu \)M?

**Dr. Jöbsis:** No. The \( K_m \) number is 0.5 mm Hg or 1\( \mu \)MO. One can

![Fig. 11](image-url)
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., micro­molar 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 fluo­rescence. 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 $a$, 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-
fore a measurable change in the rate of oxygen uptake occurs. The reason is a perfectly straightforward one, arising from chemical kinetics. Since reduced \( a_i \) reacts with oxygen, the reaction rate is proportional to the reduced \( a_i \) concentration times the \( O_2 \) concentration. Under conditions of plentiful \( O_2 \), cytochrome \( a_i \) 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 \( a_i \). This is what occurs automatically when \( O_2 \) starts getting scarce, since it will take longer before a reduced \( a_i \) molecule and an \( O_2 \) molecule will collide. Over the range of hypoxia the reduction of \( a_i \) 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. Jöbsis:** 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.

**References**


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 ade­
nine 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 mito­
chondria, and when it is reduced, it
has very much the same absorption
and fluorescence properties as
NADH. It is also involved in cer­
tain 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 metabo­
lism, which is, of course, highly ac­
tive. I am sure this will be brought
up later on. Finally, there is an­
other fraction of NAD in the mito­
chondria which is, as far as we
know at the moment, not too di­
rectly in equilibrium with the chain.
It is not reduced directly by sub­
strates but by a so-called reversed
flow of electrons. For this reducing
equivalents flow in the opposite di­
rection; that means uphill in an energy diagram. This costs
ergy, of course, and high-energy
phosphates or high-energy inter­
mediates are needed to do this. I
don’t want to go too far into it. It
does not add to the present discus­
sion. 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 gly­
colytically active. This fraction does
not interchange freely with that
which is in the mitochondria. In
fact, NADH added to isolated mito­
chondria will not be oxidized. There
is a separate pathway for intercom­
munication of reducing equivalents,
but it doesn’t concern us here be­
cause the problem is: Since it is
NADH and becomes alternately
oxidized and reduced, how do you
know that you’re not partially meas­
uring 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 con­
tribution that we could see. This
appears puzzling, because the con­
centrations are very similar. Happily
for us, the fact is that NADH has
a tremendously increased fluores­
cence efficiency when in mitochon­
dria. 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 de­
erived, as far as I can see, from
mitochondrial NADH.

NADPH is a different problem.
It is involved in various other bio­
chemical steps, and it is also oxidi­
dizable 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 experi­
ments were closely related to the
fluorescence experiments. We mea­
sured the fluorescence in one spot.
We produced a maximal decrease
in fluorescence with strychnine ad­
ministered 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 obser­
vation. Then we analyzed with en­
zymatic 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, in­
cidentally, shows a good correla­
tion with the increase in fluores­
cence. 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 fluo­
resence is some 10 to 20 times less
than that of NADH associated with
the oxidative phosphorylation func­
tion of the respiratory chain. That
is the answer, I think.

Dr. Kiesow: You were referring to
the problem of energy-linked re­
versal of the electron transport. If
I remember correctly, Chance re­
cently published a paper where he
demonstrates that this step is af­
fected 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 elec­
tron transfer. What would you con­
sider to be hyperbaric conditions
here; hyperbaric in regard to oxy­
gen, that is?

Dr. Jöbsis: If you want a hard
number, I’m sure I cannot give it
to you. I think anything that pro­
duces a level higher than about
30 to 40 mm Hg-averaged tissue
PO2 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 atmos­
pheres) it appears that one inhibits,
the reduction of the NAD fraction
which receives its reducing equiva­
lents 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, 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 H2O 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. Jöbsis:** 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 antioxidative 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, poking 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. Jöbsis:** 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 thermometer, 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 mµ 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 mµ 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. Jöbsis:** 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. Jöbsis:** Well, that’s relative. An additional problem is a faint blue fluorescence excited by 366 mµ.

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 mµ 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 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 PO2 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 PO2 due to diffusion path lengths. There is no telling when this is the case unless you do an experiment with varying PO2 levels. The uncertainty about the diffusion is, of course, part of all considerations of this nature. Fortunately, most cell membranes do not exclude either O2 or CO2, so no significant new barriers are erected.

There is one aspect of the control of a sequence of reactions that escapes the initiated. 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, I use as reference 540 and 560 m, 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. Jöbsis: 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. Jöbsis: .2 of pH unit, or something close to that.

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

Dr. Jöbsis: 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. Jöbsis: 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 PO2 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. Jöbsis: 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. Jöbsis: We do not know enough, but we know approximately. You can observe each component of the respiratory chain with the exception of cytochrome c1. 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. Jöbsis 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 C0 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 a0 as an indicator for intracellular
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. Jöbsis:** 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. Jöbsis:** 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. Jöbsis:** 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. Jöbsis, maybe this afternoon I can provide you with an animal in which you can completely knock out $a_o$ 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 $C_o$. 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.
Intracellular CO₂ Tension: Practice and Theory*

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I have been asked to discuss methods of measuring intracellular Pco₂ and to discuss the physiology and biochemistry of intracellular CO₂ tensions. Actually, I am in a relatively fortunate position in that my difficulties in discussing the methodology of intracellular Pco₂ measurements are considerably less than those which face my two colleagues. The reason for this becomes apparent if one analyzes the following quantitative relationships.

Gas Exchange

Let us consider a simple model of a cell and its blood supply. I have arbitrarily divided this blood supply into an inspiratory portion at the arterial end of the capillary and a mixed expiratory portion at the venous end of the capillary. To simplify analysis of the relationship between blood gas tensions and intracellular gas tensions, I have made certain assumptions with respect to this model.

1. It is assumed that the fluid which circulates through the vascular channel is plasma and plasma alone. It is assumed that there are no red cells or hemoglobin contained in this plasma. Although one can derive an expression for cellular gas exchange in the presence of hemoglobin (capable of combining with O₂ and CO₂), it is a more complicated equation, and, therefore, analysis is based on hemoglobin-free plasma. This is not an unreasonable assumption, for there are living systems which receive all of their O₂ supply from plasma. For example, tunicates apparently derive required O₂ solely from plasma despite the fact that this species has "green" blood cells containing a vanadium pigment which does not function as an O₂ carrier. An Arctic fish studied by Ruud lacks hemoglobin. Other animals, like some teleost fish, have rather low hematocrits, averaging between 6% and 9%. The assumption that O₂ carriage by hemoglobin is absent is, therefore, not an unreasonable one.

2. It is assumed that gas exchange between cell and plasma occurs entirely by passive diffusion. No special mechanisms for either O₂ or CO₂ transport are involved.

3. It is assumed that the rate of blood flow through the capillary is slow enough to allow establishment of complete gas tension equilibrium between cell and plasma at the venous end of the cell.

A relationship between mean O₂ and CO₂ tensions in the cell can now be obtained as follows. The amount of CO₂ given off by the cell as the plasma flows from the arterial to the venous end of the capillary is equal to the rate of plasma flow times the solubility coefficient of CO₂, α, (which, as you know, relates content of CO₂ to tension of CO₂) times the difference in partial pressures at the expiratory and the inspiratory ends of the vessel. Expiratory and inspiratory Pco₂ may be regarded as equal to the plasma Pco₂ at the

* Presented at the Symposium on Intracellular pH, PCo₂, and PO₂, September 29, 1967, Medical College of Virginia, Richmond, Virginia.
arterial and venous ends of the vessel, respectively, in accordance with the third assumption stated above. In a similar fashion we can quantitate the oxygen uptake for the cell as being equal to the flow times the solubility coefficient for oxygen times the difference in $P_o$ in the plasma at the arterial and venous ends of the capillary. Dividing one equation by the other gives the respiratory exchange ratio (RQ). The flows cancel out, and one is left with the ratio of solubility coefficients for $CO_2$ and $O_2$ times the respiratory quotient (R). This equation may now be solved for the mean plasma $P_o$ at the expiratory side of the cell. The result is:

$$P_{E_o} = P_{I_o} - \frac{1}{R} \times \alpha CO_2 \times (P_{E_{CO_2}} - P_{I_{CO_2}}) \quad (1)$$

Inserting reasonable values for these parameters, one can then solve for the variable that we are interested in, namely $P_{E_{CO_2}}$. The mean $P_{E_o}$ is unknown. Its absolute value is not important, however, for the calculation of the $CO_2$ tension inside of the cell. We shall assume a high value, say, 40 mm Hg. By definition, $P_{I_o}$ is equal to the $P_o$ of arterial plasma, namely, 90 mm Hg. $R$ has a value very near to “one.”

The solubility for $CO_2$ and $O_2$ ratio depends upon temperature and upon ionic strength, but under the present conditions (mammalian temperature and ionic strength) this ratio equals about 20/1. The partial pressure of $CO_2$ in the inspired fluid, $P_{I_{CO_2}}$, is equal to the arterial $CO_2$ tension, which is 40 mm Hg. One may now examine the effects of changes of these independent variables on the value of $P_{E_{CO_2}}$, the dependent variable. One can show that, lowering $P_{E_o}$, from 40 mm Hg to 1 mm Hg, the $P_{E_{CO_2}} (= \text{intracellular } P_{CO_2})$ by definition) increases from about 42.5 mm Hg to approximately 45 mm Hg. Thus, for very large changes of $P_{E_o}$, the effect on $P_{E_{CO_2}}$ is very small. A change of RQ, from its maximal value of “one” in the steady state to a minimal value of around .7, changes the value of intracellular $P_{CO_2}$ by only 0.5 mm Hg. The ratio of gas solubilities is relatively constant. Therefore, despite a wide spectrum of changes in the relationship of all of the other factors, there is a very close relationship between the $P_{CO_2}$ of venous blood and $P_{E_{CO_2}}$, i.e., the $P_{CO_2}$ inside the cell. In other words, $CO_2$ is so soluble and its diffusibility so high in comparison with oxygen that measurements of $P_{CO_2}$ in venous blood must closely approach the mean $P_{CO_2}$ inside the cell. If one can obtain an approximate value for $P_{I_{CO_2}}$, this is given organ, that is to say, if one can measure the $P_{CO_2}$ of the venous blood, which drains that organ, one can approximate the value of intracellular $P_{CO_2}$. This approximation is likewise acceptable for calculation of whole body intracellular $P_{CO_2}$ if the calculations are based on measurements of mixed venous blood $P_{CO_2}$. Since venous plasma is a homogeneous fluid in which measurements can be made with reasonable accuracy, one can closely approximate a value for mean intracellular $P_{CO_2}$.

I would like to contrast this situation with that faced by both of my colleagues in this seminar. Dr. Jöbtsis has implied that, from the standpoint of cellular metabolic processes, “mean” oxygen tension probably signifies little, because one needs to know the values of the $O_2$ tension at those sites in the cell where the various $O_2$-consuming reactions are taking place. Precise analysis of the quantitative aspects of oxidative metabolism within the cell are very difficult.

There are two possible mechanisms by which $O_2$ enters from capillary blood into the cell. One possibility is that $O_2$ diffuses into the cell as a result of partial pressure difference between arterial capillary plasma and intracellular fluid. Those portions of the cell with the most extensive $O_2$ consumption receive the most $O_2$ because they are consuming it more rapidly, thereby producing a greater partial pressure difference, which in turn insures an adequate supply to the involved sites. If this is true, then there must be parts of the cell, for example, the cytoplasm, which have a partial pressure of $O_2$ close to that of arterial plasma, and other areas, such as the mitochondrion, that may have a partial pressure of $O_2$ of approximately 1 mm Hg. Although the concept of “mean $O_2$ tension” may be useful for some purposes, it has little validity in describing the nature of $O_2$ exchange at those intracellular sites where $O_2$ exchange occurs.

The second possibility to explain oxygen entry is that simple diffusion does not explain the transport of $O_2$ into the cell. Even under these circumstances, say, the existence of a special $O_2$ carrier, the bulk of evidence indicates that there is a spectrum of $O_2$ tensions within the cell.

The situation is as difficult when one deals with $H^+$ concentrations inside the cell. One may agree with Dr. Carter that $H^+$ inside the cell is in thermodynamic equilibrium with $H^+$ in extracellular fluid; or one may agree with others who take the viewpoint that thermodynamic equilibrium for $H^+$ exists in some cells, like the red cell, but that in other cells the mechanism for $H^+$ distribution is obscure. At any rate, it seems quite clear that the $H^+$ concentration inside the cell is different from that of plasma. The main problem in understanding $H^+$ relationships within the cell is the problem of $H^+$ concentrations at particular sites where $H^+$ is involved in modifying protein structure or reaction rates. It is interesting to note that an important issue in contemporary biochemistry involves the question of whether intramitochondrial pH is 2.0 or 6.0. At present such questions cannot be answered definitively.

It appears, then, that, in considering intracellular gas tensions, one is on more solid ground with the
very diffusible gas CO₂ because the differences between it inside cell water and venous blood cannot be large.

The Pco₂ in venous plasma depends upon, among other things, the amount of CO₂ produced and the amount of blood ventilating the cell. One of the important factors which determines the value of venous plasma Pco₂ is the level of inspired Pco₂, which is, of course, the arterial Pco₂ level. The latter, in turn, is determined by the total CO₂ output of the animal or human subject and the alveolar ventilation. One must now raise the questions: What is it that sets the level at Pco₂ = 40 mm Hg in man living under normal circumstances? and Why is it that in certain animals the Pco₂ may be as low as 1 mm Hg? A survey of Pco₂ data obtained on a variety of animals is given in Table 1. For a bony fish, a shark has a large.

The African lung fish, which, despite its name, is an air breather, has a variety of animals is given in Table 1. For a bony fish, a shark has a large.

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<th>TABLE 1</th>
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<td>Pco₂ Tensions In Vertebrates</td>
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<td><strong>I. Aquatic Gas Exchangers</strong></td>
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<td>Teleost</td>
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<tr>
<td>Elasmobranch</td>
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<td>Tadpole</td>
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<td><strong>II. Aerial Gas Exchangers</strong></td>
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<td>Turtle</td>
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To explain this bimodal distribution of CO₂ one may use the gas exchange equation (1). Instead of applying the equation to a single cell, however, let us apply it to the whole organism, and let us assume for simplicity that Picο₂ is equal to zero. Equation 1 may then be written as follows:

\[
P_{\text{Eco}_2} = (P_{\text{Io}} - P_{\text{Eco}_2}) \times \frac{\alpha O_2}{\alpha CO_2} \times R \quad (2)
\]

Assuming reasonable values applicable to: a) aquatic gas exchangers, and b) aerial gas exchangers (man), one finds:

a) \(P_{\text{Eco}_2} = (150-100) \times 1 \times 1/35 = 1.4 \text{ mm Hg} (18^\circ \text{C})\)
b) \(P_{\text{Eco}_2} = (150-110) \times 1 = 40 \text{ mm Hg} (37^\circ \text{C})\)

For aerial gas exchangers the gas solubility ratio does not, of course, enter into the calculations.

It follows from this that the very low Pco₂ tensions found in aquatic gas exchangers is dictated by an obligatory requirement for O₂ and the ratio of the solubility of CO₂ to the solubility of O₂ in water.

Electrolyte, pH Relationships

Let us now consider the relationship between Pco₂, HCO₃⁻, and pH. This is expressed by the well-known Henderson-Hasselbach equation:

\[
pH = pK' + \log \frac{[\text{HCO}_3^-]}{\alpha \text{Pico}_2}
\]

There is only a relatively narrow range of pH which is compatible with life. Therefore, for a fixed pH, [HCO₃⁻] must be high or low if Pco₂ is high or low. The highest [HCO₃⁻] found in aquatic animals with a low Pco₂ is approximately 9 to 10 mEq/1, as compared to man and other mammals with a relatively high Pco₂. Here [HCO₃⁻] is, on the average, 25 mEq/1 and can be as high as 35 mEq/1 (Table 2). Thus, the bimodal distribution of Pco₂ referred to earlier is accompanied by a bimodal distribution of [HCO₃⁻].

Once the level of [HCO₃⁻] is determined, it becomes clear that the level of the other major extracellular anion, Cl⁻, must be determined. Since electroneutrality must be maintained, aerial gas exchanging animals with a high (HCO₃⁻) must have a low [Cl⁻] in contrast to aquatic gas exchanging animals in which one finds a low [HCO₃⁻] and a high [Cl⁻]. It therefore seems that the anion pattern of plasma in lower animals as well as in man is dependent on an obligatory requirement for O₂ and the form of gas exchange necessary to provide the O₂.

Let us now look at what the consequences of those relationships are on renal function. The kidneys are chiefly responsible for establishing and maintaining in plasma the proper [HCO₃⁻] and [Cl⁻], which are dictated by the law of electroneutrality. The renal tubule operates so that there is, more or less, an inverse relationship between the amount of [Cl⁻] and the amount of [HCO₃⁻] which is reabsorbed from the glomerular filtrate. Therefore, it appears that the renal pattern of conservation of the main anions depends, in the last analysis, on the

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<td>Bicarbonate Concentrations in Body Fluids of Vertebrates</td>
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<tr>
<td><strong>I. Aquatic Gas Exchangers</strong></td>
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mode of gas exchange and on an obligatory requirement for O₂.

One is tempted now to present certain biochemical features of the evolution of animals, as follows. The most primitive form of energy production is anaerobic glycolysis. Under these circumstances significant amounts of CO₂ are not produced, and the CO₂-[HCO₃⁻] pair is not an important buffer system. As animals developed O₂-consuming pathways, they required O₂ for energy production to maintain life. A definite relationship between Pco₂ and Pao₂ had to be established. Under some circumstances this led to a high Pco₂, while other circumstances resulted in a low Pco₂ of body fluids. This, in turn, determined the electrolyte pattern of extracellular fluid and, presumably, intracellular fluid. It also determined specific patterns of renal function. It, thus, appears that the metabolic pathways that were so beautifully outlined by Dr. Jöbiss have an interesting general biological meaning. Many functions of animals which, a priori, seem to be isolated phenomena, depend on the simple fact that a given amount of O₂ is required for energy metabolism, and the pattern of these functions evolves as a consequence of the operation of the physical chemistry of gas exchange between animals and their environment.

Let us now consider CO₂ as an acid. A fruitful way to examine the effects of CO₂ on acid-base equilibria in animals is to analyze so-called whole-body, CO₂ titration curves. One exposes the animal to a range of Pco₂’s and then determines the effect of these Pco₂’s on the pH and [HCO₃⁻] of arterial plasma. The range of Pco₂ changes in tensions in man and animals living at an ambient O₂ tension of 150 mm Hg is extensive. Pco₂ can vary from several millimeters of mercury in the shark to at least 100 mm Hg in man and dog.

Let us consider the quantitative aspects of pH changes in dog, man and shark. First of all, if one compares acute pH changes in dog obtained from the data of Schwartz, when Pco₂ is changed from 40 mm Hg to 140 mm Hg, with pH changes occurring “chronically,” when dogs are placed into a CO₂-rich atmosphere for three weeks, one finds a smaller change of pH with changes in Pco₂. In other words, within a given Pco₂ range, the acute increases of plasma in [HCO₃⁻] are much smaller than changes that take place when the dog is chronically exposed to CO₂. It may be that the acute increase in [HCO₃⁻] during the first 12- to 16-hour period takes place largely as a result of buffering of carbonic acid, especially by OH⁻ groups in intracellular fluid. Increase in [HCO₃⁻] in animals exposed chronically to elevated Pco₂ not only reflects buffering but also reflects H⁺ excretion by the kidneys. Exposure to high Pco₂, either acutely or chronically, however, will lead to lower pH values in plasma. In other words, extracellular pH is not fully protected by an adequate generation of bicarbonate.

There are two possible general explanations of this finding. One explanation is that the ability of the animal to generate sufficient [HCO₃⁻] to normalize extracellular pH is limited by either buffer capacity or renal function, or by both. This appears unlikely, since the [HCO₃⁻] generated at high Pco₂ values would be adequate to normalize pH at intermediate Pco₂ values. The animal is clearly capable of generating a higher [HCO₃⁻] than he does at intermediate Pco₂ values. Therefore, a functional limitation involving buffering or renal H⁺ excretion does not appear likely.

The other possibility is that the regulatory mechanisms involved are not geared to maintenance of a normal extracellular pH. This possibility requires emphasis since hypercapnic human subjects, unlike dogs, may maintain extracellular pH within normal limits until a relatively high Pco₂ is reached. To explain this fact, various theories have been presented. It has been suggested that such data are unreliable. This is unlikely, because similar findings have been reported by several independent investigators. The existence of a normal extracellular pH in the face of chronic hypercapnia is not unusual in man. The second theory that has been advanced to explain the data is that a normal or even high pH suggests the presence of superimposed disease. If a patient has a high Pco₂ and a normal pH, he may have developed independent metabolic alkalosis. The difficulty with this theory is that, frequently, one is unable to find clear-cut evidence of superimposed metabolic alkalosis. A third theory (our own) suggests that patients with chronic hypercapnia may regulate intracellular and not extracellular pH. Furthermore, it is suggested that intracellular regulation may be associated with low, normal or even high extracellular pH values.

Data were obtained by a young colleague of mine, Dr. Tushan, who studied a group of patients with moderate hypercapnia using the DMO technique to calculate whole-body intracellular pH and intracellular pH of muscle on biopsy samples. It was shown that the mean values for whole body intracellular pH and muscle pH are not significantly different from each other or from normal values, regardless of the level of extracellular pH. I should emphasize that this is not incontrovertible proof, but it is at least consistent with the theory that the regulatory mechanisms called upon when conditions of high Pco₂ exist are located in areas in the body which are not readily definable but are presumably intracellular in location.

I would now like to call your attention to the data from the shark, because the findings are dramatic and because I think they illustrate a rather important point. When the shark’s Pco₂ is elevated from 4 mm Hg to 12 mm Hg, his extracel-
Anaerobic Energy Metabolism

Stimulated by the lecture of Dr. Jöbsis on oxidative, energy-liberating pathways, I would like to conclude my presentation with a discussion of anaerobic energy production, which has not been discussed in this seminar.

There are at least four reasons why animals die when they undergo severe O₂ depletion. Dr. Jöbsis has already given one reason, namely, that, with loss of O₂-dependent reactions, energy production fails or ceases. Secondly, there is the loss, at least in mammals and in most vertebrates, of central nervous system integrity. There may be something about oxygen, aside from its ability to provide energy, that is necessary in vertebrate systems for the structural and functional integrity of the nervous system. Thirdly, animals suffering from oxygen depletion lose biosynthetic ability. In many biosynthetic reactions, oxygen acts as an obligatory oxidant for the synthesis of many important compounds. Fourthly, animals can die because of acidosis, which occurs as follows. As the oxygen supply becomes limited, the animal switches to anaerobic glycolysis, the end product of which is pyruvic acid. Pyruvate accumulates, as does lactate, its reduction product, along with other organic acids of the Krebs cycle. Thus, there is an accumulation of organic acids, which are proton donors, and the animal develops severe acidosis. This, combined with severe hypoxia, may become a limiting factor in survival.

Several years ago I inaugurated studies on pond turtles (Pseudemys). This species is able to dive for periods of several days. It was formerly believed that during diving the turtle respiratory exchange through the buccal mucosa by tapping the mouth. It turned out that there was no respiratory exchange under these circumstances, and the turtle survived under water for one to two weeks despite a total absence of external oxygen supply. Within a few hours the oxygen tensions in lung, arterial and venous blood dropped to zero. The animal survived with O₂ tensions at essentially zero. The turtle was also able to survive in pure nitrogen. It likewise survived in the absence of cytochrome-dependent metabolism, as was demonstrated by blocking cytochrome c with cyanide or by blocking cytochrome b with antimycin. Blood lactate levels rose from 1 mM/l to 40 to 60 mM/l over a period of seven to ten days, indicating that the major energy source was anaerobic glycolysis.

The precise mechanisms of anaerobic glycolysis under these circumstances is not known, but they may very well involve the pyruvate-lactate system. Pyruvate occupies a key position between anaerobic glycolysis and the aerobic pathway. Pyruvate can be interconvertibly reduced to lactate through the action of a group of isoenzymes which are collectively known as LDH (lactic dehydrogenase). In most vertebrates there are two basic kinds of LDH. There is an MLDH (muscle LDH) and an HLDH (heart LDH), the synthesis of each being under separate genetic control. The five isoenzymes which are found in most vertebrates represent the five possible permutations of these two polypeptides occurring as tetramers. Thus, there is an M₁₄, an M₂H₃, an M₃H₂, an M₄H₁, and an H₅.

MLDH is biochemically quite different from HLDH in a number of respects. According to Kaplan and his co-workers, MLDH, but not HLDH, is insensitive to inhibition with pyruvate ion. This means that if MLDH is dominant over HLDH, pyruvate is converted into lactate, possibly by supplying NAD, thus augmenting anaerobic glycolysis. On the other hand, if HLDH is dominant over MLDH, lactate will be converted into pyruvate, which is fed into the Krebs cycle and used in the oxidative metabolism. Thus, dominance of MLDH favors anaerobic glycolysis; dominance of HLDH stimulates oxidative metabolism. Although the role of the two forms of LDH in anaerobic glycolysis, as outlined here, is not fully established, there is good
evidence that the amount of MLDH or HLDH that is available, at least under some circumstances, is dependent on ambient O₂ tensions. Hypoxia is associated with high MLDH; anaerobiosis with high HLDH levels. We have found that whole turtle homogenates and homogenates prepared from heart, brain, liver, or turtle serum have only one LDH, and preliminary work shows that this LDH is of the M type. This may be looked upon as a fortunate evolutionary development, because it is the presence of MLDH which, in part, makes it possible for the turtle to survive by anaerobic glycolysis without O₂ living. The question may now be raised: How does the turtle survive severe acidosis for days or weeks? The turtle has an anatomical compartment which is known as the coelomic cavity. It is equivalent to the peritoneal cavity in man, and the turtle may be likened to a patient suffering from liver cirrhosis in that the coelomic compartment has in it a volume of fluid which represents about 6% or 7% of total body weight. This fluid contains 100 to 120 mEq/1 of bicarbonate, as compared to plasma, which contains approximately 32 mEq/1. This compartment is also fairly permeable to lactate ion. The coelomic fluid, then, represents a relatively large reservoir or buffering bicarbonate solution, which provides for adequate protection against acidosis during diving. Indeed, one finds that, after diving, the bicarbonate concentration of the coelomic fluid decreases, while the lactate concentration increases.

**DISCUSSION**

**Dr. Patterson:** Dr. Robin is open for quizzing.

**Dr. Jôbís:** As the lactate is freed by the body, it reacts with the very high concentration of bicarbonate here. Before that time there must have been an equilibrium. In other words, does the pH change vary greatly, or is CO₂ excreted by the animal?

**Dr. Robin:** No, you see he is div­­ing. He cannot excrete CO₂, so the system is not as efficient as if he had open egress to air to get rid of the excess CO₂. On the other hand, in real life I imagine that pond turtles do not very often dive for as long as one to two weeks, and, therefore, the ability to come up for air and to get rid of CO₂ is present in life.

**Dr. Jôbís:** What is the normal pH of the coelomic fluid?

**Dr. Robin:** Control values are ~8.2. The fluid is like a solution of three-tenths molar Na + bicarbonate. During prolonged diving, the pH falls; lactate rises; bicarbonate decreases; and the PCO₂ rises sharply so that the animal has two sources of PCO₂, the most important of which is due to the buffering of hydrogen ions by bicarbonate.

**Dr. Regelson:** It cannot only be that, though, because Sanford Sie­­gal, exobiology researcher of Union Carbide, using the red-eared turtle for survival studies, found that, at atmospheric pressures of 20,000 feet with near zero oxygen (0.1%), there is 24-hour turtle survival in this system. He claims that there is no circulation in the turtle under these circumstances, yet it stays alive; the blood sludges, and there is no movement of blood in the blood vessels. There is also a species of ocean perch, the Arctic perch, which has no hemoglobin but gets along.

**Dr. Robin:** But that does not apply to the diving turtle.

**Dr. Regelson:** No, not to the diving turtle, but the turtle can still live 24 hours with no circulation at all before it dies.

**Dr. Robin:** Well, what I really tried to imply by describing the coelomic compartment and anaerobic glycolysis is that, in animals which adapt to living under conditions of oxygen depletion, one seldom finds a single mechanism to explain survival. Generally there are a number of adaptations. There is another adaptation in the turtle. When the animal dives, he decreases blood flow to his pulmonary circulation, and the blood flow then goes to what would be the equivalent of the left ventricle in man. He thereby supplies more substrate to his tissues. Apparently, to get around the problem of inefficient energy generation, one needs a number of different tricks.

**Dr. Carter:** I am not sure I have any quizzing. With reference to bicarbonate concentrate in the kidney, as Dr. Robin mentioned, in the final analysis the kidney must set the concentration. This has some meaning in the curves that you showed regarding acute and chronic. That is, it has been shown, for example, that the bicarbonate Tm, or the maximum amount of bicarbonate that is reabsorbed, will go up in a dog exposed chronically to increased CO₂ atmospheres. In other words, that is an adaptive mechanism. We assume, and I do not think anyone has any measurements to prove it, that the same thing occurs in man. The reason we do not have measurements that are very good is that experimentally it is very difficult to put man in a CO₂ environment. The only people that have been really successful at this are the submariners in situations where CO₂ concentration in submarines has been up around 6%. In case you have not tried it, just picking up a mask full of CO₂ is a hilarious experience, to put it mildly and that is not the least of your worries. As soon as you stop, you will feel like you are about to die. So, this is a tough human experiment.
In considering the shark, perhaps the most pertinent animal is the rat; because the rat is not like the dog. The bicarbonate Tm in the rat normally is close to 18 mM higher than the serum bicarbonate normally maintained. That is a peculiar circumstance, but it seems to be quite true. In other words, in the case of man and dog, the bicarbonate Tm is at any given moment pretty well equivalent to what the serum bicarbonate is. This is not true in the rat, where the Tm approaches the equivalent of 38 to 40 mM bicarbonate in the plasma. For this reason, when you give a rat CO₂ rebreathing, he immediately has a very rapid increase in serum bicarbonate and tends to have a somewhat blunter range of pH change, although it ultimately does become acidotic. The whole thing is over in 24 hours, whereas it may take as long as several days in the dog. I wonder whether the same thing is not true in the shark—that the Tm for bicarbonate must be at all times higher than what the bicarbonate is in the plasma. Otherwise the shark would not retain the bicarbonate when one raised the Pco₂ unless one wanted to make an inordinate curve for the action of Pco₂ in the bicarbonate Tm. Do you have any measurement of that?

Dr. Robin: The shark kidney is insensitive to any measured parameter to changes in either CO₂ tension, bicarbonate or hydrogen ion. That is to say, if one takes the animal and loads him with bicarbonate, this changes neither the urinary pH nor urinary bicarbonate concentration nor urinary ammonium excretion or urinary titratable acidity. The presumption is that, since his pH under these circumstances returns to normal, excretion takes place through the gill and, for reasons which are not entirely clear, the shark kidney essentially does not respond to changes in extracellular pH by “appropriate” activity. There is no possibility of defining Tm under these circumstances.

Dr. Carter: Did I understand you to say that the gill excretes bicarbonate?

Dr. Robin: Yes.

Dr. Carter: Does it do this continuously or only when bicarbonate concentration is raised? And if it does it only when the concentration is raised, at what point does one have to raise it to see bicarbonate in the gill effluent? In other words, the Tm in the gill conceivably is in excess of the serum concentration, which would amount to the same thing as the condition in the rat.

Dr. Robin: The gill does not appear to respond by an increased Tm. That is to say, the difference between the mixed venous and arterial bicarbonate concentrations multiplied by cardiac output does not increase under circumstances in which plasma bicarbonate becomes markedly raised through inhalation of CO₂. I would not want to push these data too far, because they are preliminary. It is much more simple to measure Tm in a dog kidney than in a dogfish gill, but our impression is that this is not a very sharply regulated mechanism; and I have no observations of chronic changes.

Dr. Kiesow: In regard to your data on the pond turtle, would you have, by any chance, quotients on anaerobic glycolysis in various tissues, so that one could get an idea about the possible energy production by glycolytic pathways?

Dr. Robin: The RQ for the whole animal rapidly approaches infinity. He is producing CO₂ because of buffering of hydrogen ion by HCO₃⁻, and he is not using oxygen, so he is producing buffer CO₂. RQ measurements under these circumstances are not useful for the whole animal. We have some preliminary data for brain slices, and our impression is that, even under circumstances of adequate oxygen supply, the preferential route of energy generation is through anaerobic glycolysis.

Dr. Kiesow: Would you then say that turtle tissues are pretty much similar to cancer tissues?

Dr. Robin: This is a useful analogy. They act like Ehrlich acetes tumor cells.

Dr. Kiesow: The fetal tissue, however, cannot survive under anaerobic conditions.

Dr. Robin: Nor can the turtle for, say, longer than two weeks. I should have made that clear. You are extending the three minutes of anoxic survival in man, to two weeks in the turtle, but he: a) runs out of buffer, and that may be one factor; and b) he may run out of substrate; and c) he may have some absolute oxygen requirement which produces structural deterioration after a longer time.

Dr. Regelson: There is a lipid chemist who did a study of cardiolipin, going on up through the animal kingdom in an effort to determine mitochondrial patterns and development of respiratory pathways in the phylogenetic sense. He came across a salamander, amphiuma, which I always wanted to work with and which has no mitochondria at all. There is deposition of a melanin pigment and crystalloid aggregates in the liver when amphiuma aestivates, the implication being that the pigment in some way has a respiratory role. Several investigators have postulated that melanin could play a role in oxidation-reduction systems to provide an alternative source of energy.

Dr. Robin: That is very interesting. I am not aware of that data.

Dr. Huf: In your earlier discussion you emphasized the need for a low Pco₂ and bicarbonate in aquatic animals because they have to maintain their oxygen requirement. I take it, then, that if one elevated the CO₂ tension, the animal would die because of interference with the oxygen metabolism.

Dr. Robin: In the limiting case, yes. If one is living on ambient oxygen so that there is an ambient PO₂ of 150, there is a Pco₂ so high that the oxygen tension must be so correspondingly low that one cannot survive.

Dr. Huf: Could one extrapolate
from here and conclude that if one infused animals with bicarbonate/CO₂ mixtures at elevated Pco₂ but not in such a manner as would interfere with their general acid-base balance, the animals could survive if one also elevated the PO₂?

Dr. Robin: This is under equilibrium circumstances, and all this relationship says is that, since nitrogen is fixed in an air-breathing animal, in essence whatever is left must be distributed between oxygen and CO₂ in the steady-state circumstance. Hence, I do not think one could extrapolate these quantitative relationships therapeutically.

Dr. Brackett: The curve you showed for chronic hypercapnia—between 40 and 60 mm Hg—showed a normal pH. Certainly it is difficult in clinical material to be sure about associated acid-base disorders. I assume that these were reasonably excluded in your patients. On the other hand, dog, as you pointed out, never reaches “normal compensation,” so this may be a semantic problem, in part, but I would wonder. We, too, have been interested in studying chronic, steady-state hypercapnia in carefully selected patients. If this same linear relationship obtains and one knows, in fact, the “normal pH” in an individual, at any degree of hypercapnia he would not have the same hydrogen ion activity. I think that, certainly above a Pco₂ of 55, there might be a discernible difference. Below that it is very difficult to know when paired data are not available.

Dr. Robin: If one finds a range of pH’s, and one finds pH’s as high as 7.47 and 7.48, it would be hard to believe that a patient, when he was normal and his Pco₂ was 40, had a pH of 7.53 or 7.54. Secondly, as was shown originally by Dr. Schwartz, if one takes such patients and infuses bicarbonate so that one normalizes arterial pH, they promptly excrete the infused HCO₃⁻. That is to say, they return to an extracellular pH which seems, for whatever regulating mechanisms there are, to be satisfactory to them. We have looked meticulously for changes in chloride balance in some of these patients to see what happens to exogenously administered chloride and looked for evidence of potassium depletion inside of muscle. While in some patients it is possible to find some kind of complicating metabolic lesion, a number of these patients with normal or elevated pH’s do not show such abnormalities. The implication of the shark studies is that there is another animal that is able to generate enough bicarbonate to maintain a baseline or a normal value of pH in the face of sharp hypercapnia. Our extrapolation would be that if it can occur in some animals, then presumably it could occur in man.

Dr. Brackett: The dog data do not demonstrate complete compensation when starting with each individual dog’s control pH, and in our human data, on the basis of 20 patients and 40 steady-state points carefully selected, man behaves very much like the dog, with 95% significance bonds falling about two nanomoles lower. This suggests that man reaches for but never quite attains complete compensation with Pco₂’s over 55 mm Hg.

Dr. Robin: Thank you. I think this makes a very important point. In a sense, it highlights the very real differences that exist in this area. People who are very much intrigued with whole body titration curves have been intrigued with statistical analyses. It seems to me, though, that if you are interested in mechanisms, it is more important to explain the exception, the man who has the pH of 7.48 when his Pco₂ is 55, because if you fit his values in with statistics, it will just be a point buried in the standard deviation of your whole body titration curve. Yet it may mean that he has hyperventilated, that he is potassium depleted or that he has special kinds of regulatory mechanisms at work. So, in a sense, I think both approaches are important, but I like to think, although this is a subjective judgment, that one of the ways of progressing in this area is to analyze these patients in a very individual, mechanistic sense. Of course, the thing that impressed us when we did this for intracellular pH was the evidence that they regulated their intracellular pH more closely than they regulated extracellular pH.
Intracellular pH Determination by Means of pH Glass Microelectrodes*

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A number of years ago I decided to attempt to measure the intracellular pH of skeletal muscle by a more direct method than those in current use, that is, by an electrode. I excluded all forms of pH electrodes except the glass electrode and then set up a certain number of characteristics or requirements that this electrode should have.

First, the electrode should be insulated in such a manner that only the pH-sensitive portion is in the cell cytoplasm. Second, the reference side of the electrode group should also be within the cell, because the cell has a transmembrane potential; and the only way to circumvent this transmembrane potential in actual electrical measurements is to have the reference electrode within the cell. Finally, the tip diameter of the combined pH and reference electrode should be less than 1 µ, in order to reduce damage of the single cell by penetration or impalement.

Insulation of Electrodes

I soon discovered that one of the major problems in constructing an electrode was the method of insulating. My associates and I tried a number of methods for obtaining the required amount of insulation. One method we developed at about the same time as Kostyuk and Sorokin (1961) was the so-called concentric capillary method in which the pH capillary is run down a lead glass shield. This method has certain deficiencies. First, the insulated portion of the lead glass shield is too thick to enter the cell, so that one can never be sure that some pH sensitive glass is not on the outside of the cell membrane. Second, the numerous organic materials we tried as sealants between the pH glass and the lead glass imbibed solution and ultimately lost their high specific resistance. In addition, although listed as industrial adhesives, many of these materials do not adhere well to very small glass capillaries. These organic materials, then, although usually very satisfactory for electrical insulation, are not very satisfactory for the electrodes used in the concentric capillary method. For these reasons, none of the organic coverings were found useful as insulating materials.

Our first successful insulating technique, which was first reported in 1961, was the use of a glass stain (Carter). Of the several methods of staining glass, we chose the silver glass stain, which is a good method for insulation unless one wants to make a large number of electrodes. It has been known for a long time that, if one takes a silver salt and lays it on glass and then brings the glass to a temperature close to its annealing point, some of the alkaline cations within the glass will exchange with the silver ions. Furthermore, the silver ions that enter the glass will nucleate or form a colloidal suspension. Thus, in essence, what this glass stain does is reduce the sensitivity of the glass to hydrogen ion. Theoretically it is a good method, but it is not a very practical one.

The final method we tried is the one we are currently using and have been using for a number of years. This consists of covering the pH glass with a glaze or fired-on enamel. The principal drawback with this method is that one has to find a glaze or enamel that very closely matches the thermal characteristics of the pH glass. Furthermore, this glaze, which is really glass after being fused, has to have high chemical resistance so that it does not dissolve away from the pH glass. We have developed a ceramic glaze which we believe has the two properties just mentioned, and it is now commercially available. It is a high titanium, low melting point, lead glass. If one takes a small capillary of pH glass and coats it with this glaze, fuses the glaze on to the capillary and then pulls the capillary out to a tapered tip, one finds that, depending upon several factors mentioned below, it is possible to pull out a small length of pH-sensitive glass that has no glaze. The length of this pH-sensitive tip is determined by: 1) the initial thickness of the glaze on the capillary; 2) the thickness of the wall of the capillary or the diameter of the capillary; 3) the ultimate length of the overall taper. These factors can be worked out empirically. For example, it is possible not only to select a capillary size that will allow the tip to be pulled closed, but to select a capillary size which, when
coated with a certain thickness of glaze, will result in a tip of pH-sensitive glass that varies between 15 and 20 µ in length. It is also possible to add a small capillary of lead glass parallel to the glazed pH capillary and pull these capillaries simultaneously so that a single tip made up of two components will result and the reference side of the electrode will remain open. If the glaze is applied correctly, the pH side can be what we call "insulated" down to within a few microns of the tip. This is the ideal electrode.

**Testing of Electrodes**

The electrode is placed in a Teflon holder which contains a silver-silver chloride electrode and the mechanical attachments for mounting it in a micromanipulator. With this type of construction a number of tests must be made before any given electrode can be used to make a biologic measurement. Figure 1 shows the results obtained from an electrode which was checked in several buffers against its millivolt reading. The electrode had a fairly linear relationship over the range from pH 4.0 to pH 10.0 and was quite sensitive in the pH range in which we were most interested. The tips of these electrodes are extremely delicate, of course. Not all types of pH glasses are very durable by present day standards. It is not good practice to leave the electrodes in either pH 4.0 or pH 10.0 for any length of time, because they will deteriorate. Contrary to what had been previously published about Corning 0150 pH glass, we found it maintained its properties best, although not very well, in distilled water. Therefore, once one obtains a workable electrode, it is mandatory to perform all necessary tests and then use it without delay. The electrode depicted in Figure 1 had, at 25°C, a slope of 58.7 mv for a tenfold change in H⁺ activity. Most of the electrodes have somewhat smaller than theoretical slope factor. We have selected electrodes that have slopes above 40 mv per pH unit.

Electrodes are tested in several ways. One is to check the electrode's ability to correctly read pH of whole blood measured with a Beckman blood pH electrode. Another way is to test the electrode in a muscle homogenate by homogenizing muscle in as small an amount of water as possible, measuring the pH with a microelectrode and comparing the results with those of the pH electrode. What we are looking for, principally, is not a real fault of the glass membrane, i.e., its "protein error." As a matter of fact, we believe it is possible to reduce the so-called protein error to nearly zero by lightly siliconizing the surface of the microtips. What we are interested in is what the various micromolecules, and particularly the various anions, do to the tip potential of the reference side of the electrode. An electrode which otherwise seems to be a very good pH electrode may, because of its reference electrode, be worthless in a biologic solution. For example, the Beckman electrode may read pH 7.4, whereas the reference side, for reasons unknown, may give an apparent pH of 9.0. When you put the electrode into the muscle homogenate, it may pick up a new tip potential of perhaps 40 or 50 mv, which it never loses. The electrode, then, has to be discarded.

The tip potential of the reference side is generally quite low, and, as you will see later, we bucked the tip potential to where we wanted it to be and kept checking to make certain that it had not shifted. There are electrodes that go through all the checks and seem to be good electrodes until one starts making muscle punctures. Then the slope of the pH side of the electrode or the tip potential of the reference side changes tremendously. When this happens, the electrode usually is not recoverable. There is no consistent direction in which bad electrodes change. Providing there is
no mechanical injury to the electrode, the operational life of a truly good electrode varies somewhere between several hours to three weeks.

As I indicated earlier, insulation is one of the most important aspects of the entire operation of building and using microelectrodes, and for a long time we did not have an in vitro method of testing insulation. Several years ago we developed a method of testing insulation which, because it entailed micropuncture and perfusion of the rat kidney tubule, would never have gained acceptance. (Rector, Carter, and Seldin, 1965) If one is fairly adept at perfusion in single tubules and puncturing the tubule, this is a satisfactory way of checking the insulation of electrodes. Finally, an in vitro method was developed. We found it possible to make a membrane out of the latex used in comparative anatomy laboratories for injecting cats. The latex was poured on a piece of paraffin® film that had a hole cut in it. We obtained a membrane about 1 µ thick that sealed around the microtip well enough to prevent the buffer running down between the membrane and into a buffered agar below the membrane. We used agar in the lower buffer to give mechanical strength to the membrane. It was possible by direct microscopic observation to measure how far the microtip had penetrated the membrane into the agar. It was therefore possible to determine how much of the electrode tip was pH sensitive.

Table 1 shows the use of both insulated and uninsulated electrodes in the latex membrane method. When tested in buffered solutions of pH 6.0 and pH 7.4, these electrodes both read pH reasonably well. One had a slope of 58 mv; the other, 55 mv. These measurements were made at room temperature. When the insulated electrode was placed so that its tip was 10 µ below the latex, and, thus, inserted into the pH 6.0 buffered agar, it gave approximately the same

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<td><strong>Insulated Electrode</strong></td>
<td><strong>Uninsulated Electrode</strong></td>
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<td>Depth of Electrometer Penetration µ</td>
<td>Apparent Reading mv</td>
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<tr>
<td>Buffer pH 6.0</td>
<td>+157</td>
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<tr>
<td>Buffer pH 7.4</td>
<td>+76</td>
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<tr>
<td>Agar Buffer pH 6.0</td>
<td>+155</td>
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<td>Overlaid Buffer pH 7.4</td>
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<td>Transmembrane Potential of 90 mv Imposed</td>
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reading as it did when tested previously in a solution of pH 6.0 buffer. When we overlaid with pH 7.4 buffer on the upper surface of the latex membrane, there was no change in the electrometer reading; and the apparent pH still stood at approximately pH 6.0. Finally, when we imposed the 90 mv synthetic membrane potential, there was no effect whatsoever on the electrometer reading.

In the case of the uninsulated electrode, the tip penetrated 42 µ, an inordinate distance. Even with 42 µ below the surface of the latex membrane, however, we got an apparent pH change when we overlaid pH 7.4 buffer, which suggested that there was a significant amount of uninsulated pH glass above the membrane, or above the 42 µ point.

Finally, when we imposed the 90 mv transmembrane potential, we obtained a further change in the pH reading. Each electrode has to go through this test. Although this test is not technically difficult, it requires a large number of manipulations. One usually breaks the electrode at this point and has to make a new start.

### Intracellular pH and Transmembrane Potential

With the use of these double-barrelled electrodes and two electrometers, one can simultaneously make readings of intracellular pH and record transmembrane potential. Figure 2 shows how this is done, namely, by connecting the reference side of the microelectrode to both an independent electrometer and the low impedance side of a pH electrometer.

During a series of measurements we removed the electrode and rechecked it by dipping the electrode in our test solutions to determine not only whether the electrode was still reading the same pH as previously in any given buffer, but whether the reference of the pH electrode has developed any significant change in tip potential. By utilizing the shunt circuit on the input of the electrometer, we were able to measure at will the resistance of both the reference and pH sides of the electrode even while they were inside of a muscle cell. It was possible by this means to ascertain whether we were dealing with an intact electrode or one that had been accidentally broken off by impalement. (Carter et al., 1967b)

In Table 2 we show data obtained in normal rats. Most scientists who study transmembrane potentials consider the highest transmembrane potential to be the correct one. Hence, we arbitrarily took a value of 89 mv plus or minus one standard deviation, which was 3 mv, a value that we obtained from almost 300 impalements in 24 rats, using Ling electrodes to measure normal transmembrane potential. We examined those pH measurements that fell within the emf range from 86 to 92 mv. In this range we had 38 measurements from seven rats. The blood pH of the rats was 7.41. The average potential of these 38 readings was 88.7 mv, which was close to the 88.9 mv potential previously found. The mean intracellular pH was 5.99. If this result means that hydrogen ions are in electrochemical equilibrium across the skeletal mus-

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**TABLE 2**

**Intracellular pH of Normal Rat Skeletal Muscle**

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<th>Mean ± S.D.</th>
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<td>Blood pH</td>
<td>7</td>
<td>7.38-7.44</td>
<td>7.41 ± 0.06</td>
</tr>
<tr>
<td>Transmembrane Potential</td>
<td>38</td>
<td>85-92 mv</td>
<td>88.7 ± 2.2 mv</td>
</tr>
<tr>
<td>Intracellular pH</td>
<td>38</td>
<td>5.77-6.29</td>
<td>5.99 ± 0.14</td>
</tr>
</tbody>
</table>

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**Fig. 3**—Measured intracellular pH of rat skeletal muscle (with a simultaneous Em measurement of -85mv or higher) with electrodes having various lengths of pH-sensitive tips. (Reprinted with permission from *J. Clin. Invest.*, 1967b.)
cle membrane, then we should be able to determine mathematically the accuracy of our method.

If there is electrochemical equilibrium between blood pH and intracellular pH, then these results should fit the Nernst equation:

\[ E_m = 61.5 \, (\text{pH blood} - \text{pH}_i) \]

where \( E_m \) is the transmembrane potential in mV and \( \text{pH}_i \) intracellular pH. If the observed \( E_m \) (−88.7 mV) and blood pH (7.41) are used, the calculated \( \text{pH}_i \) is 5.97, which is very close to the observed value of 5.99, strongly suggesting electrochemical equilibrium for H⁺ activity.

In Figure 3 one sees the results of a group of experiments which show the importance of insulation and its relationship to intracellular pH. Originally this work was deliberately undertaken with poorly insulated electrodes because of the findings of Dr. Peter Caldwell from Bristol, England, and Kostyuk and Sorokina in Russia. We now feel it was not poor insulation that affected the data obtained by Dr. Caldwell (1958). The only reason we believe the Russian workers' electrodes were poorly insulated is that they used as insulators organic materials which we have never found satisfactory. Both Caldwell in his work with the crab muscle, and Kostyuk and Sorokina with the frog muscle have shown by their electrode methods that there is no electrochemical equilibrium between extracellular and intracellular compartments for H⁺. This figure shows that, if insulation is less than adequate, one will obtain a reading for intracellular pH that increases steadily until the length of the exposed tip is about 50 μ. From that point on the values will mostly fall around a pH of 7.0.

**Single-Barrelled pH Electrodes**

Despite the fact that we are getting very small tip diameters with our double-barrelled electrodes, it was thought necessary to use a single-barrelled pH electrode which can be pulled to a tip diameter well under .5 μ, thus reducing the possibility of cell damage by impalement. If one had a pH electrode outside the cell in the extracellular fluid, read its potential against a reference electrode in this fluid, and set this potential to zero, there would be no potential change when the cell membrane was impaled if H⁺ were in electrochemical equilibrium. The reason for this is that the potential across the membrane would exactly balance the reading obtained from the pH electrode because of the change in H⁺ activity inside. This only holds for electrodes that have a theoretic slope of 61.5 mV at 37°C. If the slope is less than theoretic, one reads a potential greater than zero, but this potential can be calculated.

In Figure 4 we show the predicted potential difference that we
would expect to get with the varying electrode slopes. We have drawn in the line that should be generated at various slopes with hydrogen ion equilibrium at an intracellular pH of about 5.95. The top line is what we would expect with non-equilibrium at an intracellular pH of 7.0 and different electrode slopes. We made the impalements and measured the potential. It is fairly clear that the points are clustering about the lower line. Certainly none of them are near the upper line. Hence, the data obtained with the single-barrelled electrode is in agreement with that obtained with the double-barrelled electrode; namely, H⁺ appears to be in electrochemical equilibrium across the cell membrane.

Triple-Barrelled pH Electrodes

In Figure 5 we show the results of a single experiment made with a triple-barrelled electrode. In this electrode a third barrel was placed parallel to the other two, and the third barrel was used to impose a current across the muscle cell membrane. In other words, we applied electric current to alter the transmembrane potential and observed simultaneously changes in measured intracellular pH. We varied the transmembrane potential, at least in the area of the tip of the reference electrode, from a value of about +20 mV to -240 mV. The actual pH measurements we obtained at these various membrane potentials appear to fall more closely along what we call membrane slope, referring to the muscle cell, than along the electrode slope (the dotted line). We conducted five of these experiments with five different electrodes. Results were similar to those in Figure 5.

The results with these triple-barrelled electrode experiments further suggest that H⁺ across the skeletal muscle cell membrane is in electrochemical equilibrium.

REFERENCES


DISCUSSION

Dr. Patterson: Dr. Carter's paper is now open for discussion.

Dr. Robin: Some biologists claim that they have data which show that the hydrogen ions move very slowly into cells. It would seem to me that a simple way of testing your interpretation would be to administer hydrogen ions in the form of hydrogen chloride and show that hydrogen ions are in electrochemical equilibrium.

Dr. Carter: Yes. We've done this with hydrogen chloride, and also by producing respiratory acidosis and respiratory alkalosis. From intracellular pH measurements with the pH electrode, it appears that hydrogen ions are in electrochemical equilibrium.

Dr. Jöbsis: I remember that Con-

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Fig. 5—Triple-barrelled pH electrode study wherein Em of cell could be changed by imposing a current through the third barrel of the electrode. When Em was so changed, measured pH, was noted to change predictably in accordance with the "cell membrane slope," e.g., 1 pH unit for every 61.5 mV change in Em. (Reprinted with permission from J. Clin. Invest., 1967b.)
way, defending himself against criticism raised by A. V. Hill, made some calculations on the speed of hydrogen ion movement into muscle. He came to the conclusion that these ions move only very slowly.

**Dr. Carter:** In his calculations, Conway made the rather arbitrary assumption that the mobility of hydrogen ion potassium in solution was the same. It turned out that it would take about 360 days to bring about one-half of a pH change in skeletal muscle. Nevertheless, many people hold to the view that hydrogen ions move very slowly, and they base their argument principally on the observation that Dr. Robin wanted us to look at, i.e., that metabolic acidosis does not seem to change the intracellular pH as measured by other than the pH electrode techniques.

**Dr. Robin:** Dr. Carter's work is obviously technically magnificent, and he has been put in the unfortunate position of being attacked from all sides because the methods that he uses are so advanced and technically so difficult. It is, therefore, hard to find experimenters who are capable of either confirming or denying his results. There are, however, other methods for measuring intracellular pH. I would like to mention three results obtained by independent methods which would be very difficult to reconcile with Dr. Carter's results, but are in good agreement with results obtained by indicator systems. I am not mentioning these findings to indicate that I think that they resolve the question. Firstly, if one assumes that the intracellular pH in the muscle is 5.95 to 6.00, then one can calculate the total amount of CO$_2$ which is present in the muscle; obviously, this would be very small in amount. If one uses the intracellular pH of 6.9 as obtained by indicator methods, then the amount of CO$_2$ which is in the body is considerably larger. One can measure CO$_2$ stores by independent methods, essentially dilution methods, that have nothing to do with the measurement of intracellular pH. Thus, one finds experimentally for the total body CO$_2$ a value which is much closer to the one predicted by calculation, assuming a value of 7, rather than 6, for intracellular pH. There is, however, the unresolved question whether a part of the CO$_2$ is present in the body as the "barium soluble" form. Secondly, in view of the difficulty just mentioned, another technique for measuring CO$_2$ stores has been used, which is as follows. The animal or human increases or decreases his CO$_2$ stores by hypo- or hyperventilation, and under these circumstances, by observing the amount of CO$_2$ which has been added to or subtracted from the body, one can estimate the CO$_2$ stores. If this calculated value for whole body CO$_2$ is compared with the value obtained by application of the DMO technique, or the H$_2$CO$_3$-bicarbonate indicator technique, the results are in good agreement. On the other hand, if one makes use of an intracellular pH value of 5.99 or 6.00, the results are quite disparate. This would mean either that the value of 5.9 for intracellular pH in muscle is low, or that all the CO$_2$ which has been added to the body was instantly converted into the barium soluble form. This is a rather unlikely possibility. Thirdly, if one administers mineral acid or base to an animal and then measures total CO$_2$ in muscle by gasometric procedures which are independent of any assumption about how that CO$_2$ is compartmentalized between dissolved CO$_2$, carbonic acid and bicarbonate, one can show that there is no evidence in terms of total CO$_2$ that the muscle is being titrated either by hydrogen ion or by bicarbonate. This leaves one in the rather uncomfortable position, accepting a pH value of 6.0, of saying that the gasometric techniques, which, as I emphasized, are independent of any assumptions about CO$_2$ compartmentalization, are fortuitously making precisely the same errors as are being made by measurements with pH indicators. That's not a question; that is a comment.

**Dr. Carter:** Unfortunately, one comment deserves another. We do not have time to go into our experiments with DMO, but suffice it to say that we think, as probably some of you have heard, that DMO is bound intracellularly. And although I have no data on the subject that is worth reporting here, it would appear that bicarbonate and DMO are located in the same space; perhaps one replaces the other. I'm not sure that this would explain all of the observations which you mentioned. But if allowance is made for some peculiar site, I think that the data could be reconciled on this basis. I have great reservations about the idea of the acid-labile CO$_3$, as put forth by Conway, for a variety of reasons. I don't think that new bicarbonate is formed either when the muscle is treated with potassium hydroxide for a short period of time, or when it is released with strong acid. I rather believe that some of the bicarbonate is not measured in the compartment that we call the bulk phase of the cell where we are getting the pH of 6. I don't think that negates the fact that there may be some other place where it is definitely measured and where, under a variety of physiologic alterations, it is purposefully utilized. I would agree with you that this is the meat of the whole issue. One might even go further and clarify it in very definite terms. At the current time there are almost 400 publications on intracellular pH utilizing either DMO or bicarbonate partition. With one exception, all of these agree in principle that the pH is near 7. It may vary from pH 6.7 to 7.2, but that is near pH 7. No one agrees with us that it is near pH 6, with one exception. We do have a problem indeed, but I can only show the data that we have. We are aware of the problem and anxious to reconcile the conflicting results.
intracellular pH determination

Dr. Ramsey: Dr. Carter, is that one exception Fenn and Maurer?
Dr. Carter: Conway and Fearon (J. Physiol. (London) 103: 274 289, 1944).
Dr. Ramsey: I thought that around 1935 Fenn and Maurer, using the bicarbonate method, found that muscle had a pH around 6.
Dr. Carter: I know Fenn's various methods for measuring bicarbonate. I was not aware of the fact that he had a value of pH 6. He has several values of pH 7. Now he may well have one of 6, but he has several of 7. The only other pH measurement which agrees with our data is the one published by Netter, who used ammonia. I am inclined, however, to disregard his data, as I do Conway's result. Netter's data are quoted by Peter Caldwell in his review, and he gives a value of pH 7. However, if one looks at the original article, one finds out that the 7 value is arrived at by making certain chemical assumptions which may or may not be true. The absolute method of partition gives a value closer to 5.6, which is of some interest, particularly in regard to pK's of indicators. I think there are too many complications with ammonia, e.g., its binding, production, etc., applied to the whole cell; ammonia does not give a valid measurement.

Dr. Robin: There is hope with an ammonia technique different from the one that Netter used. One can load the animal with sufficient exogenous ammonia to swamp out endogenous ammonia. This would be critical, because if the indicator methods using negatively charged species give falsely high values because of heterogeneity within the cell, then positively charged species would be expected to give falsely low values. We have tested this in one system, the red cell. This is a bad one to use, because it is easy to measure pH in the red cell by any technique, and one can easily agree that it is in electrochemical equilibrium. By using the swamping-out technique, one can arrive at intracellular pH's measured in the red cell by the ammonia technique, by the DMO technique, by the CO₂ technique, if one corrects for carbamino CO₂ by chloride ratios and freeze-thaw, which are not significantly different.

Dr. Carter: We have given the swamping-out technique some thought. The trouble with its application to the whole animal is that we got into much difficulty with what we assume are effects of ammonium ion, particularly on the central nervous system. I am not sure of this, but, anyway, it is difficult to apply this technique. We probably came nowhere near a condition of swamping-out. Let me hasten to say that as far as the red cell is concerned, we would very much like to put the pH electrode into the red cell, in the absence of DMO, and see whether the pH is the same as the rather good pH values obtained with the other methods.

Dr. Robin: Let me caution you before you start, although you are much more skilled at puncturing red cells than we are. We have spent four years getting to the point where we can insert an electrode for measuring transmembrane potential. About 15 to 20 seconds after insertion of the electrode, the cells disrupt. Furthermore, baseline stability is reached very quickly. If you decide to try red cells, I suggest using the red cell of the bullfrog, because this cell is much larger and wider than the human. For two years we have also tried to use red cells of amphiuma or cryptobranchus. They are very big (90 µ, but they have a very thin membrane, and there is no way at all of preserving the transmembrane potential.

Dr. Carter: Yes. Could you measure the transmembrane potential?
Dr. Robin: We believe we have obtained some useful preliminary measurements which agree with chloride distribution.

Dr. Carter: It would be nice if we could insert the pH electrode into these cells. In a circumstantial sort of way, that would be at least one area for agreement, hopefully. On the other hand, if we obtained different measurements, we would really be in trouble.

Dr. Huf: I want to take one step back. I am not sure, but I think the problem is still more complex. I am speaking here about the transmembrane potential. The question is: What is the source of the potential? Speaking about muscle and biological membranes in general, I am surprised that you haven't considered the possibility that there is a sodium pump effect. We begin to realize more and more that the membrane potential is generated by an electrogenic sodium pump. Other ions are distributed in accordance with the laws of thermodynamics. If one looks at the rate at which changes in the passive distribution of ions occurs, it seems to me that this would also depend on the rate at which the driving force, the sodium pump potential, changes. Alkalosis and acidosis are known to change the rate of active sodium transport. So maybe this is a determining factor, and my question is: Has this been considered?

Dr. Carter: It has, yes. I was not going to go into that in any great detail, because of time limitation. Certainly, as you state, currently there are many people who believe that the membrane potential arises from an electrogenic pump, pumping sodium out of the cell. It is certainly conceivable that changes in external pH would affect the pump. That should also affect the potential. Now I realize that there have been some people who claimed that not all of the electrogenic status is seen in the transmembrane potential, and I suppose that is a loophole. Stimulated by the ideas of Hodgkin and his workers on the origin of the transmembrane potential under steady-state conditions, we became interested in observing changes in transmembrane potential in experiments where we control potassium,
principally. This, certainly, is an oversimplification, but I also think that it is a point of departure. Electrogenic pumps are a convenient answer to the problem, but I also think that some of these so-called electrogenic pumps have arisen because investigators have found strange results on the ratios of various ions across the membrane. Finally, let me say that there may be yet a third mechanism for the production of the potential. It is conceivable that hydrogen ion sets the potential.

**Dr. Huf:** Then you should not be in agreement with the Nernst equation if this is the case.

**Dr. Carter:** Yes, why not? If you say potassium sets the potential, you are practically in agreement, and we are practically in agreement with hydrogen ion.

**Dr. Huf:** What I am driving at is this: Do you think that hydrogen ions are actively transported?

**Dr. Carter:** I know what you mean. No. I do not think this. I am saying this is another possibility, and you are absolutely right. If it is in absolute equilibrium, you do not have the potential, but looking at the case of potassium, hydrogen may behave exactly like the potassium.

**Dr. Robin:** There are various mechanisms for the development of transmembrane potential in biological systems. One of them is a Donnan distribution, which applies, for example, in the red cell. Here, with macromolecules present on the inside of the red cell, chloride distributes itself unequally, and under these circumstances, a potential difference arises. It has nothing to do with the sodium or potassium pump, either theoretically or actually. That is to say, one can stop the sodium or potassium pumping mechanisms, or change the sodium or potassium concentrations of the outside media, and this has no effect whatsoever on the distribution of chloride ratios or on the transmembrane potential.

**Dr. Huf:** All I am saying is that, if a pump is in operation, this pump, in effect, takes the place of a Donnan system.

**Dr. Robin:** Well, that depends on the relative mobility of the ions involved. Again, in the case of the red cell, where, presumably, chloride ions reach the thermodynamic equilibrium in microseconds—remember the half time for sodium is three weeks—one can modify the operation of the sodium pump to any extent, but it will not affect the transmembrane potential. Is it not true that, since potassium is in electrochemical equilibrium across the muscle, this suggested to Hodgkin that it wasn't a diffusion pump that was responsible for this distribution ratio?

**Dr. Huf:** Ion mobilities are important, but so is the driving force, the pump potential.

**Dr. Kiesow:** Dr. Carter, is it correct that, by altering the membrane potential, you can change the intracellular pH, assuming that there is an electrochemical equilibrium?

**Dr. Carter:** This is true if it is done by means of an electrode. Remember that, under those circumstances, the required anion or cation is supplied by the current-generating electrode. We were trying to look into this for more physiological conditions.

**Dr. Kiesow:** Since the pH changes are quite drastic ones, I would suggest using a physiological, intracellular pH indicator, which is already present and which can easily be observed—even in intact cells and tissues—by means of spectrophotometry. This is the [DPN.H] \times [H^+]:[DPN] ratio and its response to such an intracellular pH change. Since it is a change in ion activity of at least an order of magnitude, it should affect this ratio considerably.

**Dr. Carter:** Yes, it is. That should follow, I would think. The only exception is that the part that you can measure might be principally located in the mitochondria, as I understand it. Of course, nothing that we have said necessarily has anything to do with mitochondrial pH.

**Dr. Kiesow:** You could very well also measure the cytoplasmic concentration of reduced pyridine nucleotides.

**Dr. Carter:** Is there enough to see, technically?

**Dr. Jöbsis:** Yes, fluorometrically you are better off. You can see the mitochondrial part, but in spectrophotometry you see both equally.

**Dr. Carter:** You can alter the membrane potential by a drastic amount, and this does indeed seem to alter the pH that we are measuring. Under those circumstances I would think this would be possible. These have to be quite rapid measurements.

**Dr. Kiesow:** Oh, this should not be a problem here.

**Dr. Carter:** This is not a very stable situation.

**Dr. Kiesow:** Even if it is only transient, these optical or fluorometric methods are very rapid.
Contributors

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