METABOLISM AND ION TRANSPORT OF FROG SKIN:

BIOLOGICAL EFFECTS OF OUABAIN.

by

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INTRODUCTION

I. Ion Transport and Metabolism

A. Living Cells and Their Environment

It is well known that generally the internal composition of living cells is different from their surrounding medium, not only in regard to large molecules like proteins, polysaccharides and polynucleotides, but also with respect to small molecules like amino acids, carbohydrates, nucleic acids and electrolytes. The cellular constituents may be retained at their characteristic composition because they are enveloped by a highly selective living membrane. This phenomenon of differential composition has aroused intensive studies on membrane permeability. The ultimate purpose of these studies on permeability of living membranes is not purely for descriptive and morphological reasons, but for an understanding of its function within the field of cell metabolism.

B. Ionic Equilibrium

Of all the cellular constituents that have been studied, the ionic composition has received the greatest attention and very real differences in ionic composition between the cytoplasm and its milieu have long been observed (1).

Most cells possess high potassium and low sodium concentrations, while the extracellular concentrations of these ions are just the opposite. The maintenance of a balance of ionic composition becomes

an important physiological problem because of the concentration gradients that exist across the living membrane. Any single or combination of known forces such as diffusion, electric fields, solvent drag, etc., have not been able to explain the differences in ionic composition.

The term, active ion transport, has been used to describe the processes which cannot be explained by the concepts of physical forces mentioned. The summation of the effect of these physical forces in an ionic species can be evaluated by the use of the following equation (2):

$$\ln \frac{M_{in}}{M_{out}} = \ln \frac{C_o}{C_i} + zF \frac{E}{RT} + \frac{D_w}{D} \int_o^{x_o} \frac{1}{A} \, dx \qquad (1)$$

where M_{in}, M_{out} designates influx and outflux of an ionic species; C_o, C_i, concentration of the ion in the outside and inside solutions; E, the potential difference between the solution i and o; z, valence of the ionic species; F, Faraday's number; R, gas constant; T, absolute temperature; D_w, volume rate of the solvent flow through unit area of the membrane; D, free diffusion coefficient of the ion in water; A, fraction of the area available to flow; X, distance from the outside boundary of the membrane; and, x_o total thickness of the membrane. The equation indicates that the passive movement of an ion can be described by the three terms on the right side of the equation. The application of this equation is well discussed by Ussing (2).

A descriptive term "sodium pump" has been suggested (3) to indicate that sodium is pumped out of cells against its electrochemical gradient and that potassium may then flow inwards to replace sodium either by passive movement or by active participation in the

transport of Na, that is, the "coupled Na-K pump" (4). Such a coupled system was originally suggested to exist in human red cells. It was based on observations that the active outflux of sodium depended on the presence of potassium and that the extrusion of sodium and the uptake of potassium seemed to depend on the same metabolic factors (5). This idea of a coupled transport system was later extended to other cell types such as muscle (6), nerve (7), and frog skin (8). The obligatory coupling of Na and K in all forms of sodium transport is not yet completely established (2).

C. Metabolic Energy Source

If the so-called active processes of ion translocations cannot be supported by the three physical forces cited above, they must depend on energy originating from cell metabolism. There are two main schools of thought on possible mechanisms for the use of metabolic energy. The first concept of ion transport energetics was that the supply of energy was derived from electron movements without the mediation of ATP (9). According to this theory a metabolic hydrogen atom is accepted by a flavoprotein and is transported to a cytochrome, with the formation of a hydrogen ion that is spatially separated from the electrons liberated at the same time. The hydrogen ion is thought to exchange with a sodium ion, while electrons are passed to other metallo-enzymes of the respiratory chain.

A second hypothesis concerning the inter-relation of metabolism and ion transport involves the participation of ATP as the energy source. Evidence that ATP facilitates potassium uptake was provided by work with human red cell ghosts (10). ATP was introduced into the

ghosts by lysing the cells in a dilute ATP solution. The resulting ghosts were then able to accumulate potassium.

The opposite process, that is, the extrusion of sodium, was later demonstrated with red cell ghosts containing ATP (11). In this experiment, human red cells were labeled with radiosodium and were then depleted of metabolic resources by incubation for 18 hours at 37° C. They were then transferred to a sodium-free media containing inosine or adenosine or a medium to which no nucleotide had been added. Only the adenosine containing medium was found to stimulate sodium efflux and these studies led to the conclusion that adenosine generated ATP to support sodium transport in human red cells. This concept was later supported by an ingenious experiment using dinitrophenol or cyanide poisoned squid giant axon, in which the extrusion of sodium was initiated by the injection of high energy phosphate precursors of ATP, such as phosphoenolpyruvate or arginine phosphate or ATP itself (12). These observations seemingly are conclusive evidence that ATP is the main source of energy for active ion transport just as it is for many other metabolic activities.

D. Active Sodium Transport in Frog Skin

Since 1848 frog skin has been a favorite tissue for the study of ion transport, that is, the processes by which NaCl is moved from the epithelial side to the corium side of the isolated skin (13, 14). It has been found that the net inward movement of sodium across the skin is equal to the measured "short-circuit current" when the potential difference across the skin is maintained at zero (15). This net inward sodium movement can still be demonstrated when the

outside bathing solution of an isolated frog skin is 100 times more dilute than the inside bathing solution (16).

The fact that there is a dependence of active ion transport on energy from metabolism is well established for many tissues including frog skin. A linear relationship has been found between the amount of sodium transported and the amount of oxygen utilized above the resting (non-transport) consumption (16, 17). The major source of energy then is apparently from aerobiosis. However, even in anaerobiosis, a residual amount of sodium transport is maintained and there is found an increase of lactate production (18). This is in agreement with the concept that an energy source for active transport can be supplied either from aerobic or anaerobic pathways.

Supporting evidence for the dependence of active ion transport on a cellular energy supply in frog skin is provided by the effects of metabolic inhibitors such as fluoroacetate, azide, cyanide and dinitrophenol which are inhibitory to transport (19, 20, 21). With the exception of dinitrophenol the inhibitors cited reduce oxygen consumption, while dinitrophenol stimulates oxygen utilization. The inhibition of frog skin sodium transport by dinitrophenol strongly suggests the participation of energy-rich phosphate compounds in the transport process (20).

E. Effects of Ouabain on Ion Transport

The action of the cardiac glycoside, G-Strophanthin, ouabain, on cation transport was first demonstrated by Schatzmann (21). It was found that ouabain, among other cardiac glycosides and aglycones, prevents the uphill transport of K and Na in the cold-stored RBC. The passive movements during cold storage were not affected by the

glycoside, but the active re-accumulation of K and extrusion of Na were inhibited. However, metabolism was found unaltered as evaluated by the formation of lactate by RBC. Thus it was concluded that ouabain is a specific inhibitor of active ion transport. Subsequent work with different analytical methods showed that lactate production in the ouabain-poisoned RBC was indeed inhibited (22, 23).

Since then, the inhibitory effect of ouabain on ion transport has been verified in various tissues, e.g. in cardiac muscle, skeletal muscle, peripheral nerve, skin, kidney, etc., of different species (24). An additional finding was that the inhibitory effect of ouabain on ion transport decreases as the K concentration of the bathing medium of the tissue increases. This observation led to the idea that ouabain and K compete for the same carrier of active ion transport (25). An adenosine triphosphatase system was located (25, 26, 27) in the endoplasmic reticulum or in the microsomal fraction of the cell. The activity of this enzyme system, which catalyzes the hydrolysis of ATP, depends on an optimum concentration of Na, K and Mg. The effect of ouabain on ATP hydrolysis in the presence of optimum concentrations of metal ions was examined in RBC (27, 28). The effective concentration of ouabain for half maximum inhibition of hydrolysis of ATP was found comparable to that required for the inhibition of cation transport. Potassium can also reduce the inhibitory effect of ouabain on the hydrolysis of ATP. This is in agreement with the idea that potassium and ouabain compete for a site in cation transport, provided that the membrane ATPase is an integral part of the transporting system. Recently, with microsomal fractions isolated from guinea pig kidney cortex, Post et al (29) showed that ouabain inhibited a potassium

requiring dephosphorylation of a phosphorylated intermediate. On the basis of their experimental observations they suggested the following mechanism for active cation transport.

$$\text{Enzyme} + \text{ATP} \xrightarrow{Na^+} (\text{Enzyme-P})\ \text{Na} + \text{ADP} \qquad (2)$$

$$(\text{Enzyme-P})\ \text{Na} \xrightarrow{K^+} \text{Enzyme} + \text{P} + Na^+ + K^+ \qquad (3)$$

Site of ouabain action

F. Actions of Ouabain on Ion Transport of Frog Skin

Ouabain at 10^{-6} M concentration, applied to the inside facing compartment of an isolated skin reduces both the potential and the short-circuit current (30, 31, 32). When the concentration is increased to 10^{-4} M, the total sodium of the tissue increases and the potassium decreases (33). The latter effect seems to be due to an increase in the rate of potassium leakage to the corium side (30, 34). For a number of inhibitors, there appears to be two different mechanisms by which they exert their effects (35); e.g. at low concentrations, they may affect only active Na transport but at high concentrations, the K content may also be altered.

G. Effect of Ouabain on Energy Metabolism

Ouabain, a cardiac glycoside, is well known for its ability to inhibit or block active sodium transport. Knowledge concerning its effects on metabolic reactions is limited. It has been reported to inhibit the formation of lactate in human red blood cells (22, 36, 37). It is also known to reduce the rate of respiration in kidney slices and homogenates in which a portion of the oxygen consumption is known to be dependent on the presence of sodium and potassium (38). In addition, it has been recognized that there is a parallel inhibition of the hydrolysis of that ATP which is catalyzed by a

sodium-potassium activated ATPase (39). These observations prompted the suggestion that the ion transporting system, presumably the ion activated ATPase, functions as a pace-maker in energy metabolism through its production of ADP, the phosphate acceptor of oxidative phosphorylation (40, 41).

H. Objectives of This Thesis and Methods of Approach to the Problem

In spite of the frequent choice of frog skin for studies on active ion transport, knowledge of the details of its metabolic reactions is fragmentary. The dependence of transport on oxygen consumption and the effects of metabolic inhibitors point to the involvement of the energy metabolism of the tissue. The presence of the tricarboxylic acid cycle has been indicated by the ready oxidation of cycle intermediates (42). Metabolic energy derived from the oxidation of acetate and glucose has been shown to support active ion transport in frog skin (43, 44). Although these observations on metabolism and transport are of great interest, they represent only the beginnings of our knowledge in this area. Further information about each of these reactions is necessary in order to arrive at a full understanding of the mechanisms and the energetics of active transport of frog skin.

The fragmentary nature of our knowledge of the mechanism and control of ion transport and the far-reaching metabolic implications of this control were the chief motives behind the initiation of this work. The objectives and the methods of approach of the work are as follows.

First, an attempt has been made to understand better the biochemical events of frog skin. This has been achieved by the use of specific carbon-14 labeled glycolytic and tricarboxylic acid cycle intermediates which are glucose-1-^{14}C, glucose-6-^{14}C, glucose-U-^{14}C, acetate-1-^{14}C, acetate-2-^{14}C, glycerol-1,3-^{14}C, citrate-1,5-^{14}C and succinate-2,3-^{14}C. The oxygen consumption and incorporation of labeled substrates into carbon dioxide, glycogen and lipid fractions including fatty acid, sterol and glycerol, have been measured. The patterns of anabolism and catabolism of the metabolic intermediates and their end products in frog skin have been obtained.

Second, an attempt has been made to understand the mode of action of ouabain on frog skin. This has been approached by parallel experiments designed in keeping with the first objective stated above.

Third, an attempt has been made to use ouabain as an agent for studies on metabolic control. In many discussions of metabolic control systems, ATP and ADP levels have been the chief controlling elements considered for metabolism. If ouabain does inhibit ion transport through its action on the ion-activated ATPase, the levels of adenine nucleotides would likely be altered and in turn alter the flow of metabolites in the cell. This phase of the studies has been achieved by the quantitative measurement of metabolic intermediates such as ATP, NAD and NADH. At first, tissue extracts of frog skins were used for these analyses; however, it was found that the concentration of metabolic intermediates in this tissue is comparatively low. Upon examination of preliminary experimental data, it was found that the effects of ouabain on respiration of frog skin

is similar in many ways to that on kidney slices. The more easily manipulated kidney tissue was thus used for the test system to evaluate the metabolite changes caused by ouabain. At the same time glucose and succinate have been used as substrates to test other parameters of metabolism. The incorporation of glucose-U-^{14}C into $^{12}CO_2$, $^{14}CO_2$ and ^{14}C-lactate has been examined. Oxygen consumption, levels of lactate, ATP, NAD and NADH have been estimated quantitatively.

It was hoped that, within the framework of these experimental approaches, useful information would accumulate and reveal the mechanism of action of ouabain and its total effect on metabolism and ion transport.

II. Present Concepts of Metabolic Control

A. The Control of Respiration

In physiologically intact preparations, the availability of intracellular orthophosphate and phosphate acceptors has been suggested to be a basic factor influencing the rates of oxidation and glycolysis (45, 46, 47). Lardy and his colleague demonstrated that inorganic phosphate and phosphate acceptors also increased the rate of respiration in isolated mitochondria (48). Limiting concentrations of ADP or of phosphate were found to control respiration leading to the use of the term "respiratory control" (49, 50, 51). The opposite phenomenon, that of the effect of ATP, was not established until the demonstration of inhibition of respiration by the addition of ATP to skeletal muscle mitochondria (52, 53).

Studies such as those of Lardy led to the concept that the probable reversibility of oxidative phosphorylation reactions is a means of respiratory control.

The possibility that oxidative phosphorylation might be a reversible process is based on the fact that the energy of oxidation in the respiratory chain is transformed into the energy of the phosphoric anhydride bond of ATP, and that it might be possible to reverse the substrate oxidation reaction if energy is supplied. Such a reverse reaction may be pictured to consist of two carrier components, C_1 and C_2 of the respiratory chain:

$$C_{2_{red}} + C_{1_{ox}} + ATP \rightleftharpoons C_{2_{ox}} + C_{1_{red}} + ADP + Pi \qquad (4)$$

where $C_{1_{ox}}$ and $C_{1_{red}}$ are the oxidized and reduced forms of the carrier, C_1; and, $C_{2_{ox}}$ and $C_{2_{red}}$, the oxidized and reduced forms of the carrier, C_2.

In this reaction, it is assumed that the ATP consuming reaction corresponds to the reversal of the synthesis of ATP during the course of oxidative phosphorylation, that is, adenine nucleotides are considered to be in equilibrium with the respiratory chain. Attempts for a direct experimental proof of this suggested reversal have previously been unsuccessful (54). However, the reversibility of the the reaction has been claimed on the basis of isotope experiments (55, 56, 57, 58) in which it was shown that anaerobic mitochondria carry out the reaction:

$$ATP + P^{32} \rightleftharpoons ATP^{32} + P \qquad (5)$$

presumably via the reversal of reaction (4). An hypothetical high energy intermediate was suggested to be involved in the following

fashion:

$$X + ATP \rightleftharpoons \sim X + ADP + P \qquad (6)$$

and then energy transfer is coupled to the oxidation-reduction reaction:

$$C_{2_{red}} + \sim X + C_{1_{ox}} \rightleftharpoons C_{2_{ox}} + C_{1_{red}} + X. \qquad (7)$$

Reaction (7) was subsequently demonstrated when mitochondrial NAD was reduced by substrates which are dehydrogenated primarily by flavoprotein (59-62). It was soon recognized that this type of NAD reduction is a general characteristic of intact mitochondria from all sources (62). To this point, reversibility has been demonstrated only for the phosphorylation step in the NAD-flavin region of the respiratory chain. It also became possible to demonstrate the reversibility of the electron transport in the cytochrome region (63-66) by changing the experimental conditions.

1. Energy Dependent Oxidation-Reduction Equilibrium

The reversibility of electron transport in the respiratory chain has led to a postulate that the redox equilibria of respiratory carriers is controlled by the phosphate potential, that is, the ratio of ATP to ADP and inorganic phosphate (67, 68). By this concept the redox state of each carrier would depend on the redox state of other carriers along the chain and ultimately on the redox state of the NAD-specific oxidizing and reducing substrates. The coupling of electron transport to phosphorylation can be illustrated by the law of mass action according to the following equations:

$$C_{1_{red}} + C_{2_{ox}} \rightleftharpoons C_{1_{ox}} + C^{*}_{2_{red}} \quad K_1 = \frac{C_{1_{ox}} C^{*}_{2_{red}}}{C_{1_{red}} C_{2_{ox}}} \qquad (8)$$

$$C^*_{2_{red}} + ADP + P \rightleftharpoons C_{2_{red}} + ATP \quad K_2 = \frac{C_{2_{red}} \; ATP}{C^*_{2_{red}} \; ADP,P} \qquad (9)$$

where C_1 and C_2 have the same meanings as in (4) and C^*_2 is the energy rich intermediate of carrier C_2. Reactions (8) and (9) give:

$$\frac{C_{2_{red}} + C^*_{2_{red}}}{C_{2_{ox}}} = \frac{C_{1_{red}}}{C_{1_{ox}}} K_1 \left(1 + K_2 \frac{ADP,P}{ATP}\right). \qquad (10)$$

According to this expression, the redox state of the respiratory chain components in oxidative phosphorylation is a function of the phosphate potential.

2. Energy linked NAD reduction

The postulated reversibility of the respiratory chain was first observed by Chance and Hollunger in kidney mitochondria (59). Upon the addition of a non-NAD-linked substrate such as succinate, mitochondrial NAD was extensively reduced. The oxidation and reduction processes of NAD were monitored at 340 mμ. Characteristic of the spectrophotometric trace was the reversal of that caused by the addition of ADP in the resting mitochondria (50). Among the substrates tested, succinate and flavin-linked substrates in general were found to be the most effective.

The energy requirement of this reaction is apparent from two standpoints. First, the succinate-fumarate couple is 300 mV more positive than the NAD-NADH couple, and second, the sensitivity of the reaction to uncoupling was met by internal high-energy intermediates of oxidative phosphorylation.

The obligatory requirement of energy for this process was further supported by the use of skeletal muscle mitochondrial

preparations in which the nicotinamide nucleotide remained in an oxidized form if the substrate succinate or glycerol phosphate were added. The subsequent addition of ATP resulted in a reduction of NAD, which was then reoxidized upon the addition of inorganic phosphate and ADP.

These results show that added ATP can influence the redox state of the intramitochondrial components of hydrogen transfer such as NAD and flavoprotein. It is assumed that ATP exerts its influence through back reactions of oxidative phosphorylation.

3. Energy linked Oxidation of Flavoprotein and Cytochromes

If electrons have already been transferred up to the level of cytochrome b and flavoprotein along the chain, the flavoprotein involved in NAD reduction should be in reduced form. The addition of ATP should cause NAD reduction with a concomitant oxidation of flavoprotein. This was demonstrated in pigeon heart mitochondrial preparations in which the respiratory chain was terminally inhibited by sodium sulfide. This result affords strong support for the reaction:

$$\text{succ} \longrightarrow f_p \longrightarrow b \longrightarrow f_p \longrightarrow X \sim I + NAD^+ \qquad (11)$$

$$\begin{matrix} \vdots \\ C \\ \uparrow \\ C_1 \\ \uparrow \end{matrix}$$

where b, C_1 and C designate cytochromes b, C_1 and C; succ, succinate; $X \sim I$, high energy intermediate and f_p, flavoprotein.

With similar preparations of mitochondria, the addition of ATP caused the reduction of NAD and, at the same time, the

oxidation of cytochrome c. These reactions were followed by a spectrophotometric technique at 550-540 mμ. The response of cytochrome c to ATP addition was cyclic, and reduction toward the initial level proceeds rapidly. Cytochrome a had also been shown to be oxidized as well; however, cytochrome b showed little initial change and then a slow reduction which was complete at about the same time as that of NAD. Such changes are consistent with the idea that ATP is entering the respiratory chain in the flavoprotein-NAD region as well as in the cytochrome region (50).

4. Respiration Controlled by the Phosphate Potential

Evidences of reversibility of electron transfer along the respiratory chain and phosphorylation led to studies on the effects of adenine nucleotides on respiration (53). In respiration of skeletal muscle mitochondria it was found that in the absence of ATP, 0.05 mM ADP was required to achieve the half maximum value. However, in the presence of ATP, an appreciably higher concentration of ADP is required to attain a similar value, e.g. 0.1 mM ADP for 2.5 mM ATP, and 0.25 mM ADP for 5 mM ATP.

The correlation between respiratory activity and the ratio of ATP to ADP is in accord with the suggestion that respiration is controlled by an equilibrium between the ATP/ADP system and the respiratory chain. Hence, control of respiration is a function of the reversibility of oxidative phosphorylation.

B. The Control of Glycolytic and Oxidative Pathways

In addition to the control of mitochondrial respiration discussed above, another mechanism of metabolic regulation in connection with biosynthetic sequences has been observed. The evidence of this second control mechanism has been demonstrated with Escherichia coli, in which the bacterial cell's production of L-isoleucine was found to be controlled by both repression and feedback inhibition in the presence of excess L-isoleucine. That is, the synthesis of enzymes needed for isoleucine production was stopped and, at the same time, the activity of the first enzyme (L-threonine deaminase) in the reaction sequence leading to the formation of isoleucine was inhibited by isoleucine (69, 70).

Such reactions afford extraordinary economy and efficiency as a control system. As soon as the supply of L-isoleucine reaches an adequate level, the cell stops making it simply by decreasing the activity of the first enzyme. This leads to the development of the technological concept of feedback control to describe the regulation of metabolic systems in organisms.

The opposite situation of feedback control system would naturally be a positive control system, that is, the stimulation of enzymic activity by a metabolite. The positive control mechanism is examplified by the stimulation of glycolysis in liver flukes by serotonin (71). Increases in AMP and fructose-1,6-diP levels were observed with a decrease in the glucose-6-P level. The changes in levels of metabolites led to the findings that AMP and its isomer cyclic 3',5'-AMP act as activators for P-fructokinase of guinea pig heart and other tissues (72, 73).

The cell thus possesses mechanisms for both types of control of enzyme activity, negative (inhibited enzymes) and positive (activated enzymes) feedback control. The molecular basis for the modulation of an enzyme's kinetic behavior by interaction with small molecules is unknown, even though much speculation has been made on the subject (74, 75). Conformational changes have been suggested to be due to the sterically specific and functionally significant interactions between enzymes and small molecules (76). Such changes have been described as being allosteric (77).

Several enzymes that participate in energy metabolism have been reported to have allosteric properties and to be controlled by levels of adenine nucleotides, ATP, ADP and AMP. The following discussion will present properties of these rate-limiting enzymes (78) to provide an integrated scheme of metabolic control.

1. Equilibrium of Adenylate Kinase

Several enzymes in the glycolytic, oxidative and biosynthetic pathways are influenced directly or indirectly by adenine nucleotides. In most of these reactions, AMP or ADP and ATP exert inverse effects and it has been suggested that these compounds are regulators of the enzymes participating in energy metabolism (78).

The cellular concentrations of adenine nucleotides are interrelated by the following reaction catalyzed by adenylate kinase (79).

$$AMP + ATP \rightleftharpoons 2\ ADP \qquad (12)$$

The following calculation is cited to illustrate the interrelationship of these components. If the total nucleotide

concentration is 5 mM and the ADP is changed from 1 mM to 2 mM and the equilibrium constant value of 0.44 (80) is used, then the concentration of each component of the adenylate kinase catalyzed reaction becomes:

ADP	1.0	2.0
ATP	3.87	2.21
AMP	0.11	0.78
ATP/ADP	3.87	1.11.

The results clearly demonstrate the inverse relation of ADP or AMP and ATP. Since the largest change is in AMP levels, this compound has been suggested as the most probable regulator of metabolism (79). According to the estimated cellular concentration of ATP and ADP (81), the corresponding concentration of AMP is calculated to be 0.17 mM. Thus, the change in nucleotide illustrated above is quite close to a physiological reality.

2. Phosphofructokinase

P-fructokinase has long been recognized as one of the important enzymes in the regulation of glycolysis (82, 83). ATP was first found to be inhibitory to P-fructokinase (84), even though it is a component of the reactions. The properties of the enzyme from various sources have been examined in great detail and have been found to be controlled by various metabolic environments (85). Because of the relevance of this enzyme to metabolic control, its properties are discussed in some detail.

The Michaelis constants of the enzyme from guinea pig heart for the two substrates, fructose-6-P and ATP were 2.8×10^{-5} M and 4.0×10^{-5} M respectively (86). Below pH 7.5 ATP at

concentrations above 2×10^{-4} M caused inhibition which could be overcome by increasing the concentration of fructose-6-P. The stimulation of glycolysis in liver flukes by serotonin brought to light the effect of cyclic-3',5'-AMP which was found to stimulate P-fructokinase three to four fold. ADP and AMP were the only other nucleotides to give a stimulatory effect but to lesser extents. Inorganic phosphate was found to be an activator for the enzyme. The effects of nucleotides and inorganic phosphate were additive. The tricarboxylic acid, citric acid, was also reported to be inhibitory to the enzyme (87).

The above properties of P-fructokinase may be considered in light of the physiological conditions of the cell. The possible cellular concentrations of ATP and of fructose-6-P in guinea pig heart were estimated to be 7×10^{-3} M to 8×10^{-3} M for ATP and 4×10^{-5} M to 12×10^{-5} M for fructose-6-P (86). With these levels of substrate at pH 6.9, kinetic data indicates a 90% inhibition of the enzyme. It was therefore suggested that the ATP concentration in the cell is sufficient to maintain the P-fructokinase in the ATP-inhibited form. If this is the case, it would be expected that fluctuations in the levels of adenine nucleotides (AMP, cyclic 3',5'-AMP and ADP) and inorganic phosphate might result in either an activation or an inhibition of the enzyme.

3. Fructose-1,6-diphosphatase

Fructose-1,6-diphosphatase catalyzes the hydrolysis of fructose-1,6-diP to fructose-6-P. It is one of the two enzymes

that overcomes the energy barrier for gluconeogenesis, if the latter process follows exactly the reversal of glycolysis (79, 88).

At pH 7.5, the inhibition is 58% and 82% with 1 and 20 mM fructose-1,6-diP respectively. The K_m value for fructose-1,6-diP is 5.5×10^{-6} M. The level of fructose-1,6-diP changes from 3×10^{-8} M to 7×10^{-8} M per gram of liver in adrenalectomized rats. The inhibitory effect of the substrate as a controlling factor is therefore unlikely in physiological conditions (90). The enzyme is also inhibited by AMP; this inhibition is reversible and non-competitive. AMP does not change the K_m for fructose-1,6-diP, but it lowers the V_{max} of the reaction. The apparent number of AMP molecules bound per enzyme molecule was found to be 2.3 compared with an estimated number of 3. When the enzyme is treated with papain it is not inhibited by AMP. This supports the idea that AMP interacts with this enzyme at an allosteric site. No other nucleotide or metabolite has been found to have an effect on this enzyme.

The inhibition of fructose-1,6-diphosphatase activity by AMP can be interpreted as a system of control. Since the levels of AMP and ATP in the cell have an inverse relationship in the presence of adenylate kinase, the activity of the fructose-1,6-diphosphatase will change with changes in the ATP concentration.

4. Pyruvate carboxylase

The direct carboxylation of pyruvate was postulated to explain the formation of dicarboxylic acids by CO_2 fixation in

bacteria (91). However, there was no evidence for the existence of such a process until an enzyme was discovered that could catalyze the following reaction (92):

$$\text{Pyruvate} + \text{ATP} + HCO_3^- \xrightleftharpoons{\text{Acetyl CoA, Mg}} \text{Oxaloacetate} + \text{ADP} + \text{Pi}. \quad (13)$$

This enzyme is found mainly in the mitochondrial fraction of chicken livers. The properties of this enzyme are summarized as follows: it has an apparent K_m value of 4.4×10^{-4} M for pyruvate and 2.85×10^{-5} M for ATP; ADP, a product of the reaction, markedly inhibits the activity of the enzyme, but other nucleotides are without effect. The inhibition by ADP is not due to the reversibility of the overall reaction through a mass action effect but may be due to an allosteric effect (93).

Acetyl-CoA is required for the pyruvate carboxylase reaction. In the absence of acetyl-CoA the K_m values of the enzyme for ATP and pyruvate were unaltered but the V_{max} of the reaction decreased (94). The presence of acetyl-CoA, however, lowers the K_m value for HCO_3^-.

The requirement for HCO_3^- led to the discovery that pyruvate carboxylase is a biotin-containing enzyme inhibited by the addition of avidin. The pyruvate carboxylase system has now been found to be present in rat liver (95), baker's yeast (96) and bacteria of different species (97, 98).

Liver and kidney are the only tissues having both pyruvate carboxylase and P-enolpyruvate carboxykinase activities; both tissues are known to be active in gluconeogenesis, that is, in the conversion of pyruvate and lactate into glycogen (99, 100, 101). Because of this activity, it is possible to tie

together the relationship of these two enzymes in gluconeogenesis. The end product of pyruvate carboxylase is oxaloacetate (equation 13) which can be used as a reactant in the reaction catalyzed by P-enolpyruvate-carboxykinase:

$$\text{Oxaloacetate} + \text{ITP} \rightleftharpoons \text{PEP} + \text{IDP} + CO_2. \qquad (14)$$

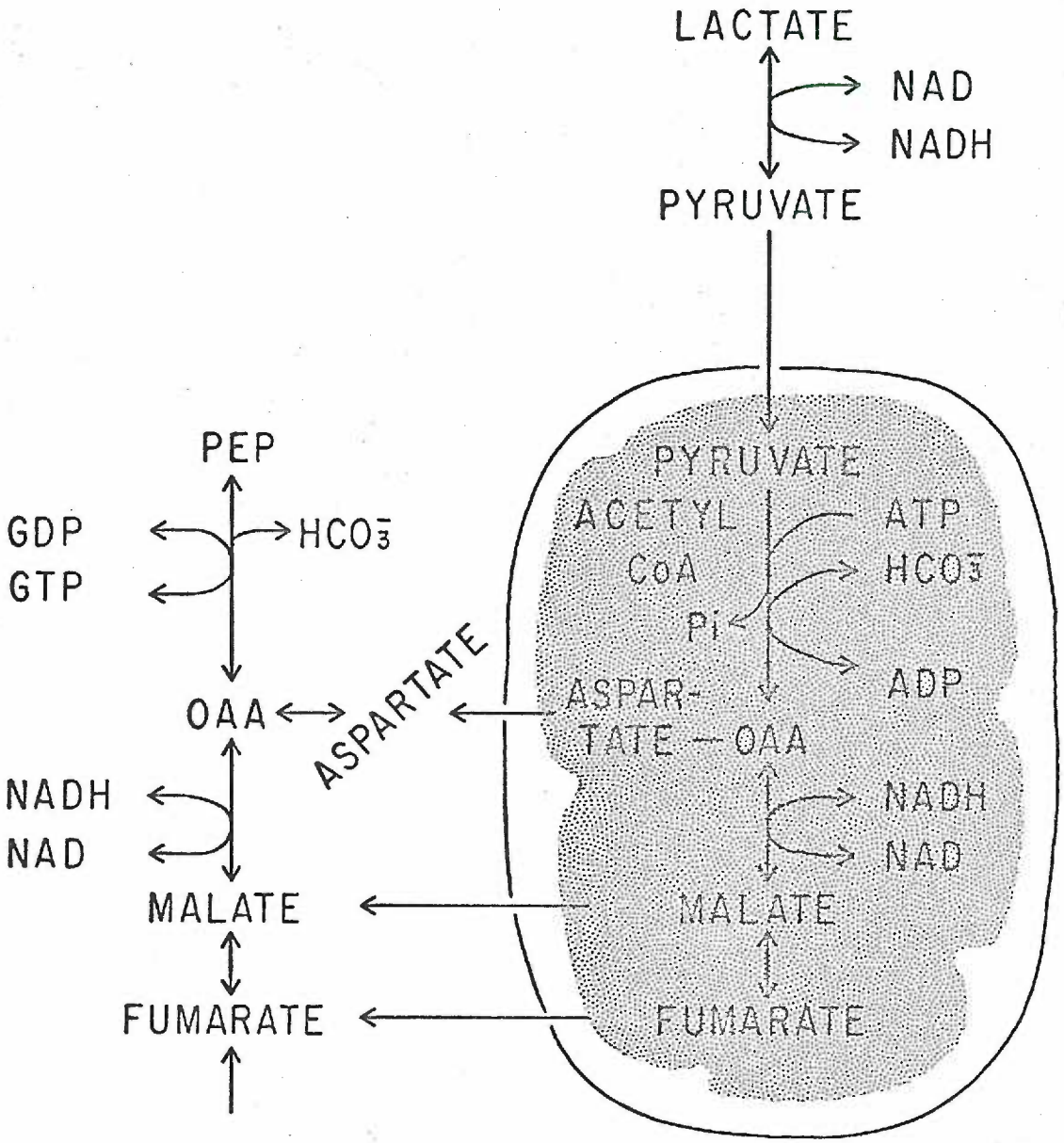
Because of the spatial separation of the two enzymes, pyruvate carboxylase being mainly found in mitochondria and P-enolpyruvate-carboxykinase in the soluble fraction, the system provides a route for the cytoplasmic synthesis of PEP from pyruvate of mitochondria. This synthesis is accomplished by the participation of another enzyme, malate dehydrogenase, which converts oxaloacetate into malate. Malate then diffuses into the cytoplasm and is converted back to oxaloacetate for gluconeogenesis as illustrated in Scheme 1 (102).

The fact that ADP can markedly inhibit pyruvate carboxylase may indicate a possible regulation of the enzyme by the cellular ratio of ATP/ADP. With a high ATP/ADP ratio, oxidative phosphorylation would be reduced. At the same time, the amount of oxaloacetate available for PEP synthesis would increase as a result of a lowered demand for the oxidative pathways of the tricarboxylic acid cycle.

The obligatory requirement for acetyl-CoA shown by the pyruvate carboxylase reaction offers another interesting control mechanism for the formation of oxaloacetate and the subsequent reaction of the tricarboxylic acid cycle. Acetyl-CoA, the product of the pyruvate oxidase reaction, would accumulate in the absence of oxaloacetate. The increased acetyl-CoA level would activate

Scheme

Proposed Scheme for Phosphoenol-pyruvate Formation.


LACTATE
NAD
NADH
PYRUVATE
PEP
GDP
GTP
HCO_3^-
OAA
ASPARTATE
NADH
NAD
MALATE
FUMARATE
PYRUVATE
ACETYL CoA
ATP
HCO_3^-
Pi
ADP
ASPAR-TATE
OAA
NADH
NAD
MALATE
FUMARATE

the pyruvate carboxylase to synthesize oxaloacetate. The newly formed oxaloacetate and acetyl-CoA may condense to form citrate. This line of reasoning is strengthened as the Michaelis constants for the acetyl-CoA of the condensing enzyme (103) and pyruvate carboxylase are of the same order of magnitude. This implies that the level of acetyl-CoA for tricarboxylic acid cycle is sufficient to activate pyruvate carboxylase. Pyruvate, thus, would be utilized for gluconeogenesis.

5. Isocitrate Dehydrogenase

There are two routes for the metabolism of isocitrate and for its conversion to α-ketoglutarate (104). A NADP-specific isocitrate dehydrogenase catalyzes the following reaction:

$$\text{Isocitrate} + \text{NADP}^+ \rightleftharpoons \alpha\text{-ketoglutarate} + CO_2 + \text{NADPH} + H^+. \quad (15)$$

This system exists mainly in the cytosol fraction of the cell. The properties of this enzyme have been extensively reviewed (105).

The alternate route for the metabolism of isocitrate requires the presence of NAD^+ as coenzyme, as illustrated in the following reaction:

$$\text{Isocitrate} + \text{NAD}^+ \longrightarrow \alpha\text{-ketoglutarate} + CO_2 + \text{NADH} + H^+. \quad (16)$$

The NAD-specific isocitrate dehydrogenase is found mainly in mitochondria and functions as a part of the tricarboxylic acid cycle. Studies of the catalytic properties of the NAD-specific enzyme, indicate that it has the characteristics of an allosteric protein, and suggest the enzyme as a possible site of regulation for the tricarboxylic acid cycle (106, 107).

It has been found that during the isolation of the NAD-specific isocitrate dehydrogenase, 5×10^{-3} M ADP would stabilize

the enzyme. ADP also stimulates the catalytic activity of the enzyme at a level of 6.7×10^{-5} M. The K_m value of the enzyme for isocitrate was lowered from 1.5×10^{-3} M to 1.4×10^{-4} M at pH 7.2 in the presence of the nucleotide. At low concentrations of isocitrate, the enzyme, therefore, shows a complete dependence on the presence of ADP for activity.

The generated NADH in reaction (16) diminishes the conversion of isocitrate to α-ketoglutarate, and causes a competitive inhibition with NAD for the enzyme. Since the NAD-specific dehydrogenase catalyzes an irreversible reaction, the effect of NADH is not expected. NADPH augmented the inhibition by NADH (106). The K_I for NADH was calculated to be 3.9×10^{-5} M. In the presence of 3.3×10^{-5} M NADPH and an equimolar amount of NADH, the apparent inhibition constant, K_I, of 0.76×10^{-5} M was obtained. ATP was also found to be inhibitory to the reaction. There is no effect of ATP or NADH on NADP-linked isocitrate dehydrogenase (106).

The facts that the NAD-specific isocitrate dehydrogenase is activated by ADP and at the same time inhibited by ATP and NADH have been suggested as a mechanism to regulate the mitochondrial oxidation of isocitrate. It has been recognized that in actively respiring mitochondria the ratio of NADH/NAD and ATP/ADP are low (50). Under these circumstances the oxidation of isocitrate by NAD-linked isocitrate dehydrogenase would be favored since ADP is stimulatory and NAD^+ competitively counteracts the inhibitory effect of NADH. Under anaerobic conditions and at low concentrations of phosphate acceptor, the NADH/NAD

and ATP/ADP ratios become high and the NAD-specific dehydrogenase becomes inhibited. Alteration of the ATP/ADP and NADH/NAD ratios in mitochondria could thus serve as a mechanism for regulating the rate of substrate oxidation at the level of isocitrate (108).

In regard to the function of the NADP-linked isocitrate dehydrogenase, it has been suggested as a pathway to regenerate NADH for biosynthetic purposes (108).

C. Metabolite Patterns

The quantitative determination of the steady state concentration of intermediates along a metabolic pathway affords an evaluation of the metabolic operation. The best comparison of two metabolic states has been achieved by the use of the "crossover theorem." The theorem was originally concerned with the respiratory chain, in which the sites of ADP phosphorylation were identified (50).

The "crossover theorem" for a metabolic pathway may be examplified with the following reaction sequence (109):

$$A \xrightarrow{E_1} B \xrightarrow{E_2} C \xrightarrow[\text{Site of Control}]{E_3} D \xrightarrow{E_4} E. \qquad (17)$$

It is assumed that in this system there is no "feedback" control. If for some reason the steady state flux from A to E is interrupted, and there is an accumulation of components B and C and a depletion of components D and E, then the step between C and D in the sequence of reactions is called a cross-over point. Such a cross-over can only occur at a rate-controlling site of a sequence.

Metabolic pathways such as glycolysis and the tricarboxylic acid cycle have been examined with the use of the theorem. Not only were controlling steps suggested but also the possible effectors were

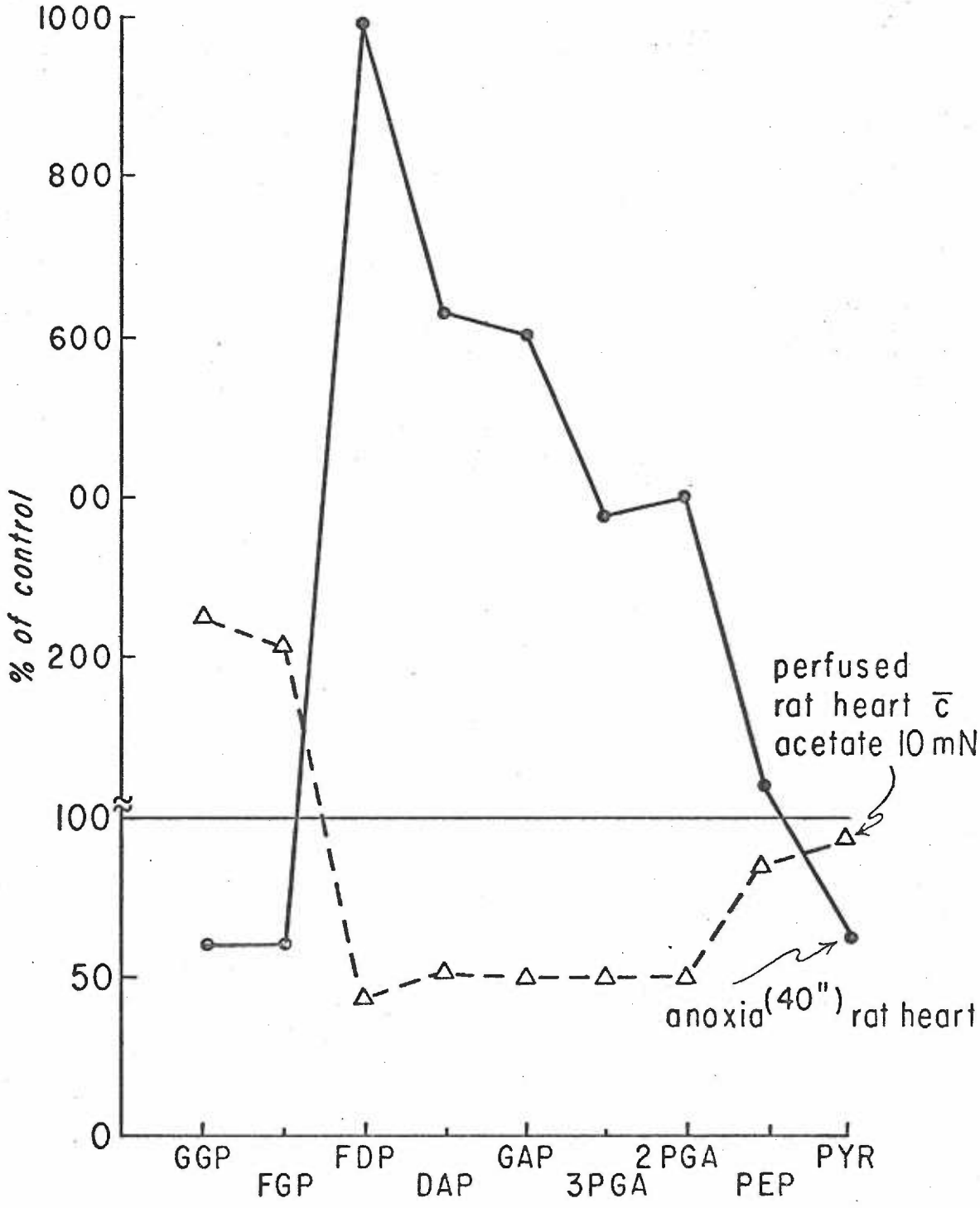
revealed for these pathways. It is the purpose of this section to discuss the operation of these pathways in view of the findings by the crossover theorem.

The effects of 10 mM acetate on glycolytic intermediates in perfused rat heart are shown in Figure 1 (110), in which the components of the glycolytic sequence are arranged along the abcissa and a comparison of the levels of the components is shown on the ordinate as a percentage of the control. Comparisons between steady state levels of intermediates are readily seen with the control values arbitrarily set at 100 per cent. After 15 minutes of perfusion with the 10 mM acetate, the glycolytic flux in the heart is reduced to 50%. The high level of fructose-6-P and low level of fructose-1,6-diP indicate that the enzyme, P-fructokinase, which catalyzes the conversion of fructose-6-P to fructose-1,6-diP is inhibited. Table I shows that there is an increase in the ratio of ATP/ADP from 8.7 to 11. Although not shown in the Figure or Table, a ten-fold increase of citrate was also found. The high level of citrate was later proved to be a major cause to the inhibition of P-fructokinase.

It is well known that a shift from anaerobic to aerobic metabolism causes a decreased rate of glucose utilization; this is known as the Pasteur effect. A shift to greater glucose utilization after 40 seconds of anoxia is shown for the perfused rat heart in Figure 1. Essentially all glycolytic components show an increase as compared to the control. The profile shows two crossover points, one of which involves the action of P-fructokinase and the other the inhibition of pyruvate kinase. It has been suggested that P-fructokinase is activated to such a degree that the rate controlling step is transferred to

Figure 1.

Relative Changes of the Glycolytic Intermediates after 40 Seconds of Anoxia, and 15 Minutes after Addition of 10 mM acetate in Perfused Rat Heart.


1000
800
600
00
200
100
50
0
% of control
perfused
rat heart c̄
acetate 10 mN
anoxia (40") rat heart
GGP
FGP
FDP
DAP
GAP
3PGA
2PGA
PEP
PYR

pyruvate kinase. The exact cause of the inhibition of pyruvate kinase is not known but is suspected to be the increased levels of inorganic phosphate.

Table I shows significant changes in the levels of adenine nucleotides under anoxia, which result in a low ATP/ADP ratio.

The metabolite profiles of these two experimental conditions compared to the control values are consistent with the idea that their changes are related to the change in the phosphate potential, that is, a change in the ATP/ADP ratio. An increase in the ATP/ADP ratio results in a decrease in glycolysis.

In Figure 2 the components of the TCA cycle of mouse brain are listed in order and the relative level of each component on the ordinate is compared in percent of the control. The previous experiment on perfused rat heart demonstrated the effect of anoxia on glycolysis. The experiments described in Figure 2 demonstrate the effect of anoxia on the tricarboxylic acid cycle (111). These levels of metabolites of the mouse brain were determined at 5 and 30 seconds after decapitation, which produces the anoxic condition. The profile of these two time periods indicates the prevailing changes of the two pathways showing stimulation of glycolysis but inhibition of the tricarboxylic acid cycle. The rapid fall of glucose and glucose-6-P, and the corresponding increase of pyruvate reflect the stimulation of the P-fructokinase step in the glycolytic pathway. However, the tricarboxylic acid cycle has been modified from a dynamic flux to a static condition, in which citrate tends to be reduced further while the α-ketoglutarate level remains low. The decreased rate of citrate synthesis is due to a 50% reduction in oxaloacetate, which is the substrate for the condensing enzyme.

Table I.

Levels of Adenylates in the Perfused Rat Heart
(μmoles/gm dry wt)

	20 mM Glucose		10 mM Glucose	
	Aerobic	Anoxic	Control	add 10mM Acetate
ATP	21.30	18.5	21.70	22.4
ADP	2.66	5.7	2.49	2.05
AMP	0.22	0.64	0.35	0.7
ATP/ADP	8.01	3.25	8.72	11.0
DPN/DPNH	731.00	136.00	---	---

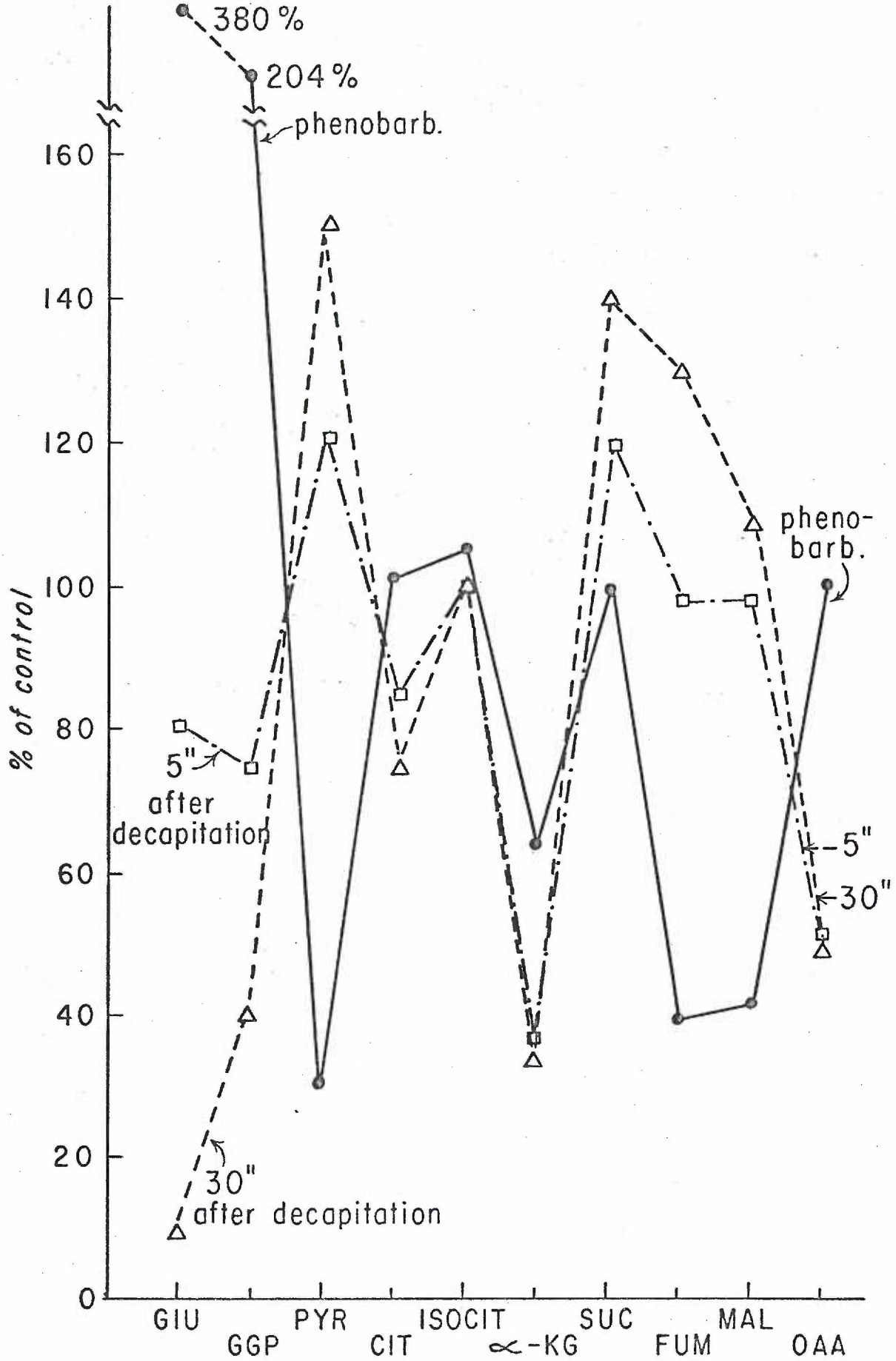
Table II.

Levels of Adenylates in the Brains of
Control and Treated Mice
(m mole/kg wet wt)

	Control	Phenobarbital	30 min after decapitation
ATP	2.95	3.21	1.67
ADP	0.33	0.27	0.65
AMP	0.05	0.03	--
ATP/ADP	8.90	12.00	2.60

Figure 2.

Changes of Substrates in the Mouse Brain after 5 or 30 Seconds of Ischemia and Anesthesia with Phenobarbital.


380 %
204 %
phenobarb.
160
140
120
100
80
60
40
20
0
% of control
5"
after
decapitation
30"
after decapitation
pheno-
barb.
5"
30"
GIU
GGP
PYR
CIT
ISOCIT
∝-KG
SUC
FUM
MAL
OAA

The reduced level of α-ketoglutarate may indicate that the NAD-linked isocitrate dehydrogenase is inhibited by the increase of NADH and ATP concentrations as discussed previously. The rise in succinate and a delayed rise in fumarate may be due to the increased succinate dehydrogenase. This is possibly enhanced by the malate dehydrogenase because of the low level of oxaloacetate observed.

When mice are treated with phenobarbital, both the glycolytic and TCA cycle activities of brain are diminished, as shown by the metabolite profile in Figure 2. Glucose and glucose-6-P accumulate; pyruvate decreases to 30% of the control; and, the tricarboxylic acid cycle is inhibited just as in the anoxic condition mentioned above.

Table II shows the levels of adenine nucleotides of mice brains under phenobarbital and/or anoxic conditions. The ATP/ADP ratio changes from 8.9 to 12 after the phenobarbital treatment and to 2.6 in the anoxic animal. These observations, therefore, have demonstrated the influence of the ATP/ADP ratio in TCA cycle activity as well as the glycolytic pathway.

MATERIALS AND METHODS

I. Animals: Source and Maintenance

A. Frogs

The animals used in the first part of these studies were male Rana pipiens. Stock animals were kept at room temperature in plastic trays containing moss and water. Usually animals were used within one week after arrival at the laboratory, however, on some occasions animals stored in the laboratory for more than one week were used.

B. Rats

The animals used in the second half of these studies were male albino rats of the Sprague Dawley strain weighing from 160 to 200 grams. They were fasted over-night and used one hour postprandial.

II. ^{14}C-Tracer Compounds

The ^{14}C tracers used in the metabolism studies were obtained from a number of suppliers. Table III lists pertinent information for each of the tracers.

III. In vitro Incubation Technique for Frog Skin

A. Warburg Procedure

These experiments were all done with a conventional Warburg incubation procedure but at 25° C. Frog Ringer's phosphate buffer was modified to consist of 115 mM NaCl, 2 mM KCl, 2 mM Na_2HPO_4, 0.05 mM NaH_2PO_4 and 0.25 mM $CaCl_2$. The buffer was gassed with oxygen before use. (2.7 ml of the buffer was added into the main compartments

Table III.

List of Carbon-14 Substrates Used

Substrate	Position of Label	Specific Activity µc/mg	Source
Acetate	C-1	208.7	Prepared here
Acetate	C-2	24.4	New England Nuclear
Citrate	C-1,5	5.5	Tracerlab
Succinate	C-2,3	6.3	Tracerlab
Glycerol	C-1,3	228.8	New England Nuclear
Glucose	C-1	17.5	New England Nuclear
Glucose	C-6	27.0	New England Nuclear
Glucose	Uniform	83.3	New England Nuclear

of 22-25 ml Warburg flasks having double side arms.) Small volumes of solutions of 2 to 3 μc of labeled substrates and/or other reagents such as ouabain were added to one side arm and 0.2 ml 10 N H_2SO_4 was added to the other side arm.

B. Tissue Sampling

The non-pigmented abdominal skins of Rana pipiens were removed from the animals and chopped into 3 mm^2 pieces with the Mickle tissue chopper (Brinkmann, Great Neck, New York). The pieces of frog skin were then randomized among 8 to 12 Warburg flasks. A number of skins was used in an experiment in order to assure a tissue weight of about 250-300 mg per flask. During the process of chopping and distribution, the tissues were kept in ice-cold buffer and the Warburg flasks were kept on ice. Tissue weights were obtained by difference, a center well containing 0.2 ml 6 N NaOH was introduced and the flasks were gassed with oxygen for 1 minute. After 10-15 minutes of equilibration at 25° C, the stoppers of the manometers were closed and substrate and reagent solutions were tipped in from a side arm which was rinsed with the buffer solution by tipping. The oxygen uptake was determined from successive manometer readings.

At the end of the incubation, biological activity was terminated by the introduction of the H_2SO_4 solution from a side arm. After 45-60 minutes of equilibration in the water bath, for the total release of CO_2, the flask was detached and the tissue was removed from the incubation medium by filtration through a nylon net. Tissues were kept for determination of the ^{14}C incorporation into glycogen and lipid fractions. The incubation medium was kept for ^{14}C lactate and total lactate determinations. The alkaline solutions in the center wells were assayed for $^{14}CO_2$.

In other experiments, when an analysis of the amounts of coenzyme was desired, the tissues were not treated with H_2SO_4, but were removed from the flasks, immediately frozen with liquid nitrogen, and stored frozen until analysis.

IV. Methods of Assay

A. Assay of $^{14}CO_2$.

The tissue carbon dioxide which evolved upon acidification of the medium was trapped in the center well containing 0.2 ml 6 N NaOH solution. After the period of acidification, the center well was removed and immersed in 9.8 ml of water in a test tube and the NaOH-Na_2CO_3 allowed to equilibrate. A 0.2 ml aliquot of the diluted alkaline solution was pipetted into a vial containing 10 ml of scintillation fluid. Carbon-14 activity was measured in a Packard Tricarb, Model 3002, Liquid Scintillation Spectrometer. The composition of the scintillation fluid was 66.7% toluene and 33.3% absolute ethanol, containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP).

B. Isolation of Lipid

1. Lipid Extraction

The lipid extraction procedure was mainly that of Folch (112). The collected pieces of frog skin were shaken for 60 minutes with 20 volumes of chloroform-methanol mixture (2:1 V/V) and allowed to stand overnight. The crude extract was then washed with 0.2 ml of 0.04% $CaCl_2$ solution. The solution separated into two phases, the upper phase of which was removed and replaced with the upper phase of a solvent mixture of chloroform-methanol-0.04% $CaCl_2$ (8:4:3 V/V). After the third washing the lower phase was

designated as the total lipid extract. An aliquot of the washed extract was analyzed in the Packard Tricarb Liquid Scintillation Spectrometer. The remaining portion of the lipid extract was saponified as described in the following section.

2. Fractionation of Lipid

The solvent of the lipid extract, contained in a screw-capped culture tube, was removed under a stream of nitrogen at 30° C. To the lipid residue, 10 ml of alcoholic KOH (11.2 gm KOH pellets in 100 ml 95% ethanol) were added. The tubes were covered with glass marbles and refluxed for two hours in a steam bath, while maintaining the alcohol level by additions of this solvent. The components of the lipid fraction were separated into the saponifiable fraction (fatty acid) the non-saponifiable fraction (sterols) (113) and the aqueous fraction containing glyceride-glycerol. Carbon-14 activities were determined by the liquid scintillation method.

C. ^{14}C-glycogen

The usual method of glycogen isolation (114) was slightly modified. The residual frog skin from section B was hydrolyzed with 2 cc 30% KOH in a boiling water bath. When a homogenous solution was achieved in 20 minutes, 5 mg of commercial "carrier" glycogen was added followed by the addition of 95% alcohol to a final concentration of 50 to 60%. The mixed solution was heated just to boiling and cooled to room temperature. The glycogen was obtained after centrifugation at 3,000 rpm for 10 minutes and was dissolved with distilled water and an aliquot was radioassayed in a liquid scintillation spectrometer.

D. Determination of Lactate Concentrations

A protein-free filtrate of the incubation medium was prepared by centrifugation after the addition of 30% trichloroacetic acid to a final concentration of 5%. An aliquot of filtrate containing 1-30 µg of lactate was pipetted into a test tube which contained 1 ml of 20% (W/V) $CuSO_4 5H_2O$ and the final volume made up to 10 ml with water. Approximately 1 gm of solid $Ca(OH)_2$ was added, mixed and allowed to stand for 30 minutes (115). The supernatant was kept after centrifugation and an additional copper-lime filtrate was made to remove possible interference by pyruvate (116). A 1 ml aliquot of the second filtrate was pipetted into a colorimeter tube and 5 ml of concentrated H_2SO_4 was introduced slowly with swirling of the tube in an ice bath. 0.05 ml 10% (W/V) $CuSO_4 5H_2O$ was added, mixed and allowed to stand for 30 minutes at 60° C. 0.1 ml p-hydroxydiphenyl (1.5%) reagent solution was pipetted into the sample, mixed and allowed to stand for 20 minutes at room temperature for color development. At the end of this period, the tube was heated in a boiling water bath for 2 minutes and immediately cooled in an ice bath. The optical density of the sample was obtained at 560 mµ against a reagent blank in a Bausch and Lomb Spectronic 20 colorimeter.

E. ^{14}C-lactate

An 0.2 ml aliquot of the second filtrate prepared for the total lactate determination was assayed in a liquid scintillation spectrometer for ^{14}C-lactate activity (117).

V. Procedures for Kidney Slices

A. Incubation Technique

The kidney slice procedure followed closely that of the frog skin, but incubation was at 37° C, and mammalian Krebs' phosphate buffer pH 7.4 was used. The usual composition of the buffer (118) was modified by reducing the concentration of calcium and magnesium to eliminate possible precipitation of these elements. The final composition of the buffer was: NaCl 154 mM, KCl 7.7 mM, $CaCl_2$ 0.4 mM, $MgSO_4$ 0.8 mM, Na_2HPO_4 15.5 mM.

B. Tissue Sampling

The kidneys were removed from a decapitated rat, placed in cold phosphate buffer, and sliced to a uniform thickness of 0.25 mm with the Mickle tissue chopper. Slices were randomized among 8 to 12 Warburg flasks containing 2.7 ml chilled phosphate buffer. The remaining manipulations were the same as for the frog skin procedure. A legend giving specific experimental conditions will accompany each illustration under the section of results.

VI. Extraction of Metabolites from Frozen Tissues

The success of extraction of a certain metabolite depends on the physical nature of the tissue, the temperature of extraction, and the pH of the extracting solution or reagent. With the exception of the reduced form of the nicotinamides, that is, NADH and NADPH, most of the metabolites can be quantitatively extracted by acidic media. For the reduced nicotinamide nucleotides, an alkaline medium has been found to be the most successful. The methods described below are the most widely used in the field and proved to be dependable (119, 120).

A. Acidic Extraction

At the end of incubation, the tissues were removed from Warburg flasks and frozen immediately with liquid nitrogen. Tissue slices were homogenized with a volume of 0.6 N perchloric acid ($HClO_4$) to give a weight ratio of tissue to $HClO_4$ of 1:5. The mixture was homogenized for three one-minute periods at ice bath temperatures. The deproteinized suspension was centrifuged at 1500 rpm for 10 minutes at 0° C. The supernatant was transferred to another tube containing 0.2 ml M K_2HPO_4 and was then neutralized to pH 7.2, slowly, with 3 N KOH. Localized areas of high pH were avoided by vigorous mixing. The sediment that formed was removed by centrifugation at 1500 rpm for 10 minutes at 0° C and the supernatant was assayed for its metabolites.

B. Alkaline Extraction

The kidney slices were homogenized with 2 ml N KOH in a glass homogenizer for three 60-second intervals while cooled by an ice bath. The suspension was heated in a 70° C water bath for 1.5 minutes, and cooled in ice immediately after. It was then neutralized to pH 7.8 with a solution of 0.5 M triethanolamine HCl, 0.4 M KH_2PO_4 and 0.1 M K_2HPO_4. It was allowed to stand at room temperature for protein precipitation, and was centrifuged at 20,000 g for 10 minutes in a Serval centrifuge at 0° C. The supernatant was used for the analysis of reduced nicotinamide nucleotides.

VII. Estimation of Nicotinamide Nucleotides and other Metabolites

The determination of the oxidized and reduced forms of the nicotinamide nucleotides was accomplished by measuring the rate of oxygen uptake with an oxygen electrode assembly (121). In this cyclic

system, the quantity of nucleotide was found to be the rate-limiting carrier in the transfer of hydrogen from a substrate to the acceptor, phenazine methosulphate. The reduced phenazine methosulphate was then oxidized by the oxygen in the reaction medium.

The oxygen sensing device (Beckman Model 777 Laboratory Oxygen Analyzer) consisted of a sensor and an amplifier. The sensor was inserted sideways into the central chamber of a water-jacketed glass cell, maintained at 25° C and a Sargent recorder was used to receive the signal from the analyzer.

A. Assay of the Oxidized and Reduced forms of NAD

A mixture (1.3 ml) containing 50 µmoles of triethanol-amine-hydrochloride buffer pH 7.6, 1 µmole EDTA and 1.7 µmoles of ethanol was placed in the cell. Up to 0.5 ml of the extract was added and finally 0.1 ml of phenazine methosulphate (PMS 2 mg/ml) was added. The contents were well mixed with a magnetic "flea." The cell was then closed with a stopper having a capillary channel for injection of enzyme solutions. After allowing 2-3 minutes for temperature equilibration and completion of any endogenous chemical reduction, 0.05 ml of alcohol dehydrogenase containing 0.3 mg of enzyme protein was injected with a Hamilton micro-syringe.

Oxygen utilization of the sample was recorded for 2-3 minutes, and the rate was compared with known amounts of nucleotide. The reactions involved in the cyclic system are:

$$\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+ \qquad (18)$$

$$\text{NADH} + \text{PMS} \longrightarrow \text{NAD}^+ + \text{PMS}_{red} \qquad (19)$$

$$\text{PMS}_{red} + \frac{1}{2}\,\text{O}_2 \longrightarrow \text{PMS}_{ox} + \text{H}_2\text{O}. \qquad (20)$$

B. Assay of ATP

The oxygen electrode technique was adapted to measure levels of ATP in tissue extracts. In the determination of nicotinamide nucleotide, the amount of coenzyme was the rate-limiting factor while the substrate for the dehydrogenase was in excess. However, in the determination of ATP, this substrate became the rate-limiting factor with excess NADP present. Thus, the absolute amount of oxygen consumed was to be measured rather than the rate of consumption.

The principles of these enzymic reactions were described by Bergmyer (119). The reaction mixture (1.3 ml) contained 50 µmoles triethanolamine-hydrochloride buffer pH 7.6, 100 µmoles glucose, 0.17 µmoles NADP and 1 µmole EDTA. After adding 0.1 ml phenazine methosulphate (2 mg/ml) and closing the cell system, a 3-5 minute period was allowed for equilibration. The injection of 0.05 ml glucose-6-phosphate dehydrogenase (0.3 mg enzyme protein) led to a utilization of oxygen proportional to the levels of glucose-6-P present. A subsequent injection of 0.05 ml hexokinase containing 0.5 mg enzyme protein further increased the amount of oxygen consumption proportional to the quantity of ATP in the reaction mixture.

The following reactions are involved:

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{GGPDH}} \text{6-P-gluconate} + \text{NADH} + \text{H}^+ \qquad (21)$$

$$\text{NADPH} + \text{PMS} \longrightarrow \text{NADP}^+ + \text{PMS}_{\text{red}} \qquad (22)$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-P} + \text{ADP}. \qquad (23)$$

C. Chemicals

NAD^+, NADH, NADP, NADPH, alcohol dehydrogenase and glucose-6-P dehydrogenase were commercial preparations purchased from Sigma Chemical Company, St. Louis, Missouri. Hexokinase and glucose-6-P were obtained from Boehringer und Soehn G.m.b.H., Manneheim, Germany.

RESULTS†

I. Metabolism of Frog Skin and the Effects of Ouabain

It is a generally accepted concept that the oxygen consumption of a cell is related to the amount of free energy being utilized by the cell in processes such as the synthesis of molecules and physical processes such as muscle contraction, glandular secretion and ion transport. Oxygen consumption is dependent on the supply of phosphate acceptor, ADP, which comes from energy-consuming processes. Tissues which have a specialized physical process are most suitable for use in studies on the dynamics of energy metabolism (2, 38, 124).

In frog skin, the establishment and maintenance of concentration gradients of sodium and potassium ions between the intracellular and extracellular fluids depends mainly on energy from respiration (16, 17). In these experiments of Zerahn and Leaf a mean value of 25% of the total oxygen consumption was found for the difference between a transporting and a control skin. This was considered to be a minimum value by both groups of workers because of the limitations of the experimental approach.

Another piece of evidence showing the dependence of ion transport on respiration was obtained from the effects of inhibitors (19, 20, 21) which blocked frog skin ion transport as well as

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†In the data presented below, a comparison of mean values has been made with the standard t test. p values of 0.05 or less are considered to indicate a significant difference.

respiration. However, in anaerobiosis, a minimum amount of ion transport remained (18) which suggested a minor dependence on energy from glycolysis.

The inhibitory effect of ouabain on ion transport of frog skin is well established (30, 31, 32). An effect on oxygen consumption has not been previously reported, but it was known that ouabain can reduce the rate of respiration for kidney cortex preparations (38, 39).

Table IV presents the results of a survey experiment designed to examine the effect of ouabain concentration on oxygen consumption and on conversion of tracer acetate-1-^{14}C to $^{14}CO_2$. The rate of oxygen utilization of control skin slices was considered to be 100%. The control slices converted approximately 21% of the acetate to $^{14}CO_2$. No effects of ouabain were observed at 10^{-8} M. However, at 10^{-6} M, ouabain caused a reduction of 22% in oxygen uptake and 33% in $^{14}CO_2$ production. At 10^{-4} M, the corresponding decreases were 46 and 44% respectively. In order to ascertain the effects of ouabain on frog skin metabolism, 10^{-4} M was used in most of the subsequent experiments.

According to a published report on effects of ouabain, active transport (SCC) decreased gradually for 30 to 60 minutes before complete inhibition was reached (32), and was directly related to the ouabain concentration (33). A time course study of oxygen uptake after ouabain treatment appeared to be desirable.

Before describing the effects of ouabain, the effect of substrates like glucose and acetate on oxygen consumption of normal tissues needs to be clarified. As shown in Table V, there is a 13% decline in the rate of oxygen consumption in the 3 hours for the control tissues without exogenous substrate (line 1). In agreement with previous observations (42), it was found that glucose and acetate (lines 3, 5

Table IV.

The Effects of Ouabain Concentrations on Oxygen Uptake and $^{14}CO_2$ Production From Acetate-1-^{14}C of Frog Skin

Ouabain Concentration M	Oxygen Uptake $\mu l\ hr^{-1}\ g^{-1}$	Acetate Converted to CO_2 $\%\ hr^{-1}\ g^{-1}$
None (Control)	189 ±11.5*	21.8 ±1.1
10^{-8}	189 ±5.3	21.4 ±0.1
10^{-6}	149 ±0.5	14.0 ±0.4
10^{-4}	102 ±2.5	12.2 ±1.0

*mean ± standard error of duplicate flasks

Table V.

A Time Course Study of the Effect of Ouabain on Oxygen Uptake by Frog Skin
($\mu l\ hr^{-1}\ g^{-1}$)

		Time in Minutes			
	Condition	30	60	120	180
(1)	No Substrate Added	196 ±4.3 (7)*	202 ±7.4 (11)	189 ±7.4 (12)	174 ±12.7 (9)
(2)	Ouabain, 10^{-4} M	134 ±6.5 (7)	150 ±4.8 (12)	128 ±3.8 (13)	116 ±7.3 (14)
	"p" values	< 0.001	< 0.001	< 0.001	< 0.01
(3)	Glucose, 1 mM	--	201 ±7.4 (7)	197 ±8.5 (7)	196 ±13.2 (7)
(4)	Ouabain, 10^{-4} M	111 ±14.0 (4)	163 ±2.5 (9)	143 ±2.9 (10)	144 ±3.0 (10)
	"p" values	--	< 0.001	< 0.001	< 0.01
(5)	Glucose, 10 mM	212 ±6.8 (11)	216 ±4.2 (11)	241 ±3.7 (5)	219 ±5.5 (11)
(6)	Ouabain, 10^{-4} M	150 ±7.3 (10)	156 ±3.4 (10)	169 ±3.3 (6)	128 ±5.9 (10)
	"p" values	< 0.001	< 0.001	< 0.001	< 0.001
(7)	Acetate, 1 mM	231 ±3.6 (2)	231 ±0.0 (2)	259 ±2.8 (2)	277 ±2.1 (2)
(8)	Ouabain, 10^{-4} M	--	155 ±12.0 (2)	173 ±2.8 (2)	192 ±0.0 (2)
	"p" values	--	< 0.05	< 0.01	< 0.01

*mean ± standard error with number of flasks in parentheses

and 7) could support the respiration of frog skin and that of the two substrates the latter appeared to be superior. From results reported below it will become obvious that labeled acetate is one of the best substrates for the production of $^{14}CO_2$ compared with other ^{14}C- substrates.

In the presence of 10^{-4} M ouabain, the rate of oxygen consumption was reduced 32% (lines 1, 2) at the end of 30 minutes of incubation. This non-transporting rate of respiration was maintained at the same rate until the end of the third hour. However, for the transporting tissue, there was actually a slight decrease at the end of the third hour (line 1). The decrease probably was due to a lower rate of ion transport during the course of incubation.

When 1 mM glucose was added to the medium (line 3), the tissue respired at the same rate throughout the 3 hours. This higher rate might indicate the need for exogenous substrate as an energy supply. The percentage of decrease due to ouabain (line 4) was consistent. The mean values for respiration at 10 mM glucose (line 5) were above those observed from endogenous substrate (line 1) and from 1 mM glucose (line 3). The increased rate of respiration might indicate that ADP, the phosphate acceptor for respiration, was not only supplied by ion transport but was also provided from the phosphorylation of glucose by hexokinase. This was supported by the mean values obtained in the presence of ouabain (line 6), for they are generally above their counterparts (lines 2, 4). Ouabain again brought about the same degree of reduction in respiration.

An additional remark should be made in regard to the basal oxygen consumptions (that is, ouabain-poisoned rate) at 1 mM and 10 mM glucose

(lines 4, 6). In 1 mM glucose basal respiration (line 4) leveled off at the second and third hour periods, while the basal rate at 10 mM glucose (line 6) continued to climb until the end of the second hour. A sharp drop of 25% resulted at the third hour. This was probably due to a higher input of glucose than its output and in turn resulted in the well known phenomenon of the Pasteur effect (125). These changes are illustrated in Figure 3.

For 1 mM acetate, the number of experiments was small; however, the mean values of the duplicate experiments were remarkably similar resulting in small standard errors. Acetate provided a higher rate of respiration (line 7) as compared to endogenous substrate (line 1) and/or glucose (lines 3, 5) and a slight increase was observed at each hourly period. In the presence of ouabain, the inhibiting effect was consistent but the basal respiratory rate increased during the course of incubation (Figure 3). Two implications of these observations need to be emphasized. First, the absolute amount of oxygen consumption supported by acetate and reduced by ouabain was larger than those supported by endogenous substrate and/or by glucose. This suggested that acetate, in essence, was a better source of energy for ion transport than the other two substrates. Second, the observed increase of the basal respiration during the three hours of incubation suggest that there was in increasing usage of acetate for biosynthetic purposes which in turn generated ADP for respiration. This was supported by later findings shown in Tables VIII and X which showed the increase in the incorporation of substrates into glycogen, glyceride-glycerol and sterols as well when ouabain was added.

The operation of the glycolytic pathway in frog skin has not been studied to any degree. With the aid of specifically labeled

glucose, it is possible to study the flow of carbon along metabolic pathways including glycolysis and the tricarboxylic acid cycle. Table VI shows the incorporation of glucose-1-^{14}C and glucose-6-^{14}C into $^{14}CO_2$. Control frog skin slices produced $^{14}CO_2$ equally well from both labeled glucoses as is indicated by the ratio in the last column. When 10^{-4} M ouabain was introduced, the 1-carbon of glucose was converted into $^{14}CO_2$ at only 50% of the control value, and that of the 6-carbon of glucose was only 24% of the control rate. These changes in the degrees of inhibition yield the higher value of 2 for the conversion ratio, 1-^{14}C/6-^{14}C as compared to the control ratio of 0.96. The significance of this change will be discussed in later sections.

In the time course study shown in Table V, it was clear that glucose was able to support respiration. Table VII presents the effects of variable glucose concentrations on $^{14}CO_2$ production. It is quite clear that a 30% stimulation of $^{14}CO_2$ production resulted from a 10-fold increase in glucose concentration. In ouabain-treated slices, the $^{14}CO_2$ productions were reduced to the same amounts at both concentrations of glucose. Because of the low metabolic rate of frog skin, the desirability of working with a higher carbon-14 specific activity, 1 mM glucose was used in later experiments.

The ratio of incorporation of glucose-1-^{14}C and glucose-6-^{14}C into $^{14}CO_2$ was changed from a value of 1.0 to 2.0 by ouabain (Table VI). A simple calculation such as this has been used by some workers in an attempt to describe the amount of participation of the pentose pathway in glucose metabolism. The insufficiency and limitations of a simple ratio for the quantitation of pathway

Table VI.

Effects of Ouabain on $^{14}CO_2$ Production from Glucose-1-^{14}C and Glucose-6-^{14}C of Frog Skin
(% hr^{-1} g^{-1})

Condition	$^{14}CO_2$ Produced		Ratio $\frac{1\text{-}^{14}C}{6\text{-}^{14}C}$
	Glucose-1-^{14}C	Glucose-6-^{14}C	
Control	9.4 ±0.6 (10)*	9.8 ±0.67 (12)	0.96
Ouabain, 10^{-4} M	4.7 ±0.13 (8)	2.3 ±0.27 (12)	2.01
$\frac{\text{Ouabain}}{\text{Control}}$ x 100	50	24	
"p" values	< 0.001	< 0.001	

*mean ± standard error with number of flasks in parentheses

Table VII.

Ouabain Effects on $^{14}CO_2$ Production of Frog Skin
(mμmoles hr^{-1} g^{-1})

Condition	$^{14}CO_2$ Produced	
	1 mM	10 mM
Control	275 ±11.3 (5)*	356 ±17 (5)
Ouabain, 10^{-4} M	133 ±8.0 (4)	137 ±4.6 (4)
"p" values	< 0.001	< 0.001

*mean ± standard error with number of flasks in parentheses

participation have been pointed out (122, 123). However, in the present study, this simple ratio has been used to indicate a qualitative change in utilization of glucose in the presence of ouabain. A more thorough study of deviations of glucose utilization was achieved by the present use of uniformly labeled and carbon-6-labeled glucose. The incorporation of these specifically labeled glucose molecules into glycogen, glyceride-glycerol and carbon dioxide was examined. The results of these experiments are shown in Table VIII. The labeling of all three metabolic products was generally higher with the uniformly labeled than with the carbon-6 labeled glucose as would be expected because of possible dilution of glucose-6-^{14}C from possible reversal of glycolysis. However, the operation of the pentose cycle does result in an enrichment of the specific activity of the triose phosphates derived from glucose-6-^{14}C due to the loss of unlabeled CO_2 in the cycle. The labeling in the lipid fractions was highest in the glyceride-glycerol moiety, the activities in other fractions being extremely low.

The effects of ouabain on the labeling of these metabolites were far reaching. First of all, when glucose-U-^{14}C was used with carrier glucose at 1 mM, ouabain caused a 75% inhibition of $^{14}CO_2$ production, but at the same time it stimulated the labeling of glycogen in one direction and of glyceride-glycerol in the other direction of glycolysis.

In glycolysis, triose-P molecules are the pathway intermediates, which are largely converted into CO_2 in mammalian tissue via the decarboxylation of pyruvate and the tricarboxylic acid cycle. At the same time the trioses can also become the glycerol moiety of glyceride or be used for the purpose of gluconeogenesis. The direction of their

Table VIII.

Effects of Ouabain on Incorporation of Glucose-U-^{14}C into Metabolites of Frog Skin ($m\mu moles\ hr^{-1}\ g^{-1}$)

Condition	$^{14}CO_2$	Lipid Fractions			
		Fatty Acid	Sterol	Glyceride-Glycerol	Glycogen
Glucose-U-^{14}C, 1 mM	297 ±7.5 (2)*	1.7 ±0.4 (2)	0.9 ±0.4 (2)	26.2 ±1.4 (2)	304 ±6.0 (2)
Ouabain, 10^{-4} M	118 ±2.9 (3)	1.5 ±0.3 (3)	0.7 ±0.2 (3)	33.8 ±0.7 (3)	967 ±47 (3)
"p" values	< 0.001			< 0.05	< 0.01
Glucose-6-^{14}C, 1 mM	189 ±11 (2)	1.8 ±0.3 (2)	1.5 ±0.2 (2)	21.8 ±0.6 (2)	245 ±43 (2)
Ouabain, 10^{-4} M	68 ±2.2 (3)	1.8 ±0.4 (3)	1.7 ±0.3 (3)	40.7 ±1.2 (3)	837 ±27 (3)
"p" values	< 0.01			< 0.02	< 0.01

*mean ± standard error with number of flasks in parentheses

uses depends on the availability of ATP. It was the underlying goal of the present work to develop this concept. The findings described above show a lower production of $^{14}CO_2$ but a greater production of labeled glyceride-glycerol from glucose-U-^{14}C was caused by ouabain. To the author, this implied that the combustion of trioses originated from the labeled glucose had been decreased but the use of trioses for biosynthesis was increased by the ion transport inhibitor. As the problem developed, later work shed much light on these observations.

Similar changes in metabolite labeling caused by ouabain were observed when glucose-6-^{14}C was used instead of glucose-U-^{14}C. As has been mentioned the lower incorporation of glucose-6-^{14}C into metabolites as compared with that of glucose-U-^{14}C was possibly due to the reversal of carbon atoms within the Embden-Meyerhof pathway. Such recycling would dilute glucose-6-^{14}C activity and in turn would show an apparent decrease in incorporation of glucose although the biological system would not discriminate between the labeled carbons of the glucose molecules.

If ouabain did stimulate gluconeogenesis, it would bring about larger differences in the labeling of metabolites between glucose-U-^{14}C and glucose-6-^{14}C. Among the two biosynthetic products, glyderide-glycerol and glycogen, the incorporation of glucose molecules into glycogen was the most active, and their results would be the most dependable. In control tissues, within an hour, 304 mμmoles of glucose were incorporated into glycogen from glucose-U-^{14}C as compared with 245 mμmoles from glucose-6-^{14}C. The difference was 59 mμmoles. In ouabain treated skins the corresponding difference was 130 mμmoles (967-837=130). Thus, a greater dilution of glucose-6-^{14}C was actually

observed. In view of the metabolic control mechanism discussed under the introductory section and the findings of later work, ouabain thus caused a reverse glycolysis in frog skin by its inhibition of the utilization of ATP for ion transport.

Furthermore, ouabain enriched the labeling of glyceride-glycerol from glucose-6-^{14}C (21.8 to 40.7). This was a larger increase than that observed with glucose-U-^{14}C (26.2 to 33.8). The enrichment of the triose derivatives from glucose-6-^{14}C indicated an increased participation of the pentose cycle as suggested by the 1-^{14}C/6-^{14}C CO_2 ratio.

The effects of ouabain on the incorporation of these labeled glucoses has been most interesting. Ouabain inhibited the production of $^{14}CO_2$ from both of the labeled glucoses but at the same time, stimulated the incorporations into glycerol and glycogen. From these findings, the problem of metabolic control evolved and ouabain became a potentially useful agent for such studies.

In many studies of ion transport, it has been a general practice to replace sodium of the incubation medium with potassium or choline. Also, when a non-transporting cell is desired, ouabain inhibition has been used. These manipulations are based on the assumption that the sodium transporting system is specific for the sodium ion and its absence will suffice to stop the functioning of the transporting system. Perhaps such an assumption is sufficient purely for the purpose of ion transport studies of physiological nature. However, the absence of sodium or the increment of potassium and choline concentrations, ultimately will cause numerous biochemical changes. The experiment presented in Tables IXa and IXb was designed to make a comparison of metabolic changes under these different ionic

Table IX (a).

Effects of Sodium, Potassium, Choline and Ouabain on the Oxygen Uptake of Frog Skin

($\mu l\ hr^{-1}\ g^{-1}$)

	Condition	Time in Minutes 60	120	180
(1)	Na 115 K5 mM Control	163 ±3.02 (4)*	155 ±2.7 (4)	148 ±3.7 (4)
(2)	Ouabain	122 ±14.0 (4)	129 ±3.4 (2)	123 ±8.2 (4)
	"p" values	< 0.05	< 0.01	> 0.05
(3)	K 115, Control	137 ±5.6 (3)	142 ±2.4 (3)	148 ±0.6 (3)
(4)	Ouabain	139 ±5.7 (2)	140 ±0.0 (2)	134 ±3.2 (2)
	"p" values	> 0.1	> 0.1	< 0.05
(5)	Choline 115, K5 mM Control	148 ±3.7 (3)	163 ±5.2 (3)	167 ±4.4 (3)
(6)	Ouabain	149 ±5.7 (2)	156 ±1.0 (2)	144 ±1.0 (2)
	"p" values	> 0.1	> 0.1	< 0.05

*mean ± standard error with number of flasks in parentheses

Table IX (b).

Effects of Sodium, Potassium, Choline and Ouabain on
Frog Skin Metabolism of Glucose-U-^{14}C

Condition	Ionic Concentrations, mM (a) Na 115, K5	(b) K 115	(c) Choline 115, K5	"p" values
$^{14}CO_2$, mμmoles Glucose hr^{-1} g^{-1}				
Glucose, 1 mM	169 ±3.6 (6)	149 ±6.0 (3)	178 ±1.7 (3)	a-b < 0.05
Ouabain, 10^{-4} M	95 ±2.2 (4)	142 ±3.6 (2)	120 ±0.4 (2)	
"p" values	< 0.001	> 0.1	< 0.01	
^{14}C-Lactate, mμmoles hr^{-1} g^{-1}				
Glucose, 1 mM	238 ±13 (6)	239 ±25 (3)	313 ±14 (3)	a-c < 0.02
Ouabain, 10^{-4} M	238 ±19 (4)	231 ±1.4 (2)	323 ±1.8 (2)	
"p" values	> 0.1	> 0.1	> 0.1	
Total Lactate μmoles hr^{-1} g^{-1}				
Glucose, 1 mM	2.55 ±0.08 (6)	2.92 ±0.03 (3)	3.15 ±0.03 (3)	a-b < 0.02 a-c < 0.01
Ouabain, 10^{-4} M	3.18 ±0.31 (4)	3.26 ±0.05 (2)	2.9 ±0.05 (2)	
"p" values	< 0.05	< 0.02	< 0.05	
^{14}C-Glycogen mμmoles hr^{-1} g^{-1}				
Glucose, 1 mM	119 ±8.8 (6)	127 ±8.4 (3)	59 ±2.7 (3)	a-c < 0.01
Ouabain, 10^{-4} M	246 ±5.8 (4)	174 ±5.7 (2)	117 ±0.0 (2)	a-b < 0.01 a-c < 0.001
"p" values	< 0.001	< 0.05	< 0.001	

environments. In the experiment reported in Tables IXa and IXb, 20 mM tris(2-amino-2(hydroxy-methyl)-1,3-propanediol)-HCl, pH 7.4 buffer was used in the bathing medium which contained 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM glucose. The test media contained either 115 mM KCl or 115 mM choline chloride in place of 115 mM NaCl.

Table IXa indicates that the QO_2 of control tissue fell 10% in three hours (line 1). The decline was not observed with normal phosphate buffer (Table V, line 3). The basal respiratory rates were similar (Table IXa, line 2 and Table V, line 4). Therefore, the use of tris buffer caused a 10% reduction in respiration as compared with control tissues in normal phosphate buffer.

With high potassium buffer, the QO_2 was lower than the control value (line 1), and there was no effect of ouabain in this medium. In fact the QO_2 was above that of the high sodium medium (line 2). The increased rate was probably due to the effect of high potassium which is known to diminish the inhibition of ouabain (25, 27). Furthermore, mitochondrial respiration is known to be stimulated by high potassium (126).

In the presence of high choline instead of sodium, the QO_2 was similar to the control values (line 5). When ouabain was added, an inhibition of oxygen consumption was not observed until the end of the second hour. The QO_2 (line 6) was above its control counterpart (lines 2, 4). Thus these data indicate a higher basal respiration in medium with high choline present.

In Table IXb, the incorporation of uniformly labeled glucose into $^{14}CO_2$ was significantly depressed when sodium in the medium was replaced by potassium, but there was no difference from the control

value when choline was substituted for sodium. Ouabain reduced values almost to one-half in a medium containing high sodium or when the latter was substituted by choline. In the presence of high potassium in the medium, the inhibitory effect of ouabain disappeared. These observations were in good agreement with the changes in QO_2 (Table IXa).

The labeling of lactate was not affected by the replacement of sodium with potassium (238 to 239 mμmole hr^{-1}) but a significant increase in ^{14}C-lactate formation resulted from the replacement of sodium by choline (238 to 313 mμmole hr^{-1}). There was no effect of ouabain on the labeling of lactate.

The amounts of lactate found in the media after incubation were slightly increased in the presence of high potassium (2.55 to 2.92 μmole hr^{-1}) or choline (2.55 to 3.15 μmole hr^{-1}). Ouabain increased the lactate content in normal high sodium medium but caused a slight increase with a high potassium medium and a decrease in the choline medium. In all cases the differences were statistically significant.

The substitution of sodium by potassium did not influence the incorporation of glucose into glycogen but the substitution by choline caused a 50% inhibition. In accord with that described proviously, in high sodium medium, ouabain stimulated the incorporation of labeled glucose into glycogen. This was equally true for the tissues incubated in a high potassium medium. The decrease of glycogen labeling by choline was reversed by ouabain but the degree of incorporation was still the lowest of the three media tested.

Additional problems were recognized in the use of tris buffer as a bathing medium. These were found in the incorporation of glucose-U-^{14}C into $^{14}CO_2$ and glycogen as compared with the corresponding values

for normal phosphate buffer (Table VIII). The rates for the incorporation of glucose into $^{14}CO_2$ were 169 and 95 mμmoles hr^{-1} in the absence and presence of ouabain (Table IX) while the corresponding rates were 297 and 118 mμmoles hr^{-1} for normal phosphate buffer (Table VIII). These data indicate that there was no change in the basal rate (ouabain present) in the oxidation of glucose, but that there was an inhibition of the total rate by tris buffer. This was in agreement with the changes in oxygen consumption caused by the use of tris buffer. In tris, 119 and 246 mμmoles hr^{-1} of glucose were incorporated into glycogen in the absence and presence of ouabain. These values are only one-third of those obtained with phosphate buffer (Table VII). The stimulating effect of ouabain was not eliminated however by the tris buffer. In conclusion, it was a poor choice to use tris buffer for this intact cell investigation.

It was shown in Table VIII that the biosynthesis of lipid in frog skin was comparatively low. Furthermore, the labeling of lipid from glucose was mainly found in the glyceride-glycerol moiety. These findings provided another key metabolite for the study of the flow of carbon into different components of metabolic pathways. In the experimental results shown in Table X, glucose-6-^{14}C, glycerol-1,3-^{14}C, and acetate-1-^{14}C were used as tracer substrates. The largest incorporations of radioactivity from glucose and glycerol were found in the glycerol fraction. With acetate-1-^{14}C as substrate, the chief incorporation of label was in the sterol fraction.

Upon the addition of 10^{-4} M ouabain with glucose-6-^{14}C as tracer substrate, the labeling of glycerol was increased three-fold as shown

Table X.

The Influence of Ouabain on Incorporation of Tracer Substrates into Lipid Fractions of Frog Skin
(% g^{-1} wet wt)

	Lipid Fractions		
Condition	Fatty Acid	Sterol	G-glycerol
Glucose-6-^{14}C			
Control	0.115 ±0.03 (4)*	0.373 ±0.06 (4)	1.27 ±0.01 (2)
Ouabain	0.090 ±0.01 (6)	0.600 ±0.08 (6)	3.13 ±0.11 (3)
"p" values	> 0.1	> 0.1	< 0.01
Glycerol-1,3-^{14}C			
Control	0.47 ±0.11 (5)	0.32 ±0.05 (5)	4.8 ±0.18 (5)
Ouabain	0.90 ±0.17 (6)	0.45 ±0.07 (6)	8.4 ±0.69 (6)
"p" values	> 0.1	> 0.1	< 0.001
Acetate-1-^{14}C			
Control	0.32 ±0.09 (4)	1.53 ±0.18 (6)	0.58 ±0.01 (2)
Ouabain	0.55 ±0.19 (6)	2.03 ±0.07 (6)	0.88 ±0.01 (2)
"p" values	> 0.1	< 0.05	< 0.01

*mean ± standard error with number of flasks in parentheses

in Table VIII. A lower utilization of triose in catabolic processes has been previously implicated and rendered an enrichment of glycerol labeling. The increased labeling of glycerol by the specific-labeled glucose indicated the pentose cycle as mentioned above. With the more direct use of glycerol-1,3-^{14}C as substrate, the labeling of glyceride-glycerol was increased as expected. The increase in glyceride-glycerol labeling by glycerol-^{14}C with ouabain was not as great as that seen with glucose-6-^{14}C. This might indicate a certain dilution of glycerol-P because of the lower utilization of other triose molecules in the presence of ouabain. Of course, quantitative measurements of trioses are required for proof of such speculations.

A slight stimulation in labeling of glycerol from acetate-1-^{14}C was observed upon the addition of ouabain. Acetate being a precursor for sterol synthesis, it was incorporated mainly into the sterol fraction of the hydrolyzed lipid and this incorporation was stimulated by ouabain.

In view of the observations described in the previous Tables, the following experiments were designed to evaluate the activity of the tricarboxylic acid cycle by using specifically labeled intermediates as tracer substrates. As indicated in Table XI, in the controls, acetate-1-^{14}C and acetate-2-^{14}C were readily converted into $^{14}CO_2$ and yield a Ac1/Ac2 ratio of 1.6. Even though to a smaller extent, citrate-1,5-^{14}C and succinate-2,3-^{14}C were oxidized by frog skin slices. The presence of ouabain caused an inhibition in the conversion of all four intermediates into carbon dioxide, however, the degree of inhibition varied. Acetate-1-^{14}C and succinate-2,3-^{14}C oxidation were reduced to 53 and 58% respectively, while the oxidation of acetate-2-^{14}C

Table XI.

Effects of Ouabain on $^{14}CO_2$ Production of Frog Skin from Tracer Tricarboxylic Acid Cycle Intermediates
(% hr^{-1} g^{-1})

Substrate	(a) Control	(b) 10^{-4} M Ouabain	% of Control $\frac{b}{a} \times 100$	"p" Value
Acetate-1-^{14}C	21.1 ±0.6 (10)*	11.1 ±0.6 (10)	53	< 0.001
Acetate-2-^{14}C	13.5 ±1.9 (6)	3.9 ±0.1 (7)	29	< 0.001
Citrate-1,5-^{14}C	0.86 ±.15 (3)	0.27 ±0.14 (4)	31	< 0.05
Succinate-2,3-^{14}C	0.93 ±.01 (3)	0.54 ±0.05 (3)	58	< 0.01

*mean ± standard error with number of flasks in parentheses

and citrate-1,5-^{14}C were further inhibited by ouabain and gave only 29 and 31% of the control values. The control ratio of 1.6 for acetate-1-^{14}C/acetate-2-^{14}C was increased to 2.8 by ouabain. These varied degrees of inhibition provide valuable clues as to the possible sites where tricarboxylic acid cycle might have been interrupted. A discussion of this aspect will be presented later.

The incorporation of labeled substrates into $^{14}CO_2$ has been shown to be much reduced by ouabain and the utilization of oxygen was also hindered. The oxidative activity of the ouabain-treated tissue was further examined by the determination of total carbon dioxide formation and the conversion of labeled glucose into $^{14}CO_2$. The results are presented in Table XII. The production of carbon dioxide was decreased by the addition of ouabain. The inhibitor also decreased the utilization of labeled glucose for the production of carbon dioxide.

II. Effects of Ouabain on Metabolism of Rat Kidney Cortex Slices

The observations made in the experiments with frog skin lead logically to an inquiry about the state of the oxidation-reduction systems of the cells during active ion transport and during the blockage of the latter process by ouabain. If the suggestion of Whittam is correct (39, 40), that the sodium-potassium activated adenosine triphosphatase functions as a mechanism to provide ADP for respiration, the levels of adenine nucleotides then would be a function of the transporting capacity of the cells. Since ouabain is known to inhibit ion transport and to cause a redistribution of carbon flow down metabolic pathways, it may well change the concentration of adenine nucleotides in the cell.

Table XII.

Effects of Ouabain on Frog Skin Production of $^{12}CO_2$ and $^{14}CO_2$ from Tracer Glucose (μmole hr^{-1} g^{-1})

Condition	$^{12}CO_2$†	$^{14}CO_2$
Control	9.54 ±0.27 (13)*	1.56 ±0.08 (3)
Ouabain, 10^{-4} M	5.71 ±0.52 (10)	1.02 ±0.09 (4)
"p" values	< 0.001	< 0.05

† performed in mounted skin condition

* mean ± standard error with number of flasks in parentheses

Furthermore, as described above in terms of the concepts of metabolic control, changes in the levels of adenine nucleotides do control the flow of carbon into various pathways. The respiratory rate of mitochondria is especially sensitive to control by adenine nucleotides (50, 53). The effects of ouabain observed here on the metabolism of frog skin is believed to be related to these changes in levels of adenine nucleotides. My attempt to estimate levels of nucleotides in frog skin was not successful because of the difficulty of preparing reproducible extracts.

A suitable tissue that has active ion transport is kidney and both slice and homogenate preparations of this tissue have been used to study the inhibitory effects of ouabain on respiration (39, 40). Rat kidney cortex slices were therefore chosen for the investigation of metabolic changes induced by ouabain and for the determination of levels of nucleotides including ATP, NAD and NADH. The results of nucleotide determinations will be presented along with oxygen consumption rates, $^{14}CO_2$, ^{14}C-lactate and ^{12}C-lactate production.

Table XIII presents the oxygen consumption of kidney cortex slices incubated with 3 mM glucose with and without 10^{-3} M ouabain. The slight decrease seen in oxygen use in the presence of 3 mM glucose was found to be not significant. Similar results were found for the combination of 3 mM glucose and succinate. These findings are contrary to those observed by Whittam with rabbit kidney cortex preparations (39, 40). Perhaps, this was due to species difference in using rat kidney in the present experiment.

$^{14}CO_2$ production (Table XIV) was reduced to 60% of the control by a 10^{-4} M concentration of the inhibitor. The concentration of the

Table XIII.

Oxygen Uptake of Rat Kidney Cortex Slices
in the Presence of Ouabain
(μmoles hr^{-1} g^{-1})

	Substrates	
Condition	Glucose, 3 mM	Glucose, 3 mM + Succinate, 3 mM
Control	106 ±5.3 (11)*	117 ±1.7 (5)
Ouabain, 10^{-3} M	92 ±4.8 (10) †	107 ±5.1 (5) †

*mean ± standard error with number of flasks in parentheses

†no significant difference

Table XIV.

Effect of Ouabain on the Metabolism of Glucose-U-^{14}C
by Rat Kidney Cortex Slices
(μmole hr^{-1} g^{-1})

Condition	$^{14}CO_2$ Produced	^{14}C-Lactate Formed	Total Lactate
Glucose-U-^{14}C, 3 mM			
Control	1.55 ±0.04 (15)*	7.9 ±0.4 (15)	11.6 ±0.8 (13)
Ouabain, 10^{-4} M	0.92 ±0.05 (4)	7.3 ±0.9 (4)	11.3 ±1.2 (4)
"p" values	< 0.05	> 0.1	> 0.1
Ouabain, 5×10^{-4} M	0.98 ±0.03 (4)	7.1 ±0.6 (4)	11.2 ±1.5 (4)
"p" values	< 0.05	> 0.1	> 0.1
Ouabain, 10^{-3} M	0.87 ±0.08 (15)	5.4 ±0.5 (15)	10.9 ±1.1 (12)
"p" values	< 0.001	< 0.001	> 0.1

*mean ± standard error with number of flasks in parentheses

inhibitor was raised to 10^{-3} M in order to produce an inhibition in ^{14}C-lactate production. With all concentrations of ouabain used, there were no significant changes found in the total lactate produced. As with frog skin, a sufficient concentration of ouabain could reverse the metabolic flow of the kidney cells as was indicated by the decrease in labeling of lactate while at the same time allow the maintenance of lactate production in the incubated kidney cortex slices.

In the presence of added succinate as additional substrate shown in Table XV, the labeling of CO_2 and of lactate were similar to that found with glucose alone. Total lactate in both cases was not changed. The effects of ouabain in the presence of added succinate were consistent with the experiments with only glucose present.

The assay of the oxidized and reduced forms of nicotinamide nucleotides provided meaningful information on the effect of ouabain. When glucose was the only substrate, ouabain caused an increase in the oxidized coenzyme and a decrease in the reduced form as is indicated in Table XVI. Thus, the ratio $NAD^+/NADH$ was changed from 3.25 to 6.22. The presence of additional succinate did not bring significant changes on either forms of nucleotide in the control tissue and also eliminated the effect of ouabain, although the ratio of $NAD^+/NADH$ was slightly changed from 2.83 to 4.17. The meanings of these findings will be explored in a later section.

The reported inhibition of Na, K-ATPase by ouabain led to the determination of ATP levels in kidney cortex slices and they are presented in Table XVII.

Table XV.

Effects of Ouabain on Metabolism of Rat Kidney Cortex Slices with Glucose and Succinate as Substrates (μmole hr^{-1} g^{-1})

Condition	Glucose Inc. $^{14}CO_2$	^{14}C-Lactate Formed	Total Lactate
Glucose, 3 mM Control	1.55 ±0.04 (15)*	7.9 ±0.04 (15)	11.6 ±0.8 (13)
Ouabain, 10^{-3} M	0.87 ±0.08 (15)	5.4 ±0.5 (15)	10.9 ±1.1 (12)
"p" values	< 0.001	< 0.001	> 0.1
Glucose, 3 mM + Succ, 3 mM Control	1.50 ±0.17 (5)	8.15 ±0.6 (5)	12.0 ±0.7 (5)
Ouabain, 10^{-3} M	0.90 ±0.07 (5)	5.2 ±0.5 (5)	11.5 ±0.75 (5)
"p" values	< 0.01	< 0.01	> 0.1

*mean ± standard error with number of flasks in parentheses

Table XVI.

Effects of Ouabain on Levels of Nicotinamide Nucleotides in Rat Kidney Cortex Slices (μmoles g^{-1})

Condition	NAD^+	NADH	$\frac{NAD^+}{NADH}$
Glucose, 3 mM Control	0.455 ±0.01 (4)*	0.140 ±0.05 (4)	3.25
Ouabain, 10^{-3} M	0.547 ±0.03 (5)	0.088 ±0.03 (3)	6.22
"p" values	< 0.05	< 0.05	
Glucose, 3mM + Succ, 3 mM Control	0.483 ±0.01 (2)	0.171 ±0.02 (5)	2.83
Ouabain, 10^{-3} M	0.555 ±0.01 (2)	0.133 ±0.01 (4)	4.17
"p" values	> 0.1	> 0.1	

*mean ± standard error with number of flasks in parentheses

Table XVII.

Effects of Ouabain on Adenosine Triphosphate Levels in Rat Kidney Cortex Slices

Condition	ATP μmoles g^{-1}	"p" value
Glucose, 3 mM, Control	0.141 ±0.01 (3)*	
Ouabain, 10^{-4} M	0.345 ±0.01 (3)	< 0.001
Ouabain, 10^{-3} M	0.463 ±0.08 (2)	< 0.05
Glucose, 3 mM + Succ, 3 mM, Control	0.324 ±0.02 (2)	
Ouabain, 10^{-3} M	0.354 ±0.03 (2)	> 0.1

*mean ± standard error with number of flasks in parentheses

In control tissues, the content of ATP was found to be 0.141 μmoles per gram. At 10^{-4} M ouabain the nucleotide content was raised to 0.345 μmoles per gram, and a further increase to 0.463 μmoles per gram was found at 10^{-3} M. The observations provide support for the previously mentioned concept that ouabain inhibits the Na, K-ATPase.

However, addition of succinate also increased the amount of ATP and the presence of ouabain did not have any other influence on the ATP.

DISCUSSION

It was early recognized that the quantities of phosphate and phosphate acceptor in the cell are small compared with the amounts of glucose utilized (125). Since the metabolism of glucose to lactic acid or the metabolism of substrate hydrogen to H_2O converts ADP and Pi to ATP, processes utilizing ATP must regenerate ADP and Pi in order for glycolysis and respiration to continue. Thus glycolytic and respiratory ATP synthesis can exceed ATP utilization only in transient periods of adjustment. The Pasteur effect is simply the manifestation of the fact that respiration consumes ADP and Pi; and, unless the ATP utilization is correspondingly increased, the rate of glycolysis must fall. The fast response of mitochondrial respiration to stimulation by ADP as compared with Pi led Chance to conclude that ADP is the major rate controlling factor (50). A similar conclusion was arrived at with whole cell studies (127, 128).

I. Effects of Ouabain on Oxygen Uptake

The variation in the rate of oxygen consumption during resting and during active ion transport has led to the general concept of a basal rate, characteristic of a resting cell, and a variable rate, determined by the demands for energy during activity. Regulation of a part of respiration by active transport has been demonstrated in frog skin (16, 17), sartorius muscle (129), toad bladder (130), avian salt gland (131) and mammalian brain slices (132, 133). The

results of the present study with frog skin slices agree with this concept of regulation.

Depending upon the supply of substrate, the basal rate of oxygen consumption of frog skin was roughly constant and was insensitive to ouabain and sodium. In 1 mM glucose and 10^{-4} M ouabain, respiration resulted in a constant basal rate over three hours (Table V, line 4 and Table IX, line 2). The replacement of sodium with potassium and choline resulted in similar rate (Table IX(a), lines 3, 4 and 6). This basal rate was about 75% of the total oxygen consumption and was in good agreement with the previous observations made in "short circuited" frog skins (16, 17). The assumption (21) that ouabain and sodium act primarily upon cation transport as suggested by Schatzman has not been too far off. However, he neglected the role of ion transport in the regulation of anaerobic metabolism in RBC. While the present study was in progress, the close relationship of ion transport and metabolism of red blood cells was rediscovered (22, 23). This concept of control was later extended to the regulation of respiration of brain slices (132, 133), kidney slices (38, 39) and frog skin as observed in the present study. In other words, respiration depends on active ion transport across cell membranes for 25% of its supply of phosphate acceptors. This interpretation has at least two limitations as pointed out by Whittam (38). The stated percentage of inhibition of respiration might have been over-estimated because ouabain might have inhibited other energy consuming processes and sodium might stimulate respiration via processes other than ion transport. On the other hand, the degree of inhibition might have been under-estimated because phosphate acceptor could have been

supplied by the increased activities of other energy consuming processes. The effect of these multiple cellular processes presents problems in the estimation of sodium transport energetics (i.e. $Na:O_2$) for various tissues (16, 17, 38) with the exception of lysed red cell ghosts (4), in which cell constituents are removed during the lysing process.

Evidence pertaining to the possible under-estimation of the energy requirement was observed in the present studies. First, there were found increasing rates of oxygen consumption in 10 mM glucose (first two hours) and in 1 mM acetate (Table V, lines 6 and 8). This under-estimation was substantiated by the results obtained in the labeling of glycogen by ^{14}C-glucose (Tables VIII and IX(b)) and the labeling of sterol and glycerol by ^{14}C-acetate (Table X). From the phosphorylation of the added glucose and the biosynthetic activities that occurred more ADP was made available for respiration.

In line with the changes in basal respiration, the sudden drop of oxygen uptake at the third hour for 10 mM glucose as compared with an increasing rate for 1 mM acetate (Table V, lines 6 and 8), indicated a Pasteur effect. It appears that at the third hour 10 mM glucose, without the functioning of membrane ion transport, had caused a competition for phosphate acceptors between glycolysis and respiration.

Active glucose transport in intestinal epithelium is closely linked to active sodium transport (134, 135). A slight inhibition of glucose transport in rat kidney slices by ouabain has also been reported (135). Unfortunately, this aspect of glucose transport could not be examined in the present study. Even though ouabain inhibition of glucose transport was possibly present, the constant rate of respiration of tissues incubated in 1 mM glucose allows interpretation of the present results.

The replacement of sodium in the bathing medium by potassium is known to cause a reduction of respiration in kidney slices (38) and brain slices (133). In the present study, a similar effect was observed with frog skin. However, effects of choline substitution vary from tissue to tissue. Choline has been shown to reduce respiration in kidney slices (38) but to have no effect on brain slices (133) or on frog skin respiration, according to the present studies. This lack of effect was supported by the $^{14}CO_2$ production shown in Table IX. In fact, a slight increase was observed in both $^{14}CO_2$ production and oxygen consumption at the end of the third hour. Unless, the actual effects of choline are known for a specific tissue under study, the use of choline to replace sodium for blockage of ion transport requires caution. On the other hand, the consistency of the potassium effects on different aspects of metabolism suggests this ion an ideal substitute for sodium if blockage of transport is desired.

II. Effect of Ouabain on the Pentose Cycle

Isotope experiments in general can afford a close approximation of tissue function and of the activity of enzymes, but they must be interpreted with caution. The results of the experiment shown in Table VI indicate that ouabain causes a differential decrease of $^{14}CO_2$ production from glucose-1-^{14}C and glucose-6-^{14}C. The conversion ratio, 1-^{14}C/6-^{14}C which was changed from 1 to 2 represents increased participation of the pentose pathway (137). The assumption underlying this evaluation is that the pentose cycle would yield $^{14}CO_2$ from glucose carbon atom 1 at a greater rate than that from carbon atom 6, whereas glycolysis plus the operation of the tricarboxylic acid cycle would yield carbon dioxide from carbon atoms 1 and 6 to the same extent.

The limitations of the assumption have been emphasized by a number of authors (112, 123). An appraisal of the merits of using the ratio for a quantitative determination of pathway contribution is beyond the scope of the present thesis, however, the qualitative value of the ratio cannot be denied.

The contribution of the pentose cycle in frog skin has not been investigated seriously in the present study. However, its participation is expected to be a small part of the total glucose utilized since it is generally understood that the function of the pentose cycle is mainly to generate NADPH for fatty acid synthesis (138), and this happens to be a minor pathway in frog skin.

III. Effects of Ouabain on Glycolysis

Glucose-U-^{14}C would label CO_2 via the decarboxylation of pyruvate and the tricarboxylic acid cycle in addition to the pentose cycle, while glucose-6-^{14}C would generate $^{14}CO_2$ via only the first two routes. It should be noted that $^{14}CO_2$ production from both glucose substrates was reduced by some 60% in the presence of ouabain. The possible increased participation of the pentose cycle discussed above must be an insignificant quantity.

A comparable drop of oxygen uptake (32%) and of total CO_2 production (36%) suggests a similar degree of inhibition of the tricarboxylic acid cycle activity and respiration. The fact that the formation of $^{14}CO_2$ from labeled glucose was decreased by 60% while total CO_2 production was decreased only some 30% suggests nearly a 60% inhibition of an extra-tricarboxylic acid cycle CO_2 forming step, the quantitatively most important one being pyruvate decarboxylation.

The decreased labeling of CO_2 from decreased pyruvate decarboxylation strongly suggests a corresponding drop in glycolytic activity.

Of the total amount of CO_2 produced in control tissue (9.54 μmole hr^{-1} g^{-1}), glucose-U-^{14}C could label only 1.8 μmole hr^{-1} g^{-1} which represents only 20% of the total CO_2 produced. This statement is based on the assumption that an endogenous supply of glucose becomes negligible with the influx of exogenous glucose. As a result, the operation of the tricarboxylic acid cycle and respiration will support more than 80% of the total energy expenditure of the tissue.

The utilization of glucose (glucose-U-^{14}C) by control tissues for glycogen synthesis was about the same magnitude as for oxidation. The increased labeling of glycogen in the presence of ouabain reflects a greater availability of an energy source. At the same time, ouabain also stimulates gluconeogenesis from non-glucose precursors as judged from a greater labeling of glycogen from glucose-U-^{14}C than from glucose-6-^{14}C. Such a difference in glycogen labeling from these specifically labeled glucoses would be expected if the tracer glucoses had labeled the pyruvate pool differentially.

The increased incorporation of labeled glucoses into glyceride-glycerol suggests an accumulation of dihydroxyacetone-P and glyceraldehyde-3-P. The accumulation of triose phosphates and the reduction of pyruvate decarboxylation point to a decreased utilization of glucose for oxidation. The controlling steps in glycolysis under the influence of ouabain cannot be located with precision in the present studies. However, the described changes are in good agreement with the conclusions obtained from the effect of ouabain on RBC (139). Minakami and Yoshikawa recently suggested that the observed elevation of the ratio ATP/ADP,Pi

in the presence of ouabain caused an inhibition of P-fructokinase in RBC. The controlling nature of this enzyme has been described in the introduction. The Japanese workers also concluded that reductions in glyceraldehyde-3-P dehydrogenase and hexokinase activities were caused by the decrease in Pi, but this would not be a limiting factor in the present study because ouabain did inhibit glycolysis of frog skin even in the presence of a medium high in phosphate. An explanation for the reduction of glyceraldehyde-3-P dehydrogenase activity may be provided by the work of Landon (140) who observed the reoxidation of NADH generated by the dehydrogenase upon the addition of the endoplasmic reticulum fraction of kidney tissue. The accumulation of the triose phosphates may also be due to the reduction of 3-P-glycerate kinase activity as suggested by both groups of workers. The inhibition of the membrane ion transport by ouabain reduces the supply of ADP for the kinase reaction.

Similar controlling mechanisms may have been exerted on the glycolytic flux in the kidney slice experiments presented in this thesis, for the utilization of glucose-U-^{14}C to produce $^{14}CO_2$ in the tissue slices was 60% of the control in the presence of 10^{-3} M ouabain. The observed increase in ATP would provide part of the evidence for the elevation of the "phosphate potential," and may lead to the respective reduction of the activities of the enzymes stated above.

At the same time, the elevated phosphate potential might have stimulated the activities of enzymes such as fructose-1,6-diphosphatase, pyruvate carboxylase, and other enzymes responsible for gluconeogenesis. There is very little evidence, if any, in the present study of increased gluconeogenesis of frog skin in the presence of ouabain. However, the

differential incorporation of glucose units into glycogen by glucose-U-^{14}C and glucose-6-^{14}C does give an indication of increased gluconeogenesis. The experiments on kidney slices were not extended to this aspect of the study. Because kidney is highly active in gluconeogenesis (79), further work of this type with kidney tissue slices may well furnish significant information. Therefore, the present thesis has demonstrated that the manipulation or control of ion transport affords a tool to study the glycolytic flow of carbohydrate metabolism.

IV. Effects of Ouabain on Tricarboxylic Acid Cycle

So far the discussion has covered the pentose cycle and pyruvate decarboxylation, the two minor routes in the production of CO_2. It was established earlier that almost 80% of the total CO_2 production originates from the tricarboxylic acid cycle. This cycle is the chief "machinery" for the assimilation of substrates and the chief source of hydrogen for respiration. Knowledge of its operation will play an important role in understanding the balance between energy production and utilization. In RBC, energy for active transport of ions depends mainly on ATP produced from glycolysis (22). However, in frog skin and mammalian tissues, transport is dependent mainly on the tricarboxylic acid cycle and the ATP produced from respiration. Any specific effect of ouabain on the tricarboxylic acid cycle has not been established. A recent report concerning the effects of ouabain on the oxidation of cycle intermediates by the ciliary body of pig eyes (141) shows that respiration supported by these substrates was inhibited by ouabain. In the control experiments, the respiratory rates in the presence of succinate, citrate and glucose were 174, 144 and 85

µl/100 mg w.w./hr respectively, while the corresponding values with 10^{-4} M ouabain were 67, 33 and 31 µl/100 mg w.w./hr.

The observations on O_2 uptake reported above are in agreement with the $^{14}CO_2$ production from these labeled intermediates in the present studies of frog skin. The labeling of CO_2 from acetate-1-^{14}C and acetate-2-^{14}C were 21.1% and 13.5% in the control, while the respective values were 11.1% and 3.9% in the presence of ouabain. The ratio acetate-1-^{14}C/acetate-2-^{14}C was changed from 1.56 to 2.85. A ratio above unity is considered to indicate the involvement of the tricarboxylic acid cycle for biosynthetic activities (142) so that the observed increase in the ratio with ouabain suggests that acetyl CoA was utilized more for synthetic than for oxidative processes. This is in agreement with the finding that the incorporation of acetate-1-^{14}C into sterol and glyceride-glycerol fractions of lipid was increased by ouabain. Increased biosynthetic labeling is probably due to a decrease in the utilization of mitochondrial ATP for ion transport and thus to a greater availability of ATP for mitochondrial synthetic purposes.

The drastic 70% decrease in $^{14}CO_2$ from citrate-1,5-^{14}C in the presence of ouabain may be interpreted as follows. According to the general knowledge of tracer methodology, a decrease in labeling of a biological product may indicate an increase in the pool size of the precursor. If that is the case, an increased citrate pool size may signify decreased utilization. This line of reasoning is very attractive to the author because it was established earlier that an increase in the ATP/ADP ratio will cause an increase in the citrate content which in turn will introduce a potent inhibitor for P-fructo-kinase.

The consequences of increased citrate and ATP concentration are consistent with both the impaired glycolysis reported for RBC (139) and with the present results on frog skin. Since, on the other hand, citrate is used as a substrate for fatty acid synthesis (143), the reduction of $^{14}CO_2$ from citrate-1,5-^{14}C might be explained by its increased removal for this synthesis. However, this possibility is unlikely because fatty acid synthesis in frog skin has been shown to be low.

The incorporation of succinate into CO_2 must also receive consideration. The reduced conversion of this substrate into CO_2 is similar to that observed with acetate-1-^{14}C. With the analogy established for the ratio acetate-1-^{14}C/acetate-2-^{14}C, a value of 1.0 for acetate-1-^{14}C/succinate-2,3-^{14}C (succinate-2,3-^{14}C is equivalent to acetate-2-^{14}C) is an indication for normal flow of carbon from succinate through fumarate, malate and oxaloacetate. The present interpretation may also explain the reason that succinate could support a higher basal rate of respiration of ciliary body than did citrate in the presence of ouabain.

If the above reasoning is valid, the location of a controlling site in the tricarboxylic acid cycle may be inferred. The different degree of reduction in the labeling of CO_2 by citrate-1,5-^{14}C and succinate-2,3-^{14}C in the presence of ouabain suggests a decreased utilization of citrate and a normal utilization of succinate for the final three conversions prior to oxaloacetate. The reactions from citrate leading to succinate thus become the possible steps responsible for the reduction of tricarboxylic acid cycle activity. The increased ATP content caused by ouabain might have limited the

decarboxylation step catalyzed by the mitochondrial NAD-specific isocitrate dehydrogenase which has been suggested previously as a rate-limiting step in the tricarboxylic acid cycle of other systems (106, 108). However, these conjectures can only be confirmed by further experiments.

V. Effects of Ouabain on the Control of Respiration

Decreased activities of glyceraldehyde-3-P dehydrogenase in glycolysis and the NAD-specific isocitrate dehydrogenase in the tricarboxylic acid cycle, may reduce the production of NADH which is normally reoxidized both by the lactic acid dehydrogenase and the respiratory chain. The present studies have shown by direct measurement of both NAD and NADH, that ouabain did cause a decrease in the content of NADH in kidney slices, and did change the ratio, NAD/NADH, from 3.2 to 6.2. Upon the addition of succinate to the incubation medium, the effect of ouabain on the nucleotides was not apparent, but a slight change in the nucleotide ratio (2.8 to 4.2) remained. Perhaps similar changes in nucleotides took place in frog skin and caused part of the reduction in oxygen uptake.

The idea that ATP can reduce the sensitivity of ADP control of mitochondrial respiration (52, 53) would be an additional explanation for the reduced oxygen consumption of frog skin. The observed increase of ATP content in kidney slices resulted from a reduced utilization of ATP for ion transport in the presence of ouabain supports this line of thought. Perhaps this is another demonstration of the reversibility of oxidative phosphorylation along the respiratory chain which is actually controlled by the "phosphate potential,"

ATP/ADP,Pi. The inhibition of the Na, K-activated ATPase by ouabain, however, has pointed to the controlling nature of ADP in respiration of frog skin characterized as "state 4" by Chance (50). In conclusion, the present thesis oriented around the concepts of metabolic control has demonstrated that the utilization of ATP for ion transport affords a controlling mechanism or pace maker for 1) glycolysis, 2) the tricarboxylic acid cycle, and 3) respiration.

SUMMARY AND CONCLUSION

The effects of ouabain on the intermediary metabolism of frog skin are summarized as follows.

1. Ouabain causes a 30% decrease of oxygen uptake.
2. The pentose cycle of glucose metabolism is increased to some extent by ouabain.
3. Glucose metabolism by glycolysis is altered by the presence of ouabain.
4. The results from the labeling of CO_2 by the cycle intermediates and the total CO_2 production indicate a 30% inhibition by ouabain of the tricarboxylic acid cycle.
5. It is concluded that glyceraldehyde-3-P dehydrogenase and NAD-specific isocitrate dehydrogenase are the possible sites at which ouabain exerts its influence and that the reduced generation of NADH from this reaction is responsible for part of the reduced oxygen uptake.
6. Ouabain elevates the ATP concentration and in turn increases the phosphate potential which limits the consumption of oxygen.
7. The decreased supply of ADP due to the blockage of ion transport is another controlling factor of the respiratory rate.
8. These studies have demonstrated the interrelation of cellular ion transport and energy metabolism.

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