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Oxidations by Rat Liver Mitochondria Under Steady-State Condition

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OXIDATIONS BY RAT LIVER MITOCHONDRIA UNDER STEADY-STATE CONDITIONS

by

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Abstract of the dissertation entitled "OXIDATIONS BY RAT LIVER MITOCHONDRIA UNDER STEADY-STATE CONDITIONS" submitted by John E. Biaglow in partial fulfillment of the requirements for the degree of Doctor of Philosophy, February, 1964.

The object of the research reported was to provide information which would lead to a better understanding of the processes which occur in living cells.

Mitochondria exist in vivo in a complex protoplasmic unit. Although we cannot, at present, be absolutely certain of the pH of the cytoplasmic matrix, it is probable that the pH does not vary over great ranges in the normal cell. Chambers and his coworkers, have consistently found that the cytoplasmic matrix has a pH of 6.8. Measurements by Behki and Melchior in this laboratory showed that the reaction mixture became alkaline when succinate was oxidized by beef heart mitochondria. These experiments were done using the currently accepted reaction medium which had an initial pH of 7.4.

Since pH 7.4 is not the cytoplasmic pH, and the mixture pH rapidly becomes even more alkaline, it is quite possible that much of the published material will not be directly applicable to understanding mitochondrial reactions in vivo. It was thought that an investigation of the effect of pH on mitochondrial oxidations would provide useful data.

It was necessary, in such a study, to employ a buffer which will not be metabolized nor influence enzymatic reactions. For this reason histidylhistidine, histidine, histamine, N-acetyl-histidine, and tris-(hydroxymethyl)-aminomethane were examined as possible buffering substances during succinate oxidation. These buffers met the criteria mentioned above. A careful study of the effect of pH on oxidative phosphorylation with succinate as the substrate was made. During this study the author found that oxidative phosphorylation with AMP (adenosine monophosphate) as the eventual phosphate acceptor, was very nearly isohydric at pH 6. The author also initiated studies, in searching for possible metabolic controls, with other citric acid cycle intermediates at pH 6 and pH 7.

Results at pH 6 are markedly different from those at pH 7. This suggests that pH dependent metabolic controls may be found by more detailed investigations between pH 6 and pH 7.

Similar considerations lead to experiments at osmotic pressures which approach those which exist in vivo. Most of the published work has been done in media which were less than 0.3 osmolar. The cellular fluid of the liver cells is calculated to be above 0.38 osmolar. In order to evaluate the possible importance of this variable, experiments were conducted at both 0.3 and 0.45 osmolar.

The author was able to demonstrate experimentally that a

change in osmotic pressure from 0.3 to 0.45 osmolar caused marked changes in oxidation rates for malate, citrate, and α-ketoglutarate but not for succinate. This is in accord with previous work which indicates that succinoxidase is located on the outside of the mitochondrial membrane, while the other dehydrogenases are within the cristae.

Another important variable is the amount of phosphate acceptor. Many workers believe that the concentration of ADP (adenosine diphosphate) controls oxidation at the cellular level. For this reason the level of phosphorylation of the adenine nucleotides added was controlled so that ATP exceeded ADP in some experiments and ADP exceeded ATP in other, otherwise comparable experiments.

Nucleotide phosphorylation was found to influence the oxidation of citrate and α-ketoglutarate oxidation at pH 7 and at low osmolarity. Possible explanations for the observed effects were that high ATP reduced manganese available to isocitric dehydrogenase (citrate added) and thus reduced the rate; high ATP reduced the amount of GDP available to succinyl CoA synthetase in the oxidation of α-ketoglutarate to succinate. Both phenomena occur in both systems, but the relative amounts of substrate make one decisive with citrate, the other with α-ketoglutarate.

Another accepted practice as reported in the literature is to add a substrate and measure oxygen consumption and phosphate

uptake. There have been few attempts reported to measure the products of oxidation of a particular substrate. For this reason the author further developed a method for the measurement of carbon-14 labeled substrates. Combining this method with enzymatic assays, it was demonstrated that all the data from experiments in which significant amounts of oxygen were absorbed are in agreement that a considerable amount of this oxygen consumption is due to the oxidation of acetyl-CoA via the Kreb's tricarboxylic acid cycle. Thus, some of the effects measured in experiments of this sort may be due to changes in the availability of acetyl-CoA.

In conclusion, the results reported in this dissertaion are compatible with the general ideas concerning the reactions which constitute the Kreb's tricarboxylic acid cycle.

These results demonstrate that changes in osmotic pressure, pH, and relative nucleotide phosphorylation, can markedly affect the oxidation of added citric acid intermediates. These variables must therefore be considered to be potential physiological controls of mitochondrial metabolism in vivo.

LIFE

John E. Biaglow was born in Cleveland, Ohio, on April 1, 1937.

He was graduated from John Adams High School, Cleveland, Ohio, in June, 1955, and from John Carroll University, Cleveland, Ohio, in June, 1959, with the degree of Bachelor of Science. The author began his graduate studies at Loyola University, Chicago, Illinois, in September, 1959. He married Mary Ann Kocab in December, 1959. The writer now has three children whose names are John, Joseph, and Mary.

The author served as a graduate teaching assistant in the Department of Biochemistry of the Stritch School of Medicine, Loyola University, for the academic years, 1959-1960 and 1960-1961. He received the degree of Master of Science from Loyola University in June, 1961, where he continued his graduate studies. In June, 1961, he was elected to associate membership in the Society of Sigma Xi. The author has been the recipient of the following fellowships: The National Science Summer Fellowship, 1961; The Standard Oil Fellowship for the academic year, 1961-1962; The National Science Summer Fellowship, 1962; The Royal E. Cabell Research Fellowship for the academic year, 1962-1963. He was supported by a Public Health Graduate Training Grant in Biochemistry (5T1 GM 698-01) for the Summer of 1963.

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John E. Biaglow

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CHAPTER I

HISTORICAL SURVEY

As in many branches of science, the growth in knowledge of cytoplasmic particles was retarded, not through lack of men with keen powers of observation and imaginative intelligence, but by the limited state of available techniques. Not until apochromatic lenses were available, in the last quarter of the nineteenth century, were cytoplasmic granules the object of intensive study (see Newcomer (1) for a review of the first observations on mitochondria). In his classic review, Cowdry (2) lists the many names given to mitochondria during the early studies when, because of inadequate fixation, they could hardly be distinguished from other particles; their description rested upon the appearance of cells fixed in fluids containing acetic acid, which is now known to destroy some mitochondria (2).

Altmann (3,4) improved the fixative and thus could see mitochondria more clearly, but even his technique left them insufficiently delineated from other granules. In this period of growing interest in mitochondria, Altmann (4) developed his concept of "bioblast" (from the Greek words for "life" and "germ"). The view that mitochondria were the ultimate in living things, "elementary organisms" like bacteria, met with a negative response and made it more difficult for the cytoplasmic parti-

cles to compete with the nucleus for the interest of investigators. The fixatives used by cytologists to bring out nuclear detail usually destroyed mitochondria.

It was almost ten years before Benda (5) modified Flemming's fixative and introduced crystal violet staining, and revived interest in mitochondria. He introduced the term mitochondria (singular, mitochondrion, from the Greek for "thread" and "grain") to describe the "fadenkornern" ("thread granules") which he saw in cells during spermatogenesis. He concluded that mitochondria were permanent cell organels which played an important part in heredity and which differentiated during histogenesis into many specialized structures such as myofibrils and the basal bodies of celia. Among the strongest supporters of Benda's views was Meves. Coining new terms for varying mitochondrial shapes, various authors (7) described their transformation into fibrils of muscle, nerve, connective tissue cells, grandular epithelia, and ciliated cells; and into secretory granules, pigment granules, and yoke spheres. Although over-enthusiastic, the claims of Benda and Meves, particularly, that they were part of the material basis of heredity, brought attention to the mitochondria in an era when chromatin still held the fascination of investigators.

Concerning the biochemical nature and basic physiological role of mitochondria, little could be learned by the techniques

then available. True, as early as 1908 Regaud (8) had drawn attention to their phosphatide and protein nature, and Kingsbury in 1912 (9) had suggested that they were a "structural expression of the reducing substances concerned in cellular respiration." Cowdry's (10) 1926 concept of "the surface film theory of the function of mitochondria," while it described an important aspect of mitochondrial structure told little of its biochemical function. The situation was essentially the same in 1934 when Sharp (11) wrote in the third edition of Introduction to Cytology, "Owing to the difficulties attending the observation of such minute objects and the determination of their relation to other cytoplasmic constituents, opinion regarding the origin, behavior, and biological significance of chondriosomes (mitochondria) is in an unsettled state.....Our knowledge of chondriosomes is far too incomplete to warrant categorical assertions concerning their function. The literature is not only complicated by conflicting statements regarding the observed behavior, but is further encumbered with a variety of hypotheses, some of which rest on very narrow foundations."

In the same year Bensley and Hoerr (12) described the isolation of mitochondria from cells of guinea pig liver by the process of differential centrifugation of ground tissues. Shortly thereafter, Claude reported the separation, by higher centrifugal forces of submicroscopic granules (for which he adopted the

old term "microsomes") after first sedimenting the "large granules" (13). Identification of the "large granules" of Claude with mitochondria was achieved by Hogeboom and co-workers (14) on the basis of their stainability with Janus green B and their elongate forms when isolated from hypertonic sucrose homogenates.

The next fifteen years saw the growth of an enormous literature devoted to the biochemistry of isolated mitochondria (this author has collected over 2,000 reprints on mitochondrial subjects of interest to him during the last two years). The outstanding discovery was the demonstration that mitochondria contained the chief (perhaps all) sites of oxidative phosphorylation, the process by which the energy of foodstuffs is made available to cell metabolism and cell function.

Reviews on the morphology and biochemistry of mitochondria which were of major importance at the time of their publication include the following: Cowdry (15,16), Wilson (17), Sharp (18), Guilliermond (19), Schneider (24), Earnster and Lindberg (25), Hogeboon (14), Schneider (26), Hackett (27), Millerd (28), Hackett (29), Goddard and Stafford (30); on the correlation of structure and biochemistry: Green and Hatefi (31), Lehninger (32), Palade (33) and Symposia: The Structure and Biochemistry of Mitochondria (34), Biochemical Structure and Function (35), Regulation of Cell Metabolism (36).

The extensive body of data on the biochemistry of mitochon-

dria has been amassed almost exclusively with fractions isolated by the technique of differential centrifugation. Although the mitochondria of rodent liver have been the most widely used, those isolated from various tissues of vertebrates, invertebrates, and plants have also been studied. The description which follows is based on results from animal tissues, particularly rat liver.

ENZYMES

Biochemical investigations indicate that mitochondria contain cytochromes a, c, and a_3 (cytochrome oxidase), and many, although perhaps not all, of the Kreb's cycle enzymes appear to be localized in these structures exclusively (26,14,24). The mitochondria are probably the chief sites of the enzyme systems linking phosphorylation to oxidation (37) and the enzyme systems which catalyze the oxidation of fatty acids (25,38,39,40), amino acids (41,42), and choline (43,44). It is generally considered that mitochondria possess the full complement of enzymes, cofactors, and accessory substrates required for these reactions (45,46). However, Schneider (24) stresses that demonstration is still lacking of the mitochondrial localization of some key enzymes such as those concerned with synthesis of fatty acids, etc.

From the evidence that mitochondrial fragments retain the capacity for electron transport and oxidative phosphorylation,

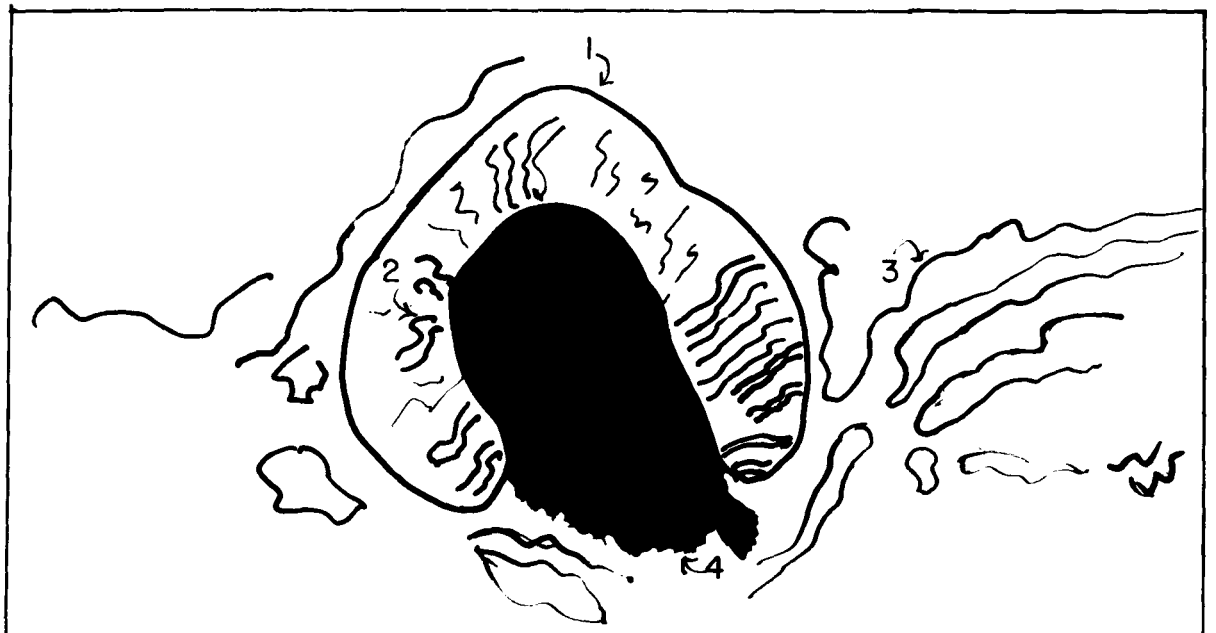

FIGURE 1

MITOCHONDRION ENGULFING LIPID

Drawn from an article by Palade (191).

it has been deduced that the enzymes involved in these processes are arranged in repetitive arrays (31,32,47,48,49,50). It is important to note that those biochemists who are engaged most actively in unravelling the sequences of these reactions are the ones who most stress the inseparability of structure and function in the mitochondrion. Rigid proof is still lacking that the cristae contain "polymeric" arrangements of multienzyme assemblies, yet there is sufficient reason to consider that some of the special properties of the enzyme systems are derived from their spatial organization in these structures, where they may function in a nonaqueous medium as "solid state" arrays, with perhaps as many as 5,000 or 20,000 such assemblies in a single liver or heart mitochondrion (31,32). Other enzymes may be present in the membranes, and there is some supporting evidence for the view that other, more-readily solubilized enzymes are localized in the inner "matrix" of the mitochondrion (see Lehninger (50) for a list of such enzymes).

By refined spectrophotometric methods, Chance and Williams (51,52,53,54) have been able to follow the oxidation-reduction of several cytochromes, step by step, in isolated mitochondrial suspensions. Their evidence suggests that the flow of electrons from NAD^+ linked substrate to oxygen occurs along the chain shown in the following reaction sequence (51-4):

Substrate $\longrightarrow$ DPN $\longrightarrow$ Flavoprotein $\longrightarrow$ Cytochrome b $\longrightarrow$ Cytochrome c_1 $\longrightarrow$ Cytochrome c $\longrightarrow$ Cytochrome a $\longrightarrow$ Cytochrome a_3 $\longrightarrow$ Oxygen

They were also able to follow the same sequence (1) in suspensions of living yeast and ascites tumor cells. Chance (52) writes: "It is, of course, of considerable interest that the isolated material[1] does not involve a serious artifact." Chance and his colleagues (55) were able to show that in living cells, as in isolated mitochondria, respiration appears to be controlled by the level of ADP, the acceptor of "activated" phosphate produced by oxidation.

As Chance and co-workers indicate, their work confirms and extends the earlier concepts of Johnson (56), Lynen (57), Potter (58) and Lardy (59). These investigators indicated that either ADP or inorganic phosphate can control the respiration rate of a mitochondrial suspension.

Although the individual steps of the electron transport system are generally established, future studies may bring some modifications. Green and co-workers (49,48,60) consider that cytochrome b is not on the main pathway. From the work of the Madison and Liverpool groups it is probable that coenzyme Q is

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1. Mitochondria

an integral part of the transport chain, but its precise location is still being discussed by these workers (48,60,61,62). An area of considerable uncertainty is the pathway of NADPH oxidation: Does it occur in mitochondria? If so, is it coupled to phosphorylation as is the pathway of NADH oxidation?

ATP generated in the mitochondria probably plays an important role in maintaining the morphological integrity of the mitochondrion. It is utilized in a great many chemical reactions, some inside and others outside the mitochondrion. Among these, at least in liver and kidney, are reactions involved in secretion and absorption of water and certain electrolytes by mitochondria. Bartley and Davies (63) and Bartley (64) consider mitochondria to be the cell's "basic units of secretory and absorptive activities" (63) and Lehninger (65) ranks this function of mitochondria as second only to oxidative phosphorylation.

Other ATP-utilizing reactions that occur within the mitochondria are the synthesis of phosphatides (66,67), protein (68, 69,70), hippuric acid (71) and citrulline (72), the phosphorylation of nucleoside diphosphates (73), carboxylations (74), and the catabolism of protein (75a,b).

A review by Schneider (24) contains a critical discussion of the role of mitochondria in most of these reactions, as well as the somewhat contradictory evidence concerning the mitochondrial localization of enzymes participating in fatty acid

synthesis (40).

Mitochondrial synthesis of phosphatides in liver has been studied largely in the laboratories of Kennedy (66). It should be pointed out that mitochondria are not established as the sole sites of phosphatide synthesis; it is possible that the microsomes (fragments of endoplasmic reticulum), for example, are also capable of such synthesis. Many synthetic reactions involving mitochondrial-generated ATP occur in other subcellular fractions.

The mitochondria of rat liver account for about 30% of the total nitrogen and about 35% of the total protein of the tissue (76). Mitochondria possess considerable lipid, about 20-30% of their dry weight. In rat liver, about half (77) or more (78) of the lipids are phosphatides. These phosphatides have been studied with newer chromatographic techniques by Marinetti (79); 70% of the total consists of lecithin and phosphatidylethanolamine (80,81,82,83).

STRUCTURAL-FUNCTIONAL INTERRELATIONSHIPS

In recent years biochemists have stressed the integrated nature of the multienzymatic systems of mitochondria. In Lehninger's (84) words, mitochondria "possess a common biochemical denominator: an organized chain of carrier enzymes which transfer electrons from substrate to oxygen and the still mysterious auxiliary enzymes which couple phosphorylation of ADP to the

exergonic electron transport chain" (47,85). Attempts have been made to correlate altered biochemical function and changed mitochondrial morphology, both *in vivo* and *in vitro*. While on the one hand efforts were made to isolate mitochondria in the best-preserved condition, in other experiments a variety of means have been used to disrupt the mitochondria in order to unravel the individual steps of the biochemical sequences.

When liver mitochondria are isolated from hypertonic solutions, they are rod shaped, as they are in many intact tissues. On the other hand, those isolated in isotonic sucrose are small spheres, yet their capacity for oxidation of added substrates is increased. This led Harman (86) to suggest that the biochemical properties of the spherical mitochondria were actually products of "structural deformity".

Linnane and Ziegler (189) showed that "morphologically intact" sarcosomes did not catalyze the oxidation of isocitrate, citrate, malate, or lactate in 0.45 M sucrose, although the spherical sarcosomes did. However, after freezing and thawing, the formerly "intact" mitochondria would catalyze these oxidations. In addition, pyruvate, α-ketoglutarate, β-hydroxybutyrate, glutamate, and succinate were also oxidized at an increased rate after the mitochondria were damaged.

For cell physiology and cell pathology the greatest interest, in the area of structural-functional interrelations,

centers on the extent of coupling of phosphorylation to oxidation, and its relationship to mitochondrial form and permeability (24, 25). In the concept that is emerging, the catalysts coupling oxidation and phosphorylation also control swelling and contraction of the mitochondrion. These enzymes may thus be intimately involved in changes of mitochondrial form that occur in vivo (32,46,50) and perhaps also in active transport and water movement in cells (50). As Earnster (87) had done earlier, Lehninger (32) compares the contractile properties of mitochondria with those of muscle. He adopts the term "mechano enzyme" used for actomyosin, for enzymes presumed to couple oxidation with phosphorylation and also to change their physical state in the presence of ATP, thus leading to changes both in the shape of mitochondria and in the permeability of their membranes.

Lehninger stresses the specificity of ATP for mitochondrial contractions and ascribes the effects described for ADP (88) to ATP formed via adenyl kinase. On the other hand, Packer considers (88) that under conditions where oxidation and phosphorylation are "tightly coupled" only ADP causes reversal of substrate-linked swelling; ATP needs to be first converted to ADP. Other workers have demonstrated swelling with all substrates as AMP is phosphorylated to ADP plus ATP (89). The resolution of this question, like many others, should come more readily now. Proteins isolated from mitochondria are being studied. Some are

capable of catalyzing reversible "partial reactions" of oxidative phosphorylation, such as the interchange of P^{32} labeled ADP with unlabeled ATP in the terminal steps of ATP formation (90,91). Boyer (92,93,94) has isolated a protein containing a phosphorylated histidine residue which he believes to be one of the intermediates in oxidative phosphorylation. Other proteins have been shown to restore phosphorylative ability to submitochondrial fragments devoid of such ability (95). One, the "C factor," can function in the swelling-contraction cycle; it may be identical with the "M factor" linking the ADP-ATP exchange enzyme and the respiratory chain (50). Lehninger's "M factor" and Boyer's phosphorylated protein may be the same, since both catalyze P^{32}-ATP exchange. Polis and Shmukler (96) isolated a hemoprotein ("mitochrome") from rat liver mitochondria. When added to fresh liver mitochondria "mitochrome" inhibited oxidative phosphorylation (decreased the P/O ratio) and increased ATP dephosphorylation. However, Hulsman (97,98) working in Slater's laboratory and using isooctane extractions, has been able to show that the active component is not the protein but a mixture of fatty acids bound to it. Wojtczak and Wojtczak (99) have demonstrated the ability of serum albumin to enhance the amount of phosphorylation associated with oxidative reactions of insect mitochondria and rat liver mitochondria. Sacktor (100) has demonstrated that this is probably due to the binding of fatty acids by albumin. Free

fatty acids present in the microsomal fraction of liver have been shown by Pressman and Lardy (101) to uncouple oxidative phosphorylation by mitochondria.

OSMOTIC EFFECTS

The existence of an outer mitochondrial membrane has long been postulated to account for certain properties of isolated mitochondria: their apparent "osmotic" behavior (14,102,103); their high content of "soluble" enzymes like adenyl kinase (104); small molecules like citrate (105); and "soluble" proteins that appeared when mitochondria were disrupted (106); and the apparent increase in permeability to NADPH upon brief exposure to water (107). Whittam and Davies (108) considered that the turnover values for sodium and potassium ions in cells indicated the presence of semipermeable membranes in addition to the plasma membrane. This was similarly suggested by investigators (63,64, 109,110) who found the concentration of certain ions to be higher in the mitochondria than in the medium. Werkheiser and Bartley (111) demonstrated that at low temperature neither nucleotides nor polyglucose penetrate into the mitochondria whereas sucrose, potassium, sodium, and chloride do. Tedeschi and Harris (112) have shown that isolated rat liver mitochondria follow osmotic laws (Boyle-van't Hoff) fairly exactly. From their permeability studies of dissolved non-electrolytes they conclude that "there

is a true semipermeable barrier which is characterized by a high degree of permeability to lipid soluble substances. In accord with the interpretation of similar data for erythrocytes and other cells (113), these findings may be taken to mean that the barrier is at least partially "lipid-like." Recknagel and Malamend (114) agree that osmotic laws govern the swelling of mitochondria. They conclude that the presence of phosphate ions or carbon tetrachloride in the isotonic sucrose increases the mitochondrial permeability membrane and results in the movement of water into the mitochondria.

Tedeschi and Harris (112) consider the osmotic dead space of rat liver mitochondria, about 50% of its volume, to be solid and thus impermeable to sucrose. Correlation of such results with mitochondrial structure will require more precise and complete electron microscopic studies than are yet available.

Uptake of various solutes by mitochondria may involve factors other than membrane structure. Amoore and Bartley (115) have suggested that manganese uptake by isolated mitochondria involves chelation of the metal ion. Gamble (116) has found that, like intact mitochondria, submitochondrial fragments presumably devoid of outer (or intact?) membranes concentrate potassium ions from solution, thus suggesting "active transport". Green (190) and Lehninger (32,46,65) have also recently described the active uptake of phosphate, calcium and magnesium by rat liver mito-

chondria. Many workers (117,118,119) believe that the action of parathyroid hormone is on the active uptake of phosphate by mitochondria. Lehninger (46,65) and Green (190) stress the importance of the respiratory enzyme assemblies in this "active" uptake.

Many investigators have described the swelling of mitochondria exposed to hypotonic salt solutions (121). This process of water imbibition can soon lead to irreversible loss of the capacity for oxidative phosphorylation. ATP or active metabolism not only prevents this initial swelling phenomena from taking place but also can reverse the process once it has taken place (121). These experiments on the osmotic behavior of mitochondria have assumed great importance in that they constitute the principal line of evidence in favor of the view that there is an active membrane enveloping the mitochondria which regulates the inflow of solutes and water.

HETEROGENEITY OF MITOCHONDRIA

Differential centrifugation as developed in Claude's laboratory has been generally used to separate four fractions: nuclear, mitochondrial, and microsomal, and a soluble phase or supernatant fluid. However, variations of his centrifugation schedules have been used increasingly to shift the point of separation between mitochondria and microsomes (122) and also to subfractionate the

main fractions (123). This led to the concept that mitochondria were biochemically heterogeneous. DeDuve and collaborators, in a series of important papers emphasized an alternative interpretation: The granules of a given group are chemically homogeneous, and the apparent heterogeneity results from the distribution of a special class of granules with intermediate sedimentation properties between those of mitochondria and microsomes (122,124,125). DeDuve and co-workers proposed the name lysosomes for this new particle because it contained high concentrations of a number of hydrolytic enzymes.

The possibility remains, however, that the mitochondria of liver are not biochemically homogeneous. Their morphology in the centrolobular cells differs from that in the peripheral cells of the lobule. There is more suggestive evidence from staining results with tetrazolium salts and substrates considered to be oxidized by mitochondria: succinate, β-hydroxybutyrate, and glutamate: the peripheral cells stain more deeply with succinate, but the centrolobular cells are more affected by β-hydroxybutyrate and glutamate. However, staining is not strong in any cells with β-hydroxybutyrate or glutamate, and there are important limitations to the staining observations (discussed in Novikoff (126)). The most decisive data presently available are those of Beaufay (127). The distributions of four mitochondrial enzyme activities--glutamic dehydrogenase, malic dehydrogenase, alkaline

deoxyribonuclease, and cytochrome oxidase were studied in subfractions obtained by density gradient centrifugation. In all cases the distributions of activities were the same, leading the authors to conclude: "In view of the numerous factors, size, density, osmotic behavior, which intervene in determining the distributions observed, such results provide strong additional evidence for the hypothesis that mitochondria are essentially homogeneous in enzymic content." In discussing these results DeDuve (128) writes: "It would indeed be very astonishing if all the mitochondria from all the liver cells had exactly the same ratio of, let us say glutamic dehydrogenase to cytochrome oxidase. In fact, such a possibility seems almost incompatible, at least for some enzymes, with the histochemical demonstration of zonal differences within hepatic lobules, as with the existence of several types of cells within the liver. The homogeneity is therefore a statistical one and the crux of the matter is to appreciate at what stage our techniques become sensitive enough to show up the heterogeneity which undoubtedly exists."

MITOCHONDRIAL CHANGES IN DIFFERENT PHYSIOLOGICAL AND PATHOLOGICAL STATES

Cowdry (2,129) stressed the great sensitivity of the mitochondria to altered cell activity, normal and abnormal; he remarked that even holding tissues between forceps may cause mito-

chondria to break into granules. He recognized three modes of reaction--"qualitative, quantitative, and topographical, which may occur singly or in combination."

In the 1920's Noel (130) studied the mitochondria in the mouse liver lobule at various intervals after feeding, and after ingestion of diets rich in fat, carbohydrate, or protein. His associations of different mitochondrial forms with the position of a given cell in the hepatic lobule reflects the thoroughness of his investigations. Kater (131) described the mitochondria patterns in the hepatic lobules of eight species of mammals and two of birds; impressive differences were noted in both form and "sensitivity" in response to blood sugar level changes.

Deane (132) also studied the heterogeneity of mitochondrial form in the hepatic lobule of the mouse; she described large spherical mitochondria in the peripheral zone; thin filaments, fewer in number, in the central cells; and intermediate forms between the two areas. She found no regular change in the mitochondria in relation to the diurnal cycle as she did with the Golgi apparatus. She concluded that there are no indications that the mitochondria play a direct role in the secretion of bile acids or in the storage of glycogen or fat: "All related zonation phenomena are more probably dependent upon the quality of the blood bathing the cells."

J. Walter Wilson (133) commented upon the work of Noel and

Deane: "Since the portal blood, carrying products of digestion, arrives at the peripheral zone first, Noel concluded that this zone is a zone of permanent activity and the central zone a zone of permanent repose, the width of the active zone depending on the amount of food materials arriving in the liver at the time. In mice that have been subjected to partial inanition or to prolonged starvation, the spherical mitochondria of the peripheral zone tend to be replaced by rods, or fine filaments. The filaments may become beaded and other pleomorphic forms may appear. It would seem reasonable to conclude, therefore, that liver mitochondria may be quite different in different parts of the same liver, and in different livers under different physiological conditions, and this should be taken into consideration as more refined methods are developed for isolating mitochondria from liver homogenates and for studying their biochemical properties. Other observations on the variations in mitochondrial form are reviewed by Dempsey (134) and Rouiller (135).

SPECIFIC REACTIONS PERTINENT TO RESULTS OBTAINED IN THIS DISSERTATION

From material already published, it is generally accepted that at least the following reactions will be encountered with "tightly coupled" rat liver mitochondria.

1. Citrate oxidation: Citrate is not oxidized as such but

is converted to isocitrate by the enzyme aconitase, via cis-aconitate.

a) $C_6H_5O_7^{-3} \rightleftharpoons C_6H_3O_6^{-3} + H_2O$

CITRATE — CIS-ACONITATE

Cis-aconitate is then hydrated in the presence of the enzyme aconitase to isocitrate.

b) $C_6H_3O_6^{-3} + H_2O \rightleftharpoons C_6H_5O_7^{-3}$

CIS-ACONITATE — ISOCITRATE

Isocitrate is then oxidized in the presence of NADP (136) or NAD^+ to α-KETOGLUTARATE.

c) $aH^+ + C_6H_5O_7^{-3} + NADP \rightleftharpoons C_5H_4O_5^{-2} + CO_2 + NADPH$

ISOCITRATE — α-KETOGLUTARATE

NADPH is then oxidized, in some unknown manner, by the cytochrome chain with the production of three ATP molecules.

$NADPH + bH^+ + 1/2O_2 + 3\ ADP + 3P_i \rightleftharpoons NADP + 4\ H_2O + 3\ ATP$

The net reaction for the oxidation of citrate is then:

d) $1/2O + C_6H_5O_7^{-3} + 3\ ADP + 3P_i + nH^+$ *

CITRATE

$= C_5H_4O_5^{-2} + CO_2 + 4H_2O + 3\ ATP$

α-KETOGLUTARATE

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* As written, n = one plus a quantity depending on the pH of the reaction medium. That is, the oxidation of isocitrate causes an increase in the pH of the medium.

2. <u>α-Ketoglutarate oxidation</u>: α-Ketoglutarate is oxidized to succinate.

a) $C_5H_4O_5^{-2}$ + NAD^+ + CoA + GDP + P_i + nH^+ ⇌

α-KETOGLUTARATE

$C_4H_4O_4^{-2}$ + CO_2 + GTP + H_2O + CoA

SUCCINATE

The guanosine triphosphate (GTP) formed reacts with ADP in the presence of a kinase to yield ATP. The NADH is reoxidized via the cytochrome chain with the production of three ATP from three ADP as already shown for NADPH.

3. <u>Succinate oxidation</u>: Succinate is oxidized to fumarate.

a) $C_4H_4O_4^{-2}$ + FAD ⇌ $FADH_2$ + $C_4H_2O_4^{-2}$

SUCCINATE FUMARATE

The reduced $FADH_2$ is then reoxidized by the cytochrome chain with the production of two ATP from two ADP.

Fumarate produced from succinate oxidation is converted to malate by the enzyme fumarase.

4. <u>Malate oxidation</u>: Malate is oxidized to oxalacetate.

a) $C_4H_4O_5^{-2}$ + NAD^+ ⇌ $C_4H_2O_5^{-2}$ + NADH + nH^+

MALATE OXALACETATE

Oxalacetate condenses with acetyl CoA yielding citrate, completing the cycle of oxidative reactions.

5a. $C_4H_2O_5^{-2}$ + C_2H_3OSCoA + H_2O ⇌ $C_6H_5O_7^{-3}$ + CoASH + H^+

The oxidation of acetyl CoA can be written:

5b. Acetyl CoA + $2O_2$ + 12 ADP + $12P_i$ + nH^+ $\rightleftharpoons$

12 ATP + 14 H_2O + $2CO_2$ + CoASH

6. Among the sources of acetyl CoA in mitochondria are the lipid components which can yield significant quantities of fatty acids.

The conversion of a straight chain fatty acid to acetyl CoA involves a number of steps which are shown schematically below:

a) $R\text{-}CH_2CH_2CO_2^- + CoASH + ATP \rightleftharpoons R\text{-}CH_2C(=O)SCoA + AMP + PP$

b) $FAD + R\text{-}CH_2CH_2C(=O)SCoA \rightleftharpoons R\text{-}C{=}C\text{-}C(=O)SCoA + FADH_2$

$FADH_2$ is oxidized via the cytochrome chain and two molecules of ATP are synthesized (eq. 3a).

The unsaturated acetyl CoA is then hydrated in the presence of the enzyme enolase.

c) $R\text{-}C{=}C\text{-}C(=O)SCoA + H_2O \rightleftharpoons R\text{-}HC(OH)\text{-}CH_2\text{-}C(=O)SCoA$

The product reacts with NAD^+.

d) $RCHOHCH_2C(=O)SCoA + NAD^+ \rightleftharpoons$

$R\text{-}C(=O)CH_2C(=O)SCoA + NADH + H^+$

The reoxidation of NADH formed results in the conversion of three ADP to three ATP. Reaction with CoA produces Acetyl CoA and a saturated Acyl CoA with two fewer carbon atoms which can enter a sequence of reactions similar to 6b-6e inclusive.

e) $CoASH + RC(=O)CH_2C(=O)SCoA \rightleftharpoons R\text{-}C(=O)SCoA$ + Acetyl CoA

The process is repeated until the R residue is one or two carbons in length. The amount of ATP produced in the conversion

of one molecule of fatty acid to acetyl CoA depends upon the length of the acid. "Activation" of each mole of acid requires that one mole of ATP be converted to AMP and pyrophosphate. Each sequence of conversions produces five ATP from five ADP so that the ATP produced in the complete conversion to acetyl CoA of a fatty acid containing n carbons will be 5(n-2)-1 moles/mole.

Acetyl CoA can be oxidized via the Kreb's cycle. Summing up reactions (5b + 6a - d inc.) for stearic acid (n = 18):

f) FATTY ACID + ATP^{α} + 30_2 + $COASH^{\alpha}$ + 17 ADP + nH^+ + $17P_i$ <u>YIELDS</u> $ACYL_{(n-2)}CoA$ + 17 ATP + $20H_2O$ + $2CO_2$ + AMP + PP^{β}

For the complete oxidation of an eighteen carbon fatty acid the reaction then becomes:

g) $CH_3(CH_2)_{16}COO^-$ + ATP^{α} + $27O_2$ + $COASH^{\alpha}$ + 148 ADP + nH^+ + $148P_i$ <u>YIELDS</u> 148 ATP + AMP + PP^{β} + $18CO_2$ + $168H_2O$

7. Pyruvate may be converted to acetyl CoA and carbon dioxide:

a) $C_3H_3O_3^-$ + NAD^+ + CoASH $\rightleftharpoons$ C_2H_3OCOA + NADH + CO_2

Reoxidation of the NADH leads to conversion of three ADP to three ATP, or:

α Initial ATP and CoASH are required to catalyze the reaction.

β Pyrophosphate is broken down in the presence of mitochondria to inorganic phosphate.

b) $1/2O_2 + C_3H_3O_3^- + 3\ ADP + 3P_i + nH^+ + CoASH \rightleftharpoons$
$C_2H_3OCoA + 3\ ATP + 4H_2O$

If the Acetyl CoA formed is oxidized via the Kreb's cycle the total reaction is:

c) $C_3H_3O_3^{-1} + 2\ 1/2O_2 + nH^+ + 15\ ADP + 15P_i \rightleftharpoons$
$3CO_2 + 17H_2O + 15\ ATP$

STATEMENT OF PROBLEM

The object of all the research which has been cited was to provide information which would lead to a better understanding of the processes which occur in living cells. Mitochondria exist *in vivo* in a complex protoplasmic unit. Although we cannot, at present, be absolutely certain of the pH of this cytoplasmic matrix, it is probable that the pH does not alter over great ranges in the normal cell. Chambers and his co-workers (137), using micro-injection techniques, have consistently found that the cytoplasmic matrix has a pH of 6.8 and the nucleoplasm a pH of 7.6 in a wide variety of cells. These include determinations on the liver. Measurements by Behki and Melchior in this laboratory showed that the reaction medium became alkaline when succinate was oxidized by beef heart mitochondria. These experiments were done using the currently accepted reaction medium, initial pH 7.4.

Since pH 7.4 is *not* the cytoplasmic pH, and the reaction

mixture rapidly becomes even more alkaline, it is quite possible that much of the published material will not be directly applicable to understanding mitochondrial reactions in vivo. It was thought that an investigation of the effect of pH on mitochondrial oxidations would provide useful data.

Similar considerations lead to experiments at osmotic pressures which approach those which exist in vivo. Most of the previous work has been done in media which were less than 0.30 osmolar. The cellular fluid of liver cells is calculated to be above 0.38 osmolar. In order to evaluate the possible importance of this variable, experiments were conducted at both 0.3 osmolar and at 0.45 osmolar.

Other variables which may have important effects include the temperature of incubation (25), the amounts and identities of alkali metal ions (138,139,140) and of alkaline earth metal ions (141,142), the concentration and kind of phosphate acceptors, proteins, lipids, CO_2 tension, substrates, the isolation medium, the age and fasting state of the rat, and modifiers of oxidative phosphorylation--oligomycin, amytal, antimycin, 2,4-dinitrophenol, quanidinium derivatives, etc. (143).

For this exploration of the effects of pH and of osmotic pressure on mitochondrial reactions it has seemed wise to stay as close as possible to other physiological conditions, and to keep as many factors constant in the medium as possible. Several

substrates have been studied in otherwise identical media, at pH's 6, 7, and 8, at low osmotic pressure (0.30 osm) and at high osmotic pressure (0.45 osm). The level of phosphorylation of the adenine nucleotides added was controlled so that ATP exceeded ADP in some experiments and ADP exceeded ATP in other, otherwise identical experiments.

Each of the factors changed affected the experimental results with at least one substrate, and the accumulated results can be fitted into a reasonable system of mitochondrial reactions.

CHAPTER II

EXPERIMENTAL

Ideally, in enzyme studies, all conditions are held constant except the factor whose effect is to be measured, and all concepts are measured continuously during the experiment. Chance (144) has measured pH, oxygen, and the state of oxidation of NAD^+ + NADP, FAD, and the cytochromes continuously. However, these measurements are limited in time and in total oxygen consumption. The elaborate spectrophotometric instrumentation does not elucidate pathways of metabolism.

All measurements employed by the author, except those of oxygen consumption, were of the sampling type. Most workers in the field who use the Warburg technique make two sampling measurements; one at zero time (or after a five or ten minute thermal equilibration) and one at the completion of the incubation period. The experimental design used by the author, permitted more than two such measurements. Obviously, the ideal situation would be the continuous measurement of all the significant components (parameters). However, it is not technically practical at the present time.

Earlier studies of the fate of carbon labeled substrates required laborious separation procedures (145). The author has found that a considerable amount of information can be obtained

by a single chromatographic separation.

REAGENTS USED

WATER, redistilled in a glass apparatus was used for preparing all solutions used in mitochondrial incubations and in enzymic reactions.

The following reagents were used:

N-Acetyl-L-histidine: California Biochemical Corporation, A grade, lot 431221.

ATP.$5H_2O$ dibarium: Pabst, lot 129-1, Sigma, lot 112-1; Na_2ATP3H_2O, Sigma, lot 112-1.

Na_2AMP, Yeast: Type II, Sigma, lot 101B648; AMP.H_2: Sigma Type 4, lot 51B-709.

Albumin, bovine serum crystallized: Armour Pharmaceutical Company, lot W693124.

1-Amino-2-napthol-4-sulphonic acid: Eastman Organic Chemicals, lot 40.

Ammonium Formate: Bakers A.R., lot 3705.

Ammonium Molybdate-$4H_2O$: Mallinckrodt, lot 3420.

Amyl Alcohol: Bakers, Reagent, lot 8061.

Citric Acid: California Biochemical Corporation, lot 504110.

Citric Acid-1,5-C^{14}: California Biochemical Corporation, 9170, lot 503048, specific activity 1.7 μc/mmole.

Citric Acid, Na Salt: Mallinckrodt, lot 0754.

Copper Sulfate: Mallinckrodt A.R., lot 6300.

Fumaric acid-2,3-C^{14}: Nuclear Chicago, lot 3, 6.15 μc/mmole.

Fumarate, Na Salt: California Biochemical Corporation, A grade, lot 108447.

Filter paper: Whatman, No. 50.

Formic acid: Mallinckrodt A.R. 88%, lot 2592.

Filter paper: Whatman, No. 41 H, Chromatography paper, Schleicher and Schuell, No. 589, orange ribbon.

Hexokinase Crystalline: Preparation made by J. Biaglow and R. Behki, by the method of Colowick and Darrow (165).

Alpha-Ketoglutaric acid: California Biochemical Corporation, A grade, lot 108896.

Alpha-Ketoglutaric acid-1,5-C^{14} sodium salt: Nuclear Chicago, CFAX139, lot 109, specific activity 3.05 μc/mmole.

Histidylhistidine: Nutritional Biochem, lot 6098.

L-Histidine (free base): California Biochemical Corporation, lot 08122.

Ion Exchange Resin-Dowex-AG-1-X10, 200-400 mesh Cl^- form (Biorad 5343-41 B-1082), styrene type, small pore size.

Lanolin, anhydrous: Mallinckrodt, lot 5654.

Magnesium Chloride-$6H_2O$: Mallinckrodt, Reagent, lot 2796.

DL Malic Acid 3-C^{14}: California Biochemical Corporation, lot 880231, specific activity 6.8 μc/mmole.

L-Malic acid: California Biochemical Corporation, lot 4424.

Perchloric acid: Mallinckrodt, Reagent, lot 601.

Potassium Chloride: Mallinckrodt, Reagent, lot 6858.

Potassium hydroxide: Bakers, Reagent, 50% solution, lot 3143, or Mallinckrodt Reagent pellets, lot 6984.

Potassium sodium tartrate: Bakers, Reagent, lot 3262.

Potassium dihydrogen phosphate: Mallinckrodt A.R., lot 7100.

Pyruvic acid: California Biochemical Corporation, lot 10343.

Na Pyruvate: Mann, lot A6753.

Pyruvic acid-C^{14}: uniformly labeled, Nuclear Chicago, CFAX230, lot 2, 2.14 μc/mmole.

Sodium Formate: Bakers, A.R., lot 3700.

Sodium Hydroxide: Bakers, Reagent 50% solution, lot 3727, or Mallinckrodt, Reagent pellets, lot 7708.

Sodium Sulfate: Mallinckrodt, Reagent, lot 2301.

Succinic acid: California Biochemical Corporation, C grade, lot 104619, or Mallinckrodt, A grade, lot 2860.

Succinic acid-2,3-C^{14}: Nuclear Chicago CFAZ142, lot 1.

Tris-(Hydroxymethyl) Aminomethane: Sigma, lot 46181 (TRIS).

Mr. Edwin Geiger prepared potassium ATP solutions from barium ATP.

PREPARATION OF MITOCHONDRIA

Mitochondria were prepared from the livers of fed albino rats of the Sprague-Dawley strain (150-250 grams) by a modifica-

tion of the method of Schneider (146). Ice-cold sucrose solution (0.25 M) was used throughout the preparation and all procedures were performed at 0-4° C.

The rats were sacrificed by a blow on the head, decapitated and the livers excised and immediately placed in ice-cold sucrose. The livers were then washed three times with 50 ml portions of cold 0.25 M sucrose (all traces of blood were removed). The livers were then minced and placed in a pre-cooled glass homogenizer containing 30 ml of ice-cold sucrose. The liver tissue (6-12 grams) was then homogenized by a triple pass with the (cold) teflon pestle. The rotation speed was 700 R.P.M. The homogenate was then centrifuged in the International Refrigerated Centrifuge, using No. 820 head, at 1900 R.P.M. (800 X gravity), for 10 minutes. The supernatant was decanted and centrifuged in a number 291 head at 17,000 R.P.M. (14,000 X gravity) for 15 minutes. The mitochondrial pellet obtained was resuspended in 10 ml of ice-cold sucrose and recentrifuged at 17,000 R.P.M. in the same head for 15 minutes. The fluffy layer obtained at the end of this centrifugation was washed off with sucrose and the mitochondria were resuspended in 10 ml of sucrose and centrifuged at 17,000 R.P.M. for the final time before use. The drained pellet was suspended in 0.25 M sucrose. There was no attempt to obtain a quantitative yield of mitochondria; the emphasis was upon obtaining mitochondria with minimum contamination by other components,

and they were used in experiments without delay. Mitochondrial protein was measured by biuret (147) before use, and appropriate dilutions prepared.

DETERMINATION OF MITOCHONDRIAL PROTEIN (147)

Procedure:

1. Dissolve one and one half grams cupric sulfate ($CuSO_4 \cdot 5H_2O$) and six grams of sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$) in 500 ml of water.

2. Add, with constant swirling, 300 ml of 10% sodium hydroxide.

3. Dilute to 1,000 ml with redistilled water--store in a plastic bottle.

4. To 1.00 ml of a solution containing 1-10 mg of protein per ml, add 4.00 ml of biuret reagent, mix by placing on a vortex mixer and read in a Klett-Summerson Colorimeter using a No. 54 filter after a few minutes. The readings obtained in Table I were after 10 minutes contact with the biuret reagent.

PROCEDURES FOR TERMINATING MITOCHONDRIAL REACTIONS

When carbon-14 was not added, two ml of the reaction mixture was pipetted, at the time selected, into four ml of ice-cold 1.5 M perchloric acid (PCA) (in cold, 12 ml centrifuge tubes), mixed well, placed in the ice-bath (or refrigerator) until time

TABLE I

MEASUREMENT OF PROTEIN

CONCENTRATION OF RECRYSTALLIZED BOVINE SERUM ALBUMIN IN MILLIGRAMS/ML	READING ON KLETT
10, 10	146, 146
8.0, 8.0	125, 125
6.0, 6.0	93, 93
4.0, 4.0	61, 61
2.0, 2.0	33, 33
1.0, 1.0	16, 16

permitted centrifugation. The tubes were then centrifuged for 5 minutes on the International Clinical Centrifuge. The supernatant liquid in each tube was decanted and saved for analyses, the pellet was discarded.

The procedure was modified if the flask contained tracer. Two ml of ice-cold 20% PCA was pipetted into the Warburg flask. The flask was stoppered and placed in an ice-bath. After all experiments were at this stage, the filter paper and KOH in the center well were transferred to a centrifuge cone with the aid of redistilled water. The acidified reaction mixture was then decanted into another centrifuge tube and centrifuged as described above.

PROCEDURE FOR THE DETERMINATION OF INORGANIC PHOSPHATE (148)

Reagents:

A. 5.0 N Sulfuric acid

B. 2.5% (w/v) ammonium molybdate

C. Reducing reagent. A fresh solution was made daily by adding 10.0 ml of H_2O to 250 mg of a mixture of 1-amino-2-napthol-4-sulfonic acid, sodium bisulfite and sodium bisulfate (1-6-6 mole ratio) mixture, prepared in bulk and stored dry.

The color reagent was prepared by mixing 40.0 ml of A, 40.0 ml of B, and 4.00 ml of C in an Erlenmeyer flask, just prior to use. The colorimeter tubes to be used were prepared by adding

7.5 ml of distilled water to them.

A measured quantity, usually 100-200 microliters of a perchloric acid supernatant was pipetted into a graduated colorimeter tube containing 7.5 ml of water. The micropipette was washed out twice with the resulting solution. After all tubes were prepared, a timer was started and 2.00 ml of the color reagent was pipetted into the first colorimeter tube. Thirty seconds later 2.00 ml of the color reagent was pipetted into the second colorimeter tube, and so on until all colorimeter tubes contained color reagent. The solutions in the colorimeter tubes were then diluted to 10.0 ml and mixed. Exactly 15 minutes from the addition of the color reagent to the first tube that tube was read on the Klett-Summerson colorimeter with a number 60 filter. Thirty seconds later the second was read, etc.

MEASUREMENT AND CONTROL OF NUCLEOTIDE DISTRIBUTION

The relative amounts of AMP, ADP, and ATP change during oxidative phosphorylation. One may modify this change by adding a predetermined concentration of hexokinase to maintain the extramitochondrial nucleotides in a steady state. Another possibility is to add ATP, AMP plus ATP, or AMP and permit the mitochondrial system to reach a steady state between synthesis and breakdown. Both of these avenues of approach were explored. The following procedure was employed for all nucleotide measure-

ments.

MEASUREMENT OF NUCLEOTIDES (Modified from Biaglow (149))

As soon as possible after the experiment, a four ml. aliquot of the perchloric acid supernatant was added to a test tube. To this aliquot 0.2 ml of a 0.1% brom thymol blue indicator was added and the resulting solution titrated with 5 N KOH, to pH 7 (brom thymol blue-green), using a 1 ml pipette graduated in 0.01 ml. The use of the potassium hydroxide permits removal of perchlorate as the sparingly soluble potassium salt. Precipitation was increased by cooling the solution to zero (centigrade). After the precipitate had settled the solutions were decanted and a measured aliquot (2-3 micromoles of nucleotide) was placed on an anion exchange column (Dowex 1-X10, 200-400 mesh, formate).

The column was eluted first with 25 ml of water. This was followed by 35 ml of 0.1 N formic acid. The effluents from the two elutions were combined and diluted to 70 ml with water to form fraction 1. The column was then eluted with 50 ml of 1.5 N formic acid. The effluent was diluted to 50 ml with eluant to form fraction 2. This was followed by 50 ml of 3.5 N formic acid containing 0.005 M ammonium formate. The effluent was diluted to 50 ml with eluant to form fraction 3. The column was then eluted with 70 ml of 4.0 N formic acid containing 0.6 M ammonium formate. The effluent was diluted to 50 ml with eluant to form

fraction 4.

An expanded elution scheme was used for certain experiments. This consisted of collecting the effluents, from the four fractions mentioned above, in five ml fractions. In this manner the elution of the various nucleotides could be followed more precisely.

The absorbancy of the fractions was then determined on a Beckman DU spectrophotometer at 260 mμ against the appropriate eluant as reference. The concentration of nucleotides in the samples was determined by using the extinction coefficient (14.2×10^3 liters/mole cm) of the adenine moiety. Fraction 1 contained free purine and pyrimidine bases, nucleosides, and any products of hydrolysis of NAD^+. Fraction 2 contained mononucleotides, fraction 3 contained FAD, FMN, products of hydrolysis of NADP, dinucleotides, and any uridine or guanidine derivatives. Fraction 4 contained all trinucleotides.

PREPARATION OF COLUMNS

All columns were constructed from pyrex glass tubing of 1.0 cm internal diameter, capillary tubing was sealed to the bottom and bent up and out to a drip tip. The design of the columns was such that the glass tip was one to one and one-half cm above the level of the resin. This prevented the resin from draining during interrupted elutions.

Specially prepared Dowex 1 X 10 resin 200-400 mesh was used to prepare the ion exchange columns. The resin, obtained in the chloride form, was washed with 0.5 N NaOH, then with 1 M HCl. This sequence was repeated three times, then the resin was washed with acetone (50-50 v/v), water-acetone, then with 1 M HCl, and finally with warm redistilled water (70 degrees C.). The resin was placed in the glass tubes in the conventional manner. The top of the resin column was protected by the insertion of a wad of fiber glass. The resin was then changed into the formate form by washing with 3 M sodium formate until no chloride was found in the eluate, then with four volumes of 7 M formic acid, and finally with water until the pH of the effluent equaled that of the eluant. The columns could be used for fourteen to fifteen analyses.

DEVELOPMENT OF METHOD FOR MEASUREMENT OF CARBON-14 LABELED SUBSTRATES

Paper Chromatography:

In any chromatographic separation it is ideal to have a solvent system which separates the solutes and is highly volatile. The upper layer of a mixture of butanol, formic acid, and water (10:2:15 volume ratio) was the first solvent tried because of its reported good separations of the acids might be present in the experiments planned (150).

Exactly 100 lambda of a mixture of acids was spotted on S and S orange ribbon acid washed paper and dried with a commercial Minerval hair dryer (cold). The standard solution spotted was 0.0075 M in each: pyruvate, citrate, malate, succinate, and fumarate. Papers were spotted 2 1/8 inches from the end. The strips were curled slightly and draped over a glass rod with one end in a glass chromatography trough. Descending chromatography for 10-12 hours was employed. The solvent system contained bromphenol blue as an internal indicator to show movement of the acids. After chromatography the strips were removed and dried in the hood. Upon drying the acids show up as yellow spots against a blue background (150).

TABLE II

CHROMATOGRAPHY OF SELECTED METABOLITES

R_f* VALUES OBSERVED

CITRATE	MALATE	PYRUVATE	SUCCINATE	FUMARATE
0.48	0.55	0.68	0.78	0.88
0.50	0.54	0.68	0.78	0.88
(.51-.60)α	(.56-.65)α	(.62-.70)α	(.76-.85)α	(.84-.95)α

* R_f calculated from front of spot.

α Literature values, (150).

After this preliminary work, known levels of radioactive succinate were spotted to test detection with a Tracerlab Chromatographic Scanner. The levels spotted were 0.04, 0.008, 0.0016, 0.00033, and 0.000066 μcuries. With the counting system used all of the spots could be detected. The most useful level appeared to be 0.04-0.008 μcuries, while the lower limit of detection was 0.00033-0.000066 μcuries. For the above work the window was opened to nearly twice the designed maximum width and the most suitable speed 12 inches per hour.

To determine possible effects of the PCA deproteinization technique employed for termination of reactions, a solution was prepared which contained 0.007 M succinate, 0.007 M α-ketoglutarate, 0.007 M malate, 0.007 M citrate, 0.007 M fumarate, 0.007 M pyruvate, 0.0025 M AMP, 0.0025 M Na_2ATP, 0.005 M $MgCl_2$, 1 mg bovine serum albumin, 0.030 M glucose, 0.015 M K_2HPO_4, and 0.115 M KOH to adjust to pH 7. Aliquot portions were removed and a selected C^{14} acid added. The volume of each sample was adjusted to 2.75 ml. Two ml of ice-cold PCA was then added followed by 0.25 ml of mitochondrial protein (20 mgs). The mixture was centrifuged and 3 ml neutralized with KOH to pH 7-7.5. Each of the resultant mixtures contained a different labeled acid (approximately 1 μcurie) and 5 unlabeled acids. Ten to twenty lambda of these mixtures were then spotted, chromatographed, and counted. It was found that the chromatography of C^{14} acids was not affected

by this procedure. However, when the PCA-treated mixture was not neutralized before being placed on the paper, significant radioactivity was found at the origin after chromatography. A similar experiment in which all the C^{14} acids available were present gave the separation shown in Figure 2.

All strips were scanned with a Tracerlab Chromatogram Scanner (SC-545) equipped with a Tracerlab Precision rate meter (SC-34B) and a Recti-riter (Texas Instrument Co.). Individual peaks were identified either by R_f or by co-chromatography of individual C^{14} Kreb's cycle acids with aliquots of the experimental sample. The areas under the peaks were determined with a Keuffel and Esser planimeter.

REPRESENTATION OF OXYGEN CONSUMPTION RATES

The amounts of oxygen consumed in each flask are sometimes shown as a function of time as a pseudo rate, defined as μatoms oxygen absorbed divided by time after flask closure divided by the milligrams of mitochondrial protein present. This notation has an advantage over merely listing total oxygen absorbed at each time interval in that different preparations may be compared, and that systems in which oxygen absorption is linear may be recognized by equal rates at each time period, whereas the mere listing of total oxygen would have to be plotted to give this information.

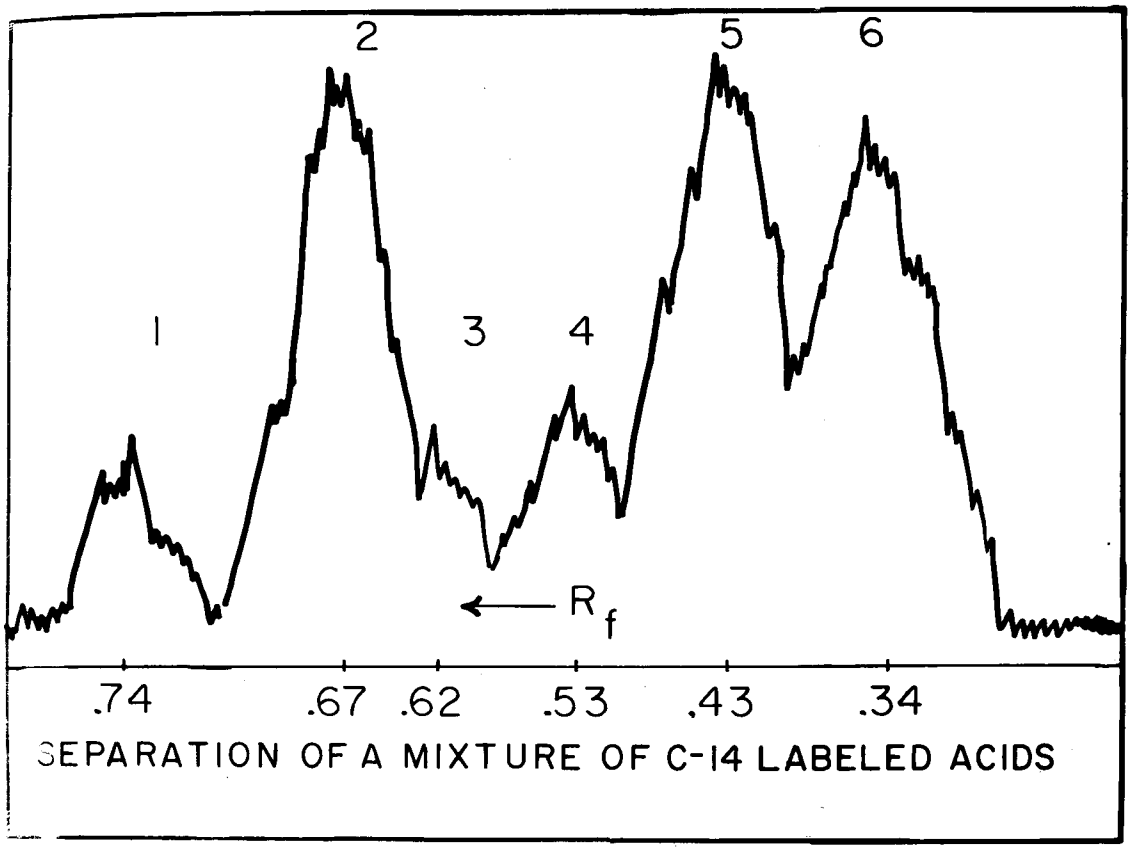

FIGURE 2

SEPARATION OF A MIXTURE OF C-14 LABELED ACIDS

The solvent system was butanol-formic acid-water. The organic phase was used for descending chromatography for 10-12 hours at 25°C. The peaks are at the predetermined R_f's of fumarate (1), succinate (2), pyruvate (3), α-ketoglutarate (4), malate (5), and citrate (6). Approximately 0.016 μcurie of each labeled acid was present in the original solution spotted on the paper.

The pseudo rate is preferred also to attempts to estimate the actual rate from the slope of a plot of oxygen consumption against time, because it preserves the actual measured quantities for later analysis if this should prove desirable.

MEASUREMENT OF LABELED CO_2 EVOLVED

After the termination of the incubation (page 35), the filter paper in the center well of the flasks was removed and placed in a graduated centrifuge tube. The remaining potassium hydroxide was removed with a pipette and placed in the tube. The center well was then washed three times with 0.5 ml aliquots of redistilled water, the wash being removed each time with a pipette and placed in the tube. The volume of solution was then adjusted to 5.0 ml with water. A two ml aliquot was then taken from the tube. To the aliquot, 5.0 ml of a 10% barium chloride solution was added and the precipitate allowed to form (one-half hour). The precipitate was collected on Whatman No. 50 paper, washed with 5.0 ml of alcohol-acetone (1:1 v/v), then 5.0 ml of acetone, dried under an infrared lamp, weighed and counted. An alternate procedure involved the adding of a small amount of solid $BaCl_2$ to the KOH containing test tube and collecting the resulting precipitate, washing with two 5.0 ml portions of water and carrying out the same procedure with acetone wash and drying.

All samples were corrected, after counting, for self-

absorption and instrument efficiency.

The counting was performed on a Tracerlab Tracermatic Scaler (SC-80M) equipped with a Tracerlab Multimatic sample changer (SC-100) and a Tracerlab Traceograph (SC-5E).

COLORIMETRIC DETERMINATION OF ACETOACETATE (151)

Procedure:

1. Add 3.00 ml of PCA supernatant (from the reaction medium) and 4.00 ml of 4 M aniline hydrochloride to 1 ml of 1 M acetate buffer, pH 4. Allow this mixture to stand for 90 minutes.

2. Add 5 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl and 10 ml of chloroform. Stopper and shake for one hour, then remove the top layer by water suction. Wash the remaining chloroform with two twenty ml portions of water and then with ten ml of 0.5 N NaOH removing each wash with the aid of water suction.

3. Read in the Klett-Summerson Colorimeter with a No. 42 filter against a standard prepared from 0.001 M acetone.

4. Color development is not proportional to acetoacetate present above 1.5 μmoles.

DETERMINATION OF ISOCITRIC ACID (152)

Procedure:

1. Dissolve 1.36 grams imidazole, and 100 mg NADP in 100 ml

of redistilled water containing 396 mg $MnCl_2$. Neutralize to pH 7.0 with 1M HCl and then add 8 mg (0.8 ml) of isocitric dehydrogenase (California Biochemical Corporation, lot 4126-503294, 3.3 enzyme units per mg of protein) and dilute to 200 ml. This solution is enough for forty determinations of isocitrate.

2. To 5.0 ml of the above solution add 200 microliters of the sample to be measured and 0.8 ml of water. Wait fifteen minutes, then measure the formation of NADPH at 340 mμ in a Beckman DU Spectrophotometer. NADPH ($\varepsilon = 6.22 \times 10^6$ liters/mole cm) produced under these conditions is equivalent to isocitrate in the sample.

DETERMINATION OF PYRUVATE AND PHOSPHOENOLPYRUVATE (153)

Procedure:

1. Dissolve 1.36 grams imidazole, 32 mg $NADH_2$, and 23 mg ADP ($NADH_2$, Sigma 1959, lot 340-102, ADP·$2H_2O$ Na, Sigma, lot 60-B-744) in 100 ml of redistilled water. Neutralize to pH 7.0 with 1 M HCl. Add 100 microliters (1 mg) of lactic dehydrogenase (Sigma type 1, lot L11B-99, 22,500 units/mg) and dilute to 200 ml in a volumetric flask. This solution is enough for forty determinations of pyruvate plus phosphoenolpyruvate.

2. To 5.0 ml of the above solution add 100-200 microliters of reaction mixture and the appropriate amount of water to 6.0 ml, mix and then wait 15 minutes. Measure decrease in NADH at

340 mμ in a Beckman DU Spectrophotometer. To measure phosphoenolpyruvate, add 10 microliters of pyruvate kinase (C.F. Boehringer and Soehne, G.M.B.H., Mannheim, Germany) and mix the contents of the cuvet.

3. NADH (ε = 6.22 X 10^6 liters/mole cm) oxidized under these conditions is equivalent to pyruvate in the sample.

DETERMINATION OF α-KETOGLUTARATE (154)

Procedure:

1. Dissolve 1.38 grams imidazole, 23 mg ADP, and 32 mg NADH in 100 ml of redistilled water, neutralize with 1 M HCl. Add 1 ml of Crystalline Glutamic dehydrogenase (in saturated ammonium sulfate (C.F. Boehringer and Soehne, Mannheim, Germany) and dilute to 200 ml in a 200 ml graduated volumetric flask. This solution is enough for forty determinations of α-ketoglutarate.

2. To 5.0 ml of the above solution add 100-200 microliters of neutralized reaction mixture and the appropriate amount of water to make 6.0 ml, mix, then wait fifteen minutes. Measure NADH disappearance at 340 mμ in a Beckman DU Spectrophotometer. The O.D. change is related to the concentration of α-ketoglutarate by the use of the molar extinction coefficient of NADH (ε = 6.22 X 10^6 liters/mole cm).

FURTHER CONSIDERATIONS ON THE USE OF A REACTION MIXTURE FOR MITOCHONDRIAL STUDIES

Because of the difficulty in pipetting separate solutions into the Warburg flasks, reaction media were made up so that only one pipetting was necessary.

The effects of alkali metal ions on respiration have been well documented (155-160). This led the author to run most of the experiments reported in the discussion section in a solution containing 0.007 M sodium ion and 0.049 M potassium. Some experiments with citrate contained a larger amount of potassium. Those run with pyruvate plus malate contained smaller amounts of potassium. Earlier pH studies on succinate oxidation were usually run in a medium containing no sodium ion, but sometimes containing positive Tris ion.

Isolated and aged mitochondria produce "mitochrome" (and "U factor") which uncouples oxidative phosphorylation (98,161-163). This degenerative action may be slowed down by adding crystalline serum albumin which removes the fatty acids by forming a lipoprotein complex (98). For this reason, most experiments reported in the results section were run in a medium containing one milligram of bovine serum albumin per milliliter.

REACTION MEDIUM A

Usually, 200 ml stock solution was made up which had the

following composition: 0.015 M AMP, 0.045 M K_2HPO_4, 0.015 M $MgCl_2$, and 0.045 M succinate. The above stock solution was employed in preparing reaction media for 10 Warburg flasks as follows:-

1. Weigh out the selected buffer to prepare 30.0 ml of 0.05 M solution.

2. Pipette a 10.0 ml aliquot of the above stock solution into this weighed-out buffer.

3. Add 5 ml of redistilled water.

4. Adjust to the desired pH with a pipette graduated in 0.01 ml, using 5 N KOH or HCl. This usually required 0.01 to 0.5 ml.

5. Calculate the osmolarity of this mixture and add sufficient sucrose so that the final solution (25 ml) will be either 0.3 or 0.45 os, as desired.

6. Rinse the above solution into a 25 ml volumetric flask, and make to volume with redistilled water.

7. During the experiment (after addition of 0.5 ml of mitochondrial suspension to 2.5 ml of reaction media), the concentrations of materials from the working solution in the reaction mixture were AMP, 0.005 M; P_i, 0.015 M; $MgCl_2$, 0.005M, Succinate, 0.015 M.

As a result of pH adjustments the concentrations of potassium and chloride were not the same in all experiments and are

shown in Table III.

TABLE III

PH EFFECT ON MEDIA "A" COMPOSITION

pH	6		7		8	
BUFFER	K^+	Cl^-	K^+	Cl^-	K^+	Cl^-
HISTIDYL-HISTIDINE	.030	.020	.055	.010	.097	.010
HISTIDINE	.030	.010	----	----	----	----
N-ACETYL HISTIDINE	----	----	.10	.010	.13	.010

MEASUREMENT OF PH

At the appropriate time one ml of the reaction mixture was pipetted into a parafin dish (with connected indentations for extension electrodes) and the pH measured with a Beckman (glass electrode) pH meter without delay.

REACTION MEDIUM B

Usually, a 200 ml stock solution was made up which was 0.0015 M AMP, 0.0135 M K_3ATP, 0.015 M $MgCl_2$, 0.045 M substrate (exception: pyruvate plus malate experiments were 0.021 M Na pyruvate and 0.006 M malate), 0.021 M NaCl, 0.045 M phosphate,

and three mg of crystalline bovine serum albumin per ml. A procedure like that on page 49 was carried out for 20 flasks. The only change is after the addition of water in step three. Hexokinase-glucose of glucose alone is added to aliquot portions. The same sequential procedure of neutralization and osmolar adjustment is then carried out. The final concentrations during the experiment are: Substrate, 0.015 M (except 0.007 M pyruvate plus 0.002 M malate); AMP, 0.0005 M; ATP, 0.0045 M; $MgCl_2$, 0.005 M, Phosphate, 0.015 M; Glucose, .03 M; Histidyl Histidine, .05 M.

A variation of the nucleotide content was used in some experiments (Medium C). This consisted of changing the nucleotide ratio in the stock solution to 0.0075 M AMP and 0.0075 M Na_2ATP. When Na_2ATP is used, NaCl is omitted.

Neutralization of the stock solutions containing 0.0015 M AMP and 0.0135 M ATP to pH 7 or pH 6 resulted in different potassium and chloride concentrations as shown in Table IV.

Neutralization of the stock solutions containing 0.0075 M AMP and 0.0075 M Na_2ATP to pH 7 resulted in different potassium concentrations as shown in Table IV-A.

EXPERIMENTAL BASIS FOR THE SELECTION OF A BUFFER SUBSTANCE FOR EXPERIMENTS AT CONTROLLED PH's

In earlier studies in this laboratory, Behki and Melchior (164) found that the pH of reaction mixtures containing mitochon-

TABLE IV

PH EFFECT ON MEDIA "B" COMPOSITION

pH	6		7	
	K^+	Cl^-	K^+	Cl^-
CITRATE	.030	.016	.080	.010
SUCCINATE	.030	.061	.049	.010
α-KETO-GLUTARATE	.030	.061	.049	.010
MALATE	.030	.061	.049	.010
PYRUVATE PLUS MALATE	.030	.018	----	----

TABLE IV-A

PH EFFECT ON MEDIA "C" COMPOSITION

pH	7	
	K^+	Cl^-
CITRATE	.085	.010
SUCCINATE	.055	.010
α-KETOGLUTARATE	.055	.010
MALATE	.055	.010
PYRUVATE PLUS MALATE	.038	.010

dria and succinate became alkaline during the period of measurement. In a search for an effective buffer for the pH range near 7, they discovered that imidazole was not satisfactory because it uncoupled phosphorylation.

In a continuation of this search for a suitable buffer, experiments were carried out with rat liver mitochondria at several pH's from 6 to 8 with several buffer substances. After consideration of the results collected in the pages which follow, histidylhistidine was selected as the most suitable buffer substance for the experiments planned.

Studies at pH 6 revealed the interesting observation that only small pH changes occurred even in the absence of added buffer. Tables V and VI show that the pH rose approximately 0.1 unit in twenty minutes at 0.45 os, and fell the same amount in the same time at 0.30 os. The differences in the direction of the pH change may be due either to the nature or to the extent of the reactions occurring in the two systems. As can be seen in the tables, net oxygen uptake at low osmolarity was nearly twice that at high osmolarity. The P/O ratio was higher (1.5 vs 1.3) at low osmolarity and the relative amounts of three adenine nucleotides present during the measurement period were significantly different. A significant amount of phosphate was incorporated into ADP/ATP during the measurement period in both cases, so that it appears that oxidative phosphorylation (succinate) is

nearly isohydric at pH 6.

The presence of L-histidine or histidylhistidine had only a small effect. At 0.45 os, oxygen uptake was reduced slightly, as was phosphate uptake. The amounts of adenine nucleotides found are as expected from a slightly lower phosphate uptake--less ADP/ATP in the "buffered" system. At low osmotic pressure, the differences are less marked, but total phosphate and AMP are lower and ATP is higher (after twenty minutes) in the "unbuffered" system. Measured P/O ratios are not significantly different in the presence of buffer.

At other pH's, however, the presence of buffers caused significant changes in the experimental results. In the absence of added buffer, the pH of the reaction mixture rose from 7.0 to 8.6 in twenty minutes (.3 os) and from 7.1 to 8.8 (.45 os), while the pH change was only 0.2 units in systems containing either histidylhistidine or N-acetylhistidine. An earlier experiment showed that most of the pH change in a system containing N-acetylhistidine occurred during the first five minutes (Figure 3). The pH change in a similar, unbuffered system was continuous (Figure 4).

At 0.3 os (Table VIII) oxygen uptake was much lower in the "unbuffered" system and the P/O ratio was only 0.6 (1.0 with histidylhistidine, and 1.1 with N-acetylhistidine). It is to be noted that inorganic phosphate and the relative amounts of each

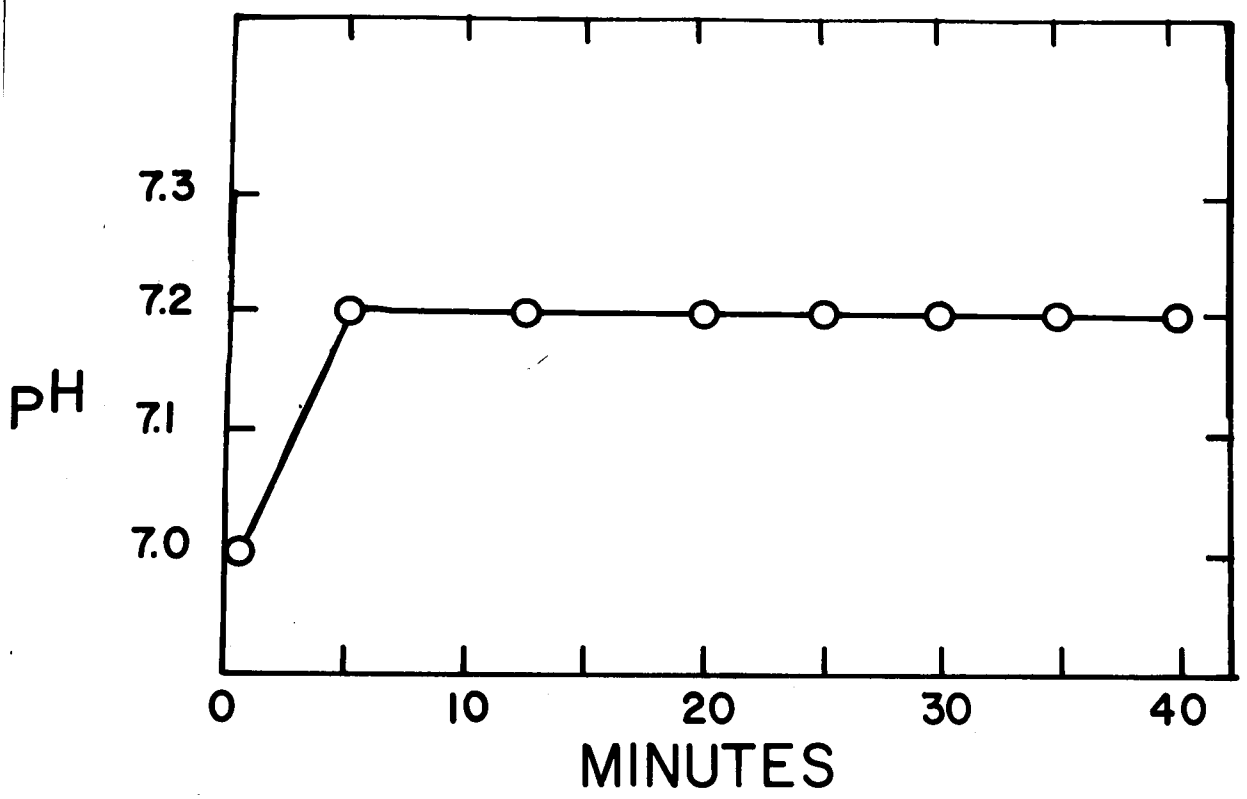

FIGURE 3

PH CHANGE DURING OXIDATIVE PHOSPHORYLATION IN HEAVILY BUFFERED MEDIUM

The above figure is a time study of the pH change during AMP phosphorylation and succinate oxidation in a medium containing 0.05 M N-acetylhistidine. The reaction medium is described on page 49 and Table III on page 50. Additional data is contained in Table LII in the appendix. Each point represents an individual Warburg flask.

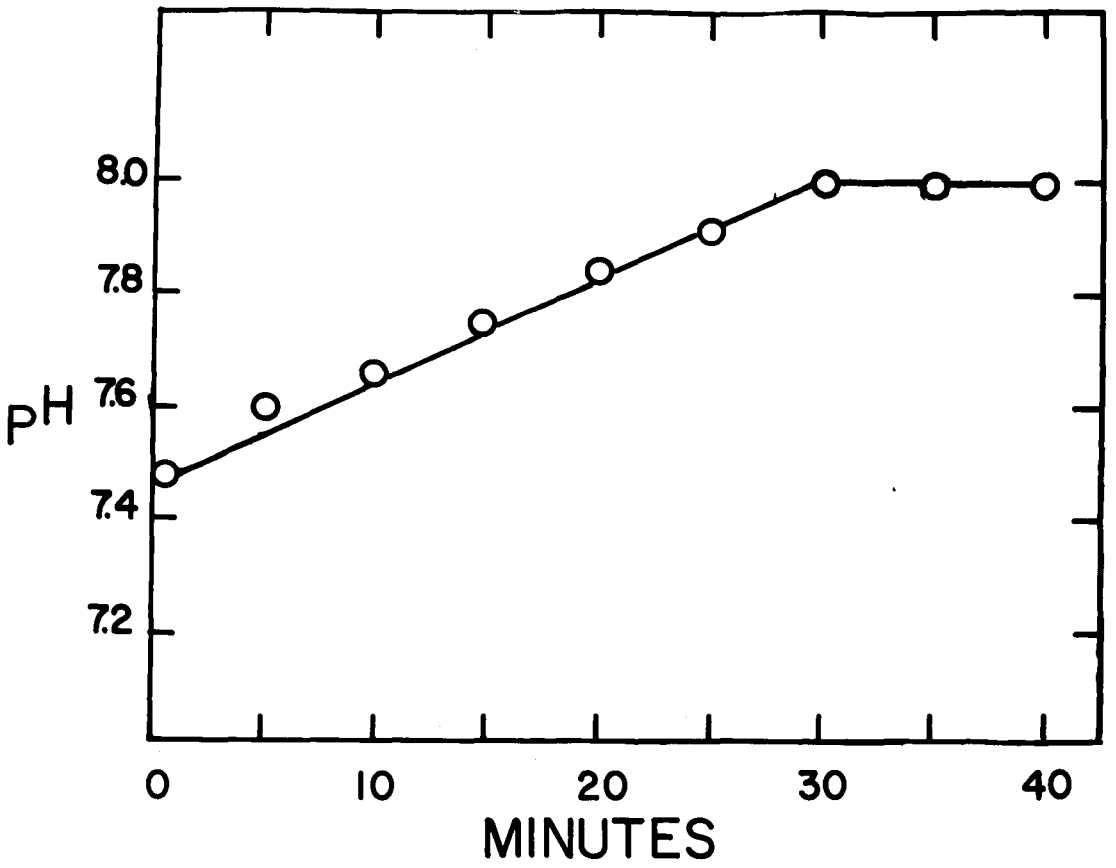

FIGURE 4

PH CHANGE DURING AMP PHOSPHORYLATION AND SUCCINATE OXIDATION

In addition to 0.013 M Tris, the reaction medium contained 0.005 M AMP, 0.005 M $MgCl_2$, 0.015 M K_2HPO_4, 0.29 M sucrose; the initial pH was 7.4. Additional data is contained in Appendix II, Table L. Each point represents an individual Warburg flask.

nucleotide present are very nearly the same in all three systems at thirty seconds and at five minutes, but are markedly different at twenty minutes. These differences appear during the period in which the pH difference between the systems is largest and are probably due to the pH change. Similar remarks apply to the results in Table VII (0.45 os) except that the differences between "unbuffered" and "buffered" systems is smaller--although still quite real. The rate of respiration dropped sharply between ten and fifteen minutes in the "unbuffered" systems; a smaller drop occurs in the "buffered" systems.

Results of measurements at "pH 8" are collected in Tables IX and X. Addition of mitochondria to "unbuffered" media, adjusted to 8.0, caused a drop in pH to 7.4-7.5. The "buffered" media sustain a smaller drop in pH; so that the initial conditions are not identical. However, it can be seen that both oxygen uptake and phosphate uptake are much lower in the "unbuffered" systems with P/O ratios significantly lower than with comparable buffered systems. As was observed with systems initially at pH 7, the observed differences in phosphate content and adenine nucleotides appear in the period between five minutes and twenty minutes--the period in which pH differences are maximal. The buffer systems used--histidylhistidine and Tris-hydroxymethylaminomethane--are very different in structure, which is additional evidence that the observed differences are due to

pH changes rather than to specific effects of the buffer substance used. The net P/O ratios are generally higher at 0.45 os. At low osmotic pressure, (Table IX-A), respiration rates are lower at all times in the "unbuffered" system. At high osmotic pressure (Table X-A), respiration rates are not markedly different at ten minutes, but are much lower at fifteen and at twenty minutes in the "unbuffered" reaction mixture than in either buffered mixture.

TABLE V

SUCCINATE OXIDATION, pH 6, LOW OSMOLARITY

	TIME AFTER MITOCHONDRIA (25 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP	ATP	TOTAL	pH
1*	30 SEC.		43.1	12.4	3.3	0.5	16.2	6.2
			43.1	13.6	2.1	0.3	16.0	6.1
	5 MIN.		38.0	9.9	4.0	1.8	15.7	
			37.4	9.9	4.0	1.6	15.5	
	20 MIN.	11.2	21.0	1.2	6.1	8.7	16.0	6.0
		11.5	20.6	1.2	5.4	10.0	16.6	6.0
2*	30 SEC.		43.4	12.5	3.1	0.3	15.9	6.0
			43.4	13.0	2.5	0.5	16.0	6.0
	5 MIN.		43.3	8.4	4.8	1.6	14.8	
			41.5	14.4	2.3	0.0	16.7	
	20 MIN.	10.8	24.7	2.9	5.8	7.0	15.7	6.0
		9.5	24.7	2.3	5.4	8.3	16.0	6.0
3*	30 SEC.		43.9	12.5	3.1	0.4	16.0	6.0
			44.0	12.7	1.6	0.4	14.7	6.0
	5 MIN.		39.1	10.9	4.7	1.2	16.8	
			39.2	11.2	4.3	1.2	16.7	
	20 MIN.	10.6	23.7	2.3	5.9	7.4	15.6	6.0
		10.6	23.7	2.0	5.8	7.4	15.2	6.0

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M L-Histidine

TABLE V-A

SUCCINATE OXIDATION, pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	RATE, μATOMS/MINUTE/MILLIGRAM[4] 1*	2*	3*
10 MIN.	.028, .019	.031, .020	.030, .030
15 MIN.	.036, .027	.025, .026	.031, .031
20 MIN.	.030, .031	.029, .025	.028, .028

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M L-Histidine

4 Pseudo rate, page 42

TABLE VI

SUCCINATE OXIDATION, pH 6, HIGH OSMOLARITY

	TIME AFTER MITOCHONDRIA (20 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL µMOLES	pH
1*	30 SEC.		44.9	15.5	0.5	0.4	16.4	6.0
			45.0	15.5	0.5	0.0	16.0	6.0
	5 MIN.		44.9	15.0	1.5	0.7	17.2	
			44.9	14.0	2.2	0.4	16.6	
	20 MIN.	6.9	36.4	8.7	4.6	2.5	15.8	6.1
		6.7	36.4	9.0	4.6	2.5	16.1	6.1
2*	30 SEC.		45.7	15.3	0.3	0.3	15.9	6.0
			46.0	15.5	0.2	0.3	16.0	6.0
	5 MIN.		44.9	14.8	0.9	0.3	16.0	
			45.2	14.8	1.0	0.3	16.1	
	20 MIN.	5.9	38.9	10.0	4.0	1.7	15.7	6.0
		6.0	38.8	10.4	4.2	2.1	16.7	6.0
3*	30 SEC.		45.3	15.5	0.5	0.3	16.3	5.9
			45.3	15.5	0.5	0.1	16.1	5.9
	5 MIN.		44.8	14.5	1.1	0.1	16.7	
			44.6	14.3	1.3	0.1	15.7	
	20 MIN.	5.9	37.6	10.3	4.0	1.7	16.0	5.9
		5.9	37.4	10.4	4.4	1.5	16.3	5.9

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M L-Histidine

TABLE VI-A

SUCCINATE OXIDATION, pH 6, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA	RATE, μATOMS/MINUTE/MILLIGRAM[4] 1*	2*	3*
10 MIN.	.013, .014	.014, .015	.014, .011
15 MIN.	.019, .020	.017, .018	.018, .011
20 MIN.	.022, .023	.020, .020	.020, .020

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M L-Histidine

4 Pseudo rate, page 42

TABLE VII

SUCCINATE OXIDATION, pH 7, HIGH OSMOLARITY

	TIME AFTER MITOCHONDRIA (23 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL µMOLES	pH
1*	30 SEC.		46.5	12.8	2.3	0.5	15.6	7.0
			46.7	13.0	2.1	0.3	15.4	7.0
	5 MIN.		39.4	12.5	3.8	2.1	18.4	
			39.4	6.8	4.7	2.0	13.5	
	20 MIN.	12.3	30.3	3.4	5.6	5.9	14.9	8.6
		10.9	30.4	3.9	5.6	5.4	14.9	8.7
2*	30 SEC.		47.0	12.2	3.0	0.4	15.6	7.0
			46.5	12.8	3.5	0.4	15.7	7.0
	5 MIN.		39.4	7.7	5.4	2.6	15.7	
			39.6	8.2	5.2	2.3	15.7	
	20 MIN.	13.7	21.1	1.6	4.7	9.2	15.5	7.2
		15.6	20.5	1.5	5.0	9.5	16.0	7.2
3*	30 SEC.		47.5	12.2	3.0	0.4	15.6	7.0
			47.5	12.4	1.5	0.4	14.3	7.0
	5 MIN.		39.6	7.8	5.4	2.2	15.4	
			39.6	7.8	5.1	2.5	15.4	
	20 MIN.	14.8	19.5	0.8	3.7	10.5	15.0	7.2
		14.5	19.5	1.2	5.4	9.1	15.7	7.2

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M N-Acetylhistidine

TABLE VII-A

SUCCINATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA	RATE, μATOMS/MINUTE/MILLIGRAM[4] 1*	2*	3*
10 MIN.	.055, .070	.048, .057 .042[α]	.052, .056 .042[α]
15 MIN.	.044, .041	.047, .054 .039[α]	.051, .049 .044[α]
20 MIN.	.036, .031	.040, .045 .032[α]	.043, .043 .041[α]

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M N-Acetylhistidine

4 Pseudo rate, page 42

α Triplicate value not included in Table VII.

TABLE VIII

SUCCINATE OXIDATION, pH 7, LOW OSMOLARITY

	TIME AFTER MITOCHONDRIA (26 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES	pH
1*	30 SEC.		45.2	10.5	4.9	0.9	16.3	7.1
			45.7	10.3	4.7	0.9	15.9	7.1
	5 MIN.		36.0	5.7	4.8	3.4	13.9	
			35.0	6.5	5.1	3.4	15.0	
	20 MIN.	7.7	30.9	4.4	6.1	2.9	13.4	8.8
		7.7	31.1	3.8	5.3	3.6	12.7	8.8
2*	30 SEC.		45.3	10.3	2.1	0.8	13.2	6.9
			45.4	12.2	2.9	0.8	15.9	6.9
	5 MIN.		33.8	6.0	6.6	4.6	17.2	
			33.6	6.0	7.0	3.9	16.9	
	20 MIN.	14.8	18.2	1.1	8.3	10.3	19.7	7.1
		15.1	18.3	1.0	7.5	10.0	18.5	7.1
3*	30 SEC.		45.1	11.2	2.8	0.8	14.8	7.0
			44.8	11.0	3.0	0.7	14.7	7.0
	5 MIN.		35.2	6.8	7.4	3.4	17.6	
			35.3	6.9	6.1	2.9	15.9	
	20 MIN.	15.6	18.4	1.0	5.0	9.7	15.7	7.2
		14.8	18.3	1.0	4.6	10.6	16.2	7.2

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M N-Acetylhistidine

TABLE VIII

SUCCINATE OXIDATION, pH 7, LOW OSMOLARITY

	TIME AFTER MITOCHONDRIA (26 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES	pH
1*	30 SEC.		45.2	10.5	4.9	0.9	16.3	7.1
			45.7	10.3	4.7	0.9	15.9	7.1
	5 MIN.		36.0	5.7	4.8	3.4	13.9	
			35.0	6.5	5.1	3.4	15.0	
	20 MIN.	7.7	30.9	4.4	6.1	2.9	13.4	8.8
		7.7	31.1	3.8	5.3	3.6	12.7	8.8
2*	30 SEC.		45.3	10.3	2.1	0.8	13.2	6.9
			45.4	12.2	2.9	0.8	15.9	6.9
	5 MIN.		33.8	6.0	6.6	4.6	17.2	
			33.6	6.0	7.0	3.9	16.9	
	20 MIN.	14.8	18.2	1.1	8.3	10.3	19.7	7.1
		15.1	18.3	1.0	7.5	10.0	18.5	7.1
3*	30 SEC.		45.1	11.2	2.8	0.8	14.8	7.0
			44.8	11.0	3.0	0.7	14.7	7.0
	5 MIN.		35.2	6.8	7.4	3.4	17.6	
			35.3	6.9	6.1	2.9	15.9	
	20 MIN.	15.6	18.4	1.0	5.0	9.7	15.7	7.2
		14.8	18.3	1.0	4.6	10.6	16.2	7.2

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M N-Acetylhistidine

TABLE VIII-A

SUCCINATE OXIDATION, pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	RATE, μATOMS/MINUTE/MILLIGRAM[4] 1*	2*	3*
10 MIN.	.028, .034 .032[α]	.048, .048 .044[α]	.034, .050 .044[α]
15 MIN.	.022, .024 .022[α]	.044, .046 .039[α]	.035, .047 .041[α]
20 MIN.	.020, .022 .020[α]	.039, .038 .033[α]	.031, .040 .038[α]

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M N-Acetylhistidine

4 Pseudo rate, page 42

α Triplicate not included in total oxygen consumption values in Table VIII.

TABLE IX

SUCCINATE OXIDATION, pH 8, LOW OSMOLARITY

	TIME AFTER MITOCHONDRIA (28 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL	pH
				µMOLES				
1*	30 SEC.		46.6	13.0	1.7	1.0	15.7	7.5
			47.0	13.0	2.0	0.4	15.4	7.5
	5 MIN.		40.2	10.5	1.8	2.4	14.7	
			40.8	10.6	2.2	2.2	15.0	
	20 MIN.	7.3	37.8	7.2	4.9	2.9	15.0	9.0
		4.7	38.7	7.0	4.9	2.9	14.8	9.0
2*	30 SEC.		45.7	11.0	3.7	0.4	15.1	7.9
			45.6	11.8	3.5	0.7	16.0	7.9
	5 MIN.		36.5	7.9	4.1	3.0	15.0	
			36.8	8.1	4.2	3.0	15.3	
	20 MIN.	15.3	18.6	0.7	2.9	11.5	15.1	8.2
		15.8	18.6	0.7	2.9	11.5	15.1	8.2
3*	30 SEC.		46.1	11.8	3.3	0.3	15.4	7.9
			45.9	11.3	3.8	0.4	15.5	7.9
	5 MIN.		37.0	8.6	3.3	3.0	14.9	
			37.5	8.2	4.2	0.4	12.8	
	20 MIN.	16.0	19.4	0.3	2.9	11.9	15.1	8.2
		15.8	19.8	0.4	2.9	12.0	15.3	8.2

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M Tris

TABLE IX-A

SUCCINATE OXIDATION, pH 8, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	RATE, μATOMS/MINUTE/MILLIGRAM[4] 1*	2*	3*
10 MIN.	.026, .015 .021[α]	.052, .054 .046[α]	.050, .052 .046[α]
15 MIN.	.021, .012 .016[α]	.042, .044 .036[α]	.044, .048 .046[α]
20 MIN.	.017, .011 .015[α]	.037, .038 .032[α]	.038, .042 .038[α]

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.050 M Tris

4 Pseudo rate, page 42

α Triplicate value not included in Table IX.

TABLE X

SUCCINATE OXIDATION, pH 8, HIGH OSMOLARITY

	TIME AFTER MITOCHONDRIA (23 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP	ATP	TOTAL	pH
1*	30 SEC.		45.9	10.0	3.4	0.8	14.2	7.4
			45.7	10.0	3.4	0.5	13.9	7.4
	5 MIN.		41.1	10.2	3.0	2.0	15.2	
			41.3	9.1	3.4	2.0	14.5	
	20 MIN.	6.6	33.6	5.7	5.8	4.0	15.5	8.8
		7.3	33.6	5.5	5.3	4.2	15.0	8.8
2*	30 SEC.		46.0	12.5	2.5	0.4	15.4	7.7
			45.8	12.5	3.0	0.4	15.9	7.7
	5 MIN.		40.3	9.1	3.4	2.0	14.5	
			40.4	9.7	3.4	2.0	15.1	
	20 MIN.	13.6	18.9	0.8	3.1	11.3	15.2	7.9
		13.1	18.9	0.9	2.9	11.1	14.9	7.9
3*	30 SEC.		45.4	11.8	3.5	0.5	15.8	7.8
			45.4					7.8
	5 MIN.		40.7	10.3	3.3	1.4	15.0	
			40.4	10.3	3.4	1.3	15.0	
	20 MIN.	13.7	21.1	1.5	4.2	8.8	14.5	8.0
		13.7	21.7	1.0	3.9	9.3	15.2	8.0

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M Tris

TABLE X-A

SUCCINATE OXIDATION, pH 8, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA	RATE, μATOMS/MINUTE/MILLIGRAM[4] 1*	2*	3*
10 MIN.	.040, .048 .046[α]	.032, .038 .037[α]	.046, .046 .038[α]
15 MIN.	.026, .029 .027[α]	.045, .040 .037[α]	.036, .045 .041[α]
20 MIN.	.019, .021 .027[α]	.039, .038 .034[α]	.040, .040 .040[α]

All media A, page 50

1* No added buffer

2* 0.05 M Histidylhistidine

3* 0.05 M Tris

4 Pseudo rate, page 42

α Triplicate values not included in Table X.

TABLES XI and XII show the effects of pH on respiration in systems buffered with histidylhistidine at different times during the measurement period. With a given mitochondrial preparation, the rate measured at pH 7.0 (initial) at each osmotic pressure was set equal to 1.00.

At 0.30 os there is very little difference in the rates observed--the results at pH 6 are somewhat erratic--but at 0.45 os it is clear that significantly lower respiration rates are found at pH 6. It also seems likely that higher respiration rates will be found at pH 7.5 to 8. These tables also demonstrate that, with a given mitochondrial preparation, the observed rate at pH 7 is higher at low osmotic pressure than at high osmotic pressure.

TABLE XI

EFFECT OF pH AND OSMOTIC PRESSURE ON RESPIRATION RATE OF MITOCHONDRIA CONTAINING ADDED SUCCINATE

Relative Rates (μAtoms/Minute/Milligram)[4]
Ten Minutes after Mitochondria were Added

pH of Media	.450 os		.300 os	
	1*	2*	2*	3*
6.0	0.5	0.7	0.8	1.3
6.5	0.7			1.0
7.0	1.0[α] (.049)	1.0[α] (.057)	1.0[α] (.069)	1.0[α] (.050)
7.5	1.2			1.0
8.0	1.1	1.1	1.0	1.1

1* 14.4 milligrams protein (See Table LXXI-B in the Appendix)

2* 20.2 milligrams protein (See Table LXXII-C in the Appendix)

3* 15.0 milligrams protein (See Table LXX-D in the Appendix)

4 Reaction Medium A, for histidylhistidine, page 50.

α Pseudo rate (page 42) ten minutes after the addition of mitochondria, at pH 7, was set equal to 1.0. The measured pseudo rate is in parentheses.

TABLE XII

EFFECT OF pH AND OSMOTIC PRESSURE ON RESPIRATION RATE OF MITOCHONDRIA CONTAINING ADDED SUCCINATE

Relative Rates (μAtoms/Minute/Milligram)
Twenty Minutes after Mitochondria were Added[4]

pH of Media	.450os		.300os	
	1*	2*	2*	3*
6.0	0.7	0.8	0.8	1.1
6.5	0.9			1.0
7.0	1.0[α] (.038)	1.0[α] (.042)	1.0[α] (.061)	1.0[α] (.045)
7.5	1.2			1.0
8.0	1.1	1.1	1.0	1.0

1* 14.4 mg protein (See Table LXXI-B in the Appendix)

2* 20.2 mg protein (See Table LXXII-C in the Appendix)

3* 15.0 mg protein (See Table LXX-D in the Appendix)

4 Reaction Medium A, for histidylhistidine, page 50.

α Pseudo rate (page 42) twenty minutes after the addition of mitochondria, at pH 7, was set equal to 1.0. The measured pseudo rate is in parentheses.

CHAPTER III

RESULTS

The existence of sequentially connected systems in mitochondria makes it difficult to determine how much of the measured oxygen consumption is due to the oxidation of the added substrate to its first oxidation product. The problem is further complicated by the known presence of endogenous substrates in the mitochondria (167,168,169), and by the possibility that ATP produced during the oxidation of added substrate may "activate" fatty acids and bring about oxygen consumption which would not have occurred in the absence of added substrate.

It was the purpose of the research reported here to determine, in so far as possible, the conditions which control the rates and the extent of the various reactions which occur in mitochondria. The problem has been approached by controlling as many variables as possible during the measurement period, and by changing certain of these variables to explore the effects of such changes. The following major variables were controlled in the reaction medium: osmolarity, pH, adenine nucleotide levels, substrate added, protein, and the ratio of sodium to potassium ion.

Experiments under these conditions gave different results with different substrates. Table XIII summarizes the rates of

oxygen consumption observed near the beginning of the measurement period. It can be seen that these rates are markedly influenced by the nature of the substrate, and by differences in osmotic pressure. Some of these rates remained substantially constant during the measurement period, others changed with time. Each of these situations must be discussed individually so that the useful information available may be recognized.

TABLE XIII

OXYGEN CONSUMPTION RATES WITH RAT LIVER MITOCHONDRIA AT pH 7

OSMOTIC PRESSURE	0.45 os		0.30 os	
HEXOKINASE	ZERO	20 μgm	ZERO	20 μgm
SUBSTRATE ADDED[1]	Pseudo Rate, μAtoms/Minute/Milligram Mitochondria Protein			
SUCCINATE .45 os, XV .30 os, XVIII	.054, .046 .050, .054	.060, .050 .054, .056	.062, .086 .036, .033	.067, .059 .056, .061
MALATE .45 os, XX .30 os, XXI	.009, .010	.013, .012	.022, .022 .022, .024	.018, .020 .020, .020
PYRUVATE PLUS MALATE .45 os, XXIII .30 os, XXIV	.002, .002 .006, .004	.008, .004 .007, .004	.024, .022 .020, .019	.022, .022 .027, .022
CITRATE .45 os, XXVIII .30 os, XXIX	.012, .013 .013, .012	.010, .008 .006, .008	.008, .011 .010	.034, .022 .020
α-KETOGLUTARATE .45 os, XXXIII .30 os, XXXIV	.011, .009	.008, .008	.012, .012 .012	.032, .034 .032

1 α-Ketoglutarate and malate at high osmotic pressure were run in reaction medium C, page 53. All other substrates were run in reaction medium B, page 52. The only difference between B and C is the initial concentrations of AMP and ATP before addition of hexokinase. The concentration of AMP is equal to the concentration of ATP in reaction medium C, whereas the concentration of ATP is much greater than that of AMP in reaction medium B.

SUCCINATE OXIDATION

At pH 7 and high osmolarity the effect of hexokinase on the rates of oxygen consumption in the various systems was small (Table XIV). Since the rates of oxygen consumption in the presence of succinate are greater than those observed with other added substrates, it is possible that the major oxygen consuming reaction is the same in each of the systems, namely the conversion of succinate to fumarate. This conclusion is supported by examination of the non-volatile carbon-14 containing substances after mitochondria had been added to reaction mixtures containing succinate-2,3-C^{14} (Table XIV-A). The major substances found were identified by co-chromatography as succinate, fumarate, and malate. The amount of succinate decreased with time, while the amounts of both fumarate and malate increase with time--all as expected. The amount of labeled carbon dioxide also increased with time, but the total amount was very small--only .001-.004% of the succinate-C^{14} added.

TABLE XIV

SUCCINATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2]			
	ZERO HEXOKINASE	5 μGRAMS HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.044, .048	.040	.044	.044, .040 .048
15 MIN.	.038	.042, .036	.044, .044	.044, .044 .046
17.5 MIN.	.047, .047	.045, .038	.045, .044	.045, .048
20 MIN.	.046, .049	.043, .040	.045, .046	.049, .053 .049

1 Reaction Media C, page 53.

2 Pseudo rate, page 42.

TABLE XIV-A

CARBON-14 DISTRIBUTION AFTER ADDITION OF

MITOCHONDRIA TO SUCCINATE-2,3-C-14

TIME AFTER MITOCHONDRIA	SUCCINATE[1] %[2]	FUMARATE %[2]	MALATE %[2]	CO_2 COUNTS/MIN.
No Hexokinase				
10 MIN.	74, 77	7, 5	19, 18	2,300
20 MIN.	62, 62	10, 7	28, 31	8,300
30 µgm Hexokinase				
10 MIN.	74, 70	5, 10	20, 20	1,800
20 MIN.	59, 55	13, 19	28, 26	9,600

1 45 µmoles succinate plus ten µcuries succinate-2,3-C-14

2 Percent of the total non-background radioactivity after chromatography.

TABLE XIV-B

SUCCINATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (19 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES* AMP	ADP	ATP	TOTAL
			µMOLES			
No Hexokinase						
10 MIN.		33.0	4.5	5.7	5.3	15.5
		37.6	4.7	6.2	5.4	16.3
		35.6				
20 MIN.	8.8	20.6	1.7	4.8	6.8	13.3
	9.4	22.2	2.4	6.6	6.6	15.6
5 µGrams Hexokinase						
10 MIN.		34.0	5.9	5.6	4.8	16.3
		32.2	5.0	5.7	4.9	15.6
20 MIN.	8.2	22.8	4.3	5.6	5.4	15.3
	7.6	23.4	3.7	5.1	5.8	14.6

con't.

TABLE XIV-B, continued

TIME AFTER MITOCHONDRIA (19 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES* AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
20 µGrams Hexokinase						
10 MIN.		36.4	10.4	5.1	1.3	16.8
		34.4	10.3	3.7	3.5	17.5
20 MIN.	8.6	17.9	12.0	3.1	1.0	16.1
	8.8	17.3	12.0	3.1	0.6	15.7
30 µGrams Hexokinase						
10 MIN.	9.4	22.4	13.0	2.5	0.3	15.8
	10.0	18.0	12.1	2.9	0.0	15.0
	9.3					

* The NAD fractions contained less than 0.1 µmole.

TABLE XV

SUCCINATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER[1] MITOCHONDRIA	RATE, µATOMS/MINUTE/MILLIGRAM[2] ZERO HEXOKINASE	20 µGRAMS HEXOKINASE
12.5 MIN.	.052*, .052*	.064*, .053*
15 MIN.	.054*, .046* .050 , .054	.060*, .050* .054 , .056
17.5 MIN.	.054*, .054*	.062*, .055*
20 MIN.	.050*, .053* .054 , .050	.054*, .055* .054 , .054
25 MIN.	.055 , .052	.050 , .048
30 MIN.	.053 , .052	.050 , .050
35 MIN.	.052 , .052	.048 , .048
40 MIN.	.053 , .048	.047 , .046

1 Reaction media B, page 52.

2 Pseudo Rate, page 42.

* 20-minute flasks started 30 minutes earlier than 40-minute flasks.

TABLE XV-A

SUCCINATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (15.5 mg protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES[2] AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
No Hexokinase						
10 MIN.		51.5	1.6	5.6	10.5	17.7
		50.4	1.6	5.6	10.9	18.1
20 MIN.	7.7	48.4	1.5	4.7	11.0	17.8
	8.2	47.5	1.2	5.8	10.4	17.2
40 MIN.	24.4	48.3	1.7	4.8	9.6	16.1
	22.5	51.0	5.9	6.1	5.9	17.9
20 µGrams Hexokinase						
10 MIN.		47.0	10.4	5.1	1.4	16.9
		46.2	10.9	5.2	1.5	17.6
20 MIN.	8.4	34.8	10.8	3.5	1.5	15.7
	8.3	35.0	11.7	3.4	1.5	16.6
40 MIN.	22.3	22.8	12.2	2.5	2.0	16.7
	21.5	23.2	11.2	2.0	1.4	14.6

1 See also Table XXXVIII

2 NAD fraction contained 0.1 µmoles or less.

A second, longer experiment was performed under the same conditions of pH and osmotic pressure, using ATP instead of a mixture of AMP and ATP to prepare the pre-mixture. As can be seen in Table XV, this experiment confirmed the finding that the rates of oxygen consumption were not significantly affected by the addition of hexokinase, and that these rates remained substantially constant from twelve through twenty minutes. In the system which contained hexokinase a small but real decrease in rate occurred after twenty five minutes. The distribution of carbon-14 material from the labeled succinate present agreed with that found previously (Table XV-B, appendix). It should be noted that oxygen consumption in ten minutes was virtually the same in all systems, in spite of the very large differences in the distribution of adenine nucleotides. This does not agree with the finding of Siekevitz (170) that high AMP inhibited oxidation. It is to be noted that the distribution of nucleotides at ten minutes in the presence of 20 μgrams hexokinase is virtually identical in the two experiments (Tables XIV-B and XV-A).

However, in the absence of hexokinase, marked differences in the two experiments are seen. At ten minutes inorganic phosphate and ATP were lower in the first experiment (Table XIV-B) while AMP was higher. ADP was approximately the same in both experiments. During the next ten minutes both systems absorbed very nearly the same amount of oxygen, but there was a large uptake of

phosphate in one, but only a small uptake in the other (Table XV-A). The distribution of nucleotides remained constant in the latter experiment, while ATP increased at the expense of AMP in the former.

There is a more subtle difference between these experiments. Inorganic phosphate was much higher at ten minutes in the second experiment in the systems containing 20 μgm hexokinase, as well as in those containing no hexokinase. In each of these reaction mixtures, the addition of the mitochondria provided enzymes which catalyze the interconversion of adenyl nucleotides.

$$2\ ADP = ATP + AMP$$

In the absence of hexokinase, the addition of the catalyst to the mixture of AMP and ATP present in Experiment 1 would increase the amount of ADP present, without reducing either AMP or ATP to low levels. In the second experiment, however, the large amount of ATP present would tend to reduce AMP levels almost to the vanishing point. Thus the two systems compared were different from the very beginning--and this appears to be a significant difference so far as the net uptake of phosphate is concerned.

None of these reactions provide any explanation for the observed fact that inorganic phosphate is much higher in the second experiment than in the first. Mitochondria prepared by the method used (Materials and Methods section, page 31) display little or no ATPase activity, so that some other explanation for

this difference must be sought. It may be a synthetic reaction which requires ATP and yields inorganic phosphate via a pyrophosphatase.

In the presence of hexokinase (20 μgram) the situation is a little more complex. Hexokinase and glucose were placed in the premixture, prepared well in advance of the addition of mitochondria. The amount added is sufficient to convert all the ATP present to ADP (and glucose-6-phosphate) prior to the addition of mitochondria. Thus, in Table XIV-B, the mitochondria are added to an equal molar mixture of AMP and ADP, with hexokinase, or AMP and ATP without hexokinase, while in Table XV-A they are added to a solution containing mostly ADP with hexokinase or mostly ATP without hexokinase. This latter solution will also contain more glucose-6-phosphate than the former. The addition of mitochondria to the AMP plus ADP mixture will result in the production of some ATP from the ADP, but not so much as in the experiment in which ADP is the predominant nucleotide.

TABLE XVI

SUCCINATE OXIDATION, pH 7.0, HIGH OSMOLARITY

% ACIDS REMAINING ON CHROMATOGRAM TRACING AND LABELED CO_2

TIME AFTER MITOCHONDRIA	% SUCCINATE[3]	% FUMARATE[3]	% MALATE[3]	CO_2(C.P.M.)
NO HEXOKINASE				
(1) 20 MIN.	77, 80	6, 4	17, 16	4,700, 5,000
(1) 40 MIN.	43, 41	18, 15	39, 44	7,000
(2) 40 MIN.	56, 59	13, 12	31, 29	15,200, 9,600
20 µGRAMS HEXOKINASE				
(1) 20 MIN.	78, 80	6, 7	16, 13	3,800, 3,800
(1) 40 MIN.	41, 50	15, 14	46, 36	3,900, 2,900
(2) 40 MIN.	61, 84	13, 8	26, 8	8,900, 2,600

1 4 µcuries of 2-3 labeled succinate was tipped from the side arm at 10 minutes and the reaction stopped either at 20 or 40 minutes (See Table XXXIX).

2 4 µcuries of 2-3 labeled succinate was tipped from the side arm at 20 minutes and the reaction stopped at 40 minutes.

3 Determined from radio-chromatograph tracings.

A third experiment at pH 7 and high osmolarity showed the fate of tracer quantities of succinate-2,3-C-14 added at various times during the mitochondrial oxidation of the usual quantities of unlabeled succinate. As before the rates of oxygen consumption are not markedly affected (Table XXXIX, appendix) by the presence or absence of hexokinase. Oxygen uptake and phosphate uptake are comparable with those in Table XV (Table XXXIX-A, appendix). The examination of radioactive substances in the reaction mixtures again showed (Tables XVI and XXXVIII, appendix) that succinate, malate and fumarate are the major non-volatile substances formed, and that the change with time of these substances indicates rapid mixing of the labeled succinate with the total succinate pool.

Again, the amount of labeled carbon dioxide formed is too small to be significant.

This last observation does not mean that the formation of carbon dioxide is zero. As Ogsten (171) has suggested, and others showed experimentally (172), the conversion of citrate into isocitrate and thence to α-ketoglutarate is stereo-specific in that the $-CH_2CO_2^-$ portion which comes from acetyl-CoA is converted exclusively to the 4 and 5 carbons of α-ketoglutarate, the one carbon of α-ketoglutarate is derived from the 4 carbon of oxalacetate, and the 2 and 3 carbons arise from the two and three carbons of oxalacetate. Since the two and three carbons of oxal-

acetate are derived (via fumarate and malate) from the two and three carbon atoms of succinate, it follows that succinate labeled in the two and three positions could be oxidized successively to fumarate, to oxalacetate, condensed with (mitochondrial) acetyl-CoA to form citrate which would be transformed to α-ketoglutarate with the loss of unlabeled carbon dioxide. The α-ketoglutarate labeled in the 2,3-position could be oxidized to succinate with further loss of non-labeled carbon dioxide. Thus four moles of oxygen could be absorbed and two moles of carbon dioxide evolved per mole of 2,3-labeled succinate transformed enzymatically via citrate into 1,2-labeled succinate without any loss of label as carbon dioxide. This newly formed succinate-1, 2-C-14 could be further oxidized to oxalacetate (presumably 1,2, 3,4-C-14) which could condense with acetyl-CoA to form citrate which would evolve some labeled carbon dioxide at the next two oxidation steps.

A somewhat different experiment was performed at pH 7, high osmotic pressure to demonstrate the formation of carbon dioxide by mitochondria in the presence of added succinate. Identical reaction mixtures were prepared in two sets of flasks. In one set KOH was placed in the center well to absorb carbon dioxide produced. In the other set the KOH was replaced by water. In Table XVII it can be seen that the amount of phosphate present was the same initially in the two sets of flasks and that changes

in both phosphate and adenine nucleotides in one set were duplicated in the other to a remarkable degree. This is not the case for "Apparent oxygen uptake" which was calculated in both cases as if no carbon dioxide were present (Figure 5). This of course, is correct for the solid points; but it is incorrect for the open points in proportion to the amount of carbon dioxide actually evolved. If we accept the evidence in Table XVII for nearly identical oxidative reactions, we must conclude that evolution of CO_2 was measurable at ten minutes and increased continuously with time.

TABLE XVII

SUCCINATE OXIDATION, pH 7.0, HIGH OSMOLARITY

NO HEXOKINASE

TIME AFTER MITOCHONDRIA[1] (20 mgs protein)	APPARENT OXYGEN UPTAKE	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			µMOLES			
KOH IN CENTERWELL						
0 MIN.		45.5				
		45.5				
5 MIN.		42.1	13.7	2.7	1.0	17.4
		42.1	13.7	2.7	1.0	17.4
15 MIN.	4.1	37.9	11.0	4.0	2.1	17.1
	3.4	37.7	11.0	4.0	2.1	17.1
30 MIN.	8.3	32.8	7.9	4.8	4.3	17.0
	8.6	32.4	7.6	4.8	4.3	16.7
60 MIN.	25.2	22.4	2.6	4.2	9.7	16.5
	24.2	21.3	1.8	3.2	10.1	15.1
NO KOH IN CENTERWELL						
0 MIN.		45.5				
		45.5				
5 MIN.		42.1	13.5	1.8	0.7	16.0
		43.3	13.4	1.8	0.7	15.9
15 MIN.	3.4	37.7	11.1	3.9	2.2	17.2
	3.4	38.8	10.8	3.5	1.8	16.1
30 MIN.	6.3	32.3	7.3	4.9	4.4	16.6
	6.2	32.3	7.3	4.9	4.4	16.6
60 MIN.	13.2	21.5	2.7	4.0	9.6	16.3
	13.0	22.2	2.7	3.9	10.0	16.6

[1] Reaction media A, page 50, N-Acetylhistidine

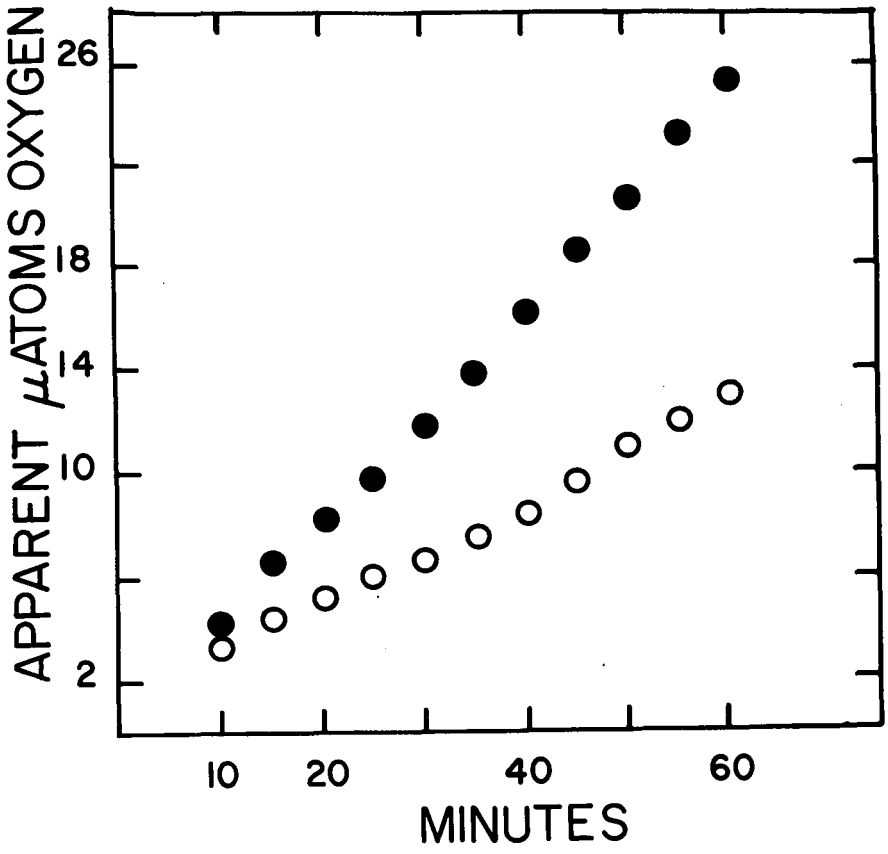

FIGURE 5

OXYGEN CONSUMPTION AND CARBON DIOXIDE PRODUCTION

BY RAT LIVER MITOCHONDRIA PLUS SUCCINATE

Typical gas changes in flasks containing aliquots of the same mitochondrial preparation and reaction medium (A, page 50, N-acetylhistidine). The filled circles show oxygen consumption in a flask with KOH in the center well. The open circles are for a flask without KOH. See also Table XVII.

OXIDATION OF SUCCINATE, pH 7, LOW OSMOTIC PRESSURE

When the osmotic pressure of the reaction medium was reduced (by omission of part of the sucrose from the premixture) below that thought to exist in the liver cell (173), certain changes could be noted in the experimental results. The rate of oxygen consumption (per milligram of mitochondrial protein) was slightly greater (fresh mitochondria) than at high osmotic pressure (Table XVIII). The addition of hexokinase did not affect this rate significantly. When mitochondria which had been stored for a longer period after preparation were used, the rate of oxygen consumption was low in the absence of hexokinase, and was increased by addition of hexokinase.

Table XVIII-A shows that, although the total oxygen uptake between ten and twenty minutes was very nearly the same as in the experiments at high osmotic pressure, there were significant differences in the nucleotide distribution. (Compare Table XIV-B, pages 81-82). In the absence of hexokinase, the distribution of nucleotides in Table XIV-B at ten minutes resembles that found in XV-A more than it does that in the experiment performed with the same initial nucleotide mixture. This may be due to a more nearly complete equilibration of the nucleotides throughout the system. Whatever the cause, the low phosphate uptake in the absence of hexokinase resembles that of Table XV-A--and this may be due to the high ATP and low AMP which is comparable in XV-A

and XVIII-A. In the presence of 20 μgm hexokinase, the total oxygen uptake at twenty minutes is about the same at both high and low osmotic pressure. The nucleotide distributions differ, but both are in the concentration range in which significant phosphate uptake is usually observed. (Biaglow, M.S. Thesis).

TABLE XVIII

SUCCINATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (12.9 mgs protein)	RATE, µATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 µGRAMS HEXOKINASE	30 µGRAMS HEXOKINASE
12.5 MIN.	.059 , .087	.068 , .062	.071 , .065
15 MIN.	.062 , .086	.067 , .059	.064 , .073
	.036[a], .033[a]	.056[a], .061[a]	.065[a], .068[a]
17.5 MIN.	.065 , .087	.067 , .061	.066 , .072
20 MIN.	.067 , .084	.067 , .059	.069 , .070
	.040[a], .038[a]	.057[a], .058[a]	.065[a], .066[a]
25 MIN.	.043[a], .040[a]	.055[a], .056[a]	.064[a], .063[a]
30 MIN.	.047[a], .044[a]	.055[a], .055[a]	.060[a], .059[a]

a Experiment started 20 minutes after the first group.

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

TABLE XVIII-A

SUCCINATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA (12.9 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE UPTAKE µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
				µMOLES		
NO HEXOKINASE						
10 MIN.		43.9	1.3	4.3	11.0	16.6
		44.7	1.1	4.1	11.0	16.2
20 MIN.	8.6	40.2	2.2	4.9	11.3	18.4
	10.9	39.4	1.6	6.0	10.0	17.6
30 MIN.	12.2	42.0	1.2	4.3	11.3	16.8
	11.3	42.3	1.2	4.0	11.5	16.7
20 µGRAMS HEXOKINASE						
10 MIN.		33.3	5.7	6.2	4.5	16.4
		31.9	5.7	5.9	4.3	15.9
20 MIN.	8.6	20.1	5.7	6.0	3.8	15.5
	7.6	20.2	5.4	6.0	3.4	14.8
30 MIN.	14.2	10.6	6.7	6.0	3.0	15.7
	14.1	10.5	9.2	5.1	1.5	15.8
30 µGRAMS HEXOKINASE						
10 MIN.		32.6	8.5	5.7	2.1	16.3
		31.2	7.1	5.1	2.0	14.2
20 MIN.	8.9	20.2	9.8	6.4	1.7	17.9
	9.1	20.1	6.2	5.8	3.2	15.2
30 MIN.	15.5	10.9				
	15.1	10.2				

SUCCINATE OXIDATION, pH 6, HIGH OSMOLARITY

Under the above conditions the rate of oxygen consumption in the presence of added succinate depended upon the nucleotides present to a startling degree. In the absence of hexokinase the rate was comparable with that observed at pH 7, but in the presence of 20 μgm hexokinase the rates of oxygen consumption were only one-sixth of this value or lower (Table XIX). In the absence of hexokinase the uptake of oxygen from ten to twenty minutes was a little larger (more mitochondrial protein) than in Table XIV-A and was accompanied by a significant phosphate uptake, which was, however, definitely smaller than that observed at pH 7 with substantially the same nucleotide distribution.

The addition of hexokinase at pH 6 had a marked effect on each of the quantities measured. At ten minutes, ATP had virtually disappeared, AMP was very large, and there appeared to be an increase in total nucleotides. AMP increased with time as did total nucleotides. ATP appeared to increase at the expense of ADP, and inorganic phosphate increased.

TABLE XIX

SUCCINATE OXIDATION, pH 6.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.064[α], .044[α]	.010[α]	.008[α], .006[α]
15 MIN.	.060 , .054	.008 , .007	.015 , .010
	.044[α], .045[α]	.009[α]	.005[α], .011[α]
17.5 MIN.	.044[α], .050[α]	.008[α], .009	.005[α], .011[α]
20 MIN.	.046 , .049	.008 , .009	.008 , .011
	.046[α]	.009[α]	.009[α], .007[α]
25 MIN.	.045 , .042	.004	.007 , .010
30 MIN.	.044 , .042	.009	.010 , .008

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

α Started 10 minutes earlier than 30 minute flasks.

TABLE XIX-A

SUCCINATE OXIDATION, pH 6.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (20.6 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE µMOLES	NUCLEOTIDES µMOLES AMP	ADP	ATP	TOTAL
NO HEXOKINASE						
10 MIN.		54.8	4.7	6.6	5.5	16.8
		52.6	4.7	6.6	5.5	16.8
20 MIN.	9.6	43.4	1.4	5.7	8.4	15.5
	10.2	44.2	1.4	4.8	9.2	15.4
30 MIN.	18.3	45.0	1.2	2.2	12.9	16.3
	17.4	44.2	0.6	2.2	12.9	15.7
20 µGRAMS HEXOKINASE						
10 MIN.		50.1	14.4	4.0	0.6	19.0
		50.4	14.4	4.1	0.4	18.9
20 MIN.	1.9	52.7	15.7	2.2	1.2	19.1
	1.8	53.6	18.6	2.8	2.3	23.7
30 MIN.	3.6	55.4	17.0	1.4	1.0	19.4
30 µGRAMS HEXOKINASE						
10 MIN.		50.6	13.8	3.3	0.5	17.6
		50.0	13.8	3.3	0.4	17.5
20 MIN.	1.6	52.7	----	---	---	----
	2.3	53.6	----	---	---	----
30 MIN.	3.9	54.9	18.8	1.6	1.8	22.2
	3.4	54.7	18.8	1.6	1.8	22.2

In summary, oxygen consumption by mitochondria in the presence of added succinate is only partly due to the conversion of succinate to fumarate. In experiments with succinate-2,3-C-14 malate and fumarate were the only non-volatile C^{14} substances found in the deproteinized reaction mixture, and only very small amounts of labeled carbon dioxide were formed. However, evolution of carbon dioxide was measurable ten minutes after addition of mitochondria, and increased with time in an experiment at 0.45 os, pH 7, buffered by N-acetylhistidine, in which AMP was the ultimate phosphate acceptor and ATP values were in the intermediate range during the measurement period.

At pH 6, addition of hexokinase to a system at high osmotic pressure results in a very marked reduction in the rate of oxygen uptake, and a release of inorganic phosphate. In the absence of hexokinase the uptake of oxygen is comparable with experiments at pH 7 (Table XIV-A), but there is a lower net phosphate uptake, and the much greater inorganic phosphate suggests a marked phosphate releasing reaction.

OXIDATION OF MALATE

At pH 7, high osmotic pressure, oxygen consumption by mitochondria in the presence of L-malate was about one fifth that of succinate under the same conditions (Table XX and XXXII, appendix). Table XX-A shows that phosphate content and nucleotide distribution were essentially identical in the two systems containing hexokinase. Phosphate uptake occurred in all systems with P/O values averaging 1.8. At low osmotic pressure, rates of oxygen consumption were much higher in all systems, but were highest in the absence of hexokinase. The rates were constant with time (Table XXI). Phosphate uptake accompanied oxygen uptake in the presence of hexokinase (P/O 1.2 av.) but no net uptake occurred in the absence of hexokinase.

Oxygen consumption at pH 6, high osmolarity, was very low in all systems (Table XXII). A considerable release of phosphate was observed which continued through the measurement period (Table XXII-A).

TABLE XX

MALATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2]		
	NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.008, .008	.014, .009	.008, .008
15 MIN.	.009, .010	.013, .012	.014, .008
17.5 MIN.	.011, .010	.015, .011	.010, .010
20 MIN.	.011, .011	.015, .013	.011, .011

1 Reaction medium C, page 53.

2 Pseudo Rate, page 42.

TABLE XX-A

MALATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (34.2 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
NO HEXOKINASE						
10 MIN.		41.4	4.3	6.8	5.9	17.0
		41.9	4.5	6.8	6.3	17.6
20 MIN.	3.6	35.1	2.2	4.6	10.2	17.0
	3.6	35.7	2.3	4.7	9.7	16.7
20 µGRAMS HEXOKINASE						
10 MIN.		40.0	14.0	3.1	1.1	18.2
		41.1	12.9	3.1	1.1	17.1
20 MIN.	5.1	29.7	12.4	3.3	0.9	16.6
	4.3	35.1	12.1	3.3	0.9	16.3
30 µGRAMS HEXOKINASE						
10 MIN.		41.2	12.4	3.6	0.6	16.6
		40.9	12.4	3.6	0.1	17.1
20 MIN.	3.9	33.7	12.5	3.2	0.8	16.5
	3.8	35.4	12.6	3.3	0.8	16.7

TABLE XXI

MALATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.021[α], .022[α]	.019[α], .020[α]	.019[α], .015[α]
15 MIN.	.022[α], .022[α] .022 , .024	.018[α], .020[α] .020 , .020	.015[α], .014[α] .020 , .020
17.5 MIN.	.023[α], .022[α]	.017[α], .019[α]	.015[α], .016[α]
20 MIN.	.021[α], .023[α] .024 , .024	.017[α], .019[α] .020 , .019	.015[α], .016[α] .019 , .018
25 MIN.	.023 , .023	.020 , .018	.018 , .018
30 MIN.	.024 , .023	.020 , .018	.019 , .018
35 MIN.	.024 , .024	.020 , .018	.019 , .018
40 MIN.	.024 , .023	.020 , .019	.019 , .019

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

α Experiment started 20 minutes after the first group.

TABLE XXI-A

MALATE OXIDATION, pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA (34.2 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
				µMOLES		
NO HEXOKINASE						
10 MIN.		44.6	3.0	5.1	9.1	17.2
		49.1	3.0	5.0	9.1	17.1
20 MIN.	7.3	45.9	3.0	5.0	9.1	17.1
	7.7	46.7	3.0	5.0	9.1	17.1
40 MIN.	24.2	44.0	2.5	4.5	9.7	16.7
	23.8	51.3	2.5	4.5	9.7	16.7
20 µGRAMS HEXOKINASE						
10 MIN.		44.8	9.6	4.0	2.0	15.6
		43.8	9.7	4.2	2.0	15.9
20 MIN.	5.8	37.6	9.8	4.0	1.5	15.3
	6.4	47.7	9.8	4.0	1.5	15.3
40 MIN.	20.6	22.1	9.9	4.0	1.4	15.3
	19.0	24.7	9.9	3.9	1.4	15.2
30 µGRAMS HEXOKINASE						
10 MIN.		44.8				
		46.0				
20 MIN.	5.2	37.6				
	5.4	37.7				
40 MIN.	19.3	21.3				
	19.6	22.0				

TABLE XXII

MALATE OXIDATION, pH 6.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (34.2 mgs protein)	RATE, µATOMS/MINUTE/MILLIGRAM[1] NO HEXOKINASE	20 µGRAMS HEXOKINASE
12.5 MIN.	.004[a], .003[a]	.002[a], .002[a]
15 MIN.	.004[a], .004[a] .003 , .003	.002[a], .002[a] .001
17.5 MIN.	.002[a], .002[a]	.002[a]
20 MIN.	.003[a], .001[a] .003 , .002	.002[a], .002[a] .002
25 MIN.	.003 , .002	.002 , .002
30 MIN.	.003 , .002	.002 , .002

1 Pseudo Rate, page 42.

a This set was started 16 minutes earlier than the 30 minute flasks.

TABLE XXII-A

MALATE OXIDATION, pH 6.0, HIGH OSMOLARITY

TIME AFTER [1] MITOCHONDRIA (26.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
NO HEXOKINASE						
10 MIN.		55.2	6.3	7.3	4.3	17.9
		55.2	6.3	7.3	5.2	18.8
20 MIN.	0.8	62.3	8.4	7.5	4.4	20.3
	0.3	60.9	8.2	6.6	4.3	19.1
30 MIN.	1.5	65.7	9.4	5.8	4.4	19.6
	1.2	66.2	9.8	5.8	2.7	18.3
20 µGRAMS HEXOKINASE						
10 MIN.		50.8	14.8	3.2	1.6	18.6
		53.4	17.7	3.5	1.7	22.9
20 MIN.	0.5	58.3	16.3	1.9	0.6	18.8
	0.4	58.3	14.3	1.5	0.6	16.4
30 MIN.	1.1	65.1	13.9	1.3	0.6	15.8
	1.0	64.6	14.8	5.9	1.0	21.7
30 µGRAMS HEXOKINASE						
10 MIN.		54.5	17.7	3.0	1.1	21.8
		51.9	10.7	1.5	4.9	17.1
20 MIN.	0.0	58.3	15.0	1.2	0.6	16.8
	0.0	58.3	9.4	5.8	4.4	19.6
30 MIN.	1.7	61.7	15.0	1.3	0.6	16.9
	0.8	63.0	14.6	1.2	0.6	16.4

1 Reaction medium B, page 52.

PYRUVATE PLUS MALATE OXIDATION

At pH 7, high osmotic pressure, the rate of oxygen consumption of mitochondria to which pyruvate and a small amount of malate have been added is quite low (Table XXIII). The rate increases slowly with time, but oxygen uptake is accompanied by phosphate release rather than uptake (Table XXIII-A). Measurements of pyruvate present show no significant decrease with time, and labeled carbon dioxide evolved from the pyruvate-1,2,3-C-14 present amounts to only 1% of that added (Table XXIII-B).

At low osmotic pressure the rates of oxygen consumption are much higher (Table XXIV) and are not affected by addition of hexokinase or by time. No phosphate uptake occurred in the absence of hexokinase, but significant phosphate uptake occurred in its presence (P/O 1.8; Table XXIV-A). The concentration of pyruvate showed no significant change with time, in spite of the evolution of labeled carbon dioxide equivalent to 4-5% of the label present (Table XXIV-B). Chromatographic separations also revealed no change in pyruvate concentration (Figure 6). They did reveal the formation of an unknown compound, but this material did not change in amount with time.

TABLE XXIII

PYRUVATE PLUS MALATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (21.5 mgs protein)	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.002[α], .004[α]	.000[α], .002[α]	.001[α], .000[α]
15 MIN.	.002[α], .002[α] .006 , .004	.008[α], .004[α] .007 , .004	.004[α], .000[α] .006 , .008
17.5 MIN.	.004[α], .001[α]	.002[α], .005[α]	.000[α], .000[α]
20 MIN.	.003[α], .004[α] .005 , .004	.005[α], .004[α] .003 , .005	.004[α], .000[α] .006 , .006
25 MIN.	.003 , .004	.005 , .006	.008 , .008
30 MIN.	.006 , .006	.005 , .007	.008 , .007
35 MIN.	.006 , .006	.007 , .007	.008 , .008
40 MIN.	.006 , .006	.008 , .008	.008 , .008

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

α These flasks were started 16 minutes later than the 40-minute flasks.

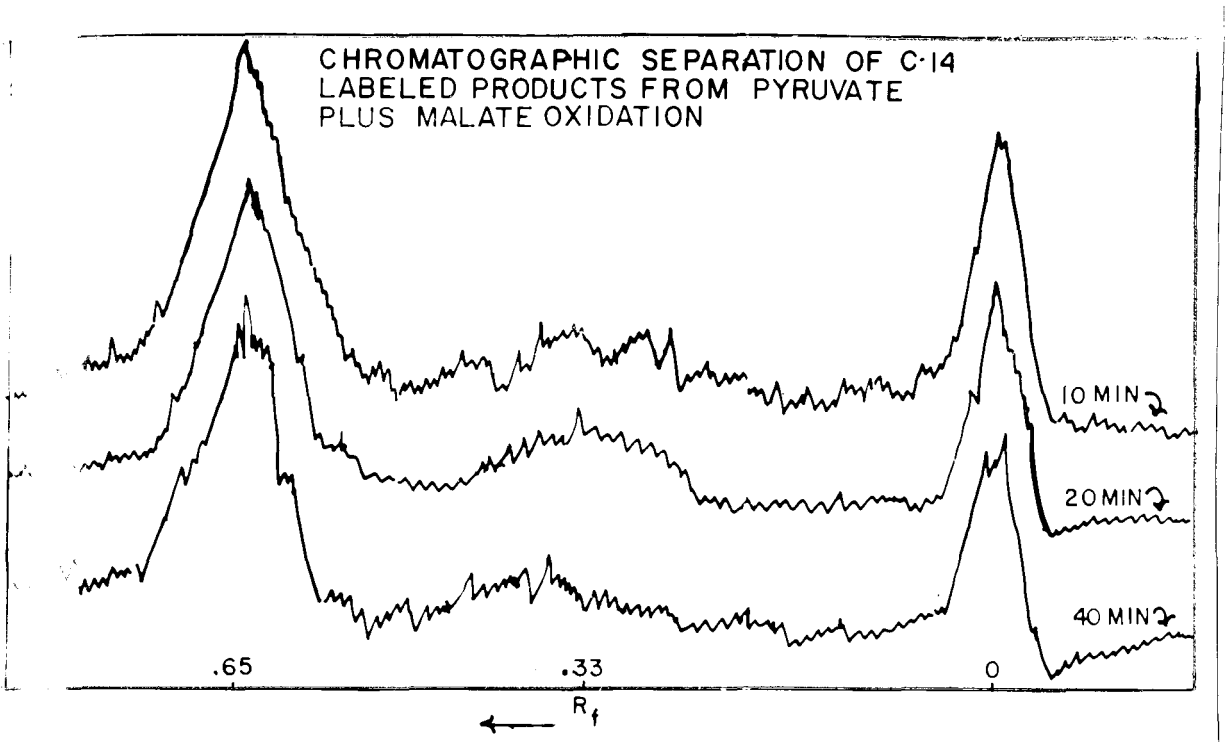

FIGURE 6

THE CHROMATOGRAPHIC SEPARATION OF C-14 LABELED PRODUCTS OF PYRUVATE PLUS MALATE OXIDATION

The reaction medium contained pyruvate plus malate as described for medium B on page 52. To this medium was added 2 μcuries of uniformly labeled pyruvate for each Warburg flask. At the appropriate time (10, 20 or 40 minutes), 2 ml of ice-cold PCA was pipetted into the Warburg flasks and the contents of the flasks analyzed as described in the material and methods chapter. The above figure is a reproduction of the recorder tracings of radiochromatograms. The peak with an R_f of 0.65 is pyruvate. The peak with an R_f of 0.0 has not been identified. Additional data is contained in Table XXIII (A-B).

TABLE XXIII-A

PYRUVATE PLUS MALATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (21.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES[1] µMOLES AMP	ADP	ATP	TOTAL
NO HEXOKINASE						
10 MIN.		51.0	1.8	3.5	13.2	18.5
		51.0	2.3	4.8	13.2	20.3
20 MIN.	1.1	54.6	1.8	4.4	15.2	21.4
	1.0	54.6	1.4	4.6	15.6	21.6
40 MIN.	3.9	56.0	2.8	3.7	12.6	19.1
	4.0	56.0	2.8	4.0	12.4	19.2
20 µGRAMS HEXOKINASE[2]						
10 MIN.		48.3	9.3	6.1	3.8	19.2
		49.0	8.6	6.5	5.4	19.5
20 MIN.	0.7	48.3	8.9	5.7	2.0	16.6
	1.3	51.9	8.9	6.3	1.3	16.5
40 MIN.	4.1	55.4	8.9	7.1	3.4	19.4
	4.0	55.7	8.9	6.9	2.8	18.6

1 Less than 0.1 µmole was found in the NAD fraction.

2 Comparable results were obtained with 30 µgrams hexokinase.

TABLE XXIII-B

PYRUVATE PLUS MALATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA	CO_2 C.P.M.	FRACTION β OF INITIAL C-14	PYRUVATE[1,2] μMOLES
NO HEXOKINASE			
10 MIN.	15,500	0.0035	22.2
	15,500	0.0035	22.2
20 MIN.	39,000	0.009	21.2
	48,000	0.011	22.5
40 MIN.	46,000	0.011	22.8
	29,000	0.007	28.5
HEXOKINASE			
10 MIN.	14,000	0.003	21.8
	18,000	0.004	21.3
20 MIN.	22,000	0.005	21.2
	28,000	0.006	21.4
40 MIN.	22,000	0.005	24.2
	28,000	0.006	23.2

1 Determined enzymically, initially 21 μmoles.

2 No detectable phosphoenol pyruvate, α-ketoglutarate or isocitrate was formed in the above system.

TABLE XXIV

PYRUVATE PLUS MALATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE
12.5 MIN.	.024[α], .022[α]	.022[α], .024[α]
15 MIN.	.024[α], .022[α] .020 , .019	.022[α], .022[α] .027 , .022
17.5 MIN.	.021[α], .022[α]	.022[α], .022[α]
20 MIN.	.020[α], .022[α] .020 , .019	.022[α], .021[α] .024 , .020
25 MIN.	.020 , .018	.022 , .019
30 MIN.	.021 , .020	.024 , .019
35 MIN.	.021 , .020	.023 , .019
40 MIN.	.020 , .021	.023 , .018

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

α Experiment started 20 minutes after the first group.

TABLE XXIV-A

PYRUVATE PLUS MALATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA (21.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES[1] µMOLES AMP	ADP	ATP	TOTAL
NO HEXOKINASE						
10 MIN.		46.7	3.8	6.6	9.2	19.6
		46.1	3.8	6.8	9.2	19.8
20 MIN.	4.4	47.0	5.9	10.5	6.5	22.9
	4.8	46.7	5.3	7.5	7.7	20.5
40 MIN.	12.7	46.4	4.4	6.4	8.5	19.3
	13.6	46.8	3.4	6.4	10.6	20.4
20 µGRAMS HEXOKINASE						
10 MIN.		----	12.7	5.8	1.2	19.7
		39.7	12.7	5.8	1.2	19.7
20 MIN.	4.8	31.6	15.6	1.8	0.5	17.9
	4.5	31.6	16.7	1.8	0.5	19.0
40 MIN.	14.9	18.1	13.8	3.0	0.1	16.9
	11.7	18.1	13.8	3.0	0.0	16.8
30 µGRAMS HEXOKINASE						
10 MIN.		38.6				
		39.5				
20 MIN.	4.6	31.6				
		31.6				
40 MIN.	14.4	23.4				
	15.6	23.4				

1 NAD fraction contained less than 0.1 µmole.

TABLE XXIV-B

PYRUVATE PLUS MALATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	CO_2 C.P.M.	FRACTION β OF INITIAL C-14	PYRUVATE[1,2] μMOLES
NO HEXOKINASE			
10 MIN.	78,000	.018	22.8
	42,000	.010	22.6
20 MIN.	210,000	.048	22.4
	210,000	.048	22.6
40 MIN.	170,000	.039	22.0
	160,000	.036	22.0
HEXOKINASE			
10 MIN.	65,000	.015	22.6
	81,000	.018	22.7
20 MIN.	230,000	.052	22.3
	58,000	.013	22.4
40 MIN.	95,000	.022	22.0
	130,000	.029	22.0

1 Determined enzymatically, initially 21 μmoles.

2 No phosphoenol pyruvate, α-ketoglutarate, or isocitrate were present as determined enzymatically.

TABLE XXV

PYRUVATE PLUS MALATE OXIDATION, pH 6.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
15 MIN.	.001 , .001 .000[α]	.000 , .000 .001[α]	.0003 , .0004 .0000[α]
20 MIN.	.003 , .003 .002[α]	.001 , .001 .001[α]	.002 , .001 .002[α]
25 MIN.	.003 , .001 .002[α]	.001 , .001 .004[α]	.003 , .002 .003[α]
30 MIN.	.003 , .002 .0002[α]	.0002, .001 .001[α]	.001 , .001 .0002[α]

No change in pyruvate concentration occurred as measured enzymically.

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

α Triplicate value not included in Table XXV-A.

TABLE XXV-A

PYRUVATE PLUS MALATE OXIDATION, pH 6.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (22.3 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NAD	NUCLEOTIDES AMP	ADP	ATP	TOTAL
				µMOLES			
NO HEXOKINASE							
10 MIN.		52.3	0.0	7.4	5.2	2.9	15.5
		52.4	0.0	6.5	5.2	2.9	14.6
		52.6					
30 MIN.	1.2	60.8	0.0	11.3	2.6	1.0	14.9
	1.1	58.9	0.0	10.8	2.9	0.6	14.3
	0.8	60.3					
20 µGRAMS HEXOKINASE							
10 MIN.		47.7	0.0	12.8	2.5	0.6	15.9
		48.9	0.0	12.9	2.6	0.6	16.1
		50.2					
30 MIN.	0.1	53.0	1.1	13.2	1.1	0.2	15.6
	0.4	50.8	1.4	12.8	1.3	0.8	16.3
	0.6	48.6					
30 µGRAMS HEXOKINASE							
10 MIN.		44.9	0.0	12.1	2.1	0.4	14.6
		46.0	0.0	12.5	2.1	0.4	15.0
		46.3					
30 MIN.	0.5	48.4	1.4	12.1	2.7	0.8	17.0
	0.5	48.4	1.4	12.5	2.0	0.9	16.8
	0.1	48.5					

At pH 6, the rates of oxygen consumption were very low at high osmolarity (Table XXV), and increased markedly at low osmotic pressure (Table XXVI). This increased rate seems to be dependent upon the concentration of ATP. Respiration is decreased with hexokinase and continues to decrease with time, with the higher hexokinase level (Table XXVI-A). Table XXVI-A shows that a marked and continuing release of phosphate occurred in each of the systems, and that significant amounts of NAD fraction, probably adenosine, were formed at 30 minutes in the hexokinase systems.

TABLE XXVI

PYRUVATE PLUS MALATE OXIDATION, pH 6.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
15 MIN.	.029, .020 .022	.010, .013 .014	.009, .013 .009
20 MIN.	.025, .020 .020	.009, .010 .011	.006, .008 .013
25 MIN.	.025, .019 .020	.007, .008 .009	.005, .007 .007
30 MIN.	.025, .018 .020	.007, .008 .009	.006, .006 .006

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

TABLE XXVI-A

PYRUVATE PLUS MALATE OXIDATION, pH 6.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (22.3 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NAD	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
NO HEXOKINASE							
10 MIN.		58.5	0.0	7.4	4.7	0.4	12.5
		60.0	0.0	8.4	5.1	0.6	14.0
		55.7					
30 MIN.	10.9	59.5	1.0	6.6	1.6	0.5	9.7
	8.2	64.2	1.3	6.6	5.1	1.2	13.1
	8.8	63.0					
20 µGRAMS HEXOKINASE							
10 MIN.		44.4	1.0	11.1	2.1	0.9	15.1
		43.3	1.3	11.1	2.6	0.8	15.8
		42.6					
30 MIN.	3.1	52.1	4.3	7.6	4.2	1.0	17.1
	3.5	53.9	4.2	7.6	1.1	3.9	16.8
	3.8	49.5					
30 µGRAMS HEXOKINASE							
10 MIN.		44.3	3.0	11.9	2.2	2.9	20.0
		42.6	0.9	10.0	1.4	0.8	13.1
		44.4					
30 MIN.	2.5	49.5	3.8	7.4	1.0	2.2	14.4
	2.6	50.6	3.4	7.4	1.0	0.8	12.6
	2.6	51.7					

1 Reaction medium B, page 52.

OXIDATION OF CITRATE

At pH 7, high osmotic pressure, the initial rate of oxygen consumption by suspensions of rat liver mitochondria was not markedly affected by addition of hexokinase (Table XXVII). This rate increases slowly with time--an increase noted more in the system without hexokinase. Phosphate uptake was observed in both systems. The P/O ratio was less than two (Table XXVIII-A and XXVII). Table XXVIII-B shows that a substantial amount of C^{14} labeled carbon dioxide was released, (five to ten percent of the initial citrate, Table XXVIII-B) and that this release increased with time. Enzymatic measurements of isocitrate revealed that only small amounts were present at ten minutes and slightly larger quantities later. Measurements of α-ketoglutarate showed none present at ten minutes and definitely larger amounts at later times--more when hexokinase was present.

At pH 7, low osmotic pressure (Table XXIX), the rate of oxygen consumption in the absence of hexokinase was essentially the same as at high osmotic pressure. In the presence of hexokinase, however, the rates at fifteen minutes were higher, and changed erratically with time. Each of the three flasks showed an "s" shaped curve of total oxygen absorbed against time, reaching actual calculated rates of 0.04 to 0.06 (μatoms oxygen/minute/milligram) and increasing to 0.01 during the last ten minutes.

The system without hexokinase demonstrated a constant rate for the entire experimental period (Table XXIX). The distribution of adenyl nucleotides was constant during the experimental period, ATP/ADP = 6-7 (Table XXIX-A). Phosphate uptake was observed (Table XXIX-A) in the presence of hexokinase, but only a slight uptake, between 30 sec and 10 minutes, was observed without hexokinase. The amounts of isocitrate present were very nearly constant with hexokinase, but without hexokinase (Table XXIX-B) there are 1.2 μmoles at 10 minutes which increases to about 2 μmoles at 30 minutes. Alpha-Ketoglutarate (Table XXIX-B) was very low both at 30 sec and at 10 minutes with or without hexokinase, but significant amounts are found after 30 minutes. Alpha-Ketoglutarate found at 30 minutes is three times greater with hexokinase than in its absence. Isocitrate is lower in the presence of hexokinase.

Paper chromatography (Table XXIX-C, appendix) showed no significant amounts of labeled material with the R_f of fumarate or malate at either high or low osmolarity. Reactions terminated at ten and at twenty minutes showed a single peak at the R_f of citrate. Experiments terminated at 40 minutes showed a small amount of material with a higher R_f than that of citrate. Co-chromatography showed migration with α-ketoglutarate.

At pH 6, oxygen consumption in the presence of citrate is quite low and erratic at high osmolarity (Table XXX). The small

uptake of oxygen at twenty minutes is accompanied by phosphate release in the absence of hexokinase, and three of the four flasks in the presence of hexokinase (Table XXX-A). At low osmolarity, moderate rates of oxygen consumption are observed, which increase somewhat with time. The net increase of inorganic phosphate with time is reduced in the presence of hexokinase, but a net uptake of phosphate was observed in only one of eleven flasks.

TABLE XXVII

CITRATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2] ZERO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.009, .007	.010, .009	.008, .010
15 MIN.	.016, .009	.010, .010	.007, .011
17.5 MIN.	.013, .009	.012, .010	.009, .011
20 MIN.	.013, .010	.013, .013	.011, .013

1 Reaction medium C, page 53.

2 Pseudo Rate, page 42.

TABLE XXVII-A

CITRATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (24.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			µMOLES			
ZERO HEXOKINASE						
10 MIN.		42.0	4.8	7.6	5.9	18.3
		41.4	4.8	5.2	7.4	17.4
20 MIN.	3.1	36.7	3.9	10.1	2.2	16.2
	2.5	37.6	2.3	8.4	5.5	16.2
20 µGRAMS HEXOKINASE						
10 MIN.		39.6	13.3	2.3	0.5	16.1
		39.3	12.6	2.4	0.3	15.3
20 MIN.	3.1	33.6	12.7	3.1	0.3	16.1
	3.2	33.7	12.2	2.7	0.5	15.4
30 µGRAMS HEXOKINASE						
10 MIN.		40.6	11.8	2.9	1.4	16.1
		40.3	11.3	2.2	0.5	16.0
20 MIN.	2.6	34.8	12.7	3.6	0.5	16.8
	3.2	34.1	12.6	3.6	0.5	16.7

1 Reaction medium C, page 53.

TABLE XXVIII

CITRATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2] ZERO HEXOKINASE	20 μGRAMS HEXOKINASE
12.5 MIN.	.010[α], .010[α]	.010[α], .009[α]
15 MIN.	.012[α], .013[α]	.010[α], .008[α]
	.013 , .012	.006 , .008
17.5 MIN.	.014[α], .014[α]	.010[α], .008[α]
20 MIN.	.014[α], .015[α]	.010[α], .008[α]
	.015 , .015	.008 , .009
25 MIN.	.017 , .017	.010 , .011
30 MIN.	.020 , .020	.011 , .012
35 MIN.	.022 , .022	.012 , .013
40 MIN.	.024 , .024	.013 , .014

1 Reaction medium C, page 53.

2 Pseudo Rate, page 42.

α Flasks were started 20 minutes later than the duplicate.

TABLE XXVIII-A

CITRATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (34.2 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES[2] AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
NO HEXOKINASE						
10 MIN.		57.0	4.5	6.2	6.2	16.9
		56.4	4.6	6.1	6.1	16.8
20 MIN.	4.8	49.4	1.4	4.9	8.5	14.8
	5.1	50.1	2.8	5.3	8.7	16.8
40 MIN.	25.1	45.1	1.2	3.8	11.4	16.4
	24.6	44.4	1.4	3.8	11.4	16.6
HEXOKINASE						
10 MIN.		53.8	12.2	2.3	1.3	16.8
		54.0	12.5	2.3	4.8	19.6
20 MIN.	3.4	53.8	13.2	1.7	0.7	15.6
	2.7	54.0	12.9	2.0	0.8	15.7
40 MIN.	13.4	44.8	11.8	2.4	1.0	15.2
	14.5	46.7	14.0	2.4	0.6	17.0

1 Reaction medium B, page 52.

2 NAD less than 0.1 µmole.

TABLE XXVIII-B

CITRATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA	CO_2[1] C.P.M.	FRACTION β OF INITIAL C-14	α-KETO-GLUTARATE[2] μMOLES	ISOCITRATE[2] μMOLES
NO HEXOKINASE				
10 MIN.	44,000	0.010	0.00	0.30
	30,000	0.007	0.00	0.05
20 MIN.	35,000	0.008	0.11	0.08
	114,000	0.026	0.11	0.01
40 MIN.	315,000	0.071	1.90	0.10
	290,000	0.065	1.80	0.11
HEXOKINASE				
10 MIN.	93,000	0.021	0.00	0.00
	142,000	0.032	0.00	0.00
20 MIN.	63,000	0.014	0.36	0.04
	78,000	0.020	0.49	0.12
40 MIN.	129,000	0.029	2.6	0.17
	135,000	0.030	2.5	0.17

1 3.48×10^6 CPM -- 1,5-C-14 citrate present.

2 Determined enzymatically.

TABLE XXIX

CITRATE OXIDATION, pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2] ZERO HEXOKINASE	20 μGRAMS HEXOKINASE
12.5 MIN.	.007, .010 .010	.032, .032 .024
15 MIN.	.008, .011 .010	.034, .022 .020
17.5 MIN.	.008, .012 .010	.034, .023 .029
20 MIN.	.008, .012 .011	.033, .028 .030
22.5 MIN.	.008, .012 .010	.033, .032 .027
25 MIN.	.009, .013	.028, .030
27.5 MIN.	.008, .011 .010	.026, .028 .023
30 MIN.	.008, .011 .011	.024, .026 .022

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

TABLE XXIX-A

CITRATE OXIDATION, pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (29 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES[2] AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
NO HEXOKINASE						
30 SEC.		50.1	1.5	2.5	12.0	17.0
		50.1	1.5	2.5	12.9	16.9
10 MIN.		48.0	1.1	2.0	13.5	16.6
		48.0	1.2	2.1	13.6	16.9
		48.0	1.4	2.0	13.5	16.9
30 MIN.	4.7	48.5	1.1	2.0	13.8	16.9
	6.4	48.5	1.1	2.3	13.9	17.3
	6.6	48.5	1.1	2.4	13.7	17.2
20 µGRAMS HEXOKINASE						
30 SEC.		50.3	4.2	14.0	0.5	18.7
		50.8	4.1	14.3	0.5	18.9
10 MIN.		27.4	9.1	5.0	1.6	15.7
		27.6	9.1	5.2	1.5	15.8
		28.1	9.1	5.1	1.6	15.8
30 MIN.	14.0	2.4	9.6	4.7	1.5	15.8
	15.2	1.5	9.6	4.7	1.4	15.7
	12.7	2.4	9.4	4.6	1.1	15.1

1 Reaction medium B, page 52.

2 NAD fraction contained less than 0.1 µmole.

TABLE XXIX-B

CITRATE OXIDATION, pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA (29 mgs protein)	ISOCITRATE* µMOLES	α-KETOGLUTARATE* µMOLES
NO HEXOKINASE		
30 SEC.	0.52	0.03
	0.52	0.03
10 MIN.	1.2	0.05
	1.2	0.03
	1.2	0.04
30 MIN.	2.2	0.73
	1.8	0.73
	1.8	0.73
20 µGRAMS HEXOKINASE		
30 SEC.	0.52	0.03
	0.58	0.03
10 MIN.	0.62	0.05
	0.54	0.05
	0.34	0.03
30 MIN.	0.69	2.4
	0.72	2.4
	0.68	2.5

* Determined enzymatically.

TABLE XXX

CITRATE OXIDATION, pH 6, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2]		
	NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
15 MIN.	.0007, .0000	.005, .000	.003, .003
20 MIN.	.004 , .000	.005, .002	.000, .004
25 MIN.	.001 , .002	.005, .003	.004, .003
30 MIN.	.003 , .004	.005, .002	.004, .003

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

TABLE XXX-A

CITRATE OXIDATION, pH 6, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (29.0 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NAD	NUCLEOTIDES AMP µMOLES	ADP	ATP	TOTAL
NO HEXOKINASE							
10 MIN.		39.3	0.6	6.3	5.4	6.0	18.3
		39.3	0.5	6.0	5.9	6.0	18.4
30 MIN.	2.0	41.7	0.2	6.3	4.1	1.0	11.6
	2.2	42.0	0.6	12.6	1.6	1.4	16.2
20 µGRAMS HEXOKINASE							
10 MIN.		39.6	0.6	10.7	5.0	1.4	16.7
		39.0	0.8	10.1	3.5	3.5	17.9
30 MIN.	2.9	37.5	0.6	11.8	1.4	1.8	15.6
	1.2	43.5	1.1	11.6	1.1	0.2	14.0
30 µGRAMS HEXOKINASE							
10 MIN.		39.9					
		39.9					
30 MIN.	2.1	42.6					
	2.0	42.6					

TABLE XXXI

CITRATE OXIDATION, pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, µATOMS/MINUTE/MILLIGRAM[2]		
	NO HEXOKINASE	20 µGRAMS HEXOKINASE	30 µGRAMS HEXOKINASE
15 MIN.	.010, .010	.006, .008	.008, .008
20 MIN.	.011, .012	.010, .012	.011, .010
25 MIN.	.012, .012	.010, .010	.011, .011
30 MIN.	.013, .013	.010, .011	.011, .011

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

TABLE XXXII

CITRATE OXIDATION, pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (37.0 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES[2] AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
NO HEXOKINASE						
10 MIN.		60.8	8.3	5.8	3.5	17.6
		60.4	8.0	5.3	3.6	16.9
20 MIN.	4.1	65.9	10.7	4.2	1.8	16.7
	4.5	66.3	10.0	3.5	1.4	14.9
30 MIN.	9.5	74.3	13.3	3.1	0.8	17.2
	9.3	67.9	13.3	2.6	0.9	16.8
20 µGRAMS HEXOKINASE						
10 MIN.		52.5	12.4	3.6	1.2	17.2
		51.8	10.9	3.3	1.9	16.1
20 MIN.	3.8	54.5	12.8	2.3	0.9	16.0
	4.6	54.4	11.4	2.2	1.1	14.7
30 MIN.	7.5	56.5	13.1	2.4	0.8	16.3
	7.9	55.3	13.4	1.8	0.9	16.1
30 µGRAMS HEXOKINASE						
10 MIN.		51.0	12.4	3.5	0.8	16.7
		51.1	11.9	3.1	1.0	16.0
20 MIN.	4.0	45.3	12.4	3.3	0.9	16.6
	3.8					
30 MIN.	8.1	53.4	13.1	3.1	0.8	17.0
	8.0	54.6	13.1	2.1	1.0	16.2

1 Reaction medium B, page 52.

2 NAD fraction contained less than 0.1 µmole.

OXIDATION OF α-KETOGLUTARATE

The rate of absorption of oxygen by mitochondria plus α-ketoglutarate is only about one fifth that observed with succinate. At high osmotic pressure (Table XXXIII) the rate is faster at first without hexokinase, but with hexokinase the rates increase with time dependent upon the amount of hexokinase present. At low osmotic pressure oxygen absorption was only slightly higher without hexokinase, but was three times faster with hexokinase. This rate was seen to decrease after 25 minutes (Table XXXIV), which was probably due to a lack of phosphate.

Phosphate uptake occurred with and without hexokinase at high osmolarity (Table XXXIII-A). At low osmolarity (Table XXXIV-A), there was only a slight uptake (between 30 sec. and 10 minutes) without hexokinase, while nearly all the phosphate disappeared from the medium with hexokinase.

ATP increased without hexokinase at pH 7, high osmolarity (Table XXXIII-A). This was to be expected from the use of reaction medium C. Nucleotide distribution was very nearly constant with hexokinase. Nucleotides were also constant with and without hexokinase at low osmolarity (Table XXXIV-B). AMP, with 20 μgrams hexokinase, is greater at high osmolarity than at low osmolarity.

Alpha-Ketoglutarate disappearance is low without hexokinase (Table XXXIV-B), but increases when hexokinase is present (Δ 3.5 μM, between 10-30 min.). The disappearance of α-keto-

glutarate, at 0.300 os is, however, too low to account for total oxygen consumption in these systems (Table XXXIV).

Isocitrate concentration (Table XXXIV-B) is relatively low and constant with hexokinase. This is not the case without hexokinase. There is appreciable isocitrate at ten minutes, somewhat less at 30 minutes.

At pH 6, high osmolarity, oxygen consumption rates are quite low and variable (Table XXXV). At low osmolarity, however, oxygen consumption rates were comparable with those observed at pH 7 (Table XXXVI). These rates were substantially constant with time. Phosphate uptake occurred in the systems containing hexokinase, but a small phosphate release was observed in the absence of hexokinase, probably due to the high ATP and low AMP (Table XXXVI-A). This table shows a net decrease in ATP during the measurement period, and an increase in ADP, but the change is not great.

TABLE XXXIII

α-KETOGLUTARATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (34.2 mgs protein)	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.010, .010	.006, .006	.009, .009
15 MIN.	.011, .009	.008, .008	.009, .009
17.5 MIN.	.011, .010	.009, .009	.012, .012
20 MIN.	.011, .010	.011, .012	.013, .012

1 Reaction medium C, page 53.

2 Pseudo Rate, page 42.

TABLE XXXIII-A

α-KETOGLUTARATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES[2] AMP μMOLES	ADP μMOLES	ATP μMOLES	TOTAL
NO HEXOKINASE						
10 MIN.		39.3	3.9	6.8	6.6	17.3
		39.4	4.3	6.7	6.3	17.3
20 MIN.	3.7	34.0	1.9	5.5	11.0	18.4
	3.5	34.3	2.2	5.5	10.3	18.0
20 μGRAMS HEXOKINASE						
10 MIN.		39.4	13.2	3.8	0.9	17.9
		39.4	14.1	3.8	1.1	18.0
20 MIN.	3.7	31.1	12.5	3.8	1.4	17.7
	4.1	31.6	13.0	3.8	1.1	17.9
30 μGRAMS HEXOKINASE						
10 MIN.		39.0	12.3	4.5	1.2	18.0
		39.3	12.7	4.2	1.2	18.1
20 MIN.	4.3	31.2	12.2	4.5	1.2	17.9
	4.2	32.5	12.8	4.5	1.4	18.7

1 Reaction medium C, page 53.

2 NAD fraction contained less than 0.1 μmole.

TABLE XXXIV

α-KETOGLUTARATE OXIDATION, pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE
12.5 MIN.	.009, .009 .009	.031, .031 .031
15 MIN.	.012, .012 .012	.032, .034 .032
17.5 MIN.	.012, .011 .012	.032, .033 .033
20 MIN.	.012, .011 .012	.032, .034 .034
22.5 MIN.	.012, .011 .011	.032, .033 .031
25 MIN.	.012, .011 .012	.031, .032 .029
27.5 MIN.	.012, .011 .012	.027, .029 .028
30 MIN.	.011, .011 .012	.026, .027 .026

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

TABLE XXXIV-A

α-KETOGLUTARATE OXIDATION, pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (29 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES[2] AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
NO HEXOKINASE						
30 SEC.		50.7	1.5	2.5	13.0	17.0
		51.2	1.5	2.5	13.0	17.0
10 MIN.		48.0	1.4	2.0	13.5	16.9
		46.9	1.2	2.2	13.6	17.0
		48.0	1.3	2.0	13.5	16.8
30 MIN.	6.6	48.0	1.1	2.0	13.8	16.9
	6.3	48.5	1.1	2.0	13.9	17.0
	6.7	48.5	1.1	2.1	13.7	16.9
20 µGRAMS HEXOKINASE						
30 SEC.		53.1	4.1	14.0	0.5	18.6
		53.4	4.2	14.1	0.5	18.8
10 MIN.		27.0	9.1	5.1	1.5	15.7
		27.6	9.2	5.2	1.6	16.0
		27.2	9.1	5.0	1.6	15.7
30 MIN.	15.0	1.1	9.5	4.8	1.4	15.7
	15.4	1.7	9.4	4.8	1.5	15.7
	14.9	1.7	9.5	4.9	1.4	15.8

1 Reaction medium B, page 52.

2 NAD fraction contained less than 0.1 µmole.

TABLE XXXIV-B

α-KETOGLUTARATE OXIDATION, pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	ISOCITRATE* μMOLES	α-KETOGLUTARATE* REMAINING μMOLES
NO HEXOKINASE		
30 SEC.	0.59	41.9
	0.59	41.9
10 MIN.	0.83	41.9
	1.96	41.9
	1.78	41.9
30 MIN.	0.59	39.4
	0.72	40.6
	0.78	39.4
20 μGRAMS HEXOKINASE		
30 SEC.	0.49	41.9
	0.59	41.9
10 MIN.	0.52	39.4
	0.52	38.7
	0.36	39.4
30 MIN.	0.64	35.6
	0.31	35.6
	0.39	35.6

* Determined enzymatically.

TABLE XXXV

α-KETOGLUTARATE OXIDATION, pH 6, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (29.0 mgs protein)	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
15 MIN.	.0007, .0003	.003, .003	.001 , .0004
20 MIN.	.004 , .003	.003, .0004	.0007, .004
25 MIN.	.005		.002 , .004
30 MIN.	.008 , .007	.003, .0002	.006 , .009

See Table XLV in appendix for nucleotides and phosphate.

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

TABLE XXXVI

α-KETOGLUTARATE OXIDATION, pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2]		
	NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.020[a], .024[a]	.024[a], .024[a]	.020[a], .020[a]
15 MIN.	.020[a], .024[a] .018 , .019	.024[a], .024[a] .024 , .022	.020[a], .020[a] .022 , .024
17.5 MIN.	.016[a], .024[a]	.024[a], .024[a]	.021[a], .020[a]
20 MIN.	.020[a], .023[a] .018 , .018	.023[a], .024[a] .024 , .022	.020[a], .018[a] .021 , .020
25 MIN.	.020 , .020	.021 , .020	.021 , .020
30 MIN.	.020 , .020	.021 , .020	.019 , .019

1 Reaction medium B, page 52.

2 Pseudo Rate, page 42.

a 20-minute flasks started 16 minutes later than 30-minute flasks.

TABLE XXXVI-A

α-KETOGLUTARATE OXIDATION, pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (37 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
NO HEXOKINASE						
10 MIN.		43.7	1.5	3.7	12.0	17.2
		44.9	1.5	2.9	12.0	16.4
20 MIN.	7.6	45.9	1.8	5.8	10.4	18.0
	8.6	44.7	3.8	4.9	10.4	19.1
30 MIN.	14.9	44.0	2.1	6.7	9.9	18.7
	14.4	45.3	1.3	4.3	9.9	17.5
20 µGRAMS HEXOKINASE						
10 MIN.		36.1	7.9	5.6	2.8	16.3
		37.1	7.9	5.2	2.9	16.0
20 MIN.	8.4	26.0	8.2	4.3	2.5	15.0
	8.8	26.1	7.9	4.6	3.5	16.0
30 MIN.	15.8	17.5	8.2	6.0	1.4	15.6
	15.1	17.8	11.6	4.6	1.6	17.8
30 µGRAMS HEXOKINASE						
10 MIN.		43.7	11.4	3.8	1.5	16.7
		42.6	10.8	4.1	1.8	16.7
20 MIN.	7.4	33.8	8.9	3.7	1.0	13.6
	6.6	35.6	8.9	3.7	1.0	13.6
30 MIN.	14.4	27.3	10.6	4.3	2.3	17.2
	13.9	27.8	11.7	3.9	1.6	17.2

1 Reaction medium B, page 52.

CHAPTER 4

DISCUSSION

General Considerations: It may be useful to review some of the factors which affect the interpretation of the experimental results which have been presented. First, it must be recognized that rat liver mitochondria are a variable factor. The procedure used is thought to be as good as any available, and has given reproducible results under a variety of conditions, but, because of the unavoidable variability, comparisons within an experiment are more likely to be appropriate than comparisons of different experiments.

As shown in the materials and methods section, the use of an adequate amount of histidylhistidine has undoubtedly limited pH changes during the measurement period to 0.1 unit or less in the majority of cases. Relatively large amounts of inorganic phosphate and substrate were used; so that changes in concentration during the measurement period were usually less than a factor of one half. Control of the relative amounts of ATP and ADP by adding hexokinase was reasonably successful in most cases, and was revealing in other cases in which a steady-state nucleotide situation was not maintained during the measurement period. This device does introduce a hidden variable--glucose-6-phosphate was present in considerable amounts when hexokinase was present, but

was absent where the enzyme was not added. There is abundant evidence that adenyl kinase activity was present in the mitochondria, but no evidence that this activity varied enough to be critical. Different results were found at 0.30 os as compared with 0.45 os in otherwise identical systems sufficiently often to justify continued attention to this variable.

Finally, it should be remembered that mitochondrial suspensions were mixed with cold medium at zero time and the mixture shaken in a bath at 30°C for ten minutes prior to measurement of most of the substances studied. It is obvious that changes will occur in both mitochondria and reaction medium during that time, and these changes should be kept in mind in the interpretations which follow.

SUCCINATE ⇌ FUMARATE ⇌ MALATE

The finding that oxygen consumption by rat liver mitochondria is generally greater in the presence of succinate is in agreement with information in the literature (174). Under special conditions rates of oxygen consumption in the presence of citrate or α-ketoglutarate have been reported to approach those achieved with succinate.

For this reason succinate is a reasonable place to begin to discuss the effects of pH and osmotic pressure on oxidation reactions which involve the Kreb's tricarboxylic acid cycle. Slater

and others (175) found that oxygen consumption due to succinate oxidation to fumarate-malate was high if the initial succinate concentration was high (.02 M), but that both the rate of oxygen consumption and the fraction of that rate due to succinate-fumarate decreased with the initial succinate concentration. Krebs (176) found that malate plus fumarate and citrate accumulated when succinate was the initial substrate. Others (177,178) have also found that succinate is converted to malate (via fumarate).

Figure 7 shows the time course of changes in radioactive materials present in the reaction mixture. It is quite clear that succinate decreases and both fumarate and malate increase. Small amounts of citrate and/or α-ketoglutarate could be obscured by the malate peak at twenty and forty minutes.

Additional evidence of the rapidity of conversion of succinate to fumarate is found in Table XLII-A where, in spite of the consumption of up to 43 micro-atoms of oxygen in a system containing initially only 45 micromoles citrate plus citrate-1, 5-C-14, no significant quantities of succinate or fumarate were found in the reaction mixture.

Evidence for the very rapid conversion of fumarate to malate is found in the fact that labeled fumarate builds up less rapidly than does labeled malate in experiments containing succinate-2,3-C-14 (Figure 7; Tables XIV-A, XV-B, XVI). The latter experiment

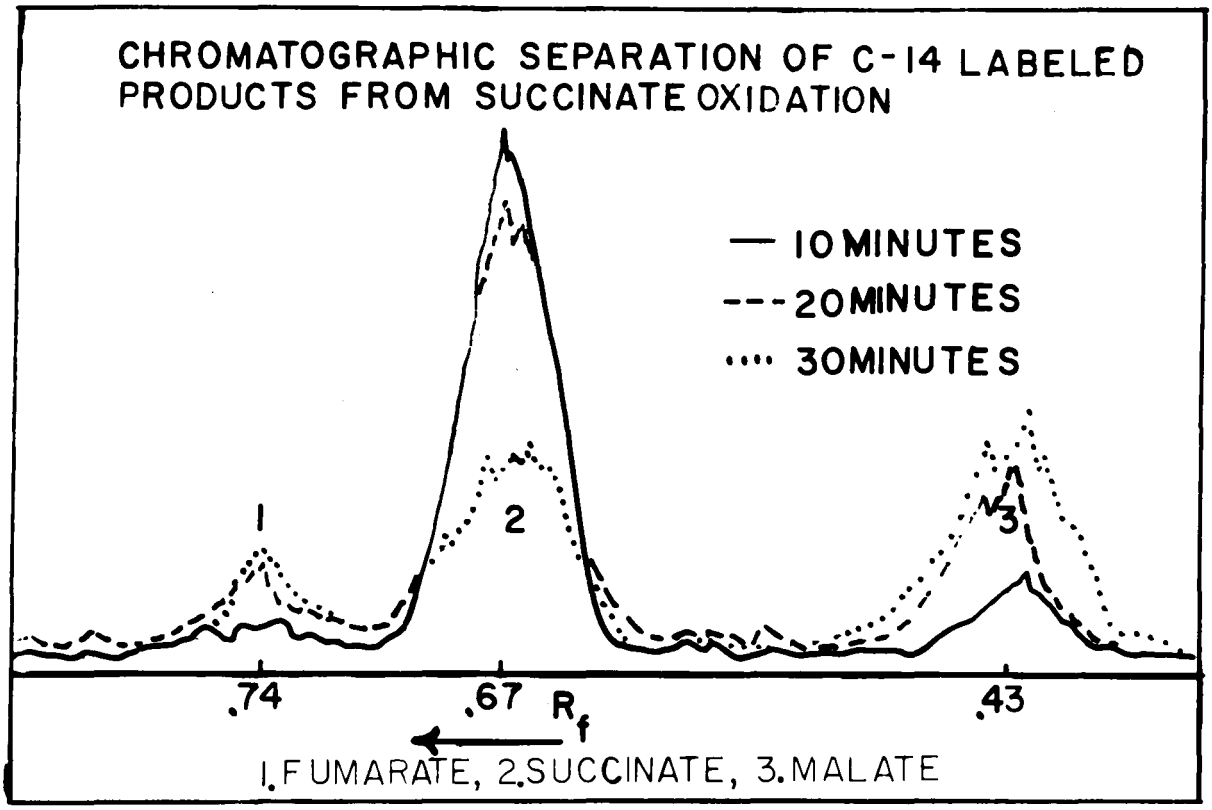

FIGURE 7

THE CHROMATOGRAPHIC SEPARATION OF C-14 LABELED PRODUCTS FROM SUCCINATE OXIDATION

Descending chromatography, 10-12 μcuries, butanol-formic acid-water, organic phase, 25°C. Each flask contained reaction medium C (with hexokinase) as described on page 53, plus 4 μcuries of 2,3-labeled succinate. See Table XXXVIII, for planimeter measurements. At the appropriate time, 10, 20 or 30 minutes, 2 ml of the reaction mixture was pipetted into 4 ml of ice-cold PCA and the supernatant examined as described for radioactive substances in the material and methods section.

(Table XVI) showed that succinate-2,3-C-14 added to reaction mixtures long after unlabeled succinate in large amounts had been mixed with the mitochondria is converted rapidly to labeled fumarate and malate. This indicates rapid mixing of the added succinate with the pool which has access to succinic dehydrogenase. This finding is in agreement with the suggestion that succinic dehydrogenase is located in the mitochondrial membrane in contrast to other dehydrogenases which are thought of as being located within the cristae (179). The findings that oxygen consumption with succinate (hexokinase present) is not markedly affected by change from 0.45 os to 0.30 os, while all other substrates show greater oxygen consumption at low osmotic pressure is also in harmony with this picture of relative enzyme locations. It is known that mitochondria swell at 0.30 os (180), but do not at 0.45 os. Swelling will open (and perhaps disrupt) the membranes, thus making "inner" enzymes more accessible--but may not affect "surface" enzymes.

It is important to note that very large differences in the relative amounts of ATP and AMP do not appreciably affect the rates of oxygen consumption with succinate at pH 7. The situation is strikingly different at pH 6. Table XIX shows that systems containing hexokinase showed oxygen uptake rates of only one tenth to one fifth that observed in systems containing moderate to high levels of ATP. Phosphate uptake and net synthesis of ATP are

noted in the absence of hexokinase, but there is a net release of inorganic phosphate with hexokinase.

Two possibilities come to mind immediately. Chance has suggested that ATP is required for oxidation of succinate (181). It may be that this requirement, coupled with a large utilization of ATP for other reactions at this pH brought about the marked differences between the system without hexokinase and that which contained the added enzyme. The only other obvious difference is that the high ATP system contained no glucose-6-phosphate, the hexokinase system contained appreciable quantities--and this compound or its reaction products have different effects at pH 6 than at pH 7.

Whatever the reason for the observed difference of pH 6 systems from pH 7 systems, it will be desirable to explore this area in more detail.

MALATE OXIDATION

L-Malate is known to be oxidized to oxalacetate by rat liver mitochondria. Ochoa (182) has demonstrated with purified malic dehydrogenase, that this reaction does not proceed to a measurable extent unless the product oxalacetate is removed--for example, by reaction with acetyl-CoA in the presence of the condensing enzyme. Another reaction which would remove oxalacetate is the formation of phosphoenolpyruvate, but Lardy (183) has recently demonstrated

the absence of the required enzyme (oxalacetate decarboxylase phosphorylase) from rat liver mitochondria. The experiments presented here are in agreement in that no phosphoenolpyruvate was found in the reaction products. Malate may also be converted to pyruvate, but no pyruvate was found.

We must then conclude that oxygen consumption in the presence of rat liver mitochondria and added malate will be appreciable only if sufficient acetyl-CoA is available to remove the oxalacetate produced. It seems desirable to consider what factors can influence the supply of acetyl-CoA. As was pointed out in the Introduction, page 23, fatty acids produced from mitochondrial lipids can be "activated" by ATP, then oxidized to acetyl-CoA in a complex sequence of reactions. The acetyl-CoA produced may be used in synthesis or can be oxidized via the tricarboxylic acid cycle to carbon dioxide and water. Since this latter sequence of reactions requires oxalacetate in the step to form citrate, but regenerates oxalacetate when the citrate formed is oxidized via α-ketoglutarate, succinate, fumarate and malate, it is quite easy to see that there will be difficulties in interpreting oxygen consumption measurements which involve the oxidation of malate.

Because of the inter-relationship of malic oxidation with the acetyl-CoA supply, two sets of experiments should be considered together--those in which 0.015 M malate was added and those which pyruvate (0.007 M) plus 0.002 M malate was added. Table XIII

shows that oxygen consumption at pH 7 was very nearly the same for either situation at 0.30 os, but was definitely different at high osmotic pressure. This finding suggests that a different rate determining process existed at 0.45 os. An obvious possibility is that the rate of entry of L-malate into the mitochondria was slow at 0.45 os. This limiting rate would be greater with 0.015 M malate than with 0.002 M malate in agreement with these measurements. Furthermore, because of the regeneration of oxalacetate within the mitochondria, one would expect the rate of oxygen consumption to increase with time as is observed at high osmolarity with both malate (Table XX) and pyruvate plus malate (Table XXIII). Moreover, one would not expect the rate of oxygen consumption to increase with time at low osmotic pressure, since malic dehydrogenase should be saturated (K_m of the isolated enzyme is 10^{-4} M) (184). The observed results (Tables XXI and XXIV) are in complete agreement with this prediction. The fact that the constant rates observed with malate at 0.30 os are slightly lower in the presence of hexokinase (Table XXI) suggests that in these systems the supply of acetyl-CoA is somewhat limited by the low ATP in these systems as compared with those which do not contain hexokinase.

From the comparative rates of oxidation at low osmolarity (Table XIII) one would predict that malate would be expected to accumulate during the oxidation of succinate because it is produced faster than its maximum rate of oxidation, and this is in

accord with the experimental findings already discussed. One would also predict that, since the oxidation of α-ketoglutarate must precede the formation of succinate-fumarate, that L-malate would not accumulate during the oxidation of either citrate, pyruvate, or α-ketoglutarate until supplies of acetyl-CoA became acutely limiting. Chromatograms of reaction mixtures involving labeled citrate or labeled pyruvate showed no evidence of the accumulation of labeled malate--although small amounts of malate could be obscured by the large amounts of citrate in that experiment.

At pH 6, the rates of oxygen consumption with malate at 0.45 os were quite low at all times, and there was no measurable increase with time (Table XXII). Pyruvate plus malate did show an increase of oxygen consumption with time at 0.45 os (Table XXV)--but again the rates are so low as to leave room for many uncertainties in interpretation. However, at 0.30 os the results with pyruvate plus malate indicate a significant role for ATP. In the absence of hexokinase, oxygen consumption levels were comparable with those observed at pH 7, but with hexokinase were only half that observed at pH 7 and decreased with time. As has already been mentioned in the case of succinate, this pH area should be further explored.

CITRATE ⇌ ISOCITRATE ⇌ α-KETOGLUTARATE

The equilibrium ratio of citrate to isocitrate is approximately 20:1 (185). The data in Table XXVIII-B suggests that equilibrium between citrate added and isocitrate formed is not attained rapidly at 0.45 os. This does not mean that this equilibrium is not attained within the mitochondrion--merely that the bulk of the citrate added cannot rapidly reach the enzyme site. Or, in other words, the rate of penetration of citrate into the mitochondrion limits the conversion to isocitrate and thus subsequent oxygen consumption, as has been suggested for each of the added substrates except succinate.

Table XXVIII shows that the rate of oxygen uptake increases with time more rapidly in the absence of hexokinase. In the period from ten to forty minutes, twenty-five microatoms of oxygen were taken up in the absence of hexokinase, but less than two micromoles of α-ketoglutarate are found in the reaction mixture (Table XXVIII-B). In the presence of hexokinase, more than thirteen microatoms of oxygen were absorbed, but α-ketoglutarate found amounted to less than three micromols. From this we must conclude that most of the oxygen absorbed was due to the further oxidation of α-ketoglutarate, and from the fact that no significant amounts of labeled succinate or fumarate were found (Table XLII-C) this oxidation must have proceeded rather rapidly as far as malate in both cases. As has been pointed out before, oxidation beyond

malate depends upon a supply of acetyl-CoA. In situations in which this supply depends upon the activation of fatty acids by ATP, further oxidation would be greater in the system with the greatest ATP, as is observed here. As has been pointed out in the case of malate, at high osmotic pressure, the citrate formed is inside the mitochondria and is expected to be oxidized sequentially to α-ketoglutarate ⟶ succinate ⟶ fumarate ⟶ malate ⟶ oxalacetate so that a continuously increasing rate of oxygen consumption is possible until the supply of fatty acids is exhausted. In the presence of hexokinase, the rate of this increase is limited by the low level of ATP maintained. The slightly larger, slightly more rapid accumulation of α-ketoglutarate in the system in which oxygen consumption rates alone might suggest it is formed less rapidly are in harmony with this general picture, since oxalacetate should accumulate more in the system with low acetyl-CoA--the system containing hexokinase. Oxalacetate inhibits 2-oxoglutarate:FAD-lipoate-oxidoreductase (186) and its presence would lead to an accumulation of 2-oxoglutarate (α-ketoglutarate).

At low osmolarity the situation is considerably different. Citrate can reach cis-aconitase rapidly, as shown by the amounts of isocitrate found after only thirty seconds (Table XXIX-B). It is to be noted that no appreciable α-ketoglutarate is found even after ten minutes. Significant changes do occur during the equilibration period as indicated by the fact that oxygen consump-

tion at fifteen minutes is nearly three times as rapid in the presence of hexokinase than in its absence (Table XXIX, page 130). Isocitrate is higher in the absence of hexokinase than in its presence, which suggests that oxidation of isocitrate is faster in the presence of hexokinase than in its absence. This may be due to complexing of manganese by the high ATP (Table XXIX-A, page 131). Manganese is more efficient than magnesium with isocitrate dehydrogenase, but is strongly bound by ATP (187).

In the absence of hexokinase, approximately six μatoms of oxygen are absorbed during the measurement period (Figure 8), but only 0.7 μmols of α-ketoglutarate are found at the end of this time. It would appear that the removal of α-ketoglutarate is very efficient, as was indicated at 0.45 os and will be discussed under α-ketoglutarate oxidation.

The presence of hexokinase at 0.30 os leads to a very rapid phosphate uptake--so rapid that 23 micromols are taken up between 30 seconds and ten minutes, and 25 more in the next twenty minutes (Table XXIX-A, page 131). There was a considerable change in the nucleotides during the first ten minutes--due to adenyl kinase added with the mitochondria and hexokinase present, but no change during the twenty minute measurement period. In spite of the constancy of nucleotides and of pH, the rate of oxygen consumption changed during the measurement period (Figure 9). Table XXIX-B, page 132, shows that there was no appreciable α-ketoglutarate

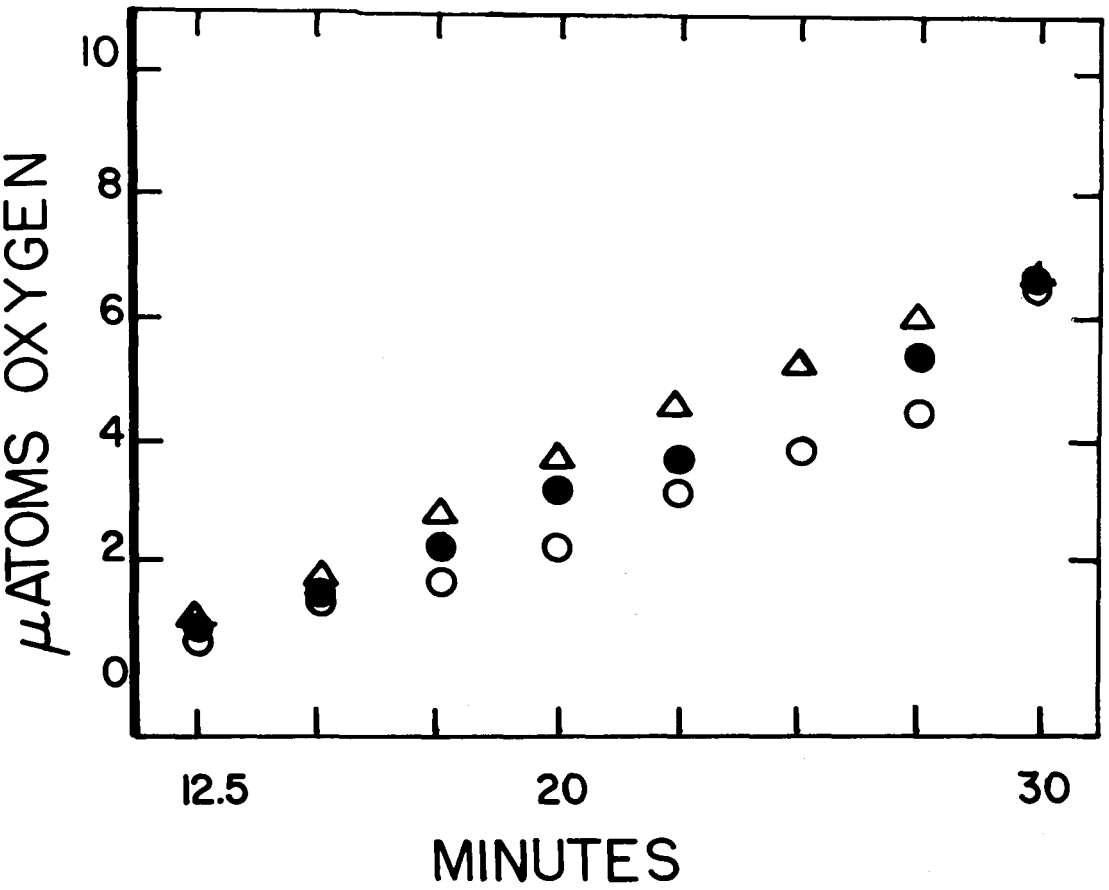

FIGURE 8

OXYGEN CONSUMPTION BY RAT LIVER MITOCHONDRIA

The reaction medium is the same as described on page 52 for reaction medium B with citrate. This medium contained no hexokinase. The experiment was performed at pH 7, .30 osmolar. Additional data is contained in Table XXIX; the symbols indicate the individual flasks.

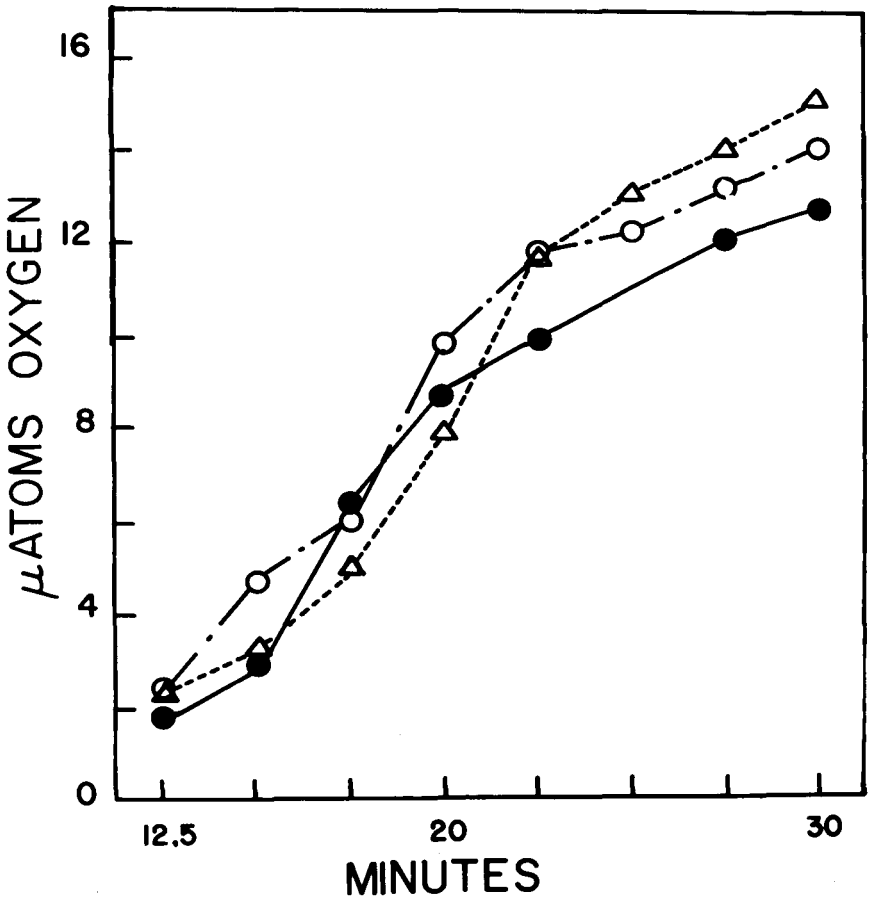

FIGURE 9

OXYGEN CONSUMPTION BY RAT LIVER MITOCHONDRIA

The reaction medium is the same as described on page 52 for reaction medium B, with citrate. This medium contained 20 μgm hexokinase. The experiment was performed at pH 7, .30 osmolar. Additional data is contained in Table XXIX.

found at ten minutes, and only 2.5 μmols after thirty minutes--a time during which at least 14 μatoms of oxygen were absorbed. Since the oxidation of α-ketoglutarate requires phosphate, it seems possible that most of this accumulation occurred during the latter five to ten minutes of the measurement period when oxygen consumption was as low as 0.01 μatoms/minute/milligram protein, and inorganic phosphate was quite low.

From the ten minute phosphate uptake and the oxygen consumption rates measured at twelve minutes, it seems likely that at least some material had been oxidized as far as malate by that time. Oxidation beyond malate is limited by acetyl-CoA which, in turn is limited by ATP. In this system total ATP is kept low by hexokinase, but internal ATP is continually supplied. It is possible that maximal acetyl-CoA was not yet attained at ten minutes, and that the amount of acetyl-CoA increased during the measurement period--thus causing an increasing rate of oxygen consumption until the supply of phosphate was nearly exhausted.

α-KETOGLUTARATE ⟶ SUCCINATE

Some of the factors which affect the oxidation of α-ketoglutarate have already been mentioned. When this compound is added to mitochondria at 0.45 os, pH 7, the initial rate of oxygen consumption is moderate in the absence of hexokinase, and remains constant with time. In the presence of hexokinase, the first

measured rates are lower than in its absence, but increase with time so that the rate at twenty minutes is definitely greater in the presence of hexokinase. The differences are small, however, and will not be considered further at this time.

At low osmotic pressure, pH 7, the observed rate of oxygen consumption in the absence of hexokinase is low--comparable to that observed at 0.45 os--and remains constant during the twenty minute measurement period (Table XXXIV, Figure 10). Table XXXIV-B indicates that only about two μmols of α-ketoglutarate are removed during the consumption of more than six μatoms of oxygen (Table XXXIV-A); so that oxidation beyond malate is indicated. In this case the oxygen consumption rate is presumably not limited by access to enzymes, and the rather high ATP maintained throughout the experiment should be able to affect the internal GTP/GDP. Since GDP is required for the conversion of succinyl CoA to succinate plus CoA, the relatively low GDP may be the limiting factor in the total oxygen consumption. In the case discussed earlier, the oxidation of added citrate, it would appear that the effect of high ATP is more marked on the oxidation of isocitrate than on the following oxidation of α-ketoglutarate, because α-ketoglutarate does not accumulate to a large extent. However, the observed rate of oxygen consumption is essentially identical with α-ketoglutarate and with citrate (Tables XXIX and XXXIV), and since these data were obtained with the same mitochondrial preparation, the com-

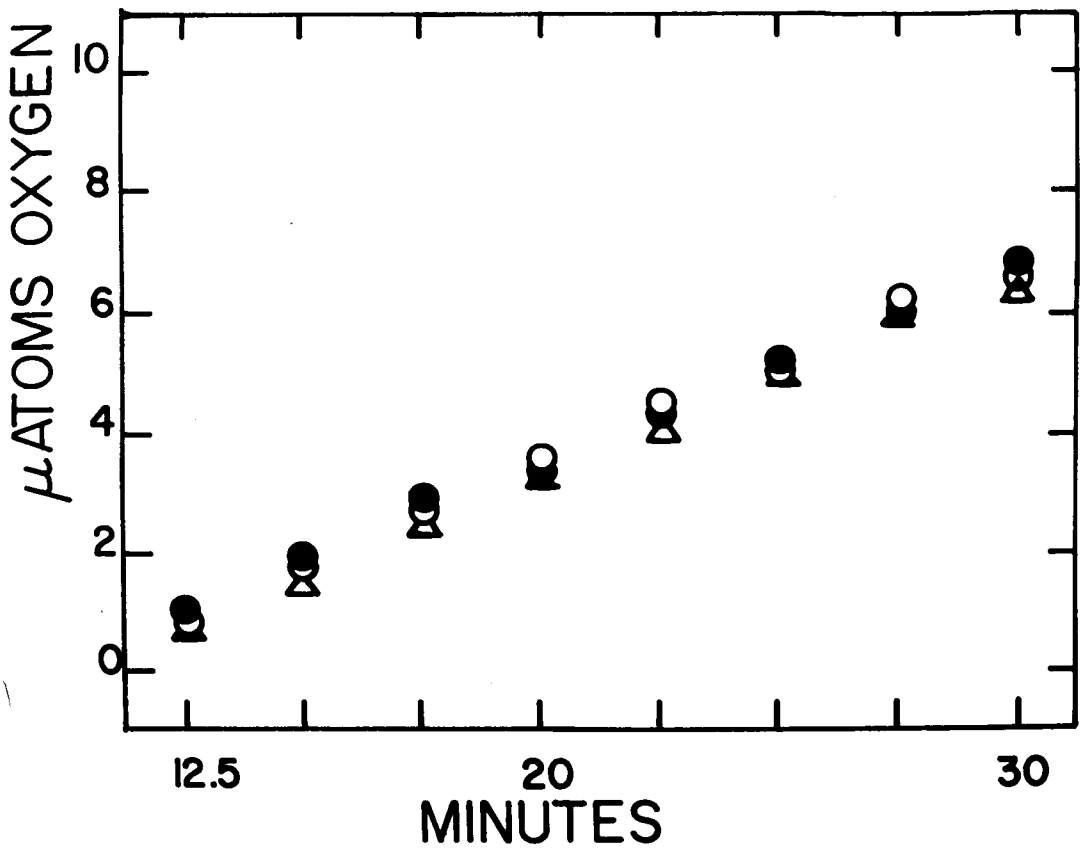

FIGURE 10

OXYGEN CONSUMPTION BY RAT LIVER MITOCHONDRIA

IN THE PRESENCE OF ALPHA-KETOGLUTARATE

The reaction medium is the same as described on page 52 for reaction medium B, with alpha-ketoglutarate. This medium contained no hexokinase. The experiment was performed at pH 7, .30 osmolar. Additional data is contained in Tables XXXIV; the symbols indicate the individual flasks.

parison is meaningful.

When hexokinase is present at low osmotic pressure, pH 7, the rate of oxygen consumption with α-ketoglutarate is high and nearly constant from ten to twenty minutes (Figure 11). As in the case of citrate with the same mitochondria and conditions (Tables XXIX and XXXIV), the rate drops markedly after twenty-five minutes--probably because of the very low phosphate. The erratic changes between ten and twenty minutes observed with citrate are not found with α-ketoglutarate. Since the preparation, the pH, the level of adenine nucleotides, the phosphate concentration, etc. were all nearly identical in both systems, the cause of the erratic oxygen consumption with added citrate must be sought in the differences imposed by high citrate on one hand, and high α-ketoglutarate on the other. The K_m for α-ketoglutarate is reported to be about 10^{-5} M (188). The amounts of α-ketoglutarate present at ten minutes in the citrate system are of this order of magnitude--1 to 1.6×10^{-5}. It is quite likely that maximum velocity of α-ketoglutarate oxidation (in the citrate system) was not reached at ten minutes, but was reached later--at a slightly different time in each system. This would account for the erratic oxygen uptake rates observed. The suggestion made earlier that the oxygen uptake changes (citrate added) might be due to change in acetyl-CoA is still a possibility, but both factors must be considered.

Table XXXIV-A shows that a very considerable uptake of phos-

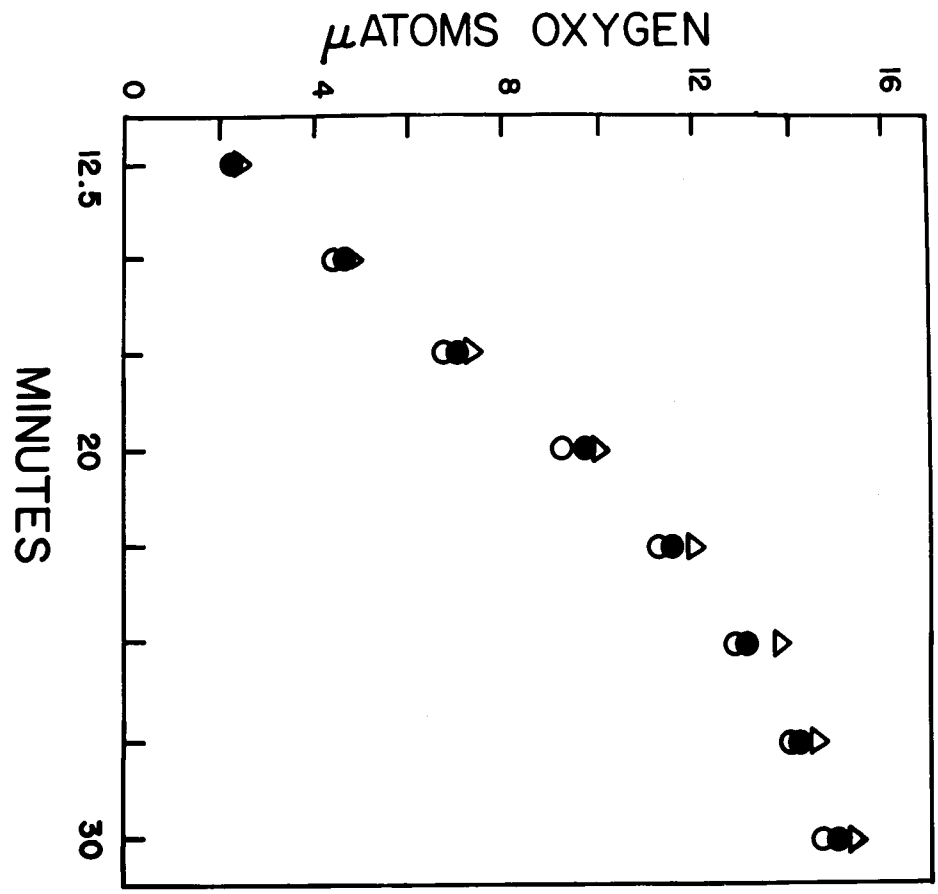

FIGURE 11

OXYGEN CONSUMPTION BY RAT LIVER MITOCHONDRIA

IN THE PRESENCE OF ALPHA-KETOGLUTARATE

The reaction medium is the same as described on page 52 for reaction medium B, with alpha-ketoglutarate. This medium contained 20 μgm hexokinase. The experiment was performed at pH 7, .30 osmolar. Additional data is contained in Tables XXXIV; the symbols indicate the individual flasks.

phate occurred during the first ten minutes, and that this continued until practically all the inorganic phosphate was taken up. (It is possible that all the inorganic phosphate was gone at the end of the reaction--the 1.1 to 1.7 μmols found may be produced by hydrolysis under the experimental conditions for deproteinization and phosphate measurement.) From this it is clear that oxidation-phosphorylation processes are well underway at the beginning of the measurement period. From ten to twenty minutes four μmols of α-ketoglutarate disappear and fifteen μatoms of oxygen are used. This is nearly four μatoms of oxygen per μmol of α-ketoglutarate, and indicates oxidation beyond malate. Since this oxidation will require acetyl-CoA, it would appear that the supply of acetyl-CoA is adequate to maintain the rate of oxygen consumption observed. It is, however, important to note that this system may not be as sensitive to acetyl-CoA as one with an equal steady-state oxygen consumption starting with succinate or malate, since some oxygen uptake is due to the conversion of α-ketoglutarate to succinate, some to succinate ⟶ fumarate, and some to malate ⟶ oxalacetate; and only the last reaction is limited by acetyl-CoA.

SUMMARY

Many facets of the oxidation of added substrates by rat liver mitochondria have been studied under controlled conditions of pH, osmotic pressure, and adenine nucleotide phosphorylation. In a

number of cases it has been possible to obtain a significant amount of oxygen consumption under steady-state conditions.

A change in osmotic pressure from 0.30 (which is equal to or higher than that in most mitochondrial experiments which have been reported) to 0.45 os (which approximates that inside the liver cell) caused marked changes in oxidation rates for all substrates except succinate. This is in accord with previous work which indicates that succinic oxidase is located on the outside of the mitochondrial membrane, while the other dehydrogenases are within the cristae.

Results at pH 6 are markedly different from those at pH 7. This suggests that pH dependent metabolic controls may be found by more detailed investigations between pH 6 and pH 7.

At 0.30 os, pH 7, oxygen consumption was markedly affected by the presence of hexokinase only in the case of citrate and α-ketoglutarate. Different reasons were advanced in each case--high ATP may have reduced manganese available to isocitric dehydrogenase (citrate added) and thus reduced the rate; high ATP may have reduced the amount of GDP available to succinyl CoA synthetase in the oxidation of α-ketoglutarate to succinate. Both effects are present in both systems, but the relative amounts of substrate make one decisive with citrate, the other with α-ketoglutarate.

At pH 7, high osmolarity, a limiting factor in the case of

malate, malate plus pyruvate, citrate, and α-ketoglutarate appeared to be entry into the mitochondrion.

All the data from experiments in which significant amounts of oxygen were absorbed are in agreement that a considerable portion of this oxygen consumption is due to the oxidation of acetyl-CoA via the Kreb's tricarboxylic acid cycle. Thus, some of the effects measured in experiments of this sort may be due to changes in the availability of acetyl-CoA.

APPENDIX 1

THE FOLLOWING EXPERIMENTS ARE PRELIMINARY EXPERIMENTS AND DUPLICATIONS OF THE EXPERIMENTS REPORTED IN THE RESULTS AND DISCUSSION SECTION.

TABLE XXXVII

SUCCINATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2]		
	NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.033, .036	.048, .040	.056, .044
15 MIN.	.030, .032	.040, .034	.034, .042
17.5 MIN.	.031, .031	.036, .033	.033, .040
20 MIN.	.031, .029	.035, .032	.033, .039

1 Reaction medium C, page 53.

2 Pseudo Rate, page 42.

TABLE XXXVII-A

SUCCINATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (24.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES[2] AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
NO HEXOKINASE						
10 MIN.		32.9	3.0	9.5	3.7	16.2
		32.8	2.0	7.6	5.9	15.5
20 MIN.	7.6	32.5	2.0	9.6	5.9	17.5
	7.2	32.5	2.2	7.8	7.4	17.4
20 µGRAMS HEXOKINASE						
10 MIN.		31.4	12.8	3.1	0.5	16.4
		30.9	12.6	3.3	0.8	16.7
20 MIN.	8.6	20.1	13.5	2.8	0.5	16.8
	7.9	20.3	14.0	2.4	0.3	16.7
30 µGRAMS HEXOKINASE						
10 MIN.		31.5	11.5	4.6	0.6	16.7
		32.2	11.9	4.4	0.8	17.1
20 MIN.	8.1	22.3	12.4	4.4	1.4	18.2
	9.5	17.2	11.0	4.4	0.5	15.9

1 Reaction medium C, page 53.

2 NAD fraction contained less than 0.1 µmole.

TABLE XXXVIII[1]

SUCCINATE OXIDATION, pH 7.0, HIGH OSMOLARITY

MEASUREMENTS OF SUBSTRATES CONTAINING CARBON-14

TIME AFTER MITOCHONDRIA	% SUCCINATE[2]	% FUMARATE[2]	% MALATE[2]	CO_2 (C.P.M.)
NO HEXOKINASE				
10 MIN.	83, 83	3, 3	13, 13	NOT MEASURED
20 MIN.	72, 65	8, 10	21, 25	4,300, 5,800
40 MIN.	40, 45	14, 38	46, 38	75,000, 5,100
20 µGRAMS HEXOKINASE				
10 MIN.	82, 85	6, 4	12, 11	2,600, 2,300
20 MIN.	70, 87	10, 2	20, 8	16,000, 6,300
40 MIN.	44, 59	12, 4	48, 37	10,200, 13,900

1 For other data, see Tables XV and XV-A.

2 Total area under recording tracing minus background = 100%. The initial tracer present in each flask was 8.9×10^6 cpm.

TABLE XXXIX[1]

SUCCINATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (15.5 mgs protein)	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE
12.5 MIN.	.034 , .036	.039 , .036
15 MIN.	.038 , .050	.054 , .048
	$.052^{\alpha}$, $.050^{\alpha}$	.052 , .050
17.5 MIN.	.051 , .051	.052 , .052
20 MIN.	.049 , .054	.056 , .054
	$.060^{\alpha}$, $.053^{\alpha}$	.054 , .050
22.5 MIN.	$.013^{\beta}$, $.052^{\beta}$	$.042^{\beta}$
25 MIN.	$.031^{\beta}$, $.054^{\beta}$	$.048^{\beta}$
	$.053^{\alpha}$, $.055^{\alpha}$	.052 , .054
27.5 MIN.	$.040^{\beta}$, $.055^{\alpha}$	$.048^{\alpha}$
30 MIN.	$.047^{\beta}$, $.054^{\beta}$	$.048^{\beta}$
	$.054^{\alpha}$, $.055^{\alpha}$	$.056^{\alpha}$, $.054^{\alpha}$
32.5 MIN.	$.050^{\beta}$, $.053^{\beta}$	$.050^{\beta}$
35 MIN.	$.051^{\beta}$, $.053^{\beta}$	$.051^{\beta}$
	$.057^{\alpha}$, $.055^{\alpha}$	$.058^{\alpha}$, $.055^{\alpha}$
37.5 MIN.	$.056^{\beta}$, $.054^{\beta}$	$.050^{\beta}$
40 MIN.	$.053^{\beta}$, $.053^{\beta}$	$.050^{\beta}$
	$.053^{\alpha}$, $.054^{\alpha}$	$.052^{\alpha}$, $.054^{\alpha}$

1 Same mitochondrial preparation as Table XVI.
2 Pseudo Rate, page 42.
α Flask closed and tracer added at 10 minutes.
β " " " " added at 20 minutes.

TABLE XXXIX-A[1]

SUCCINATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (15.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES µMOLES AMP	ADP	ATP	TOTAL
NO HEXOKINASE						
(2) 20 MIN.	7.5	48.2	2.3	5.7	9.1	17.1
	8.4	48.7	1.8	4.3	11.0	17.1
(3) 40 MIN.	16.3[4]	44.0	1.2	4.0	13.0	18.2
	16.9	45.6	2.2	5.6	9.2	17.0
(2) 40 MIN.	24.5	47.5	1.8	4.1	9.8	15.7
	25.0	47.5	2.3	5.2	9.0	16.5
20 µGRAMS HEXOKINASE						
(2) 20 MIN.	8.6	27.9	11.2	3.7	2.7	17.6
	8.3	30.6	11.3	3.5	2.6	17.4
(3) 40 MIN.	15.3[4]	18.8	11.4	2.7	2.3	16.4
	----	18.2	12.6	2.4	1.9	16.9
(2) 40 MIN.	24.3	19.6	12.4	2.9	1.6	16.9
	25.1	19.6	12.4	2.9	1.3	16.6

1 Same mitochondrial preparation as Table XVI.

2 Four µcuries of 2-3 labeled succinate was tipped from the side arm at 10 min. and the reaction stopped either at 20 or 40 min.

3 Four µcuries of 2-3 labeled succinate was tipped from the side arm at 20 min. and the reaction stopped at 40 min.

4 Oxygen consumed for 20 minutes to 40 minutes.

TABLE XL

MALATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (24.5 mgs protein)	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.008, .008	.006, .004	.002, .0008
15 MIN.	.012, .009	----, .008	.001
17.5 MIN.	.011, .007	.007, .008	.003, .005
20 MIN.	.011, .008	.007, .008	.005, .006

1 Reaction medium C, page 53.

2 Pseudo Rate, page 42.

TABLE XL-A

MALATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (24.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
NO HEXOKINASE						
10 MIN.		51.9	6.9	8.7	5.5	21.1
		52.6	6.9	8.8	5.7	21.4
20 MIN.	2.8	50.1	16.0	2.2	0.0	18.2
	2.0	50.4	16.0	2.2	0.0	18.2
20 µGRAMS HEXOKINASE						
10 MIN.		40.3	13.7	2.3	0.5	16.5
		35.6	13.6	2.4	0.3	16.3
20 MIN.	1.7	34.5	13.2	2.4	0.8	16.4
	1.9	35.6	13.2	2.7	0.9	16.8
30 µGRAMS HEXOKINASE						
10 MIN.		38.0	16.0	2.2	0.0	18.2
		39.0	16.0	2.2	0.0	18.2
20 MIN.	1.2	34.5	11.8	3.6	0.5	15.9
	1.4	35.6	11.7	3.1	0.3	15.1

1 Reaction medium C, page 53.

TABLE XLI

PYRUVATE PLUS MALATE OXIDATION, pH 6.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (29 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NAD	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
NO HEXOKINASE							
10 MIN.		43.5	0.6	6.5	5.3	5.7	19.1
		43.5	0.6	6.6	7.4	5.7	20.3
30 MIN.	1.4	48.6	0.7	7.8	4.3	1.0	13.8
	1.5	46.5	0.8	11.6	2.4	0.3	14.1
20 µGRAMS HEXOKINASE							
10 MIN.		43.5	1.1	15.0	3.2	0.5	19.8
		43.8	1.0	14.0	2.1	1.1	18.2
30 MIN.	0.0	46.5	0.8	13.0	1.7	0.9	16.4
	0.0	48.4	1.0	12.6	1.6	0.6	15.8

1 Reaction medium B, page 52.

TABLE XLII

CITRATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (34.2 mgs protein)	RATE, μATOMS/MINUTE/MILLIGRAM[2] NO HEXOKINASE	20 μGRAMS HEXOKINASE
12.5 MIN.	.037[α], .035[α]	.028[α], .023[α]
15 MIN.	.038[α], .038[α] .036 , .036	.027[α], .024[α] .024 , .024
17.5 MIN.	.037[α], .036[α]	.027[α], .024[α]
20 MIN.	.038[α], .037[α] .038 , .040	.027[α], .025[α] .026 , .026
25 MIN.	.044 , .047	.027 , .027
30 MIN.	.040 , .043	.027 , .027
35 MIN.	.039 , .042	.026 , .026
40 MIN.	.039 , .042	.027 , .026

1 Reaction medium C, page 53.

2 Pseudo Rate, page 42.

α Flasks were started 20 minutes later than the duplicate.

TABLE XLII-A

CITRATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (34.2 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES[2] AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
NO HEXOKINASE						
10 MIN.		49.8	1.9	5.1	10.7	17.7
		49.2	2.3	4.9	10.0	17.2
20 MIN.	13.0	49.8	1.6	4.3	10.6	16.5
	12.8	47.6	1.8	4.7	11.0	17.5
40 MIN.	39.6	53.9	2.9	5.8	7.5	16.2
	42.8	53.9	3.2	5.6	7.9	16.7
20 µGRAMS HEXOKINASE						
10 MIN.		43.0	10.8	3.9	1.7	16.4
		44.7	11.3	3.9	1.5	16.7
20 MIN.	9.3	30.1	9.2	3.3	1.5	14.0
	8.4	30.2	9.6	4.9	2.1	16.6
40 MIN.	27.3	11.3	9.6	3.9	1.4	14.9
	26.3	14.0	9.4	3.3	1.3	14.0

1 Reaction medium B, page 52.

2 NAD fraction contained less than 0.1 µmole.

TABLE XLII-B

CITRATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	CO_2 C.P.M.	FRACTION β OF INITIAL C-14	α-KETO-GLUTARATE[β] μMOLES	ISOCITRATE[β] μMOLES
NO HEXOKINASE				
10 MIN.	222,000	0.051	0.38	0.16
	142,000	0.032	0.32	0.14
20 MIN.	280,000	0.063	0.12	0.17
	190,000	0.043	0.17	0.17
40 MIN.	490,000	0.11	1.1	0.10
	490,000	0.11	0.7	0.28
HEXOKINASE				
10 MIN.	105,000	0.024	0.35	0.14
	220,000	0.050	0.20	0.16
20 MIN.	140,000	0.031	0.70	0.17
	65,000	0.015	0.70	0.17
40 MIN.	330,000	0.075	4.7	0.20
	320,000	0.075	4.7	0.18

β Enzymatically determined.

TABLE XLII-C

CITRATE OXIDATION, pH 7.0, LOW OSMOLARITY

(Planimeter Areas for C-14 Citrate Chromatograms)

TIME AFTER MITOCHONDRIA	PLANIMETER AREAS[1] NO HEXOKINASE	20 μGRAMS HEXOKINASE
LOW OSMOLARITY		
10 MIN.	5.8, 6.0	6.8, 5.6
20 MIN.	6.5, 5.2	6.5, 5.5
40 MIN.	4.2, 2.6	4.0, 4.3
HIGH OSMOLARITY[2]		
10 MIN.	5.4	4.5
20 MIN.	5.4	6.0, 6.0
40 MIN.	4.5	5.2, 6.3

1 Determined from chromatograph tracings.

2 The same mitochondrial preparation as Table XXIX.

TABLE XLIII

α-KETOGLUTARATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2]		
	NO HEXOKINASE	20 μGRAMS HEXOKINASE	30 μGRAMS HEXOKINASE
12.5 MIN.	.006, .007	.007, .011	.010, .013
15 MIN.	.010, .008	.012, .013	.011, .011
17.5 MIN.	.009, .009	.011, .012	.011, .011
20 MIN.	.011, .010	.012, .014	.011, .012

1 Reaction medium C, page 53.

2 Pseudo Rate, page 42.

TABLE XLIII-A

α-KETOGLUTARATE OXIDATION, pH 7.0, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (34.2 mgs protein)	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES AMP μMOLES	ADP μMOLES	ATP μMOLES	TOTAL
NO HEXOKINASE						
10 MIN.		44.3	7.3	6.9	6.0	20.2
		44.6	5.7	7.0	4.8	17.5
20 MIN.	3.9	38.6	3.1	5.9	8.5	17.5
	3.5	38.6	3.1	7.3	6.6	17.0
20 μGRAMS HEXOKINASE						
10 MIN.		41.9	13.2	3.1	1.1	17.4
		43.6	13.6	3.1	0.9	17.6
20 MIN.	4.2	34.4	13.1	5.0	1.7	18.8
	4.7	34.1	13.1	3.2	1.2	17.5
30 μGRAMS HEXOKINASE						
10 MIN.		43.6	12.9	3.6	0.8	17.3
		43.3	13.1	3.8	1.4	17.3
20 MIN.	3.9	36.2	11.8	4.2	1.4	17.4
	4.1	34.0	11.7	4.2	1.4	17.3

1 Reaction medium C, page 53.

TABLE XLIV

α-KETOGLUTARATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	RATE, μATOMS/MINUTE/MILLIGRAM[2]	
	NO HEXOKINASE	20 μGRAMS HEXOKINASE
12.5 MIN.	.022, .024	.026, .031
15 MIN.	.024, .024	.030, .038
17.5 MIN.	.025, .025	.030, .041
20 MIN.	.026, .026	.037, .041

1 Reaction medium A, page 50--contained N-acetylhistidine.

2 Pseudo Rate, page 42.

TABLE XLIV-A

α-KETOGLUTARATE OXIDATION, pH 7.0, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (22.2 mgs protein)	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES
NO HEXOKINASE		
10 MIN.		37.7 37.7
20 MIN.	5.8 5.7	35.9 36.1
20 μGRAMS HEXOKINASE		
10 MIN.		32.7 32.2
20 MIN.	8.1 9.0	16.4 14.0

1 Reaction medium A, page 50--contained N-acetylhistidine.

TABLE XLV

α-KETOGLUTARATE OXIDATION, pH 6, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (29.0 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP	ATP	TOTAL
NO HEXOKINASE						
10 MIN.		64.6	4.8	7.1	0.2	12.1
		65.0	---	---	---	----
30 MIN.	4.4	58.0	6.8	5.0	5.3	17.1
	4.0	58.0	5.7	5.6	5.2	16.5
20 µGRAMS HEXOKINASE[2]						
10 MIN.		39.3	12.6	5.8	0.6	19.0
		39.7	12.8	2.0	0.5	15.3
30 MIN.	1.6	38.7	14.0	1.7	0.8	16.5
	---	39.3	11.0	1.9	3.4	16.3

1 Reaction medium B, page 52.

2 Comparable results were obtained with 30 µgrams hexokinase.

TABLE XLVI

COMPARATIVE RATES OF OXIDATION, pH 6

OSMOLARITY	.45 os		.30 os	
HEXOKINASE	ZERO	20 µgm	ZERO	20 µgm
	RATE, µATOMS OXYGEN/MINUTE/MILLIGRAM PROTEIN*			
PYRUVATE PLUS MALATE (1,2)	.001 , .001 .000	.000, .000 .001	.029, .020 .022	.010, .013 .014
CITRATE (3,4)	.0007, .0000	.005, .000	.010, .010	.006, .008
α-KETOGLUTARATE (5,6)	.0007 .0003	.003, .003	.020, .024 .018, .019	.024, .024 .022
SUCCINATE (7)	.060 , .054 .044 , .045	.008, .007 .009		
MALATE (8)	.004 , .004 .003 , .003	.002, .002 .001		

All substrates were run in reaction medium C, page 53.

* Pseudo Rate, page 42--15 minutes after addition of mitochondria.

1.....Data taken from Table XXV, high osmolarity.
2.....Data taken from Table XXVI, low osmolarity.
3.....Data taken from Table XXX, high osmolarity.
4.....Data taken from Table XXXI, low osmolarity.
5.....Data taken from Table XXXV, high osmolarity.
6.....Data taken from Table XXXVI, low osmolarity.
7.....Data taken from Table XIX, high osmolarity.
8.....Data taken from Table XXII, high osmolarity.

TABLE XLVII

COMPARISON OF ATP/ADP RATIOS, pH 7, HIGH OSMOLARITY

SUBSTRATE[1]	TIME 10 MINUTES HEXOKINASE ZERO	20 µGM	TIME 20 MINUTES HEXOKINASE ZERO	20 µGM	TIME 40 MINUTES HEXOKINASE ZERO	20 µGM
PYRUVATE PLUS[2]	3.9	0.6	3.5	0.4	3.4	0.5
MALATE	2.8	0.7	3.5	0.2	3.1	0.4
CITRATE[3]	1.0	0.6	1.7	0.4	3.0	0.4
	1.0	0.5	1.6	0.4	3.0	0.3
α-KETOGLUTARATE[4]	1.0	0.2	2.0	0.4		
	0.9	0.4	2.1	0.3		
SUCCINATE[5]	1.9	0.3	2.3	0.4	2.0	0.8
	1.9	0.3	1.8	0.4	1.0	1.4
MALATE[6]	0.9	0.4	2.2	0.3		
	0.9	0.4	2.1	0.3		

1 α-Ketoglutarate and malate substrates were run with reaction medium C, page 53. Reaction medium B, page 52, was used for all other substrates.

2 See Table XXIII-A.

3 See Table XXVIII-A.

4 See Table XXXIII-A.

5 See Table XV-A.

6 See Table XX-A.

TABLE XLVIII

COMPARISON OF ATP/ADP RATIOS, pH 7, LOW OSMOLARITY

SUBSTRATE[1]	TIME 10 MINUTES HEXOKINASE ZERO	20 μGM	TIME 20 MINUTES HEXOKINASE ZERO	20 μGM	TIME 40 MINUTES HEXOKINASE ZERO	20 μGM
PYRUVATE PLUS[2] MALATE	1.4	0.2	0.6	0.3	1.3	0.3
	1.4	0.2	1.0	0.3	1.6	0.3
CITRATE[3]	2.1	0.4	2.5	0.5	1.3	0.4
	2.0	0.4	2.3	0.4	1.4	0.4
SUCCINATE[4]	2.6	0.4	2.3	0.3	2.7	0.3
	2.7	0.4	1.7	0.6	2.9	0.3

1 All substrates were run in reaction medium B, page 52.

2 See Table XXIV-A.

3 See Table XLII-A.

4 See Table XVIII-A.

TABLE XLVIII-A

COMPARISON OF ATP/ADP RATIOS, pH 6

HIGH OSMOLARITY AND LOW OSMOLARITY

SUBSTRATE*	TIME 10 MINUTES HEXOKINASE ZERO	20 µGM	TIME 20 MINUTES HEXOKINASE ZERO	20 µGM	TIME 30 MINUTES HEXOKINASE ZERO	20 µGM
LOW OSMOLARITY						
PYRUVATE PLUS MALATE	0.10	0.40			0.30	0.20
	1.20	0.30			0.20	0.20
Table XXVI						
CITRATE	0.60	0.30			0.30	0.30
Table XXXI	0.70	0.60			0.30	0.50
α-KETOGLUTARATE	3.2	0.50			1.5	0.20
Table XXXVI	4.1	0.60			2.3	0.40
HIGH OSMOLARITY						
PYRUVATE PLUS MALATE	0.60	0.30			0.30	0.20
	0.60	0.20			0.20	0.60
Table XXV						
CITRATE	1.1				0.20	
Table XXX	1.0				0.90	
α-KETOGLUTARATE	0.03	0.10			1.10	0.50
Table XXXV		0.30			0.90	
SUCCINATE	0.80	0.20	1.5	0.6	5.9	0.4
Table XIX	0.80	0.10	1.9	0.8	5.9	0.7
MALATE	0.60	0.50	0.60	0.30	0.80	0.50
Table XXII	0.71	0.50	0.70	0.40	0.50	0.20

* All substrates were run in reaction medium B, page 52.

APPENDIX II

EXPERIMENTS PERFORMED ON AMP PHOSPHORYLATION, ADDITIONAL BUFFER EXPERIMENTS. TIME STUDIES WITH BETA-HYDROXYBUTYRATE AS THE INITIAL SUBSTRATE. THE EFFECTS OF MAGNESIUM AND DINITROPHENOL ON BETA-HYDROXYBUTYRATE OXIDATION. THE EFFECT OF IMIDAZOLE ON SUCCINATE OXIDATION.

TABLE XLIX

SUCCINATE OXIDATION, pH 7.4, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (12.5 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
4 MIN.		39.8	12.9	0.6	2.3	14.8
		40.2	13.2	0.3	1.6	15.1
8 MIN.		35.6	10.3	1.0	4.1	15.4
		36.0	9.7	1.5	3.9	15.1
12 MIN.	2.9	27.7	4.1	4.8	7.3	16.2
	2.6	32.2	8.7	2.3	5.0	16.0
16 MIN.	3.8	31.6	7.1	3.3	5.7	16.1
	3.8	32.1	7.5	3.0	5.3	15.8
20 MIN.	5.5	29.4	3.0	4.1	7.3	15.4
	5.5	28.9	6.6	3.7	5.2	15.5
24 MIN.	6.3	34.6	7.2	2.1	5.8	15.1
	6.3	28.0	4.3	3.9	7.1	14.3
28 MIN.	6.6	26.9	3.9	4.6	8.6	16.1
	6.6	26.0	3.2	4.5	7.5	15.2
32 MIN.	9.2	23.0	2.1	4.2	9.0	15.3
	7.2	24.8	2.9	3.8	7.4	14.1

1 Reaction medium contained: 0.005 M AMP, 0.005 M $MgCl_2$, 0.012 M Tris, 0.015 M K_2HPO_4, 0.25 M Sucrose, pH 7.4, 327 os.

2 Flasks closed at 8 minutes.

TABLE L

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 7.4, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (10.5 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES	pH
0 MIN.		45.0					7.5
		45.0					7.5
5 MIN.		44.1	14.3	0.2	1.6	16.1	7.6
		43.3	14.7	0.2	1.6	16.5	7.6
10 MIN.		40.7	12.4	1.4	2.7	16.5	7.7
		40.4	12.4	1.6	3.2	16.2	7.6
15 MIN.		38.9	10.4	1.9	3.5	15.8	7.7
		36.0	11.7	1.9	3.2	16.8	7.8
20 MIN.	4.2	35.8	9.6	2.1	4.9	16.6	7.9
	3.1[α]	35.5	9.9	3.0	3.2	16.1	7.9
25 MIN.	6.1	33.8	7.6	5.0	4.0	16.6	7.9
	4.6[α]	35.5	6.5	4.2	3.2	15.9	7.9
30 MIN.	8.7	31.4	6.6	4.4	4.6	15.6	8.0
	8.3[α]	31.9	5.0	4.9	5.8	15.7	8.0
35 MIN.	9.4	30.3	5.2	6.0	4.4	15.6	8.1
	8.7[α]	28.5	3.9	4.9	6.3	15.1	8.0
40 MIN.	8.1	28.0	2.4	6.1	5.8	14.3	8.1
	10.0[α]	27.2	2.6	5.5	6.6	16.7	8.1

1 Same reaction medium as page 192.

2 Flasks closed at 5 minutes.

α Flasks started 60 minutes after duplicate.

TABLE LI

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (11.7 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES	pH
0 MIN.		45.0					6.0
		45.0					6.0
5 MIN.		42.2	8.9	5.2	1.7	15.8	6.0
		42.7	8.9	7.5	2.4	18.8	6.0
10 MIN.		38.1	8.3	5.7	2.0	16.0	6.0
		38.7	8.1	6.0	2.0	16.1	6.0
15 MIN.		37.3	7.7	6.0	2.1	15.8	6.0
		37.7	7.6	5.0	1.8	14.4	6.0
20 MIN.	3.6	26.7	6.6	6.3	2.1	15.0	6.0
	3.1[α]	26.4	6.9	4.8	1.9	13.6	6.0
25 MIN.	5.4	34.1	7.9	5.3	2.3	15.5	6.0
	5.1[α]	34.6	7.4	5.4	2.3	15.1	6.0
30 MIN.	8.2	31.2	7.3	6.8	1.9	16.0	6.0
	7.3[α]	30.9	6.8	5.8	2.3	14.9	6.0
35 MIN.	9.3	31.6	6.8	6.3	2.3	15.4	6.0
	7.3[α]	31.8	6.5	5.6	3.0	15.1	6.0
40 MIN.	10.0	28.3	6.2	5.7	3.1	15.0	6.0
	9.3[α]	30.3	7.0	5.2	3.0	15.2	6.0

1 Reaction medium A, page 50.

2 Flasks closed at 5 minutes.

α Flasks started 60 minutes after duplicates.

TABLE LII

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (15 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL	pH
			µMOLES				
0 MIN.		45.0					7.0
		45.0					7.0
5 MIN.		36.5	8.5	4.7	2.2	15.4	7.2
		36.1	7.7	5.2	2.0	15.0	7.2
10 MIN.		27.6	4.0	5.8	4.8	14.6	7.2
		30.1	5.3	7.5	4.2	17.0	7.2
15 MIN.		25.6	3.0	5.7	6.1	14.8	7.2
		24.5	3.3	7.0	6.7	17.0	7.2
20 MIN.	8.0	22.9	2.4	5.4	7.0	14.8	7.2
	5.2[α]	22.9	3.7	4.9	6.3	14.9	7.2
25 MIN.	8.8	19.3	1.1	4.4	9.6	15.1	7.2
	7.4[α]	19.1	1.6	5.1	9.3	16.0	7.3
30 MIN.	12.7	12.7	0.6	2.0	11.3	13.9	7.2
	10.6[α]	16.3	1.1	2.0	10.8	15.9	7.2
35 MIN.	13.1	13.6	0.5	2.2	13.1	15.8	7.2
	14.8[α]	14.8	0.5	1.9	12.8	15.2	7.2
40 MIN.	14.7	13.4	0.7	1.5	13.7	15.9	7.2
	13.8[α]	13.1	0.8	1.9	13.6	16.3	7.2

1 Reaction medium A, page 50, for N-acetylhistidine buffer.

2 Flasks closed at 5 minutes.

α Duplicate flasks started 60 minutes later.

TABLE LIII

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 8, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (14.4 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES	pH
0 MIN.		39.0					7.8
		39.0					7.8
5 MIN.		37.0	6.8	7.3	0.1	14.2	7.8
		37.5	6.7	7.8	0.1	14.6	7.8
10 MIN.		32.6	5.3	7.8	2.1	15.2	7.8
		27.8	5.8	6.4	2.6	14.8	7.8
15 MIN.		30.8	4.2	6.4	4.3	15.9	7.8
		26.4	3.8	7.0	4.9	15.7	7.8
20 MIN.	7.4	26.6	2.9	6.5	5.4	14.8	7.9
	7.4[α]	22.5	2.9	6.5	5.4	14.8	7.9
25 MIN.	10.9	25.6	1.9	5.6	7.4	14.9	7.9
	10.9[α]	20.6	1.9	5.6	7.4	14.9	7.9
30 MIN.	10.6	20.6	1.6	4.6	8.4	14.6	8.0
	12.9[α]	20.6	1.1	4.5	8.0	13.6	8.0
35 MIN.	8.5	19.0	1.1	4.0	10.1	15.2	8.0
	10.0[α]	19.0	0.8	3.8	9.7	14.3	8.0
40 MIN.	10.0	17.6	0.8	3.2	10.5	14.5	8.0
	10.5[α]	18.2	1.0	3.7	10.0	14.7	8.0

1 Reaction medium A, page 50, for Tris.

2 Flasks closed at 5 minutes.

α Duplicate flasks started 60 minutes later.

TABLE LIV

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (14.4 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES µMOLES AMP	ADP	ATP	TOTAL	pH
0 MIN.		45.0					6.0
		45.0					6.0
5 MIN.		36.8	7.1	6.1	2.1	15.3	6.0
		35.0	6.6	5.9	2.2	14.7	6.0
10 MIN.		31.2	4.1	6.7	4.7	15.5	6.0
		27.8	4.0	6.4	4.8	15.2	6.0
15 MIN.		26.0	3.1	6.0	5.6	14.7	6.0
		26.0	2.1	5.6	6.9	14.6	6.0
20 MIN.	10.5	25.4	2.4	5.7	6.9	15.0	6.0
	8.6[α]	24.4	1.5	4.6	8.4	14.5	6.0
25 MIN.	11.2	20.8	1.3	4.8	8.5	14.6	6.1
	11.2[α]	20.9	0.8	3.5	10.4	14.7	6.0
30 MIN.	15.2	12.8	0.3	1.6	12.8	14.7	6.0
	15.6[α]	14.2	0.4	1.4	12.7	14.5	6.0
35 MIN.	17.5	12.1	0.3	0.2	13.6	14.1	6.0
	17.5[α]	13.1	0.4	0.5	13.2	14.1	6.0
40 MIN.	20.0	12.1	0.4	0.0	14.0	14.4	6.0
	20.0[α]	12.4	0.4	0.8	13.3	14.5	6.0

1 Reaction medium A, page 50, for L-histidine.

2 Flasks closed at 5 minutes.

α Flasks started 60 minutes after duplicates.

TABLE LVI

SUCCINATE OXIDATION, pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (7.5 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			µMOLES			
5 MIN.		41.1	10.8	2.9	0.6	14.3
		40.6	10.5	3.2	0.5	14.2
10 MIN.		35.3	8.0	4.9	2.1	15.0
		34.8	9.3	4.3	2.1	15.7
15 MIN.	6.1	31.6	5.0	6.3	3.9	15.2
	6.0	34.0	7.1	5.0	2.9	15.0
20 MIN.	9.0	27.2	3.9	5.6	4.4	13.9
	9.0	28.6	4.2	5.1	4.3	13.6

1 Reaction medium A, page 50, for L-histidine.

2 Flasks closed at 5 minutes.

TABLE LVI-A

SUCCINATE OXIDATION, pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (7.5 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			µMOLES			
0 MIN.		48.0				
		49.4				
5 MIN.		43.5	11.1	1.9	0.8	13.8
		43.5	10.5	3.0	1.0	14.5
10 MIN.		34.0	7.8	3.3	0.8	11.9
		35.8	7.9	4.5	2.3	14.7
15 MIN.	6.1	29.5	5.7	5.6	4.1	14.4
	6.0	29.2	5.3	3.9	1.2	10.4
20 MIN.	7.5	28.9	5.9	5.6	4.1	15.6
	7.3	29.9	5.1	5.5	4.7	15.3

1 Reaction medium A, page 50, for N-acetylhistidine.

2 Flasks closed at 5 minutes.

TABLE LVI-B

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 8, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (7.5 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
0 MIN.		41.5				
		41.5				
5 MIN.		40.1	11.7	2.8	0.2	14.7
		40.0	11.1	2.9	0.0	14.0
10 MIN.		36.0	10.0	2.4	1.7	14.1
		38.1	9.3	3.0	2.8	14.1
15 MIN.	6.1	33.1	9.0	3.5	2.7	15.2
	6.0	33.7	8.3	3.8	3.0	15.1
20 MIN.	7.5	31.5	7.3	3.4	3.9	14.6
	7.3	30.4	7.1	3.4	4.3	14.8

1 Reaction medium A, page 50, for Tris.

2 Flasks closed at 5 minutes.

TABLE LVII

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (14.4 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
				µMOLES		
0 MIN.		45.8				
		45.8				
5 MIN.		42.4	6.9	5.7	0.8	13.3
		43.0	6.6	7.1	1.4	15.1
10 MIN.		39.0	5.7	6.0	2.6	14.3
		38.9	5.7	6.7	2.3	14.7
15 MIN.	9.3	33.4	3.7	5.7	3.7	13.1
	9.3	33.4	3.7	5.7	3.7	13.1
20 MIN.	9.9	32.8	4.8	6.4	5.2	16.4
	9.9	32.8	4.8	6.4	5.2	16.4

1 Reaction medium A, page 50, for L-histidine.

2 Flasks closed 5 minutes after thermal equilibration.

TABLE LVII-A

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 8, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (14.4 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
				µMOLES		
0 MIN.		45.3				
		45.3				
5 MIN.		41.5	9.2	6.7	0.5	16.4
		41.0	7.1	6.1	1.5	14.7
10 MIN.		37.1	7.1	5.3	2.4	14.8
		39.3	7.1	5.3	2.4	14.8
15 MIN.	7.2	34.2	4.4	5.4	3.9	13.7
	6.8	34.2	5.5	5.4	3.8	14.7
20 MIN.	12.6	29.7	3.2	6.0	5.6	14.8
	13.2	29.7	3.3	6.1	6.0	15.4

1 Reaction medium A, page 50, for Tris.

2 Flasks closed at 5 minutes.

TABLE LVII-3

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 7, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (14.4 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
0 MIN.		45.8				
		45.8				
5 MIN.		43.4	6.9	7.7	0.6	15.2
		43.3	7.9	7.4	0.5	15.8
10 MIN.		39.9	5.9	7.1	2.0	15.0
		41.6	7.1	6.9	2.3	16.3
15 MIN.	7.2	35.6	6.4	6.5	2.4	15.3
	7.2	35.6	7.8	6.0	2.5	16.3
20 MIN.	10.3	32.5	4.8	7.3	4.3	16.4
	9.7	33.1	3.7	7.7	4.3	15.7

1 Reaction medium A, page 50, for N-acetylhistidine.

2 Flasks closed at 5 minutes.

TABLE LIX

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (14.4 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			µMOLES			
0 MIN.		44.3	12.8	3.0	0.1	15.9
		44.3	12.6	3.0	0.1	15.7
5 MIN.		38.2	10.7	4.1	0.5	15.3
		36.0	9.1	4.1	0.5	13.7
10 MIN.		33.8	9.1	5.3	0.9	15.3
		34.7	8.6	4.8	0.9	14.3
15 MIN.		27.5	8.6	7.4	1.2	17.2
		29.2	6.2	5.7	1.2	13.1
20 MIN.	11.6	25.3	4.9	5.7	2.6	13.2
	10.8	26.4	5.8	6.9	2.7	15.4
pH 7 LOW OSMOLARITY						
0 MIN.		46.5	13.5	1.7	0.0	15.2
		46.5	14.2	2.1	0.0	16.3
5 MIN.		37.4	9.6	4.8	0.9	15.3
		36.9	10.6	4.6	0.7	15.9
10 MIN.		34.7	8.8	5.5	1.2	15.5
		29.4	8.2	4.6	1.5	14.3
15 MIN.		21.5	3.9	8.1	2.6	14.6
		20.6	4.8	6.2	3.0	14.0
20 MIN.	15.1	18.4	2.5	4.1	9.1	15.7
	12.6	20.9	1.5	3.7	10.6	15.8

1 Reaction medium A, page 50, for L-histidine. pH 7 reaction medium was neutralized with 0.027 M Tris.

2 Flasks closed at 5 minutes.

TABLE LIX-A

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 8, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (14.4 mgs protein)	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES µMOLES AMP	ADP	ATP	TOTAL
0 MIN.		43.7				
		43.7				
5 MIN.		39.9	12.5	3.7	0.1	16.3
		38.8	13.5	1.6	0.0	15.1
10 MIN.		32.3	10.7	5.3	0.3	16.3
		30.5	10.7	6.3	0.8	17.8
15 MIN.		24.5	6.7	6.1	2.0	14.8
		22.3	7.4	3.9	2.0	14.3
20 MIN.	12.7	22.5	2.6	9.2	4.0	15.8
	12.1	22.6	3.4	8.3	2.4	14.1
pH 7 LOW OSMOLARITY N-ACETYLHISTIDINE						
0 MIN.		44.6	11.7	4.0	0.0	15.7
		43.2	11.4	4.0	0.0	15.4
5 MIN.		36.6	9.3	4.8	0.7	14.8
		36.9	8.6	5.8	0.6	15.0
10 MIN.		27.0	7.7	6.4	1.6	15.7
		26.1	7.0	5.8	2.2	15.0
15 MIN.		22.8	3.7	8.5	2.3	14.5
		29.2	5.7	6.9	2.3	14.9
20 MIN.	13.8	19.5	3.1	5.0	4.7	12.8
	13.8	21.5	2.8	7.6	4.3	14.7

1 Reaction medium A, page 50, for Tris at pH 8 and N-acetylhistidine at pH 7.

2 Flasks closed at 5 minutes.

TABLE LX

β-HYDROXYBUTYRATE OXIDATION, pH 7.4, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (25 mgs protein)	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			μMOLES			
2)						
0 MIN.		50.0				
		50.0				
8 MIN.		37.2	8.9	6.1	0.6	15.6
		36.7	8.9	6.0	0.8	15.7
38 MIN.	5.3	22.1	8.3	6.3	0.8	15.4
	5.3	22.4	8.6	6.0	0.8	15.4
3)						
0 MIN.		50.0				
		50.0				
8 MIN.		42.1	7.8	5.8	1.9	15.5
		41.9	9.0	6.0	0.9	15.9
38 MIN.	5.3	33.6	9.1	5.9	0.7	15.7
	5.3	33.8	9.1	6.0	0.8	15.9

1 All reaction mixtures contained: .005 M AMP, .005 M $MgCl_2$, .015 M K_2HPO_4, .020 M Tris, .003 M KOH, .05 M Histidine, .03 M Glucose, 20 μg hexokinase.

2 Substrate: 0.020 M sodium β-hydroxybutyrate, Mann.

3 Substrate: 0.20 M β-hydroxybutyric acid, Matheson-Coleman, plus .028 M Tris.

TABLE LXI

β-HYDROXYBUTYRATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (14.4 mgs protein)	ACETO-ACETATE µMOLES	OXYGEN[2] UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL
0 MIN.	0.00		46.0	15.6	1.0	0.2	16.8
	0.00		46.0	15.6	1.0	0.2	16.8
5 MIN.	1.1		40.7	13.0	3.0	0.7	16.7
	0.9		40.4	13.1	2.4	0.7	16.2
10 MIN.	2.4		39.4	10.3	4.4	1.3	16.0
	2.5		40.2	10.9	3.4	1.2	15.5
15 MIN.	2.5	2.0	37.6	8.5	5.8	1.5	15.8
	2.5	1.9	37.9	9.4	5.7	1.8	16.9
20 MIN.	2.5	3.8	38.2	9.5	4.1	2.7	16.3
	3.0	2.3	37.1	9.4	5.3	2.4	16.1
30 MIN.	3.9	4.2	35.2	7.9	5.0	3.4	16.3
	3.8	3.1	34.2	7.3	5.3	3.4	16.0
40 MIN.	4.2	5.3	32.4	7.1	4.3	4.5	15.9
	4.8	5.6	33.2	7.6	4.5	4.1	16.1
75 MIN.	7.0	8.7	28.9	5.6	5.5	4.7	15.8
	7.2	8.9	29.9	4.3	5.6	6.0	15.9

1 Reaction medium contained: 0.005 M AMP, 0.15 M HK_2PO_4, 0.005 M $MgCl_2$, 0.050 M N-acetylhistidine, 0.25 M sucrose, 0.028 M Tris, pH 7, .020 M Na betahydroxybutyrate, Mann.

2 Flasks closed at 5 minutes, after thermal equilibration.

TABLE LXI-A

β-HYDROXYBUTYRATE OXIDATION, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA	RATE, μATOMS/MINUTE/MILLIGRAM[1]
10 MIN.	.011 , .010 , .016 , .014 , .015 .014 , .010 , .014 , .010 , .014
15 MIN.	.014 , .013 , .017[α], .011[α], .014[α] .010[α], .010[α], .012[α], .009[α], .009[α]
20 MIN.	.018 , .011 , .013[α], .009[α] .011[α], .011[α], .009[α], .009[α]
25 MIN.	.012 , .008 , .010[α] .010[α], .010[α], .010[α]
30 MIN.	.012 , .009 , .011[α] .011[α], .010[α], .010[α]
35 MIN.	.011 , .011 , .011 , .010
40 MIN.	.011 , .011 , .010[α], .011[α]
45 MIN.	.010 , .010
50 MIN.	.010 , .010
55 MIN.	.010 , .010
60 MIN.	.010 , .010
65 MIN.	.009 , .009
70 MIN.	.009 , .009

1 Pseudo Rate, page 42.

α Values not included in Table LXI.

TABLE LXII

β-HYDROXYBUTYRATE OXIDATION, pH 7, HIGH OSMOLARITY

WITHOUT MAGNESIUM

TIME AFTER MITOCHONDRIA[1] (31 mgs protein)	ACETO-ACETATE μMOLES	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES AMP μMOLES	ADP	ATP	TOTAL
0 MIN.			46.0				
			46.0				
5 MIN.	0.9		40.3	13.1	2.9	0.8	16.8
	1.5		40.0	10.8	4.5	1.5	16.8
10 MIN.	2.0		40.0	10.0	5.7	1.6	17.3
	2.0		39.6	10.0	5.7	1.6	17.3
15 MIN.	2.4		40.0	9.8	5.5	1.7	17.0
	2.4		39.6	10.7	5.9	1.9	18.5
20 MIN.	2.4	5.8	38.2	9.8	5.5	1.7	17.0
	2.4	4.8	37.1	10.7	5.9	1.9	18.5

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.010, .008
15 MIN.	.012, .009
20 MIN.	.013, .011

1 Reaction medium contained 0.015 M Na β-hydroxybutyrate, 0.050 M N-acetylhistidine, 0.015 M K_2HPO_4, 0.25 M Sucrose, 0.005 M AMP, 0.026 M Tris, no magnesium.

2 Pseudo Rate, page 42.

TABLE LXII-A

β-HYDROXYBUTYRATE OXIDATION, pH 7, HIGH OSMOLARITY

WITH 0.0016 M MAGNESIUM CHLORIDE

TIME AFTER MITOCHONDRIA[1] (31 mgs protein)	ACETO-ACETATE µMOLES	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL µMOLES
0 MIN.			46.2				
			46.2				
5 MIN.	0.8		41.9	11.5	4.5	0.9	16.9
	1.0		44.1	11.5	4.5	0.9	16.9
10 MIN.	1.7		40.0	11.2	4.1	1.5	16.8
	1.9		40.4	11.2	4.1	1.5	16.8
15 MIN.	2.4		37.6	9.9	5.2	1.5	16.6
	2.3		37.1	9.9	5.2	1.5	16.6
20 MIN.	2.7	4.7	36.2	9.8	5.3	1.9	17.0
	2.2	4.6	37.1	8.8	6.3	2.7	17.8

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION µATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.007, .008
15 MIN.	.010, .009
20 MIN.	.010, .010

1 Same reaction medium as LXII plus 0.0016 M magnesium

2 Pseudo Rate, page 42.

TABLE LXII-B

β-HYDROXYBUTYRATE OXIDATION, pH 7, HIGH OSMOLARITY

WITH 0.005 M MAGNESIUM CHLORIDE

TIME AFTER MITOCHONDRIA[1] (31 mgs protein)	ACETO-ACETATE µMOLES	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP	ATP	TOTAL
0 MIN.			45.8 45.7				
5 MIN.	0.8 0.8		43.3 43.3	13.1 13.1	2.9 2.9	0.8 0.8	16.8 16.8
10 MIN.	1.8 1.8		40.2 41.3	10.7 10.7	4.5 4.5	1.1 1.1	16.3 16.3
15 MIN.	2.3 2.0		39.6 39.6	12.3	4.8	1.7	18.8
20 MIN.	2.2 2.6	4.2 3.5	37.9 36.8	10.8 10.8	4.9 4.8	1.7 1.8	17.4 17.4

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION µATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.006, .005
15 MIN.	.008, .007
20 MIN.	.009, .008

1 Same reaction medium as LXII plus 0.005 M magnesium.

2 Pseudo Rate, page 42.

TABLE LXII-C

β-HYDROXYBUTYRATE OXIDATION, pH 7, HIGH OSMOLARITY

WITH 0.010 M MAGNESIUM CHLORIDE

TIME AFTER MITOCHONDRIA[1] (31 mgs protein)	ACETO-ACETATE µMOLES	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP	ATP	TOTAL
0 MIN.			46.4 46.4				
5 MIN.	0.7 0.8		43.0 43.0	12.0	3.6	0.7	16.3
10 MIN.	1.4 1.4		41.0 40.5	12.3	3.7	1.0	17.0
15 MIN.	1.5 1.8		40.2 39.2	9.8	5.2	1.6	16.6
20 MIN.	1.9 1.9	3.8 3.8	37.9 37.9	9.3	5.3	1.8	16.4

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION µATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.007, .008
15 MIN.	.008, .008
20 MIN.	.009, .009

1 Same as reaction medium in Table LXII plus 0.010 M magnesium.

2 Pseudo Rate, page 42.

TABLE LXIII

β-HYDROXYBUTYRATE OXIDATION, pH 7, HIGH OSMOLARITY

WITHOUT MAGNESIUM

TIME AFTER MITOCHONDRIA[1] (29 mgs protein)	PHOSPHATE REMAINING μMOLES	OXYGEN UPTAKE μATOMS	ACETOACETATE μMOLES
0 MIN.	45.0 45.0		
5 MIN.	42.7 40.3		1.0 1.0
10 MIN.	41.6 41.9		1.5 1.8
15 MIN.	40.2 40.2		1.7 1.6
20 MIN.	40.5 40.8	3.3 4.3	1.7 1.8

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.006, .007
15 MIN.	.007, .006
20 MIN.	.008, .010

1 Same reaction medium as LXII, without magnesium

2 Pseudo Rate, page 42.

TABLE LXIII-A

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION IN A MAGNESIUM FREE MEDIUM, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (29 mgs protein)	PHOSPHATE REMAINING μMOLES	OXYGEN UPTAKE μATOMS	ACETOACETATE μMOLES
0 MIN.	45.2 45.2		
5 MIN.	44.3 44.9		0.7 0.7
10 MIN.	47.3 46.2		1.0 0.9
15 MIN.	47.0 46.5		1.1 0.7
20 MIN.	48.4 50.3	1.4 2.6	0.8 0.9

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.002, .003
15 MIN.	.002, .005
20 MIN.	.003, .006

1 Same reaction medium as LXIII, with .0003 M dinitrophenol.

2 Pseudo Rate, page 42.

TABLE LXIII-B

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION IN A MAGNESIUM FREE MEDIUM, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (29 mgs protein)	PHOSPHATE REMAINING μMOLES	OXYGEN UPTAKE μATOMS	ACETOACETATE μMOLES
0 MIN.	44.7		0.0
	44.8		0.0
5 MIN.	46.5		0.6
	45.4		0.7
10 MIN.	46.5		0.5
	47.1		0.7
15 MIN.	46.0		0.7
	47.6		0.9
20 MIN.	45.2	0.4	0.8
	48.7	0.6	0.8

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.002, .000
15 MIN.	.001, .001
20 MIN.	.001, .001

1 Same reaction medium as LXIII, with 0.00003 M dinitrophenol.

2 Pseudo Rate, page 42.

TABLE LXIII-C

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION IN A MAGNESIUM FREE MEDIUM, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (29 mgs protein)	PHOSPHATE REMAINING μMOLES	OXYGEN UPTAKE μATOMS	ACETOACETATE μMOLES
0 MIN.	46.0 46.0		
5 MIN.	43.5 43.0		1.0 0.7
10 MIN.	44.1 44.1		1.0 0.9
15 MIN.	43.3 44.6		1.5 0.7
20 MIN.	44.6 44.9	2.9 2.6	1.7 1.7

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.004, .005
15 MIN.	.005, .005
20 MIN.	.007, .006

1 Same reaction medium as LXIII, with 0.000003 M dinitrophenol.

2 Pseudo Rate, page 42.

TABLE LXIV

THE EFFECT OF TIPPING IN DINITROPHENOL ON

β-HYDROXYBUTYRATE OXIDATION, pH 7, HIGH OSMOLARITY

	TIME AFTER MITOCHONDRIA[5] (10 mgs protein)	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL μMOLES	ACETO-ACETATE μMOLES
	0 MIN.		49.0	16.1	1.0	0.6	17.7	
			49.0	15.7	1.0	0.6	17.3	
	5 MIN.		43.2	12.9	5.5	0.8	19.2	
			43.2	13.1	4.7	0.8	18.6	
	10 MIN.		43.2	12.2	5.1	1.2	18.5	
			43.7	12.2	6.1	1.0	19.3	
	15 MIN.		41.5	11.6	5.7	1.5	17.8	
			41.5	11.4	6.3	1.3	19.0	
	20 MIN.		41.5	11.1	6.3	1.6	19.0	
			41.5	10.0	5.5	2.0	17.5	
1*	40 MIN.	8.7	38.6	9.6	6.5	2.5	18.6	8.7
		9.0	40.0	9.6	6.8	2.5	18.9	9.0
2*	40 MIN.	6.8	44.7	12.8	5.1	0.9	18.8	5.8
		5.6	46.8	12.9	5.0	0.9	18.8	5.6
3*	40 MIN.	6.2	46.4	13.4	5.0	0.8	19.2	6.2
		6.8	45.5	13.8	6.2	0.7	20.7	6.8
4*	40 MIN.	4.5	42.8	11.3	6.2	1.3	18.8	4.5
		6.8	42.8	11.3	6.0	1.3	18.6	6.8

1* 0.5 cc water was tipped from the side arm at 20 minutes.
2* 1 μmole dinitrophenol was tipped from the side arm at 20 minutes.
3* 0.1 μmole dinitrophenol was tipped from the side arm at 20 minutes.
4* 0.01 μmole dinitrophenol was tipped from the side arm at 20 minutes.
5 Same reaction medium as LXIII.

TABLE LXV

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION

pH 7, HIGH OSMOLARITY, WITHOUT DINITROPHENOL

TIME AFTER MITOCHONDRIA[1] (36 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES	ACETO-ACETATE µMOLES
0 MIN.		44.8	17.7	0.7	0.0	18.4	0.0
		44.8	17.7	0.7	0.0	18.4	0.0
5 MIN.		38.0	12.5	3.6	1.7	17.8	1.4
		38.0	12.0	4.1	1.7	17.8	1.4
10 MIN.		33.3	9.7	5.0	2.7	17.4	3.0
		33.3	9.7	5.5	2.9	18.1	3.1
15 MIN.		32.2	8.6	6.0	3.6	18.2	3.3
		31.0	8.8	5.6	3.6	18.0	3.6
20 MIN.	6.5	31.0	6.8	6.3	4.5	17.6	4.9
	7.6	30.8	7.2	6.7	4.6	18.5	5.1
	5.6	29.1	8.2	6.3	3.9	18.4	4.3

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION µATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.011, .012, .006
15 MIN.	.012, .014, .012
20 MIN.	.012, .014, .010

1 Same reaction medium as LXIII.

2 Pseudo Rate, page 42.

TABLE LXV-A

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION

pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (36 mgs protein)	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL μMOLES	ACETO-ACETATE μMOLES
0 MIN.		45.2	17.7	0.9	0.0	18.6	0.0
		45.2					0.0
5 MIN.		40.7	13.2	4.1	1.2	18.5	1.0
		41.8	14.3	3.2	0.9	18.4	1.3
10 MIN.		34.4	11.6	4.8	1.3	17.7	2.1
		35.8	11.7	4.8	1.8	18.3	2.0
15 MIN.		35.0	9.9	6.1	2.6	18.6	3.2
		34.4	9.9	6.1	2.3	18.3	4.2
20 MIN.	5.8	35.0	9.5	6.2	2.6	18.3	5.4
	6.5	35.8	10.4	5.8	2.6	18.8	4.0

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.008[α], .010, .010
15 MIN.	.004[α], .006, .006
20 MIN.	.008[α], .011, .012

1 Same reaction medium as LXIII, plus 0.003 mM dinitrophenol.

2 Pseudo Rate, page 42.

α Triplicate value not included in oxygen uptake values.

TABLE LXV-B

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION

pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (36 mgs protein)	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES AMP μMOLES	ADP μMOLES	ATP μMOLES	TOTAL μMOLES	ACETO-ACETATE μMOLES
0 MIN.		45.5	17.4	1.1	0.0	18.5	
		45.5					
5 MIN.		45.8	17.6	0.8	0.0	18.4	0.7
		45.8	17.0	1.0	0.1	18.1	0.7
10 MIN.		46.8	17.7	0.8	0.1	18.6	1.6
		47.7	17.6	1.0	0.2	18.8	1.6
15 MIN.		46.6	17.0	1.4	0.1	18.5	3.5
		46.6	17.7	1.0	0.1	18.8	4.2
20 MIN.	4.8	46.5	17.8	1.5	0.1	19.4	3.0
	5.7	46.0	17.6	1.2	0.1	18.9	3.0

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.005, .005, .007[α]
15 MIN.	.006, .009, .009[α]
20 MIN.	.009, .011, .010[α]

1 Same reaction medium as LXIII, plus 0.03 mM dinitrophenol.

2 Pseudo Rate, page 42.

α Triplicate value not included in total oxygen consumption.

TABLE LXV-C

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION

pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (36 mgs protein)	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL	ACETO-ACETATE μMOLES
			μMOLES				
0 MIN.		45.0	17.7	0.7	0.0	18.4	0.0
		45.0					0.0
5 MIN.		43.5	16.7	1.1	0.0	17.8	0.7
		45.5	17.5	0.6	0.0	18.1	0.6
10 MIN.		44.7	17.4	0.6	0.0	18.0	1.4
		44.7	17.5	0.7	0.0	18.2	1.4
15 MIN.		46.0	17.4	0.6	0.0	18.0	3.2
		46.2	17.1	0.6	0.0	17.7	3.2
20 MIN.	5.0	46.0	17.7	0.9	0.1	18.7	3.4
	5.2	46.0	17.6	0.6	0.1	18.3	4.0

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.006, .004α, .006
15 MIN.	.007, .008α, .009
20 MIN.	.009, .008α, .010

1 Same reaction medium as LXIII, plus 0.33 mM dinitrophenol.

2 Pseudo Rate, page 42.

α Triplicate value not included in total oxygen consumption.

TABLE LXVI

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION IN THE PRESENCE OF ADP, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (28.8 mgs protein)	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES μMOLES AMP	ADP	ATP	TOTAL	ACETO-ACETATE μMOLES
0 MIN.		49.5	2.9	10.5	1.4	14.8	0.0
		49.5	2.9	10.5	1.4	14.8	0.0
5 MIN.		42.1	2.5	8.7	4.5	15.7	1.4
		42.1	2.5	8.7	4.5	15.7	1.4
10 MIN.		40.7	1.1	7.2	7.1	15.4	2.6
		42.1	1.2	8.0	6.0	15.2	3.2
15 MIN.		40.3	1.0	9.1	5.2	15.3	3.3
		41.2	1.2	8.7	4.2	14.1	3.9
20 MIN.	7.1	40.3	0.9	8.4	5.9	15.2	3.8
	7.0	40.7	0.9	8.8	4.8	14.6	3.9

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.014, .018, .016[α]
15 MIN.	.016, .016, .016[α]
20 MIN.	.016, .016, .016[α]

1 Reaction medium contained: 0.015 M Na β-hydroxybutyrate, 0.050 M N-acetylhistidine, .015 M K_2HPO_4, 0.25 M sucrose, 0.005 M $MgCl_2$, 0.005 M NaADP, 2 H_2O, 0.025 M Tris.

2 Pseudo Rate, page 42.

α Triplicate value not included in total oxygen consumption.

TABLE LXVI-A

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION IN THE PRESENCE OF ADP, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL μMOLES	ACETO-ACETATE μMOLES
0 MIN.		50.7	5.1	8.3	2.5	15.9	0.0
		50.7	4.8	8.0	2.2	15.0	0.0
5 MIN.		45.0	6.9	6.6	1.8	14.3	2.1
		45.0	7.4	6.8	1.6	15.8	2.1
10 MIN.		44.2	6.4	7.8	1.4	15.6	3.9
		45.0	5.9	8.2	1.7	15.8	3.5
15 MIN.		45.3	4.8	7.4	3.2	15.4	4.9
		45.0	4.1	7.1	3.9	15.1	4.6
20 MIN.	6.3	46.6	4.2	7.8	4.7	16.7	5.3
	5.3	46.2	5.1	7.7	4.1	16.9	5.1

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.017, .014, .018[α]
15 MIN.	.015, .014, .017[α]
20 MIN.	.015, .012, .017[α]

1 Same reaction medium as LXVI, plus 0.000003 M dinitrophenol.

2 Pseudo Rate, page 42.

α Triplicate value not included in total oxygen consumption.

TABLE LXVI-B

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION IN THE PRESENCE OF ADP, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES μMOLES AMP	ADP	ATP	TOTAL	ACETO-ACETATE μMOLES
0 MIN.		50.0	7.6	7.8	2.1	17.5	0.0
		50.0	6.2	8.6	2.0	16.8	0.0
5 MIN.		50.4	8.1	6.9	0.6	15.6	2.1
		50.9	9.8	6.3	0.6	16.7	2.1
10 MIN.		52.9	9.8	5.2	1.4	16.4	3.9
		51.2	9.0	6.6	0.9	16.5	3.5
15 MIN.		53.4	9.9	5.9	0.7	16.5	4.9
		53.4	10.2	5.9	0.7	16.8	4.6
20 MIN.	6.2	58.5	10.2	5.8	0.6	16.6	5.3
	6.2	57.5	10.5	5.7	0.5	16.7	5.4

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.020, .020, .020[α]
15 MIN.	.016, .017, .019[α]
20 MIN.	.015, .015, .016[α]

1 Same reaction medium as LXVI, plus 0.00003 M dinitrophenol.

2 Pseudo Rate, page 42.

α Triplicate value not included in total oxygen consumption.

TABLE LXVI-C

THE EFFECT OF DINITROPHENOL ON β-HYDROXYBUTYRATE OXIDATION IN THE PRESENCE OF ADP, pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1]	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES μMOLES AMP	ADP	ATP	TOTAL	ACETO-ACETATE μMOLES
0 MIN.		48.2	4.4	9.6	2.7	16.7	0.0
		48.2	4.6	9.9	3.1	17.6	0.0
5 MIN.		51.7	4.8	10.0	3.2	18.0	1.5
		51.4	5.2	9.9	3.2	18.3	1.4
10 MIN.		54.6	6.5	9.2	2.1	17.8	3.1
		54.6	6.8	7.6	2.3	16.7	2.5
15 MIN.		55.5	7.0	7.7	2.1	16.8	2.6
		56.2	8.3	7.2	1.6	17.1	3.6
20 MIN.	5.0	57.2	8.4	7.4	1.5	17.3	4.3
	6.5	57.5	8.5	6.6	1.4	16.5	4.3

TIME AFTER MITOCHONDRIA	RATE OF OXIDATION μATOMS/MINUTE/MILLIGRAM PROTEIN[2]
10 MIN.	.012, .014
15 MIN.	.012, .017
20 MIN.	.012, .015

1 Same reaction medium as LXVI, plus 0.0003 M dinitrophenol.

2 Pseudo Rate, page 42.

TABLE LXVII

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES µMOLES AMP	ADP	ATP	TOTAL	NAD
1)							
30 SEC.		44.6	12.9	4.1	0.0	17.0	0.2
		44.8	11.9	3.1	0.0	15.0	0.2
10 MIN.		41.1	9.5	5.6	1.0	16.1	0.5
		41.4	8.9	5.4	1.4	15.7	1.0
40 MIN.	18.0	27.1	1.1	7.0	9.5	16.6	1.0
	17.5	27.6	1.4	7.0	9.6	18.0	1.4
2)							
30 SEC.		45.4	11.7	4.3	0.0	16.0	
		45.4	11.0	4.9	1.5	15.4	
10 MIN.		35.9	5.7	6.8	1.3	13.8	0.0
		35.9	4.3	7.1	1.8	13.2	0.0
40 MIN.	19.0	22.1	0.0	6.0	8.6	14.6	1.2
		22.3	0.6	3.9	12.0	16.5	1.2

1 Reaction medium A, page 50, for N-acetylhistidine.

2 Reaction medium A, page 50, for histidylhistidine.

TABLE LXVII-A

THE EFFECT OF DINITROPHENOL ON SUCCINATE OXIDATION

pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES µMOLES AMP	ADP	ATP	TOTAL	NAD
1)							
30 SEC.		44.8	14.1	1.7	0.0	15.8	0.0
		44.8	14.1	3.0	0.0	17.1	0.0
10 MIN.		49.6	9.3	5.6	1.0	15.9	0.5
		49.6	9.4	5.0	1.0	15.4	0.7
40 MIN.	17.1	51.5	12.5	4.1	0.0	16.6	0.7
	16.0	51.5	12.5	4.1	0.0	16.6	0.7
2)							
30 SEC.		50.8	13.8	2.5	0.0	16.3	0.1
		50.8	13.8	3.2	0.0	17.0	0.6
10 MIN.		50.8	13.4	2.2	0.3	15.9	0.8
		51.8	14.2	2.1	0.0	16.3	0.8
40 MIN.	11.5	51.7	12.5	3.9	0.3	16.7	2.0
	11.4	51.7	12.6	4.0	0.0	15.6	2.7
3)							
30 SEC.		48.8	16.6	2.9	0.0	19.5	0.6
		48.5	16.8	5.0	0.0	21.8	0.6
10 MIN.		52.0	16.0	2.2	0.0	18.2	1.2
		52.0	15.9	2.4	0.0	18.3	1.1
40 MIN.	7.3	50.0	13.7	2.6	0.0	16.3	3.6
	7.3	49.0	13.7	2.6	0.0	16.3	3.6

1 Reaction medium A, page 50, plus 0.000003 M dinitrophenol.
2 Reaction medium A, page 50, plus 0.00003 M dinitrophenol.
3 Reaction medium A, page 50, plus 0.0003 M dinitrophenol.

TABLE LXVII-B

THE EFFECT OF DINITROPHENOL ON SUCCINATE OXIDATION

pH 7, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA		μATOMS OXYGEN CONSUMED				
		1*	2*	3*	4*	5*
15	MIN.	4.1	4.3	5.0	1.5	1.2
		2.3		5.1	2.3	1.2
		3.6		5.1		
20	MIN.	8.1	11.0	9.3	3.3	2.2
		5.5	8.1	10.2	4.6	2.1
		7.4		7.5		
25	MIN.	12.3	11.0	10.7	5.4	4.1
		11.8		12.6	6.4	3.1
		9.0		12.6		
30	MIN.	16.0	13.5	12.0	7.4	4.8
		14.8		14.5	8.5	4.2
		13.3		13.9		
35	MIN.	18.5	16.0	12.9	9.4	5.7
		17.0		15.9	10.7	5.2
		15.6		14.8		
40	MIN.	20.0	19.0	14.3	11.5	7.3
		18.0		17.1	11.4	7.3
		17.5		16.0		

1* Reaction medium A, page 50, for N-acetylhistidine.
2* Reaction medium A, page 50, for Histidylhistidine.
3* Same as 2 plus 0.000003 M dinitrophenol.
4* Same as 2 plus 0.00003 M dinitrophenol.
5* Same as 2 plus 0.0003 M dinitrophenol.

TABLE LXVIII

SUCCINATE OXIDATION, pH 6, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA[1] (7.5 mg/ml)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			µMOLES			
0 MIN.		45.0	12.2	3.7	0.0	15.9
		45.1	13.3	2.0	0.0	15.3
5 MIN.		40.0	9.9	4.0	1.8	15.7
		41.1	9.9	4.0	1.6	15.5
20 MIN.	15.5	25.0	1.4	4.5	8.8	14.7
	14.8	25.2	2.0	4.7	8.8	15.5
40 MIN.	28.9	23.0	0.9	4.9	9.3	15.1
	28.8	23.2	0.9	4.9	9.1	14.9

1 Reaction medium A, page 50, for histidylhistidine.

TABLE LXVIII-A

SUCCINATE OXIDATION, pH 6, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE μATOMS
10 MIN.	5.8, 6.3[α], 6.0 5.6, 5.1 , 5.2[α]
15 MIN.	11.2, 11.5[α], 11.0 11.4, 11.3 , 10.3[α]
20 MIN.	15.5, 16.2[α], 14.8 15.7, 16.1 , 14.3[α]
25 MIN.	10.0, 19.7 , 17.5[α]
30 MIN.	22.5, 22.5 , 20.5[α]
35 MIN.	25.7, 26.8 , 23.0[α]
40 MIN.	28.9, 28.8 , 25.3[α]

α Triplicate value not included in total oxygen consumption, Table LXVIII.

TABLE IXIX

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL	pH
			µMOLES				
1)							
30 SEC.		45.0	12.0	1.2	0.4	13.6	6.1
		45.0	12.5	1.0	0.3	13.8	6.1
5 MIN.		37.3	7.5	3.8	3.2	14.5	
		37.3	7.3	3.9	2.6	13.8	
20 MIN.	15.1	16.0	0.2	2.0	12.1	14.3	6.15
	17.7	16.9	0.0	2.2	12.1	14.3	6.15
2)							
30 SEC.		45.0	13.2	2.1	0.2	15.5	6.0
		45.0	13.0	2.0	0.3	15.3	6.0
5 MIN.		30.0	6.1	3.4	1.8	11.3	
		30.0	7.4	4.9	2.7	15.0	
20 MIN.	14.8	17.6	0.1	2.2	12.2	14.5	6.1
	12.7	17.3	0.1	2.2	12.1	14.5	6.1
3)							
30 SEC.		45.0	11.8	3.1	0.3	15.2	6.0
		45.0	11.5	3.8	2.5	15.8	6.0
5 MIN.		35.3	7.3	4.4	2.9	14.6	
		35.3	7.8	3.8	2.5	14.1	
20 MIN.	14.8	18.0	0.6	2.7	10.2	13.5	6.0
		18.1	0.0	1.5	12.8	14.3	6.0

1 Reaction medium A, page 50, for no buffer.
2 Reaction medium A, page 50, for histidylhistidine.
3 Reaction medium A, page 50, for L-histidine.

TABLE LXIX-A

SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

pH 6, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE, μATOMS		
	1*	2*	3*
10 MIN.	2.1[α], 4.3 4.2	5.3, 4.8[α] 4.0	5.7
15 MIN.	5.8[α], 9.1 12.8	10.7, 8.4[α] 9.1	10.6
20 MIN.	5.7[α], 15.1 17.7	14.8, 11.0[α] 12.7	14.8

1* Reaction medium A, page 50, for no buffer.

2* Reaction medium A, page 50, for Histidylhistidine.

3* Reaction medium A, page 50, for L-histidine.

α Data not included in Table LXIX.

TABLE LXX

pH STUDY OF SUCCINATE OXIDATION, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA (15 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			µMOLES			
pH 6, 1*						
0 MIN.		45.8				
		45.8				
5 MIN.		33.6	6.9	5.1	3.4	15.4
		33.6	5.8	4.0	2.0	12.8
20 MIN.	11.4	19.3	1.3	3.6	9.0	13.9
	10.7	19.0	1.3	3.9	9.3	14.5
	10.7					
pH 6.5, 1*						
0 MIN.		44.8				
		44.8				
5 MIN.		34.1	7.1	4.4	2.9	14.4
		34.1	6.4	5.1	2.9	14.4
20 MIN.	10.0	20.0	2.9	5.5	6.0	14.4
	10.2	20.1	2.2	4.1	9.1	15.4
	10.9					
pH 7, 1*						
0 MIN.		45.2				
		45.2				
5 MIN.		33.6	7.1	5.1	3.8	16.0
		33.7	6.3	5.4	3.3	15.0
20 MIN.	10.0	20.3	1.7	4.0	8.7	14.4
	10.3		1.7	3.8	9.1	14.6
	9.6					

1* Reaction medium A, page 50, for histidylhistidine.

TABLE LXX-A

pH STUDY OF SUCCINATE OXIDATION, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			µMOLES			
pH 7.5, 1*						
0 MIN.		45.1				
		45.1				
5 MIN.		33.5	6.6	5.6	3.9	16.1
		32.7	7.3	5.3	3.5	16.1
20 MIN.	10.7	18.2	1.1	2.8	11.8	15.7
	9.1	18.8	1.1	2.8	11.8	15.7
	10.3					
pH 8, 1*						
0 MIN.		44.2				
		44.2				
5 MIN.		34.7	7.1	5.4	2.9	15.4
		35.0	7.5	4.6	3.2	15.3
20 MIN.	11.1	18.2	1.9	3.5	11.8	17.2
	11.1	18.2	1.2	2.9	13.4	16.5
	9.4					

1* Reaction medium A, page 50, for histidylhistidine.

TABLE LXX-B

pH STUDY OF SUCCINATE OXIDATION, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
pH 6, 2*						
0 MIN.		45.4				
		45.4				
5 MIN.		33.6	5.8	5.5	3.2	14.5
		33.6	6.5	5.3	3.2	15.0
20 MIN.	10.7	20.2	1.4	3.9	10.2	15.5
	10.5	20.7	1.9	3.6	10.4	14.9
pH 6, 3*						
0 MIN.		47.1				
		47.1				
5 MIN.		34.7	6.7	4.6	2.7	14.0
		34.1	6.5	5.0	2.8	14.3
20 MIN.	10.0	17.3	1.5	3.6	9.5	14.6
	10.0	17.7	1.8	3.6	9.0	14.4
	10.0					
pH 7, 4*						
0 MIN.		44.4				
		44.4				
5 MIN.		33.6	6.9	5.4	3.3	15.6
		33.7	6.7	4.0	3.8	14.5
20 MIN.	10.9	18.1	2.1	3.8	8.2	14.1
	10.7	18.1	2.3	5.1	6.9	14.3
	10.4					

2* Reaction medium A, page 50, for L-histidine.

3* Reaction medium A, page 50, for no buffer.

4* Reaction medium A, page 50, for N-acetylhistidine.

TABLE LXX-C

pH STUDY OF SUCCINATE OXIDATION, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			µMOLES			
pH 8, 5*						
0 MIN.		44.0				
		44.0				
5 MIN.		34.3	7.5	4.6	3.6	15.7
		34.1	7.4	4.8	3.6	15.8
20 MIN.	11.4	19.6	1.8	3.5	11.3	16.6
	11.4	20.2	1.9	4.0	11.4	17.3
	9.1					

5* Reaction medium A, page 50, for Tris.

TABLE LXX-D

pH STUDY OF SUCCINATE OXIDATION, LOW OSMOLARITY

TIME AFTER MITOCHONDRIA		OXYGEN UPTAKE[α] pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8
1*						
10	MIN.	.066	.050	.048	.054	.058
		.062	.052	.052	.046	.056
		.060	.052	.050	.050	.058
15	MIN.	.055	.054	.049	.053	.057
		.055	.053	.051	.056	.056
		.054	.053	.047	.052	.049
20	MIN.	.051	.045	.045	.048	.050
		.048	.048	.046	.041	.050
		.048	.049	.043	.046	.042
		pH 6 2*	pH 6 3*	pH 7 4*	pH 8 5*	
10	MIN.	.058	.044	.048	.070	
		.064	.052	.054	.052	
			.050	.062	.052	
15	MIN.	.060	.047	.051	.053	
		.056	.052	.054	.054	
			.050	.054	.057	
20	MIN.	.048	.045	.049	.051	
		.047	.045	.048	.051	
			.045	.047	.041	

1* Reaction medium A, page 50, for Histidylhistidine.
2* Reaction medium A, page 50, for no buffer.
3* Reaction medium A, page 50, for Histidine.
4* Reaction medium A, page 50, for N-acetylhistidine.
5* Reaction medium A, page 50, for Tris.
α Pseudo Rate, page 42.

TABLE LXXI

pH STUDY OF SUCCINATE OXIDATION, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (14.4 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
pH 6, 1*						
0 MIN.		46.3				
		46.0				
5 MIN.		43.8	7.8	4.2	2.2	14.2
		43.0	7.9	3.7	2.1	13.7
20 MIN.	5.0	35.0	5.7	4.9	5.6	15.2
	6.0	34.8	2.3	4.4	9.0	15.7
	4.3					
pH 6.5, 1*						
0 MIN.		46.7				
		46.1				
5 MIN.		41.8	9.7	2.0	1.0	12.7
		41.1	11.1	2.5	0.8	14.4
20 MIN.	7.1	30.8	1.9	4.7	9.0	15.6
	7.1	30.8	1.0	4.0	11.2	16.2
	7.1					
pH 7, 1*						
0 MIN.		45.8				
		45.7				
5 MIN.		39.8	6.5	4.5	3.1	14.1
		39.5	6.5	4.2	3.0	13.7
20 MIN.	8.0	27.1	1.5	4.6	8.1	14.2
	8.2	26.2	1.6	4.5	8.2	14.3
	11.2					

1* Reaction medium A, page 50, for histidylhistidine.

TABLE LXXI-A

pH STUDY OF SUCCINATE OXIDATION, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (14.4 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
pH 7.5, 1*						
0 MIN.		44.9				
		45.0				
5 MIN.		39.0	6.1	6.7	3.0	15.8
		40.1	8.1	4.4	2.0	14.5
20 MIN.	9.3	20.0	1.3	2.6	9.2	13.1
	8.5	19.6	1.8	4.4	9.2	15.4
	9.7					
pH 8, 1*						
0 MIN.		38.3				
		39.4				
5 MIN.		33.8	7.9	4.5	2.5	14.9
		33.5	6.9	3.9	2.6	13.4
20 MIN.	9.3	18.0	2.3	4.0	9.4	15.7
	8.9	18.4	2.9	4.0	10.7	17.6
	8.9					

1* Reaction medium A, page 50, for histidylhistidine.

TABLE LXXI-B

pH STUDY OF SUCCINATE OXIDATION, HIGH OSMOLARITY

TIME AFTER MITOCHONDRIA (14.4 mgs protein)	RATE, μATOMS/MINUTE/MILLIGRAM[1] pH 6	pH 6.5	pH 7	pH 7.5	pH 8
10 MIN.	.024[α]	.034	.050	.054[α]	.054
	.026	.036	.048	.062	.058
	.016[α]	.036	.064[α]	.060	.054
15 MIN.	.026	.037	.042	.049	.052
	.028	.037	.044	.049	.048
	.020	.036	.057	.051	.048
20 MIN.	.023	.033	.037	.043[α]	.043
	.028	.033	.038	.044	.041
	.020[α]	.033	.052[α]	.045	.041

1 Pseudo Rate, page 42.

α Not included in average as shown in Table XI.

TABLE LXXII

THE EFFECT OF pH ON SUCCINATE OXIDATION

AT HIGH AND LOW OSMOLARITY

TIME AFTER MITOCHONDRIA (20.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL µMOLES
pH 6, 1* HIGH OSMOLARITY						
0 MIN.		45.6				
		45.9				
		45.5				
5 MIN.		37.7				
		36.7				
		36.8				
20 MIN.	11.0	19.8				
	10.1	21.4				
	10.0	21.3				
pH 6, 1* LOW OSMOLARITY						
0 MIN.		45.0				
		45.9				
		45.0				
5 MIN.		38.4				
		36.7				
		36.8				
20 MIN.	15.7	21.4				
	14.9	21.2				
	14.9	24.7				

1* Reaction medium A, page 50, for histidylhistidine.

TABLE LXXII-A

THE EFFECT OF pH ON SUCCINATE OXIDATION

AT HIGH AND LOW OSMOLARITY

TIME AFTER MITOCHONDRIA (20.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL µMOLES
pH 7, 2* LOW OSMOLARITY						
0 MIN.		46.0				
		44.4				
5 MIN.		32.8				
		34.4				
		34.6				
20 MIN.	19.0	18.6				
	18.4	19.2				
	19.1	19.0				
pH 7, 2* HIGH OSMOLARITY						
0 MIN.		43.8				
		44.4				
5 MIN.		37.7				
		37.7				
		37.6				
20 MIN.	13.0	23.6				
	13.9	23.4				
	13.0	23.6				

2* Reaction medium A, page 50, for histidylhistidine.

TABLE LXXII-B

THE EFFECT OF pH ON SUCCINATE OXIDATION

AT HIGH AND LOW OSMOLARITY

TIME AFTER MITOCHONDRIA (20.5 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL µMOLES
pH 8, 3* HIGH OSMOLARITY						
0 MIN.		44.4				
		45.5				
		44.4				
5 MIN.		36.7				
		36.5				
		36.2				
20 MIN.	15.1	19.6				
	14.9	19.8				
	12.9	20.1				
pH 8, 3* LOW OSMOLARITY						
0 MIN.		40.7				
		41.0				
5 MIN.		33.2				
		34.2				
		32.7				
20 MIN.	17.5	18.6				
	19.4	17.0				
	18.5	17.6				

3* Reaction medium A, page 50, for histidylhistidine.

TABLE LXXII-C

THE EFFECT OF pH ON SUCCINATE OXIDATION

AT HIGH AND LOW OSMOLARITY

TIME AFTER MITOCHONDRIA (20.5 mgs protein)	RATE, µATOMS/MINUTE/MILLIGRAM	
	pH 6, HIGH OSMOLARITY	pH 6, LOW OSMOLARITY
10 MIN.	.040, .036, .040	.058, .058, .054
15 MIN.	.039, .039, .036	.056, .053, .051
20 MIN.	.035, .033, .032	.051, .047, .047
	pH 7, HIGH OSMOLARITY	pH 7, LOW OSMOLARITY
10 MIN.	.054, .056, .060	.070, .064, .072
15 MIN.	.050, .052, .050	.063, .066, .067
20 MIN.	.042, .045, .042	.062, .060, .062
	pH 8, HIGH OSMOLARITY	pH 8, LOW OSMOLARITY
10 MIN.	.062, .060, .058	.070, .072, .072
15 MIN.	.056, .055, .048	.059, .072, .063
20 MIN.	.049, .047, .042	.057, .063, .060

TABLE LXXIII

THE EFFECT OF HISTAMINE ON SUCCINATE OXIDATION

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE µATOMS	PHOSPHATE[4] UPTAKE µMOLES
1*		
16 MIN.	3.0, 3.1	34.0, 33.8 11.6
2*		
16 MIN.	2.9, 3.7	33.2, 33.8 12.2, 11.6
3*		
16 MIN.	2.3, 2.9	34.8, 35.8 10.6, 9.6
1* SUMMARY OF OXYGEN UPTAKE FOR THREE OTHER EXPERIMENTS		
16 MIN.	3.2, 3.6 2.9, 3.0 3.1	
2*		
16 MIN.	4.1, 4.1 4.4, 3.7	
3*		
16 MIN.	2.4, 2.1, 2.3 2.5, 2.0, 2.3	

1* Reaction medium contained: .005 M AMP, .005 M $MgCl_2$, .015 M succinate, .015 M K_2HPO_4, .013 M Tris, pH 7.4, 25 mgs mito. protein.
2* Same as 1*, plus .006 M Histamine and .019 M Tris.
3* Same as 1*, plus .060 M Histamine and .066 M Tris.
4 Initial phosphate = 45.4 µmoles.

TABLE LXXIV

THE EFFECT OF HISTAMINE ON SUCCINATE OXIDATION

AND ADENINE NUCLEOTIDE DISTRIBUTION

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES
1*						
4 MIN.		38.3	12.3	1.6	2.4	16.3
		39.8	12.1	2.4	2.3	16.8
8 MIN.		33.5	9.5	2.4	4.0	15.9
		34.4	7.3	3.7	5.6	16.6
12 MIN.	3.0	30.2	5.6	5.1	5.3	16.0
	3.8	29.8	6.6	4.3	5.3	16.2
16 MIN.	4.9	37.2	4.7	6.2	6.4	17.3
	6.4	37.6	4.4	5.1	7.8	17.3
2*						
4 MIN.		37.0	12.3	2.1	2.3	16.7
		39.2	12.5	2.0	2.6	17.1
8 MIN.		34.4	6.0	4.0	6.2	16.2
		32.4	8.2	3.7	5.0	16.9
13 MIN.	2.4	32.4	5.4	4.6	6.0	16.0
	2.3	28.4	5.4	4.2	6.6	16.2
16 MIN.	5.0	28.2	3.9	5.6	7.2	16.7
	5.4	26.0	4.3	4.9	7.8	17.0

TABLE LXXIV, CONTINUED

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP	ATP	TOTAL
3*						
4 MIN.		40.2	9.8	4.6	2.7	17.1
		40.2	12.6	2.8	1.8	17.2
8 MIN.		33.4	7.4	5.9	3.7	17.0
		35.5	9.1	4.4	2.7	16.2
12 MIN.	3.4	31.0	5.5	6.0	5.3	16.8
	2.4	31.0	6.6	5.1	4.9	16.6
16 MIN.	4.9	27.0	3.3	7.9	6.2	17.4
	5.7	28.6	5.4	5.5	5.9	16.9

1* Reaction medium contained: .005 M AMP, .005 M $MgCl_2$, .015 M succinate, .015 M K_2HPO_4, .013 M Tris, pH 7.4, 25 mgs mito. protein.
2* Same as 1*, plus .006 M Histamine and .019 M Tris.
3* Same as 1*, plus .060 M Histamine and .066 M Tris.

TABLE LXXV

THE EFFECT OF IMIDAZOLE ON β-HYDROXYBUTYRATE OXIDATION

TIME AFTER MITOCHONDRIA	OXYGEN UPTAKE μATOMS	PHOSPHATE REMAINING μMOLES	NUCLEOTIDES AMP	ADP	ATP	TOTAL
			μMOLES			
1*						
8 MIN.		34.8	5.9	4.7	3.8	14.4
		32.6	6.2	4.9	3.7	14.8
18 MIN.		37.8	7.4	5.3	2.3	14.9
		35.2	4.9	4.8	3.6	13.4
2*						
8 MIN.		35.2	5.6	5.3	4.7	15.6
		34.6	6.0	4.5	4.5	14.9
18 MIN.		37.7	7.7	4.4	2.8	15.0
		37.7	8.5	3.2	2.6	14.3
3*						
8 MIN.		35.6	7.4	4.6	2.5	14.5
		36.9	7.5	4.6	2.5	14.6
18 MIN.		39.3	8.2	4.6	2.6	15.5
		39.4	7.1	4.5	2.8	14.4

1* Reaction medium contained: 0.005 M AMP, 0.005 M $MgCl_2$, .027 M Na β-hydroxybutyrate, .015 M Phosphate (K_2), .020 M L-histidine, .028 M Tris, pH 7.4.

2* Same as 1*, plus .006 M Imidazole hydrochloride.

3* Same as 1*, plus .060 M Imidazole hydrochloride.

TABLE LXXVI

THE EFFECT OF IMIDAZOLE ON SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

TIME AFTER MITOCHONDRIA (22.3 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES	P/O
1*							
5 MIN.		38.1	7.0	4.4	3.9	15.3	
		38.1	7.3	4.4	3.7	15.4	
15 MIN.	9.2	22.0	1.6	4.5	9.7	15.8	1.8
	9.1	22.0	1.6	4.2	9.7	15.5	1.8
20 MIN.	12.1	18.1	0.8	3.1	12.2	16.1	1.7
	12.7	18.8	0.7	2.4	13.0	16.1	1.5
40 MIN.	26.3	17.9	0.9	4.5	13.7	19.1	
	26.5	18.1	0.6	4.5	13.7	18.8	
2*							
5 MIN.		37.7	7.5	3.7	3.8	15.0	
		38.3	7.2	4.1	3.9	15.2	
15 MIN.	9.8	20.7	1.1	3.8	10.9	15.8	1.8
	9.9	20.7	0.7	2.6	13.2	16.5	1.8
20 MIN.	11.9	17.9	0.6	3.4	11.1	15.1	1.7
	14.7	18.1	0.6	1.8	13.3	15.7	1.4
40 MIN.	30.7	18.1	0.6	1.8	12.1	14.5	
	31.2	18.1	0.4	1.7	13.1	15.2	

All systems contained 0.005 M AMP, 0.005 M $MgCl_2$, 0.015 M K_2HPO_4, .015 M succinate, 0.05 M buffer; pH 7.4, 0.45 os.

1* .05 M Histidylhistidine plus 0.038 M Tris.

2* .05 M L-Histidine plus 0.038 M Tris.

TABLE LXXVI, CONTINUED

TIME AFTER MITOCHONDRIA (22.3 mgs protein)	OXYGEN UPTAKE µATOMS	PHOSPHATE REMAINING µMOLES	NUCLEOTIDES AMP µMOLES	ADP µMOLES	ATP µMOLES	TOTAL µMOLES	P/O
3*							
5 MIN.		43.1	9.8	3.1	2.4	15.3	
		42.5	9.5	3.5	2.9	15.9	
15 MIN.	10.4	30.3	4.6	5.1	5.0	14.7	1.2
	11.0	31.2	4.6	4.7	5.3	14.6	1.0
20 MIN.	13.5	29.4	3.6	5.2	6.8	15.6	1.0
	13.8	29.4	3.5	5.6	7.1	16.2	1.0
40 MIN.	27.3	20.9	0.9	3.1	11.4	15.4	
	28.8	20.7	0.6	2.7	12.0	15.3	

All systems contained 0.005 M AMP, 0.005 M $MgCl_2$, 0.015 M K_2HPO_4, .015 M succinate, 0.05 M buffer; pH 7.4, 0.45 os.

3* .050 M Imidazole plus .017 M Tris, pH 7.4.

TABLE LXXVI-A

THE EFFECT OF IMIDAZOLE ON SUCCINATE OXIDATION AND AMP PHOSPHORYLATION

BUFFER	MICROATOMS OF OXYGEN CONSUMED TIME - MINUTES						
	10	15	20	25	30	35	40
HISTIDYLHISTIDINE	6.1	9.2					
	5.9	9.1					
	5.7	9.2	12.1				
	5.7	9.6	12.7				
	5.2	9.3	12.2	15.1	18.4	22.5	26.3
	5.8	9.1	11.9	15.0	18.5	22.5	26.7
	6.2	10.1	12.7	16.0	18.8	22.8	26.5
HISTIDINE	5.7	9.8					
	5.9	9.9					
	5.4	8.9	11.9				
	5.7	10.4	14.7				
	6.3	10.0	13.6	17.7	21.8	25.9	30.7
	6.0	10.5	14.2	18.2	22.2	26.3	31.2
	5.8	9.1	12.3	15.7	20.0	22.7	28.6
IMIDAZOLE	6.9	10.4					
	6.6	11.0					
	6.8	11.0	13.5				
	7.1	11.0	13.8				
	6.5	11.8	15.1	18.1	21.6	25.7	33.7
	6.3	10.7	13.1	16.2	19.6	23.0	27.3
	7.5	11.5	14.0	17.3	20.5	24.6	28.8

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ABBREVIATIONS

AMP	ADENOSINE MONOPHOSPHATE
ADP	ADENOSINE DIPHOSPHATE
ATP	ADENOSINE TRIPHOSPHATE
CoASH	COENZYME A
NAD	NICOTINE ADENINE DINUCLEOTIDE
NADH	NICOTINE ADENINE DINUCLEOTIDE REDUCED
NADP	NICOTINE ADENINE TRINUCLEOTIDE
NADPH	NICOTINE ADENINE TRINUCLEOTIDE REDUCED
FAD	FLAVINE ADENINE DINUCLEOTIDE
$FADH_2$	FLAVINE ADENINE DINUCLEOTIDE REDUCED
P_i	INORGANIC PHOSPHATE
GDP	GUANOSINE DIPHOSPHATE
GTP	GUANOSINE TRIPHOSPHATE
TRIS	TRIS (HYDROXYMETHYL) AMINOMETHANE

APPROVAL SHEET

The dissertation submitted by John Earl Biaglow has been read and approved by five members of the Faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given approval as regards content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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DATE

SIGNATURE OF ADVISOR