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Interconversions of the Adenosine-5'-Phosphates During Oxidative Phosphorylation by Rat-Liver Mitochondria

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INTERCONVERSIONS OF THE ADENOSINE-
5'-PHOSPHATES DURING OXIDATIVE PHOSPHORYLATION
BY RAT-LIVER MITOCHONDRIA

by

JOHN EARL BIAGLOW



A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

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LIFE

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

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CHAPTER I

INTRODUCTION

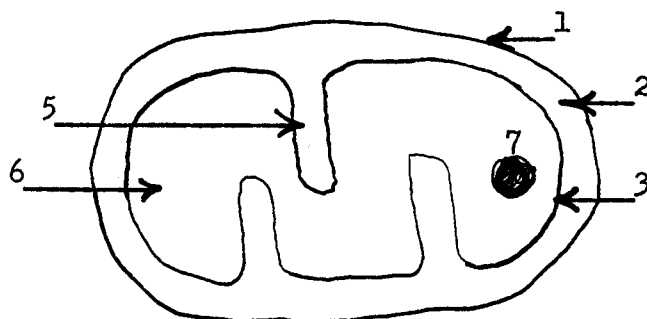
Living organisms need energy to maintain a steady state. The main source of this energy is the oxidation of carbon compounds (fatty acids, dicarboxylic acids, amino acids, -etc.) by molecular oxygen with the formation of CO_2 and the release of energy. The energy released may be stored in the form of high energy phosphate compounds, such as adenosine triphosphate (ATP). Localized within the cytoplasm of each cell are structures in which respiration is coupled to the synthesis of ATP. This "Powerhouse" is the mitochondrion.

A mitochondrion (from the Greek: mitos: filament, and kondria: granule; (1) is morphologically defined as a cell organelle bounded by a double membrane within which are membranous structures (inner membranes, cristae), either villous or visicular, a ground substance or granular matrix, and occasionally, very dense granulations.

From the numerous works issued during the last few years [comprehensive reviews (2-7)] it is reasonably well established that the mitochondrion has the following gross structure:

1. It is bounded by a continuous membrane consisting of two dense layers divided by a less dense outer chamber. The total membrane thickness ranges from 140-180 A., that of the dense layers from 35-60 A., and that of

the outer chamber from 40-70 A. (2).



1. Limiting membrane
2. Outer chamber
3. Inner chamber
4. (1,2, and 3 form surface) surface of mitochondrial membrane
5. Cristae
6. Inner chamber
7. Dense granulations

FIGURE 1. Diagrammatic representation of a mitochondrion.

The external layer (surface membrane) is to be considered as the limiting membrane of the mitochondrion while the cristae are connected to the inner surface (4).

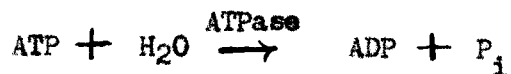
2. These membranes enclose two chambers; the outer (between the two layers), and the inner, bounded by the inner membrane. These two chambers are not inter-connected. The outer chamber is not so dense as the inner, which contains the matrix consisting of small 40-50 A., diameter granulations (8)

3. Inside the mitochondrion is a laminated system, usually at right angles to the main axis of the cell organelle and termed "cristae mitochondrials" and "Inner membranes" (2,4).

4. The dense layers of the cristae and mitochondrial membrane consist of three dense layers: two outer (15-17 A.) and another, less dense, between the two (20-23 A.) (8-11).

Many authors emphasize the relationship between morphological integrity of the mitochondria and the biochemical activity (12-26). Conversely, when the oxidative phosphorylation process occurs normally it is conducive to the preservation of the structural organization of particles (18,19). Owing to the interaction of function and morphology, much research work has been done these last few years to obtain media in which mitochondria may retain more closely their aspect as described in situ.

At the present time it is conceded that aqueous sucrose (27), particularly at 0.44M concentration is the medium wherein mitochondria exhibit the least agglutination and are preserved the best (28) although the matrix appears to be denser than that of mitochondria in situ (29). Conservation of sarcosomes is still further improved when Versene (30,31), citric acid (28), or high molecular weight polymers such as dextran (32) or polyvinyl-pyrrolidone (33) are added to the sucrose or saline medium. One criterion for ultrastructural conservation is low adenosine triphosphatase activity (34).



Ziegler and Linnane (35) have made a comprehensive study on the effect

of the isolation medium on the biochemical activity of beef heart sarcosomes. These are difficult to isolate intact because of the localization of the sarcosome in the sarcoplasm between dense muscular protein and the requirement of additional substances to preserve integrity (31). They found that the isolation medium greatly affected the activity of the sarcosomes. Morphologically intact sarcosomes did not catalize the oxidation of isocitrate, citrate, malate, or lactate. The enzymes involved in the oxidation of these substances could be demonstrated only after the mitochondria were damaged (35).

Pyruvate, alpha-ketoglutarate, beta-hydroxybutyrate, glutamate, and succinate were each oxidized at an increased rate after the mitochondria were damaged (35).

Although the above authors have shown a correlation between morphological integrity and biochemical activity the search still progresses to determine relations between reactions obtained on isolated material and those actually taking place in living tissues.

MITOCHONDRIAL CHANGES ACCORDING TO MEDIUM

1. The size of mitochondria varies according to the tonicity of the medium. In a hypertonic sucrose medium the size decreases whereas the opposite occurs in hypotonic sucrose medium (36-42).

2. Swelling of mitochondria is quickened and/or accentuated by pH rise or addition to the medium of various substrates such as calcium, phosphate, potassium, succinate, etc. (43-46).

3. Conversely, swelling of mitochondria is slowed down by addition to the medium of ATP, citrate, dinitrophenol, magnesium, fluoride, etc. (43-47).

4. Changes in the solute content of mitochondria have been demonstrated by Siekevitz and Potter (47) and others (48), who showed that mitochondria lost materials absorbing at 260 millimicrons, either on storage at zero degrees or more rapidly on incubation. As judged by the phosphate levels in the medium in which mitochondria are stored (48,49) an equilibrium is set up between the concentration of nucleotide in the medium and that in the mitochondria. The attainment of this equilibrium will be hastened by shaking, since diffusion will no longer limit the rate of transfer of nucleotides from the mitochondrial surface to the medium. The level of cofactors will be lower in a dilute suspension than in a concentrated suspension, since relatively more of the mitochondrial cofactors will be lost in raising their level in the medium to the equilibrium level. In some instances the rate of respiration of mitochondria is dependant on the level of internal cofactors, because aged or water treated mitochondria require additional cofactors such as diphosphopyridine nucleotide (DPN) and ATP for maximal rates of respiration (50).

5. Mitochondria, the major site of synthesis for energy rich phosphate compounds in animal tissues, have been alluded to as ionic pumps in cells because of the action of energy rich compounds in the control of osmotic properties (51,52). Since the work of Raaflaub (42,53) there have been several reports (51,54,58) that ATP plus magnesium or manganous ions can reverse or prevent swelling of isolated mitochondria. Swelling and shrinking is also known to be accompanied by changes in intramitochondrial water and

ions (51,52,56), by changes in light scattering properties (53,54,58) and by functional changes such as the ability to carry out oxidative phosphorylation (54,56,57,18). These findings suggest that a close relationship exists between mitochondrial size and metabolism. Packer (59) has shown that ADP causes contraction, while AMP and ATP cause swelling when mitochondria are incubated with oxidizable substrate. The swelling and shrinking changes may be followed by the commonly used practice of measuring turbidity or percentage of light transmission at a wave length where the absorption band of a specific pigment does not interfere. Packer demonstrated that the amount of contraction is proportional to the sucrose concentration up to 100mm after which there is no further contraction (59).

6. The increase in diameter of mitochondria in dilute solutions and the reversibility of the phenomena suggest that these organelles function as osmometers (60-63). However, it would be wrong to consider mitochondria as mere osmometers since numerous facts point to the contrary; when immersed in hyperosmotic solution of potassium chloride (0.5M), mitochondria swell more than in distilled water (62), in hypertonic media, swelling after the addition of succinate is increased (44). Calcium ion augments the swelling effect of dilute sucrose, whereas ATP, Versene, and magnesium, even at low concentrations prevent swelling.

SUCCINOXIDASE

Succinoxidase is a mitochondrial enzyme, located in the membrane which causes the reversible oxidation of succinic acid to fumaric acid.

In intact mitochondria it is coupled to the phosphorylation of ADP with a phosphorus to oxygen ratio approaching two. Succinic dehydrogenase (isolated from succinoxidase) is a flavoprotein containing 4 gram atoms of iron per 200,000 grams protein in non-dialysable form. Hopkins has demonstrated the presence of -SH groups essential for enzymatic activity (64).

NUCLEOTIDES AND MITOCHONDRIA

Siekevitz and Potter studied the extramitochondrial adenine-5'-phosphates and their relation to intramitochondrial concentrations (47,65,66). They developed the method of analysis used in this thesis (65). With the exception of Slater (67,68), who measured the oxidative phosphorylation by phosphate disappearance and glucose-6-phosphate formation, most authors have been content with the measurement of phosphate uptake. Siekevitz and Potter (47,65) and Pressman (68) have studied the intramitochondrial contents under various conditions. Radioactive phosphorus was used to investigate the interconversions of the nucleotides during oxidative phosphorylation. They concluded that there may be two sites of adenyl kinase activity, ($2\text{ADP} \rightleftharpoons \text{AMP} + \text{ATP}$) one at the surface of the mitochondria and the other at the site of oxidative phosphorylation within the mitochondria. They also demonstrate that added yeast hexokinase appears to act at the surface of the mitochondrion upon the ATP which has been formed within the mitochondria and is being released into the reaction medium. The role of adenyl kinase is discussed in relation to regulation of mitochondrial

oxidation through an "active" AMP. Pressman (68), in a continuation of the work begun by Siekevitz and Potter, has also concerned himself with intramitochondrial reaction mechanisms. He has studied the effects of several incubation conditions on the intramitochondrial nucleotide distributions with the hope of finding an explanation for the adenyl effect (adenyl effect-upon the addition of AMP or ADP to a preincubation medium respiration and phosphorylation stop after 5 minutes). From these studies he has concluded that the intramitochondrial nucleotide distributions and concentrations are not a function of the extramitochondrial distributions and concentrations, but depend for the most part on other factors within the mitochondria.

Both of these investigations were run under conditions favorable for measuring intramitochondrial nucleotide distributions and concentrations. In order to obtain a large enough sample they used the mitochondria obtained from at least 10 grams of rat liver. The nucleotides in this amount would be 4-5 micromoles. The above authors made certain modifications of the accepted Warburg technique in order to obtain enough intramitochondrial nucleotide to measure. (The generally accepted procedure in studying oxidative phosphorylation is to use the mitochondria from 0.5 grams of rat liver, the final reaction mixture volume being 2-3 ml.). The procedure of Siekevitz and Potter and that of Pressman involves the use of the entire mitochondrial pellet isolated from 1-3 (10-30 grams) rat livers in a final volume of 24-26 ml. This medium forms the basis of their work. They either preincubate the mitochondria and then add a phosphate acceptor system, or preincubate and then withdraw aliquots for the conventional

Warburg and phosphate determinations. In some instances the intramitochondrial nucleotide distribution was studied by adding the mitochondria to the reaction mixture and immediately centrifuging the particles from the medium. This type of experiment was run at zero degrees.

ADENYL KINASE

The enzyme adeny1 kinase or myokinase (69-82), which catalyses the establishment of an equilibrium among the three nucleotides ATP, ADP, and AMP, has been localized in rat liver mitochondria (78,79,83). The equilibrium constant for this enzyme is between 0.3-0.6 (not corrected for magnesium complex formation) (76,83,84).

The presence of this enzyme causes great uncertainty as to the state of nucleotides used in oxidative phosphorylation. Slater and co-workers (85), along with Siekevitz and Potter (65), have considered that this enzyme plays a significant role in oxidative phosphorylation while the majority of workers tend to neglect its presence and function in the mitochondrion. For the most part there is great confusion as to what is happening during oxidative phosphorylation. Siekevitz and Potter publish statements which appear to conflict as to the role of adeny1 kinase — in one paper (65) they claim it has no activity and in another (66) they expound it as controlling the entry of phosphate acceptor into the mitochondrion — a re-evaluation of this enzyme's role in oxidative phosphorylation is necessary.

OXIDATIVE PHOSPHORYLATION

It is well established that the respiratory carriers are present in or on the mitochondrial membrane(s) (86-88). With the possible exception of cytochrome c, the carriers are tightly bound to the lipoprotein structure. The enzymes necessary for energy coupling seem also to be bound to the membranes since they remained attached after treatment with digitonin (89,88,90) or ultra sonic radiation (91,92).

There may be a biological necessity for structural organization of these catalysts in a moderately rigid, geometrical organized constellation in the membrane, to minimize the path distance between slowly diffusing large molecules and to maximize the probability of interaction. This fact thus will make it necessary to identify the types of reactions occurring at "active sites" of these proteins before the mechanism of oxidative phosphorylation can be understood; the active sites of these enzymes become in effect the substrates or "intermediates" in oxidative phosphorylation (106).

While the elucidation of the energy-conserving reactions of the respiratory chain in the mitochondrial membrane may be regarded as a formidable technical problem, on the other hand it may provide important clues to the molecular structure and cellular functions of membranes in general.

The respiratory carriers and coupled enzymes appear to exist as organized "assemblies" in the membranes, in which the carriers are present in simple finite molar ratios, as indicated by calculations based on

difference spectra (86,87,88), the nitrogen content of mitochondria, and mitochondrial counts and volume indicate that a single rat liver may contain at least 5,000-10,000 sets of respiratory "assemblies", possibly more depending on the assumptions made (94,95). Fragmentation and centrifugal studies indicate these assemblies to be distributed fairly evenly over the membrane (88,89,96). Such calculations also suggest that a large fraction of the protein mass of the membrane may be made up of these carrier assemblies: 40% is not an unreasonable estimate, and it could be higher (86).

More detailed analysis is limited by the currently changing views on the structure of the outer and inner mitochondrial membranes (97) and the manner of the stratification of lipid and protein molecules. These considerations, however, show the mitochondrial membranes to be more than passive limiting structure; they may more appropriately be thought of as complex multienzyme systems (94). Changes in permeability and physical state of the membranes could be expressions of changes in the condition or function of the respiratory catalysts in the membrane.

The exact mechanism for the phosphorylation of nucleotides coupled with oxidations is unknown. There has been much speculation as to the mechanism (98-104).

Two main routes to understanding the mechanism of the energy-coupled reactions are being followed in current work with submitochondrial systems (93-105). The first is the direct approach (93), namely, attempts to identify the specific energy-coupled carriers and the chemical species of such a carrier which represents the first form in which energy of electron transfer is conserved. The second route to the mechanism of coupling

is an indirect one — the Lehninger "back-door approach" (106). This exploits the possibility that the terminal or perhaps all of the reactions of the coupled mechanisms are reversible, which would provide the possibility that the individual reaction steps may be isolated and identified by proceeding from the known end product(ATP) step by step.

Certain functional groups of respiratory carriers, such as -SH groups (106) the amide of diphosphopyridine nucleotide, the carbonyl group of the isoalloxine ring and the formyl of cytochrome a and a₃ porphyrin have been suggested as participants in formation of such energy conserving derivatives of the carriers during electron transport (106) but as yet no convincing evidence has been presented to support these suggestions.

Recent development in the possible participation of reversible quinol-quinone systems in the phosphorylating respiratory chain, such as vitamin K (109-115) and coenzyme Q (117,118), provide chemically attractive potentialities for a mechanism of energy conservation.

This research was initiated with the hope of obtaining additional information about the oxidative phosphorylation process through nucleotide and phosphate studies.

CHAPTER II

MATERIALS AND METHODS

PREPARATION OF SOLUTIONS

Water, redistilled in a glass apparatus was used for preparing all solutions used in the enzyme determinations. Carbon dioxide was removed from the redistilled water by purging with nitrogen gas for at least 12 hours, and the storage bottle was protected with a tube filled with "Ascarite".

The following materials were used in the preparations of the reagents.

AMP—Sigma, lot 43-46.

ADP—Sigma, lot 129 (mono-sodium).

ATP—Sigma, lot 74-95 (di-sodium).

Albumin—Armour, lot J-4902 Crystalline Albumin.

Ammonium Formate—made by neutralization of formic acid to pH 6.

Ammonium Molybdate—Mallinckrodt Analytical Reagent 3126.

Ammonium Sulfate—Mallinckrodt Analytical Reagent 3512.

Brom Thymol Blue—Harleco, 119-60.

Cresol Red—Harleco, 879-11.

Formic Acid—Mallinckrodt, C.P. lot YBZ.

Hydrochloric acid—Concentrated HCl, Mallinckrodt Analytical Reagent 2612, lot GPY

Magnesium Chloride—Mallinckrodt Analytical Reagent 5958, lot CSP.

Potassium Hydroxide—Baker Analytical USP 2117.

Perchloric Acid—70% Mallinckrodt Analytical Reagent 2766.

Sodium Fluoride—Mallinckrodt Analytical Reagent 7636.

Sodium Formate—Baker Analyzed Reagent Reagent 20559.

Sucrose—Mallinckrodt Analytical Reagent 8360.

Tris—(hydroxymethyl)aminomethane—Sigma 121, lot 46-160.

PREPARATION OF MITOCHONDRIA

Mitochondria were prepared from the livers of fed male rats (150-200 grams) by a modification of the method of Schneider (12). Ice-cold sucrose solution (0.25 Molar) was used throughout the preparation and all procedures were done 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 weighed and minced then placed in a precooled glass homogenizer containing 30 ml. of ice-cold sucrose. The liver (6-10 grams) was then homogenized by a double 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 the number 820 head, at 800 X gravity, for 15 minutes. The supernatant was decanted and centrifuged in the number 291 head at 14,000 X gravity for 15 minutes. The mitochondrial pellet obtained was then resuspended in 10 ml. of ice-cold sucrose and recentrifuged at full speed 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 10ml. of sucrose and centrifuged at full speed for the

final time before use. The drained pellet was suspended in 0.25M 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 with minimum delay.

QUALITY OF THE PREPARATION

One of the tests of a "tightly coupled" mitochondrial preparation is the absence of ATPase activity. Table 4 contains evidence that this sample contained no ATPase detectable in a total of 18 minutes incubation. Several preparations were thus tested and no ATPase activity was found in the absence of known uncouplers. A second test is the inability of the preparation to oxidize added DPNH although they were able to oxidize succinate with acceptable P/O ratios.

REACTION MIXTURES USED FOR MITOCHONDRIA STUDIES

The stock solution of succinate-phosphate was made up as follows: 50 ml. of 0.494M phosphoric acid plus 4.425 grams succinic acid was neutralized with 14.2 ml. of 0.845M KOH with the addition of water to a final volume of 250 ml. From this stock solution 40ml. was placed in a 100 ml. volumetric which contained 34.2 grams sucrose. The solution was made to volume with redistilled water. The resulting solution was 0.060M succinate, 0.0395M phosphate, and 0.19M potassium in 1.0M sucrose. These solutions were stored in a refrigerator.

Nucleotides used were weighed out and added to MgCl_2 and the mixture was then neutralized with TRIS to pH 7.4.

During the centrifugation periods of the preparation of the mitochondria, a "pre-mix" was prepared which could be pipetted accurately into each reaction vessel. This solution contained each of the components which would be constant during a series of measurements in quantities such that the correct final concentration would be attained when the mitochondrial suspension was added.

Unless otherwise specified, all mitochondrial experiments were carried out in a media which contained the following constituents per milliliter. The final volume in each reaction mixture was 2.0 or 3.0ml.

0.005 molar MgCl_2
 0.005 molar AMP, ²ADP, ATP
 0.015 molar succinate
 0.010 molar phosphate
 0.250 molar sucrose
 0.095 molar potassium
 0.025 molar glucose (when used)
 TRIS—sufficient to neutralize the Nucleotide- MgCl_2 mixture.
 0.2 mg/ml Hexokinase, capable of reacting with 14 umoles of ATP per minute. (when used)
 Two tenths of a milliliter of 5M KOH was placed in the center well of the flask.

The conventional Warburg technique was employed in the measurement of oxygen consumption (119). The experiments were planned to require minimum total time. A typical time sheet looked like this:

FLASK NUMBER	MANOMETER NUMBER	START MINUTES	TIME MINUTES	TIME MINUTES	TIME MINUTES
A	9	0	5 CLOSE	10 READ	15 READ, TAKE-OFF
Q	21	1	6 CLOSE	11 READ	16 READ, TAKE-OFF
E	57	2	7 TAKE-OFF		
F	67	3	8 TAKE-OFF		

The procedure was to pipet the mitochondria into a cold reaction mixture contained within the Warburg flask, swirl to mix, then fasten the flask to the manometer. The manometer with flask was placed in a Warburg water bath for 30 degree temperature equilibration while shaken. This process of "thermal equilibration" requires 5-10 minutes. The machine was stopped for each manometer closing. Manometer readings were taken at appropriate time intervals. The machine was stopped only to add, to close, or to remove flasks. Flasks were removed at appropriate times and aliquots deproteinized for analysis.

Conflicting reports are found in the literature about the length of time required to attain thermal equilibrium for the measurement of oxygen consumption. The experiment in Table 1 illustrates an error which might be encountered if the measurement of oxygen consumption with our equipment were begun as early as five minutes after adding the mitochondria. It is clear that the finding that less oxygen is taken up when the taps are closed at five minutes than when they are closed at 8 minutes must be incorrect--since the mixtures were identical and the total time of incubation was identical.

When the oxygen consumption for the 5 minute closure is calculated by extrapolation of later readings, as described by Slater (134), more realistic values are obtained. In every case the oxygen measurements have been extrapolated and the P/O ratios are obtained from the extrapolated values.

TABLE I

COMPARISON OF OXYGEN CALCULATIONS RUN ON FLASKS CLOSED AT 5 AND 8 MINUTES. MEASUREMENT WAS CARRIED OUT ON EXTRAMITOCHONDRIAL NUCLEOTIDES DURING THE OXIDATION OF SUCCINATE BY RAT-LIVER MITOCHONDRIA WITH ADP AS THE INITIAL NUCLEOTIDE.

(Conditions as given in materials and methods. The reaction volume was 3.0ml.)

CONDI- TION	OXYGEN UPTAKE μ ATOMS		P _i UPTAKE P/O μ MOLES	AMP	ADP	ATP	TOTAL	RATIO*
	MEASURED	EXTRAPOLATED		μ MOLES				
5 min. closure				2.7	7.3	5.8	15.8	0.30
5 min. blank				2.7	5.8	6.5	15.0	0.52

21 min.	4.5	6.0	8.4	1.4	tr.	3.0	12.3	15.4
	6.2	7.9	8.4	1.1	tr.	2.1	12.4	14.5

8 min. closure				2.7	6.9	6.4	16.0	0.36
8 min. blank				2.7	5.8	6.5	15.0	0.64

21 min.	6.0	6.3	7.7	1.2	tr.	3.3	12.3	15.6
	5.7	5.7	7.0	1.2	tr.	2.4	12.8	15.2

* RATIO = $\frac{\text{AMP} \times \text{ATP}}{\text{ADP} \times \text{ADP}}$

Phosphate determinations were performed on an aliquot of a TCA (trichloroacetic acid) supernatant after centrifugation of the precipitated protein. A modified Fiske-Subbarow method was used. The reducing agent was prepared daily from a solid mixture of 0.4 grams 1-amino-2-naphthol-4-sulfonic acid, 2.4 grams of sodium bisulfate and 2.4 grams of sodium sulfite. Two hundred mgs. was dissolved in 10 cc. of water and used as described (120).

For nucleotide analysis an aliquot of the reaction mixture was pipetted into 2.0ml. of 1.5 normal PCA (perchloric acid). The precipitated protein from the PCA was centrifuged in the International refrigerated centrifuge at 4,000 x gravity for 15 minutes. An aliquot of the resultant supernatant was taken for nucleotide analysis: to this sample 0.2ml. of ^{0.1%} brom thymol blue was added (The use of an internal indicator had no experimental effect on the elution of nucleotides from the column. The indicator is bound tightly to the resin.) and the resultant solution was titrated with 5 normal KOH until a light-blue-green color is obtained (pH 7-7.5). Potassium perchlorate is sparingly soluble (0.75 grams/100 ml. at 25° C.). Precipitation was enhanced by allowing the solution to approach the freezing point. The resultant mixture was centrifuged at 4,000 x gravity for 15 minutes at 0° C.

Bio-Rad resin (CalBiochem) was used to prepare the ion-exchange columns. The resin was analytical grade anion exchange, AG-1-X 10, 100-200 mesh. The resin was obtained in the chloride form and was placed in the glass tubes in the conventional manner (121).

The top of the column was protected from channeling by insertion of a wad of fiber glass on top of which was placed 20 glass beads (small diameter).

The resin was then changed into the formate form by washing with 3 molar sodium formate until no chloride was found in the eluate, then with 4 volumes of 7N formic acid, then with distilled water until the effluent did not show a color change with blue litmus (pH 5-7).

An aliquot of the supernatant was placed on the column and washed into the resin with 10-15 ml. of water. Usually 2-3 umoles of nucleotides were placed on the columns—this has proven to be a desirable range. Nucleotide content was calculated from the absorbancy measured at 260 millimicrons after correcting for the measured absorbancy of the eluant. Each 5 ml. fraction is read in the Beckman D. U. spectrophotometer, 1 cm. cell, slit width of 0.1mm. The concentrations of the nucleotides were calculated by using the extinction coefficient (14.2/umole/ml.) for the adenine moiety.

During the course of elution it is necessary to change the eluant concentration. A large number of elution steps is required to prevent channeling due to changes in water activity. Usually it takes 15 ml. to wash the previous eluant off the column. The pH changes from approximately 4 to 1.5 in changing from 0.2 to 2.5 normal formic acid. Then there is no appreciable pH change until the 2.5 normal formic acid is replaced by the solutions containing ammonium formate. The pH then increases to 2. At the end of the elution the columns are washed with 3 molar ammonium formate. This serves a two fold purpose, first it assures the removal of all other nucleotides which may have been present and converts the resin back to the formate form for the next analysis.

THE STANDARD ELUTION SCHEME USED WAS:

TUBE NUMBER	FORMIC ACID MOLES/LITER	AMMONIUM FORMATE MOLES/LITER
1-3	ZERO	ZERO
3-5	0.005	"
6-8	0.05	"
9-18	0.20	"
19-21	0.40	"
22-24	1.70	"
25-27	2.50	"
28-37	3.00	"
38-40	4.00	"
41-43	4.00	0.05
42-44	4.00	0.15
45-54	4.00	0.30
55-64	4.00	0.50
65-67	ZERO	3.00

EACH TUBE CONTAINED 5 MILLILITERS

Perchlorate ion has no effect on the elution of the nucleotides even when the concentration is 0.025 molar (which is greater than the solubility of KClO_4 at 0°C. , which is 0.25 grams/100ml.) No appreciable hydrolysis of AMP, ADP, or ATP occurred in the neutral, cold potassium perchlorate solution in a 24 hour period at 0°C.

ISOLATION AND PURIFICATION OF YEAST HEXOKINASE

(From the thesis by R. A. Darrow (122). A preprint of this method was obtained through the courtesy of Dr. S. P. Colowick.)

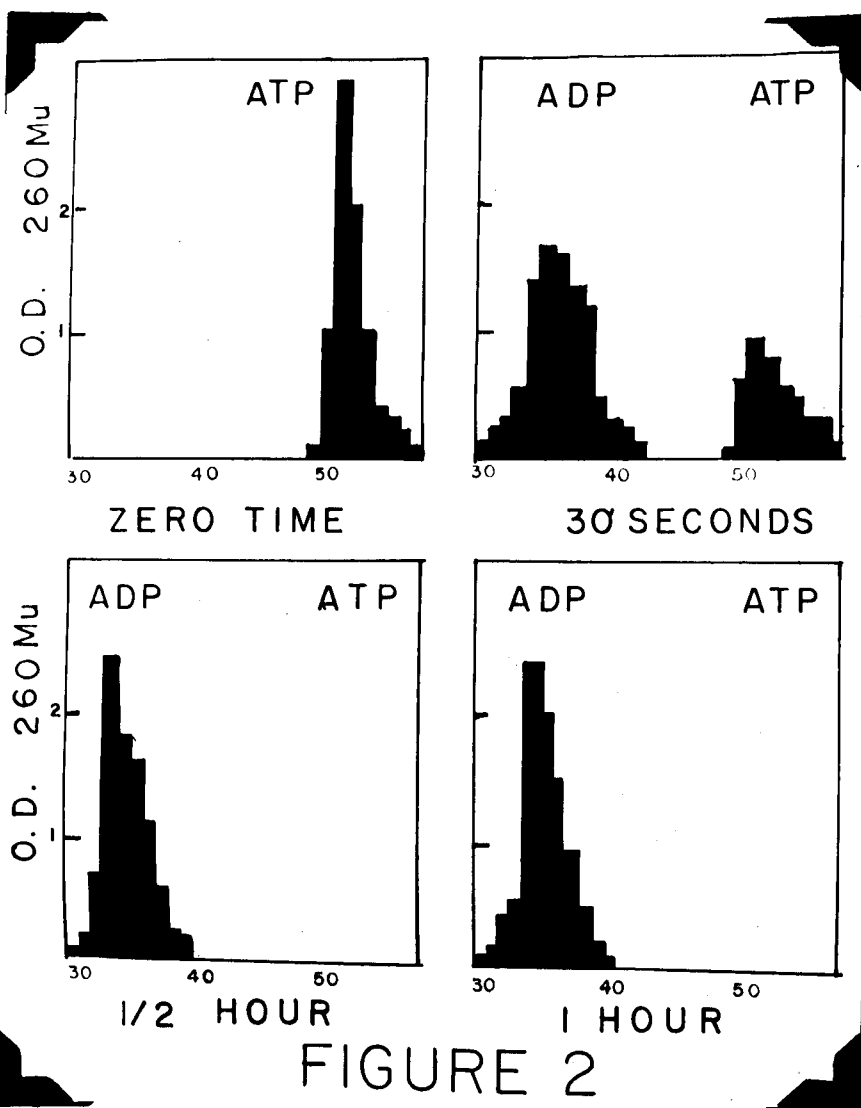
Purified Yeast hexokinase was prepared and assayed according to Darrow. The partial purified hexokinase showed an activity of 34.2 micromoles/min/mg.

protein. Twentyfive ml. of the hexokinase preparation (3.74 mg./ml.) was added to 25 ml. of 0.5M glucose and the solution dispensed in 2 ml. lots which were stored frozen.

The method which has been described for measuring adenine nucleotides was applied to a typical pre-mix containing ATP before adding hexokinase. Figure 2 shows that there was no detectable nucleotide except ATP. Thirty seconds after the addition of the usual quantity of hexokinase, 75% of the ATP (75 umoles) had been converted to ADP. After 30 minutes and 60 minutes the reaction mixture contained ADP but no AMP or ATP. The preparation of hexokinase is therefore free of adenyl kinase. This experiment provides additional evidence that the described method separates the nucleotides efficiently. (Note that because of the scale of the drawings early fractions are omitted. These fractions were analyzed for AMP and none was found.)

FIGURE 2

Examination of products of hexokinase reaction mixture. Fractions 1-30 were analysed for AMP in each case and because none was present they were omitted from this diagram.



CHAPTER III

RESULTS AND DISCUSSION OF BALANCE STUDIES ON EXTRA-MITOCHONDERIAL ADENOSINE-5'-PHOSPHATES DURING OXIDATIVE PHOSPHORYLATION

Because of the extremely complex nature of the experimental system it should be made clear at the onset that the results and the inferences to be drawn from them may be limited in their application. For example, results obtained from rat heart sarcosomes may not be completely applicable to rat liver mitochondria. Further, different methods of preparation of mitochondria from rat liver may yield significantly different samples. Even with identical preparations, there are so many differences in the protocol of published experiments as to limit the generalizations which can be drawn from any group of experiments.

The most striking results were obtained in the experiments (Figures 3 and 4, Tables 1,2,3,5, and 8) in which hexokinase was not used. When ADP was the initial nucleotide, there was a very rapid formation of AMP and ATP, presumably by the action of adenylyl kinase (myokinase). This has occurred during the time required to bring the flasks and contents near to equilibrium with the bath temperature. Consequently there has been a considerable oxidation of endogenous substrate, succinate, and possibly the products of succinate oxidation (123,124). There is therefore some uncertainty

FIGURE 3

Figure 3 shows the results of an experiment run with mitochondria plus ADP. The reaction mixtures were examined 5 minutes and 20 minutes after adding mitochondria. Note that AMP disappears before ADP does.

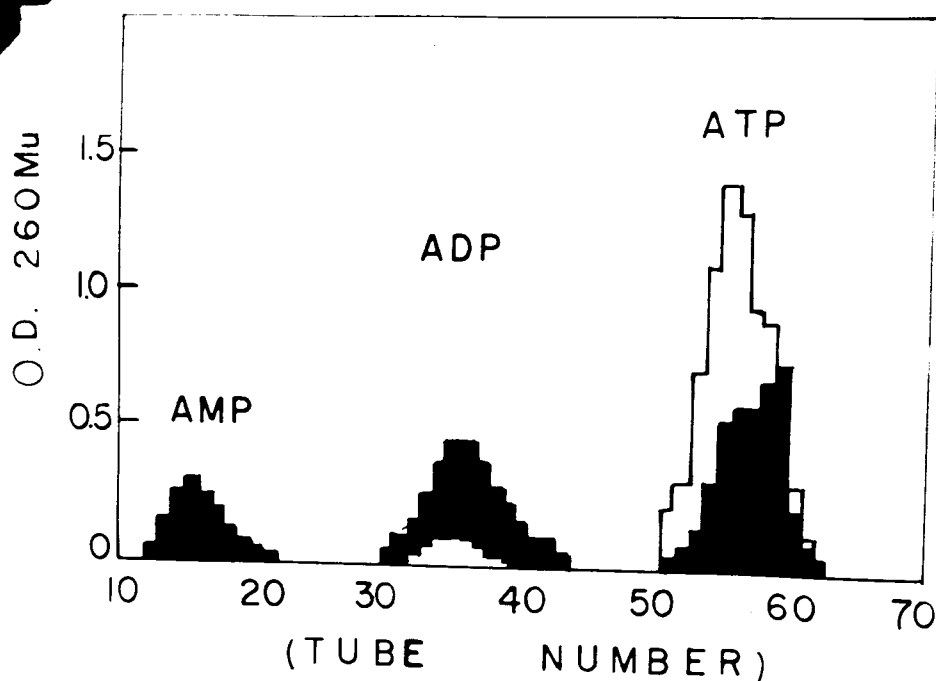
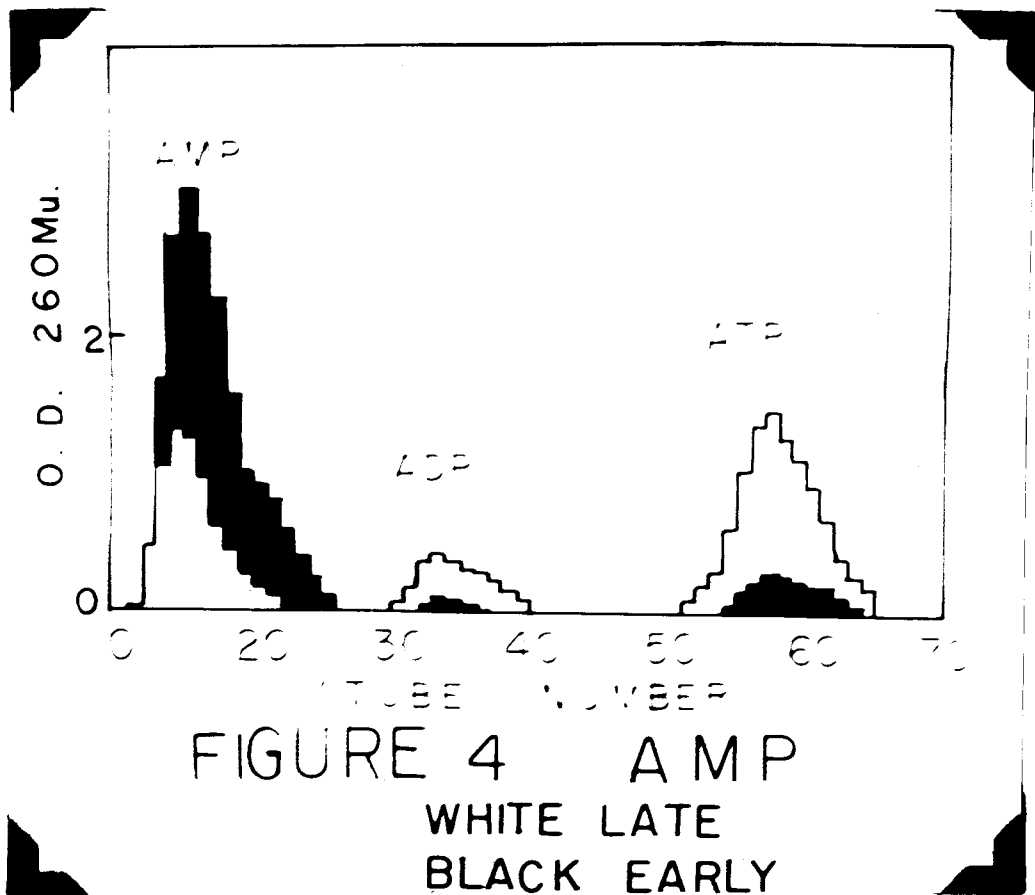


FIGURE 3 ADP

WHITE LATE
BLACK EARLY

FIGURE 4

Figure 4 shows the results of an experiment run with mitochondria plus AMP. The reaction mixtures were examined at 5 and 13 minute time intervals. Note that ADP increases only slightly as compared to ATP.



as to the materials oxidized during the measurement period which follows. The experimental P/O ratios are usually high (1.9-2.4). This is evidence for a preparation with low ATPase activity, a result which is confirmed by the experiment in which added ATP was recovered and no ADP or AMP was found (Table 4).

During the measurement period, the amount of AMP and ADP decreases, while the amount of ATP increases. In each case, however, the ratio of nucleotides found appears to be comparable with the attainment of the myokinase equilibrium



$$K = \frac{\text{AMP} \times \text{ATP}}{\text{ADP} \times \text{ADP}}$$

It will be noted that specific ionic species are not indicated in the equation. The ratio of total nucleotides in the tables will be equal to the constant appropriate for the ionic strength of the medium only when a single ionic species of each is present. This ideal situation is not found here, since a considerable amount of magnesium is also present, and magnesium is known to form complexes with each of the nucleotides (125) as well as with succinate (126) and with phosphate (127). However, when the formation of such complexes is taken into account, there is only a slight (0.39-0.19) change in the value of the ratio.

This finding is in accord with the experiments reported by Siekevitz and Potter (65). These authors postulate that ADP reacts with a surface adenyl kinase to form an active AMP, and that this AMP enters the mitochondria and is phosphorylated by ATP at a second myokinase site. Chance (128) has shown that the

addition of ADP to mitochondria in what he calls "state 4" (substrate present, oxygen present, but DPN and cytochrome b almost completely reduced) causes a more rapid oxidation of DPNH than does addition of an equal concentration of AMP. Chance therefore concludes that adenyl kinase is not rate limiting in this system. It must be pointed out, however, that the addition of AMP alone is not equivalent to the addition of ADP in the presence of adenyl kinase. To be meaningful, the comparison would have to be made with a mixture of ATP and AMP--and one would still not have eliminated the possibility of an "active AMP". This far these data indicate an active adenyl kinase, but do not imply anything about its relative location.

When AMP is the initial nucleotide (Tables 1,3) the distribution of nucleotides after five minutes is markedly different than when ADP is the initial nucleotide. After five minutes a small amount of ATP is found and a barely detectable amount of ADP. The amounts of both of these nucleotides increases with further incubation, but AMP is the predominant nucleotide throughout. The nucleotide ratio is very different from the approximate equilibrium value of 0.4, even after correcting as accurately as possible for the effect of the formation of magnesium complexes present. This finding requires very careful consideration, since it implies that the external nucleotides are not in equilibrium via adenyl kinase.

In the experiments shown in Tables 1,4 a large excess of purified hexokinase was used. In preliminary experiments, the P/O ratios were quite satisfactory (1.7-2.2) and no consideration was given to using a preparation of lower specific activity--which would have been necessary because of problems of enzyme stability. As the results of the nucleotide distribution became

TABLE 3

EXTRAMITOCHONDRIAL NUCLEOTIDES DURING THE OXIDATION OF SUCCINATE BY RAT LIVER MITOCHONDRIA WHEN ADP IS THE INITIAL NUCLEOTIDE.
(Conditions as given in materials and methods chapter. Final volume 2.0ml.)

CONDI- TION	OXYGEN UPTAKE μMOLES	P _i UPTAKE μMOLES	P/O	AMP μMOLES	ADP	ATP	TOTAL	$\frac{\text{AMP} \times \text{ATP}}{\text{ADP} \times \text{ADP}}$
no hexokinase 5 min.				1.5	4.6	3.9	10.0	0.21

20 min.	4.2	5.1	1.2	0.0	0.9	8.5	9.4	
	5.0	5.3	1.1	0.0	0.6	8.4	9.0	
hexokinase 5 min.				3.1	5.6	0.0	8.7	

20 min.	7.3	11.7	1.6	5.4	4.0	0.0	9.4	
	7.9	12.9	1.6	6.0	2.8	0.0	8.9	

TABLE 4

EXTRAMITTOCHONDRIA NUCLEOTIDES DURING THE OXIDATION OF SUCCINATE BY
 RAT-LIVER MITOCHONDRIA PREPARATION.
 (Conditions as given in materials and methods chapter. Final volume 2.0ml.)

CONDI- TION	OXYGEN UPTAKE μMOLES	P _i UPTAKE μMOLES	P/O	AMP μMOLES	ADP	ATP	TOTAL	$\frac{\text{AMP} \times \text{ATP}}{\text{ADP} \times \text{ADP}}$
AMP-5 min.				10.6	tr.	0.9	11.5	
				9.3	tr.	0.7	10.0	

13 min.	2.3	4.1	1.8	8.1	0.8	2.7	11.6	34
	2.3	—	—	7.4	1.6	1.8	10.8	5.3

ADP-5 min.				1.9	3.8	3.5	9.2	0.40
				2.0	3.6	2.7	8.3	0.53

13 min.	1.9	6.6	2.4	0.5	2.3	6.5	9.3	0.61
	—	—	—	0.5	2.1	6.3	8.9	0.55

ATP-5 min.*				0.0	0.0	9.7	9.7	

ATP-13 min.*	0.0	0.0	0.0	0.0	0.0	9.8	9.8	

* Averages of agreeing duplicates.

TABLE 5

EXTRAMITOCHONDRIAL NUCLEOTIDES DURING THE OXIDATION OF SUCCINATE BY RAT-LIVER MITOCHONDRIA WITH THE HEXOKINASE-GLUCOSE TRAP.
(Conditions as given in materials and methods chapter. Final volume 2.0ml.)

NUCLEO- TIDE	OXYGEN UPTAKE μATOMS	P _i UPTAKE μMOLES	P/O	AMP μMOLES	ADP	ATP	TOTAL
<hr/>							
AMP-5 min.				9.5	0.6	0.0	10.1
				10.0	0.6	0.0	10.6
<hr/>							
20-min.	4.9	7.4	1.5	9.5	1.1	0.0	10.6
	4.4	7.0	1.6	8.7	1.1	0.0	9.8
<hr/>							
ADP-5 min.				3.6	6.3	0.0	9.9
				3.0	6.1	0.0	9.1
<hr/>							
20-min.	4.3	7.9	1.8	5.5	4.1	0.0	9.6
	3.6	7.3	2.0	6.5	3.9	0.0	10.4
<hr/>							
ATP-5 [*] min.				4.2	5.2	0.0	9.5
				3.3	5.9	0.0	9.2
<hr/>							
ATP-20 min.	3.3	8.0	2.4	5.9	3.7	0.0	9.6
	4.5	9.0	2.0	6.2	2.7	0.0	9.1

*Converted to ADP plus glucose-6-phosphate prior to addition of mitochondria.
See Figure 1.

TABLE 6

EXTRAMITOCHONDRIAL NUCLEOTIDES DURING THE OXIDATION OF SUCCINATE BY RAT LIVER MITOCHONDRIA WITH A MIXTURE OF AMP AND ATP AS THE INITIAL NUCLEOTIDES.

(Conditions as given in materials and methods chapter. Final volume 3 ml.)

TIME	OXYGEN UPTAKE μATOMS	P UPTAKE μMOLES	P/O	AMP μMOLES	ADP	ATP	TOTAL	$\frac{\text{AMP} \times \text{ATP}}{\text{ADP} \times \text{ADP}}$
8 min.				3.6	4.6	10.6	18.8	1.8
				3.8	4.4	9.7	17.9	1.9

18 min.	5.1	9.5	1.8	0.8	3.7	12.5	17.0	0.73
	4.7	9.5	2.0	0.7	3.3	13.7	17.7	0.85
23 min.	6.2	12.3	2.0	tr.	1.5	15.7	17.2	
	6.2	11.6	1.9	tr.	1.5	16.6	18.1	

Effects of Dinitrophenol:—Table 7 shows the results of an exploratory experiment with a relatively high concentration of DNP ($8 \times 10^{-5}M$). When hexokinase is present there is a measureable decrease in inorganic phosphate in every case. Respiration is markedly inhibited except in the case of AMP. When hexokinase is absent, the three nucleotides give rise to significantly different results. With AMP, there is moderate respiration, and a small increase in inorganic phosphate. With ADP there is no oxygen uptake and a small increase in inorganic phosphate. While with ATP there is also no oxygen uptake but a large increase in inorganic phosphate. The marked difference in the effect of DNP in the experiments with initial ATP and initial ADP is to be noted. There is significant ATPase activity only when ATP is the major nucleotide present. A further experiment involved ADP under various conditions (Table 8); in the absence of DNP, the respiration was moderate with a high P/O ratio. With DNP present ($2.5 \times 10^{-5}M$) respiration was not greatly affected, and phosphate uptake was diminished in the presence of hexokinase and abolished in the absence of hexokinase.

THE EFFECT OF DINITROPHENOL ON THE EXTRAMITOCHONDRIAL NUCLEOTIDES
DURING THE OXIDATION OF SUCCINATE BY RAT-LIVER MITOCHONDRIA.
(Conditions as given in materials and methods, DNP- 8×10^{-5} M, volume 2.0 ml.)

CONDI- TION	OXYGEN UPTAKE μATOMS	P _i UPTAKE μMOLES	P/O	AMP μMOLES	ADP	ATP	TOTAL	AMP x ATP ADP x ADP
Hexokinase present AMP-5 min.				9.5	0.0	0.0	9.5	
AMP- 20 min.	1.8	1.8	1.0	9.2	0.1	0.0	9.3	
ADP- 5 min.				2.9	5.2	0.2	8.3	
20 min.	0.2	0.8	4.0	5.2	3.8	0.0	9.0	
ADP- G-6-P 5 min.				4.6	4.4	0.0	9.0	
20 min.	0.3	0.9	3.0	6.2	2.6	0.0	9.0	
No hexokinase AMP-5 min.				10.3	tr.	tr.	10.3	
AMP- 20 min.	1.3	1.0		10.1	0.1	tr.	10.2	
ADP- 5 min.				2.7	3.6	2.8	9.1	0.59
I3- min.	0.0	0.6		2.8	4.3	2.5	9.6	0.39
ATP- 5 min.				0.1	2.2	7.8	10.1	0.33
I3- min.	0.0	5.1		0.5	3.3	6.9	9.7	0.16

TABLE 8

THE EFFECT OF DINITROPHENOL ON THE EXTRAMITOCHONDRIAL NUCLEOTIDES DURING THE OXIDATION OF SUCCINATE BY RAT-LIVER MITOCHONDRIA WHEN ADP IS THE INITIAL NUCLEOTIDE WITH AND WITHOUT DNP AND HEXOKINASE.
(Conditions as given in materials and methods. Final volume 2.0 ml.)

CONDI- TION	OXYGEN UPTAKE μ ATOMS	P _i UPTAKE μ MOLES	P/O	AMP μ MOLES	ADP	ATP	TOTAL	$\frac{\text{AMP} \times \text{ATP}}{\text{ADP} \times \text{ADP}}$
ADP no hexokinase or DNP 5 min.				1.4	3.7	3.8	8.9	0.39
				1.5	4.0	2.9	8.4	0.28
666								
13 min.	1.0	3.4	2.4	0.4	3.0	5.3	8.8	0.24
	1.9	3.5	1.9	0.4	2.6	5.4	8.4	0.28
ADP-DNP-hexokinase 5 min.				2.3	6.8	0.0	9.1	
				2.7	6.4	0.0	9.1	
13 min.								
	0.8*	0.7	0.9	4.1	5.2	0.0	9.3	
	0.8*	1.3	1.4	4.3	4.8	0.0	9.1	
ADP-DNP 5 min.				1.5	4.7	2.9	9.2	0.20
				1.8	4.6	2.4	8.8	0.20
13 min.								
	1.8*	-0.6		1.6	4.6	2.8	9.0	0.21
	1.2*	-0.2		1.9	4.0	2.6	8.5	0.31

*Oxidation stopped after 10 minutes in all cases.

THE PHOSPHORUS-OXYGEN RATIO: Because of the difficulty in preparing mitochondria with uniform activity it has become customary to report the ratio of μ moles of inorganic phosphate removed from the medium in a given time to μ moles of oxygen absorbed in the same period. For the oxidation of succinate to fumarate the expected value is 2 (129,130). The values obtained in most of the experiments reported here are in the expected range. However, in a few experiments a comparison is possible with a single mitochondrial preparation and different relative amounts of external nucleotides. For example, in the Table 5 the observed P/O ratios increase from 1.6 with AMP to 1.9 with ADP, 2.2 with ADP plus G-6-P. This would cause no particular comment except that in Table 4 the observed ratio is 1.8 with AMP and 2.4 with ADP. From this data it would appear that in the experiments in which AMP is the dominant nucleotide, that the efficiency of phosphorylation is less. This is in harmony with the suggestion made by Lindberg and Ernster (131) and echoed by Siekevitz and Potter (66). An equally important conclusion is that succinate is far from an ideal substrate for measurement of oxidative phosphorylation phenomena because of the possibility that the mixture of substances being oxidized may change with time during the course of a measurement. This phenomenon did not appear conclusively in earlier work since many of the preparations had to be propped up with hexokinase-glucose and other additions to attain a P/O ratio greater than 1. Additional work will be necessary to find a more suitable substrate or to measure the contribution of succinate oxidation products in these systems.

Any explanation for the phenomena observed must, ofcourse, provide a mechanism which will account for all of the observed results,

The confidence which one may place in the explanation is decreased in proportion to the number of assumptions which have to be made, and increased with the number of phenomena consistent with the explanation. The changes in the total amounts of adenine nucleotides in the system may be considered to be the resultant of the following simultaneous reactions:

1. $2\text{ADP} \xrightleftharpoons[k'_1]{k_1} \text{AMP} + \text{ATP}$ (Adenyl Kinase)
2. $\text{ADP} + \text{P}_i \xrightarrow{k_2} \text{ATP}$ (Oxidative Phosphorylation)
3. $\text{ATP} + \text{HOH} \xrightarrow{k_3} \text{ADP} + \text{P}_i$ (Hydrolysis)
4. $\text{ATP} + \text{X} \xrightleftharpoons[k'_4]{k_4} \text{ADP} + \text{XP}$ (Metabolic utilization, including hexokinase trap)

One must then consider each of the systems studied to see whether the reactions postulated will account for the experimental results. In the presence of an excess of hexokinase, which has been shown to be free from adenyl kinase (Fig. 1), the process of oxidation proceeds at quite comparable rates whether AMP or ADP was the initial nucleotide, but ADP increases slowly from a low level at the expense of AMP, when AMP is the initial nucleotide; while when ADP is the initial nucleotide, AMP increases at the expense of ADP. No appreciable ATP is present in either system.

If one attempts to construct a balance of the nucleotides on the basis of the equations listed one finds that the assumptions made are inadequate to explain the fact that AMP is decreasing in one case, but increasing in

another. The following equations express the effects of reactions 1,2,3, and 4 on the various nucleotides.

$$5. d(\text{AMP})/dt = k_1(\text{ADP})^2 - k'_1(\text{ATP})(\text{AMP})$$

$$6. d(\text{ADP})/dt = -2k_1(\text{ADP})^2 + 2k'_1(\text{ATP})(\text{AMP}) - k_2(\text{ADP})(\text{P}_1) + k_3(\text{ATP}) + k_4(\text{ATP})(\text{X})$$

$$7. d(\text{ATP})/dt = k_1(\text{ADP})^2 + k_2(\text{ADP})(\text{P}_1) - k_3(\text{ATP}) - k_4(\text{ATP})(\text{X}) - k'_1(\text{ATP})(\text{AMP})$$

CASE I: (Initial AMP, excess hexokinase-glucose) During the measurement period there was no net change in ATP, therefore $d(\text{ATP})/dt$ was zero, and assuming that hydrolysis of ATP was negligible:

$$8. k_1(\text{ADP})^2 - k'_1(\text{ATP})(\text{AMP}) = k_4(\text{ATP})(\text{X}) - k_2(\text{ADP})(\text{P}_1), \text{ and,}$$

since ATP is zero equation 8 combined with equation 6 gives:

$$d(\text{ADP})/dt = -k_1(\text{ADP})^2$$

The factor $k_1(\text{ADP})^2$ is negative and the equation predicts that ADP should decrease. This is not found in the experiment.

CASE II: (Initial ADP, excess hexokinase-glucose) An exactly identical argument leads to the same equation--but it is consistent with the experimental results. To resolve this contradiction, it is necessary to add some restriction

to the system. Slater (85) suggested that, "...in the presence of hexokinase in the amounts normally used in oxidative phosphorylation experiments, the myokinase reaction will proceed only from left to right." He further suggested that AMP was an inhibitor for adenyl kinase (myokinase). This formulation will not account for the change in sign of $d(\text{ADP})/dt$ under only slightly different conditions. It is clear that some additions must be made to the description of the system. Siekevitz and Potter suggested that the mitochondrial membrane was permeable only to AMP and that at least two loci of adenyl kinase activity existed in the mitochondrion—one in the membrane and the other at or very near the site of oxidative phosphorylation (65). This suggestion has been attacked by Chance (128) who showed quite clearly that the oxidation of DPNH of mitochondria in "State 4" is more rapid when ADP is added to the medium than when AMP is added. Regardless of the fine details of the suggestion, there is much evidence of compartmentalization of enzymic activities and it remains to be determined how many and what kind of compartments are required for the results presented above.

The modified description of the system must also account for the results obtained in the absence of hexokinase.

A system which consists of three compartments: the medium, the adenyl kinase area and the oxidation area seems to be adequate. This kind of system would provide the following course of events in CASE I: AMP would be relatively high in all three areas. ADP would be formed at the adenyl kinase (A.K.) site and would have a diffusion gradient toward the oxidation (O.P.) site. This formulation is in accord with the concept

that ADP is the specific phosphate acceptor. ATP will be formed mainly at the O.P. site and will move into the medium as well as toward the A.K. site where it reacts with AMP to form ADP. That portion which enters the medium is converted immediately to ADP by the action of the large quantity of active hexokinase bound to the membrane (65). This medium ADP will have a diffusion gradient toward both the A.K. site and the O.P. site—but this only recycles ADP. ADP will accumulate as observed.

How does the situation change when ADP is the initial nucleotide? Nucleotide reaching the A.K. site is converted to a mixture of AMP, ADP, and ATP. There is a considerable amount of each of the nucleotides in the mitochondrion from the earliest period in the experiment. It is therefore probable that the gradient of ADP to the oxidation site is very much smaller in this case than in CASE I. All other reactions proceed very much as in CASE I: AMP increases at the expense of ADP and the same steady state will be approached as the amount of ADP in the system decreases, as is observed.

CASE III: AMP is the initial nucleotide, and no hexokinase is present. The intramitochondrial situation is the same as in CASE I. However, there is an accumulation of ATP in the external medium. To the extent that ATP reaches the A.K. site, the formation of ADP will occur, but cannot go beyond the limits permitted by the AMP and ATP concentrations at the A.K. site. Therefore, during most of the period of measurement the medium will contain excess ATP and AMP over the A.K. equilibrium because ADP is formed only at the A.K. site and is divided between the medium and the oxidation site—

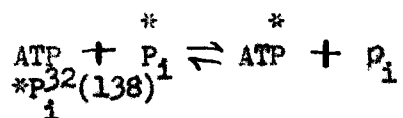
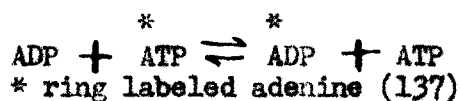
at which it is converted to ATP. This is the distribution actually observed. And the non-equilibrium distribution slowly approaches an apparent adenyl kinase equilibrium.

CASE IV: (ADP is the initial nucleotide, no hexokinase present)

As in CASE II, all three nucleotides are well distributed within the mitochondrion and the gradient of ADP from the A.K. site is much lower than in CASE III. ATP is formed at O.P. from ADP and may enter the medium or move to the A.K. site. Its entry to the medium is opposed more than in CASE III, consequently more may move to A.K. where it can react with AMP to generate more ADP. Since the initial entry of ADP produces the equilibrium concentrations of nucleotides, and this distribution is fairly well established at the beginning of the measurement period, rather large differences in the relative rates of movement of the nucleotides between the compartments would be required to cause any large change in the nucleotide ratio. However it must be stressed that this ratio is undoubtedly a steady state rather than an equilibrium involving a single reaction.

Before discussing the effects of dinitrophenol (DNP) in this system, it seems desirable to relate these ideas to the experimental results obtained by previous workers in this field. In measuring intramitochondrial nucleotides the experimenter must markedly modify the experimental conditions from those used in the ordinary measurement of oxidative phosphorylation. For example, Siekevitz and Potter used a ratio of mitochondria to reaction medium at least twice that used in the experiments recorded here (65). This fact alone might account for the differences in interpretation from that given above and

the hypothesis of Siekevitz and Potter already mentioned that there is an "external adenyl kinase". In the system postulated above, external ATP generated in the CASE III AMP system must pass into the mitochondrion to the A.K. site before it can react with AMP to form ADP in the equilibrium ratio. In the Siekevitz and Potter experimental system, the probability of this happening in a given interval is twice that which exists in the experimental system used in this research. The intramitochondrial nucleotides in the Siekevitz and Potter system amounted to about 8% of the total nucleotides, therefore changes in the 8% would also be reflected more rapidly in the 92% external. There are findings of Siekevitz and Potter which cannot be explained by the system postulated here—the most important of which is the finding that the specific activity of the external ATP in one experiment (starting with labeled inorganic phosphate and AMP) is considerably higher than expected from the specific activity of the internal phosphate. Since the formation of ATP from inorganic phosphate is presumed to occur inside the mitochondrion, one would expect that the specific activity of the internal inorganic phosphate to limit that of the ATP formed. This seems to be a severe limitation to the complete acceptance of the data of Siekevitz and Potter. Further complications in the interpretation of their P^{32} data are the exchange reactions which have been discovered in mitochondria:



these complications lead one to examine the relative values of measurements of external nucleotides versus internal nucleotides. The external nucleotides can be obtained at a given time during an experiment without significant contamination by internal nucleotides. The internal nucleotides can be obtained only after a separation process which takes at least several minutes during which all the conditions surrounding the particle have been changed, and which provide considerable opportunity for changes in the nucleotide content. Obviously, it would be better to measure the internal nucleotides—if it could be done, but it seems to be quite difficult experimentally.

EFFECTS OF DINITROPHENOL:

According to a number of authors (134,135,65), this compound acts on one of the early intermediate substances in a series which culminates in the transfer of inorganic phosphate to ADP to form ATP. It is also reported to stimulate the respiration (136), and to activate the ATPase of mitochondria (65). The data in Tables 7 and 8 indicate that this may not be the whole story of the action of DNP. The "stimulatory" action on respiration is conspicuously absent at $8 \times 10^{-5} M$ when ADP is present in significant amounts. In the presence of hexokinase and AMP there is quite a significant uptake of oxygen and a measurable uptake of inorganic phosphate. Even in the absence of hexokinase, some oxidation occurs in the presence of AMP and very small amounts of ADP and ATP escape to the medium. These differences in action may well repay further investigation. At a lower

level $2.5 \times 10^{-5} \text{ M}$ DNP effectively stops phosphorylation in the absence of hexokinase. No significant ATPase activity is noted. Oxidation is low, and the data showed a decrease in oxidation rate during the measurement period. This may indicate a slow rate of action of the DNP added.

If the site of action of DNP is within the mitochondrion—at the site of oxidative phosphorylation (65,134,135,136), one might expect that ADP would not be removed from the oxidation site as fast as when DNP is absent, and that the consequences would therefore depend upon the other conditions of the experiment. In CASE I, the amount of ATP formed would be reduced, therefore the amount of ADP which could be formed at A.K. would be markedly decreased, and the system may never reach a state comparable with that in the absence of DNP. This is observed in the results in TABLE 7. In Case II, if DNP were added, the amount of ATP which can be formed would be decreased. This will decrease the amount which escapes to the external medium to be converted to ADP and return to the A.K. site to form AMP and ATP, but it will also reduce the amount of ATP which goes directly to the A.K. site. The results in table 7 are very similar to those observed with no DNP except that very little oxygen uptake is noted.

In CASE III plus DNP, again the reduction of formation of ATP severely limits formation of ADP, and the system never approaches that observed in the absence of DNP. It is worthy of note that significant oxidation is observed in the presence of DNP only when AMP is the major nucleotide. This is of considerable interest in light of the recent paper by Borst and Slater (136) in which they have compared the oxidation of various substrates in the presence of DNP and DNP plus phosphate in mitochondria deficient in phosphate. Under

their conditions, intramitochondrial ADP is very low.

In CASE IV, the addition of DNP effectively prevents formation of ATP, but without any action which would greatly disturb the adenyl kinase equilibrium reached. In the case of ATP, hydrolysis due to DNP occurs, presumably at the oxidation site. Siekevitz and Potter found under the conditions of no substrate that ATP was dephosphorylated until adenyl kinase had adjusted the nucleotides to approximately equal concentrations. They also show (with no substrate present) that ADP inhibits DNP stimulated ATPase and that no hydrolysis occurred with ADP plus DNP, even though ATP was present due to the adenyl kinase reaction (139). Their results are therefore compatible with the results and the interpretations presented in this Thesis.

CHAPTER VI

SUMMARY

A study has been initiated of the extramitochondrial nucleotides under various conditions of oxidative phosphorylation by rat-liver mitochondria. Some of the variables which affect these processes in tightly coupled mitochondria have been revealed.

1. When AMP is present in large amounts during the measurement period, oxidation is greater with a given preparation, and the P/O ratio is smaller.

2. The results of a number of experiments point to a compartmented system of enzymes in which the major phosphorylating enzymes are separated from the major site of adenylyl kinase activity and both sites are separated from the medium. This compartmentization is entirely compatible with modern knowledge of mitochondrial structure.

3. Preliminary experiments with dinitrophenol have provided additional information which must be considered in individual experiments with this substance, but most of the results are compatible with an action of this substance early in the sequence of reactions involved in the formation of ATP from ADP. It is worthy of remark that dinitrophenol stopped respiration in every case in which significant amounts of ADP were present.

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APPROVAL SHEET

The thesis submitted by John Earl Biaglow has been read and approved by three members of the faculty of the Graduate School.

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

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

Date

May 31, 1961

Signature of Adviser

M. C. Melchior