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Title EVIDENCE FOR AN INTERMEDIATE RELATED TO OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM BLOWFLIES, PHORMIA REGINA

Abstract approved
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A large number of experiments were done to detect and identify one or more intermediates involved in coupling ATP synthesis to electron transport. Using mitochondria from blowflies, addition of ADP after a period of incubation with Pi^{32} was found to give rise to a temporarily rapid esterification (the "ATP jump"), inhibited by antimycin A, DNP, oligomycin and others. This briefly rapid esterification may indicate accumulation of an intermediate containing P^{32}, before the addition of ADP.

The supernatant fluid from blowfly mitochondria, incubated with Pi^{32} and substrate, was found to contain material which reacted upon addition of ADP to give increased acid-stable P^{32} ester, which by all tests to date appears to be ATP^{32}. Inclusion of oligomycin during incubation of the mitochondria appears to increase the amount of acid-stable P^{32} ester in the extracts, but abolishes the change on adding

ADP; oligomycin only partially inhibits the ADP effect when added to the extracts. Extracts from mitochondria incubated with DNP (and Pi^{32}) gave no detectable reaction upon addition of ADP.

Addition of ADP and Pi^{32} to extracts from blowfly mitochondria, previously incubated with substrate (without Pi^{32}), was also found to give acid-stable P^{32} ester, suggesting that part (at least) of the "intermediate" may not be phosphorylated before this addition. The amount of P^{32} ester measured was reduced by addition of DNP and by omission of substrate during the initial incubation.

The nature of the two intermediates suggested by these results, and the possibility that they may be identical, can be determined only by additional research.

EVIDENCE FOR AN INTERMEDIATE RELATED TO OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM BLOWFLIES, *PHORMIA REGINA*

by

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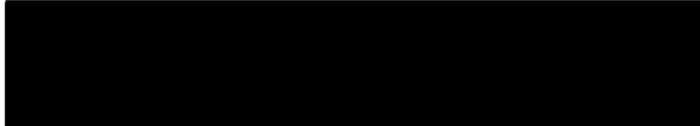

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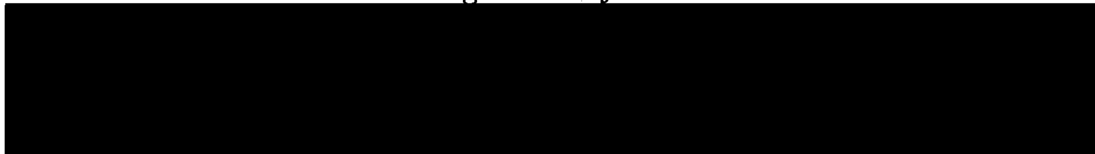

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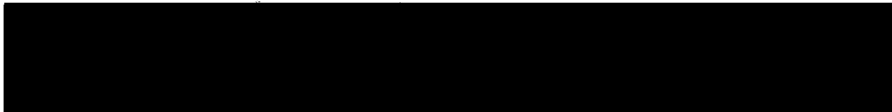

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EVIDENCE FOR AN INTERMEDIATE RELATED TO OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM BLOWFLIES, PHORMIA REGINA

INTRODUCTION

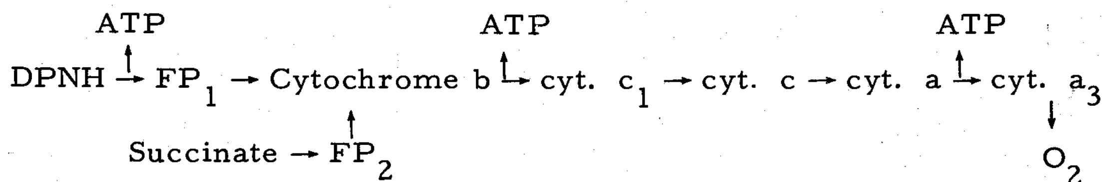
It is well known that adenosine triphosphate (ATP) is the most important compound to supply energy derived from the oxidation of foodstuffs. In cells which utilize oxygen, most of the ATP is formed from adenosine diphosphate (ADP) and inorganic phosphate (Pi) by the process of oxidative phosphorylation. Three molecules of ATP are generated in coupled reactions during the exergonic passage of a pair of electrons from substrate to molecular oxygen in the respiratory chain (47, p. 450-456).

Both electron transport and oxidative phosphorylation take place in mitochondria (60, p. 565-570), which are also the site of other important biochemical processes such as the citric acid cycle (64, p. 893-903) and fatty acid oxidation (40, p. 957-972; 32, p. 1). Thus, each mitochondrion is a complex mixture of enzymes and other materials, capable of converting the energy in many foods to a generally-utilizable form.

The Respiratory Chain

At present, the sequence and the identity of the members of the electron transport chain in higher animal mitochondria, suggested by

kinetic studies, can be pictured as follows (46, p. 47; 21, p. 67-71; 62, p. 347):



An additional member which may function in the respiratory chain is ubiquinone (coenzyme Q)(46, p. 48). Hatefi et al. (38, p. 441-446; 37, p. 447-453) have shown that ubiquinone can connect the four electron-carrier complexes of the NAD- and succinate-linked respiratory chains to give high rates of nonphosphorylating electron transport.[1] It is not yet certain that ubiquinone is an obligatory member of the electron-transport chain, since the kinetic evidence does not support this (20, p. 39; 19, p. 327-340). However, Green and his co-workers clearly showed its ability to oxidize flavoprotein

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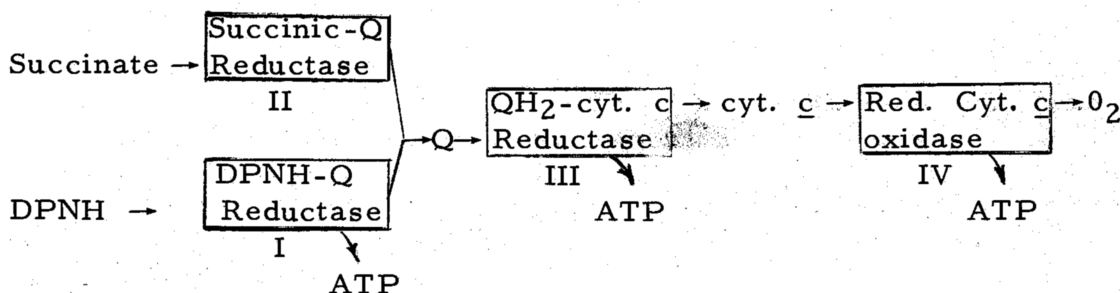
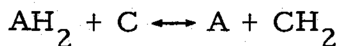
[1]The following abbreviations are used in this thesis: ADP and ATP, adenosine di- and tri- phosphate, respectively; ATPase, adenosine triphosphatase; DPN, diphosphopyridine nucleotide; FP, flavoprotein; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; cyt., cytochrome; DNP, 2,4-dinitrophenol; Pi, inorganic orthophosphate; Pi^{32}, radioactive inorganic orthophosphate; N. E. P^{32}, non-extractable radioactive phosphate; P^{32}, radioactive phosphorus, form unspecified; μg., microgram; μ, micro; μm., μmole(s); olig., oligomycin; anti A., antimycin A; MA, myristic acid; Mg^{++}, magnesium ion; Ca^{++}, calcium ion; TCA, trichloroacetic acid; conc., concentration; red., reduced; min., minute(s); %, percent.

and to transfer electrons ultimately to cytochrome c_1 via or parallel to cytochrome b (45, p. 952-963; 31, p. 987-999). Recently they proposed a scheme of the respiratory chain which consisted of four enzyme complexes (30, p. 1460-1468), arranged as follows:

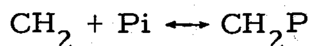


The Coupling Mechanism

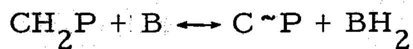
Many investigators have proposed that phosphorylation is coupled with electron transport, at each of three sites in the chain, by formation of a "high-energy" derivative of an electron carrier, either with phosphate or with some other compound (47, p. 450-456; 65, p. 975-978; 58, p. 1970-1979). As early as 1946, Lipmann proposed a hypothesis for the coupling mechanism as follows (51, p. 392-405):



$$AH_2 + C \longleftrightarrow A + CH_2$$



$$CH_2 + Pi \longleftrightarrow CH_2P$$

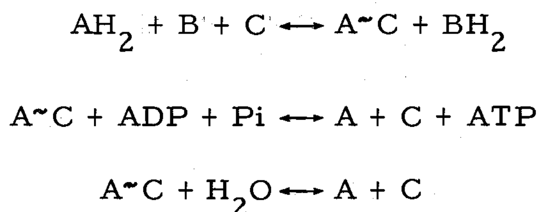


$$CH_2P + B \longleftrightarrow C{\sim}P + BH_2$$

$$C{\sim}P + ADP \longleftrightarrow C + ATP$$

In the sequence above, A and B represent an oxidation-reduction couple, and C (65, p. 975-978) is a participant compound which sometimes is designated by the symbol I (21, p. 99) or X(Y or Z) (47, p. 450-456).

Slater advanced Lipmann's hypothesis by visualizing a combination of C and A with a high energy bond, before the reaction with Pi (65, p. 975-978; 51, p. 392-405). This can be written as follows:

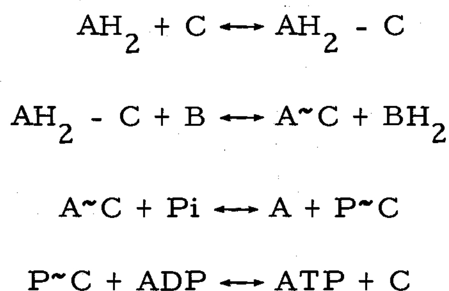


$$AH_2 + B + C \longleftrightarrow A{\sim}C + BH_2$$

$$A{\sim}C + ADP + Pi \longleftrightarrow A + C + ATP$$

$$A{\sim}C + H_2O \longleftrightarrow A + C$$

This hypothesis was extended by Cooper and Lehninger as shown in the following (22, p. 561-578):



$$AH_2 + C \longleftrightarrow AH_2 - C$$

$$AH_2 - C + B \longleftrightarrow A{\sim}C + BH_2$$

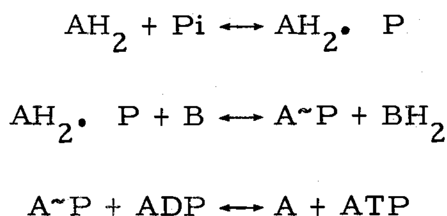
$$A{\sim}C + Pi \longleftrightarrow A + P{\sim}C$$

$$P{\sim}C + ADP \longleftrightarrow ATP + C$$

All of the schemes shown above have the same basic pattern, and contain participant C which, on passage of electrons from carrier A to carrier B, forms a "high-energy" bond either with phosphate or carrier A. Slater designated this kind as Type I (46, p. 49). Lehninger

postulated that the oxidized form of carrier A is involved in the formation of a high-energy intermediate (73, p. 681-687), while Chance and Williams claimed that it is in the reduced form and not the oxidized one (21, p. 99). It is still uncertain whether the oxidized or the reduced form is correct; reports from some laboratories, such as Pinchot (58, p. 1970-1977) and Green (30, p. 1460-1469), suggest the oxidized form, while Griffith (34, p. 1064-1070) and Eisenhardt and Schachinger (26, p. 215) assume the reduced form.

A Type II coupling mechanism was also proposed by Slater (46, p. 47-78). The scheme can be shown as follows:



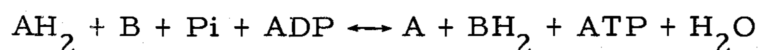
$$AH_2 + Pi \longleftrightarrow AH_2 \cdot P$$

$$AH_2 \cdot P + B \longleftrightarrow A{\sim}P + BH_2$$

$$A{\sim}P + ADP \longleftrightarrow A + ATP$$

Instead of the component C, inorganic phosphate itself is postulated to react directly with an electron carrier molecule to form a phosphorylated carrier molecule.

The sum of the partial reactions in both cases (Types I and II) is:



$$AH_2 + B + Pi + ADP \longleftrightarrow A + BH_2 + ATP + H_2O$$

Type I mechanisms appear to be more popular at present (58,

p. 1975; 30, p. 1467; 45, p. 961).

Exchange Reactions

The enzymes in the system that causes oxidative phosphorylation, or at least enzymes found in the mitochondria, also catalyze a series of characteristic exchange reactions which are thought to be related to oxidative phosphorylation (43, p. 936-939; 62, p. 355-360). The four types of exchange reaction are (43, p. 936) as follows:

(1) Inorganic orthophosphate with the terminal phosphate of ATP.

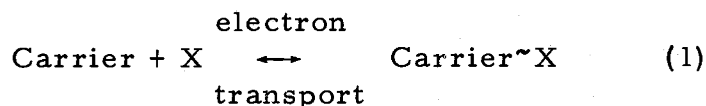
(2) Adenosine diphosphate with adenosine triphosphate.

(3) H_2O^{18} with the oxygen atoms of inorganic orthophosphate.

(4) H_2O^{18} with the oxygen atoms of the terminal phosphate of ATP.

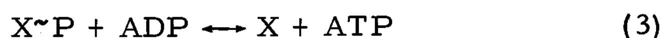
The exchange reactions have been studied very intensively in the hope that they might provide information to reveal the mechanism of oxidative phosphorylation.

Wadkins and Lehninger (72, p. 1589-1597; 23, p. 2351-2356) have reported a number of observations in accord with the proposal shown below:



$$\text{Carrier} + X \underset{\text{transport}}{\overset{\text{electron}}{\longleftrightarrow}} \text{Carrier}{\sim}X \qquad (1)$$

$$\text{Carrier}{\sim}X + Pi \longleftrightarrow \text{Carrier} + X{\sim}P \qquad (2)$$



$$X{\sim}P + ADP \longleftrightarrow X + ATP \qquad (3)$$

The Pi-ATP exchange is the sum of reactions (2) and (3), while ADP-ATP exchange is reaction (3) alone.

Several laboratories have reported that the ADP-ATP exchange is considerably faster than the Pi-ATP exchange (72, p. 1589-1597; 17, p. 369-377; 23, p. 2351-2356). This observation has been an important one, because it supports some of the mechanisms of oxidative phosphorylation outlined previously.

Wadkins and Lehninger (72, p. 1589-1597) concluded that the ATP-ADP exchange is a reflection of the terminal reaction of oxidative phosphorylation, by which ATP is formed from ADP and a "high-energy" phosphate donor:

$$ADP + P{\sim}X \longleftrightarrow ATP + X$$

X is an enzyme protein capable of undergoing phosphorylation.

By using O^{18} as a tracer, Drysdale and Cohn (24, p. 1574-1577) and Boyer (8, p. 405-421) have shown that intact mitochondria catalyze a rapid oxygen exchange reaction between inorganic phosphate and H_2O of the medium. The Pi-H_2O^{18} exchange is about nine times as fast as the ATP-Pi^{32} exchange in an ATP activated system (18, p. 1790-1795). The rate of the H_2O^{18}-ATP exchange appears to be much greater than that of H_2O^{18}-Pi exchange (24, p. 1574-1577).

Lardy, Johnson and McMurray found that the antibiotic, oligomycin, has very interesting properties; it is a potent inhibitor of oxidative

phosphorylation, of adenosine triphosphatase, and of the Pi-ATP and Pi-H_2O exchange reactions, which are thought to be associated with oxidative phosphorylation (44, p. 587-597).

It has long been recognized that 2,4-dinitrophenol (DNP) is a potent agent for "uncoupling" phosphorylation in intact mitochondria (50, p. 807-808). More recent investigations with submitochondrial particles have confirmed the uncoupling effect of DNP as well as its stimulation of ATPase activity and its inhibition of the ATP-Pi^{32} and ATP-ADP^{32} exchange reactions (17, p. 369-377). All three exchanges, ATP-Pi^{32}, ATP-ADP and O^{18} exchange are inhibited by DNP at concentrations which uncouple oxidative phosphorylation (45, p. 932-962). The facts, that oligomycin inhibits both the ADP-ATP and the Pi-ATP exchange reactions, and that DNP inhibits the three exchanges mentioned, provide rather strong evidence that these reactions take part in oxidative phosphorylation.

Coupling Factors

Recently there have been reports from many laboratories about "coupling factors," soluble components which are required for the phosphorylation process. These have been obtained from bacteria (58, p. 1970-1977; 15, p. 398-404) and from animal tissues (49, p. 469-478; 45, p. 952-962; 77, p. 259-265).

There are at least three soluble protein factors that stimulate

the phosphorylation process in submitochondrial particles from animals (63, p. 1659-1663; 74, p. 245-249; 77, p. 259-265). Each coupling factor is specific for one of the three phosphorylation sites on the electron transfer chain (30, p. 259-265).

Racker (63, p. 1659-1663) suggested the possible roles of the soluble coupling factors as follows:

(1) That coupling factor 1 is adenosine triphosphatase (ATPase).

(2) That coupling factor 2 is required for the phosphorylation of ADP associated with the oxidation of succinate, and that it is identical with the factor of Linnane and Titchener (49, p. 469-478).

(3) That coupling factor 3 is required for the ATP-Pi^{32} exchange.

Webster _et al._ (77, p. 259-266; 76, p. 399-404; 75, p. 16-20) were able to show that a soluble, high-energy substance formed during the oxidation of reduced cytochrome c by submitochondrial particles, supplemented with the specific coupling factor and magnesium ions. This substance was found to react with ADP and Pi to form ATP. The formation of the high-energy substance was prevented by DNP.

Recently, Glaze _et al._ (28, p. 194-199) have extracted the enzyme which catalyzes the ATP- ADP exchange reaction. They found that purified ATP-ADP exchange enzyme forms a kinetically detectable

complex with reduced cytochrome c when incubated with ATP. They pointed out that the ATP-ADP exchange enzyme may be identical with a highly purified protein of beef heart mitochondria which Webster (74, p. 245-249) has found to restore phosphorylation at site 3 and to catalyze an ATP-ADP exchange reaction.

Possible Intermediates

Several investigators have indicated that mitochondria, in the absence of added phosphoryl acceptor, may accumulate material which allows rapid formation of ATP when ADP is added (71, p. 2555-2563). It has been suggested, therefore, that the accumulated material must serve as an intermediate of oxidative phosphorylation. In the last few years, several laboratories have made more intensive studies in order to obtain conclusive evidence for the presence of an intermediate, and some laboratories have identified one or more of them.

Pinchot (58, p. 1970-1977) studied with the bacterium, *Alcaligenes faecalis*, and concluded that the intermediate which he observed consists of DPN bound to a protein by means of a high-energy bond. He found that this intermediate combines first with Pi to release DPN and to form a transient phosphorylated intermediate. The latter reacts with ADP to form ATP and free protein (59, p. 54). Webster *et al*. reported that they obtained a high-energy substance with similar characteristics, formed under similar conditions (57, p. 929-938).

Brierley (11, p. 237-245; 14, p. 437-442; 13, p. 3482-3489; 12, p. 1928-1935) reported the accumulation of both magnesium and calcium phosphates in beef heart mitochondria. This accumulation is inhibited by uncouplers of oxidative phosphorylation and by inhibitors of electron transport, and it is substrate dependent. It is not sensitive to oligomycin. Therefore, Brierley suggested that ion accumulation is energized by an intermediate of oxidative phosphorylation and gave the following scheme:

$$\text{Electron Transport System} \longrightarrow [\sim] \xleftarrow[\text{ADP}]{\text{Oligomycin}} \longrightarrow \text{ATP}$$

$$[\sim] \downarrow \text{Ion Accumulation}$$

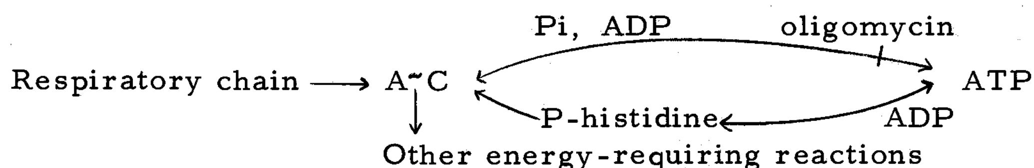
Purvis (61, p. 435-446) reported the formation of "extra DPN" upon incubation of mitochondria with phosphate, ADP or DNP. They postulated that this is due to liberation of DPN from some precursor, such as DPN~I, of an energy-rich intermediate of oxidative phosphorylation.

Brodie (16, p. 89-100) studied with _M. phlei_ and found that a naphthotocopherol-like compound appears to be an intermediate in oxidative phosphorylation.

Phosphorylated intermediates have also been identified by a number of investigators. Boyer _et al._ (68, p. 43-47; 7, p. 195) reported a possible intermediate in oxidative phosphorylation, because

they found rapid uptake of Pi^{32} into a protein-bound form, in intact mitochondria. Later, they identified phosphohistidine (9, p. 3306-3308; 55, p. PC1182; 6, p. 1080-1087) in hydrolysates of the labeled protein. This same material was also formed in intact mitochondria from adenosine triphosphate-P^{32} (56, p. PC1180-1182). Peter and Boyer (56, p. PC1180-1182) found that oligomycin does not decrease the amount of bound intermediate or uncouple oxidation from phosphorylation; oligomycin does inhibit the passage of P^{32} from either Pi or ATP into intermediate. The phosphoryl moiety is attached to an imidazole nitrogen (10, p. 1147-1153). Bound phosphohistidine is acid-labile and alkali-stable.

Recently, Slater _et al._ (66, p. 781-784) studied the incorporation of Pi^{32} into protein-bound phosphorus (presumably phosphohistidine) and into ATP; they found that the rate of incorporation into protein-bound phosphorus is only 1.5% of the rate into ATP. Oligomycin inhibited the incorporation into ATP by 96-99% while Boyer found 56% inhibition. Slater _et al._ concluded, therefore, that the protein containing phosphohistidine is not an intermediate on the main pathway of oxidative phosphorylation, but that it is involved in a slow, oligomycin-insensitive pathway. These conclusions were summarized as follows:



Ahmed and Judah (1, p. 295-304) suggested that the phosphoprotein accepts phosphate from an intermediate of the oxidative phosphorylation process, rather than from ATP, because they found that oligomycin does not inhibit phosphoprotein turnover at a concentration which greatly reduced labeling of ATP. They proposed that mitochondrial phosphoprotein is on a cyclic pathway between intermediates of the oxidative phosphorylation system and ATP. This postulation can be written as follows:

DNP Oligomycin
Electron Transport → X~I ⟷ X~P ⟷ ATP
Pi
Pi
Mg^{++}
Mn^{++}
Protein~P

Griffiths and Chaplain (35, p. 497-300; 34, 1064-1070) reported the presence of a phosphorylated intermediate in extracts of heart mitochondria which had been incubated with Pi^{32}, NAD and succinate. The intermediate reacts with ADP in the presence of submitochondrial particles to give ATP and NADH. This phosphorylated intermediate appears to be a new derivative of NAD and is not NADP. Only succinate is found to be effective as a substrate during incubation of the mitochondria, and the intermediate is not detected if electron transport is blocked by antimycin A. The formation of ATP is inhibited by

oligomycin, but not by antimycin A. This intermediate is highly labile even at neutral pH (36, p. 501-505).

Packer (54, p. 57-71) studied the swelling and shrinkage of mitochondria, and indicated that the state of least swelling is obtained with substrate alone; the maximum swelling is brought about by addition of both substrate and phosphate. This suggests the accumulation of a phosphorylated intermediate, X~P, as swelling approaches a maximum. An intermediate state of swelling is obtained upon addition of substrate, phosphate and ADP, because, it is presumed, the X~P concentration is reduced by ATP synthesis. Although oligomycin prevents ATP synthesis, it apparently does not inhibit accumulation of X~P; oligomycin prevents the partial reversal of swelling brought about by addition of ADP.

Wadkins (70, p. 411-415) reported two kinds of phosphoprotein which require respiration for their formation:

(1) An acid-stable form which can be isolated as serine phosphate after acid hydrolysis of the protein (41, p. 153-163; 27, p. 775-788). Ahmed and Judah (2, p. 245-252) have also identified phosphorylserine in digests of phosphoprotein.

(2) An acid-labile and base-stable form isolated as phosphohistidine after alkaline hydrolysis of the protein (9, p. 3306-3308).

The purpose of this thesis is to present evidence for accumulation of an intermediate of oxidative phosphorylation in mitochondria from

blowflies, *Phormia regina*, when these are incubated under approp-riate conditions. Although an intermediate has not been isolated from these preparations, the author hopes that the results reported in this thesis will assist others who attempt to purify and identify such a compound.

METHODS AND MATERIALS

The blowflies, Phormia regina, used in these studies were from a laboratory colony maintained by the Department of Entomology at Oregon State University. The diets of the breeding colony and of the adult flies have been described previously (32, p. 15). The mitochondria were isolated by modifications of the procedure used by Gregg (32, p. 15-21).

Methods for Preparation of the Mitochondria

Method A

Adult flies (about seven days old) were anesthetized by chilling at $1^{o}C$. for about one-half hour; then, 25-125 ml. of the whole flies were measured and transferred to a tray standing on ice. Another tray containing ice was used as a cover. The thorax was removed from each fly and stored in a cold beaker until all flies had been dissected. The thoraces were ground gently in an ice cold, glass mortar with 150-300 ml. of standard homogenizing medium, which contained 0.25 M sucrose, 0.05 M Tris, 0.1 M potassium phosphate, 0.003 M EDTA, 0.003 M $MgCl_2$, 0.006 M citrate, 0.006 M succinate and 0.006 M pyruvate; the pH of this medium was 7.4. The homogenate was filtered through eight layers of cheesecloth and then through glass wool, to remove pieces of chitin and other debris. It was then

centrifuged at 460 x g for three minutes in a Servall refrigerated centrifuge, to remove whole cells, muscle fibers and other unwanted materials. The upper portion of the supernatant fluid was decanted through a glass wool plug; then, the filtrate was centrifuged at 6,780 x g for five minutes to sediment the mitochondria. The supernatant fluid was decanted and the walls of the centrifuge tubes were carefully wiped with Kleenex tissue to remove fatty and incompletely sedimented materials streaked along the walls of the tubes. The mitochondrial pellets were suspended in 80 ml. of suspending medium containing 0.25 M sucrose, 0.05 M Tris, 0.003 M EDTA, 3×10^{-5} M $MgCl_2$, 0.006 M citrate, 0.006 M succinate and 0.006 M pyruvate, pH 7.4; a loosely fitting, glass, Potter-Elvehjem homogenizer, operated by hand, was used in making the suspension. This was recentrifuged to sediment the mitochondria. The supernatant liquid was again decanted, the tubes were wiped as described previously, and the mitochondrial pellets were resuspended in suspending medium to the desired volume.

Method B

The procedure for Method B was the same as Method A, except that phosphate was omitted from the homogenizing medium.

Method C

The procedure for Method C was the same as Method A, except that phosphate was omitted from the homogenizing medium, the mitochondria were washed twice (20 ml. each) in suspending medium, and then suspended in suspending medium supplemented with 1 mg. albumin per ml.

Method D

The procedure for Method D was the same as Method C, except that magnesium was omitted from all media used in isolating and suspending the mitochondria.

Method E

The procedure for Method E was the same as Method A, except that the blowfly thoraces were homogenized in "Steele homogenizing medium" (67), which contained 0.3 M sucrose, 0.05 M Tris, 0.003 M EDTA, 1×10^{-4} M $MgCl_2$, 0.005 M pyruvate, 3.3×10^{-5} M fumarate and 1 mg. albumin per ml. The mitochondria were also washed twice (40 ml. each time) and suspended in Steele homogenizing medium

Procedure for Incubation of the Mitochondria

Aliquots of the mitochondrial suspension were added to 25 ml.

Erlenmeyer flasks which already contained most or all of the additional materials to be included in the incubation medium. Unless otherwise noted, the volume of the complete incubation medium was 3.0 ml., including the mitochondrial suspension. In the experiments to be reported, the amount of Pi^{32} employed, where indicated, was equivalent to 1.3×10^5 to 4.3×10^6 counts/minute/3.0 ml. of incubation medium; the range for most of the experiments was between 3.0×10^5 and 2.0×10^6 counts/minute/3.0 ml. of medium. The other contents of the incubation medium used in each experiment are given in the appropriate table orfigure.

Unless indicated otherwise, each flask was incubated at 25°C. in a Dubnoff incubator, with constant shaking, and enzymatic reaction was stopped by addition of 1.0 ml. of 0.8 M trichloroacetic acid (TCA). Then, each TCA-incubation mixture was filtered through Whatman No. 50 filter paper. The filtrate so obtained will be called a "TCA filtrate."

Determination of Non-Extractable P^{32}

Method N

In Method N ("normal"), N.E. P^{32} was determined on each TCA filtrate by the procedure of Nielson and Lehninger (53, p. 555-570). A 1.0 ml. aliquot of each TCA filtrate was transferred to a separate

glass-stoppered centrifuge tube containing the following: 4 ml. iso-butyl alcohol-benzene reagent, 1 ml. ammonium molybdate reagent (including 4 N H_2SO_4) and 2 ml. of distilled water. Each centrifuge tube was shaken vigorously for 30 seconds, then centrifuged in an International centrifuge for 4 minutes to obtain complete separation of the two phases. The aqueous layer was then filtered into a second centrifuge tube containing 4.0 ml. of isobutyl alcohol-benzene reagent. The tube was again shaken vigorously for 30 seconds and then centrifuged as stated above. The aqueous layer was filtered again and the final filtrate was used for radioactive measurements.

The isobutyl alcohol-benzene reagent was prepared by mixing 200 ml. isobutyl alcohol, 200 ml. benzene and 40 ml. of distilled water, and shaking the mixture for 30 minutes.

The ammonium molybdate reagent was prepared as described by Martin and Doty (52, p. 965-967).

Method H

In Method H (heated), N.E. P^{32} was determined as follows: A 1.0 ml. aliquot of each TCA filtrate was mixed with 1 ml. of 2 N H_2SO_4 in a glass-stoppered centrifuge tube. The tube was covered with aluminum foil and immersed in boiling water for two minutes. After cooling the tube to room temperature, 1 ml. of distilled water, 1 ml. of special molybdate reagent (containing only 2 N H_2SO_4) and

4 ml. of isobutyl alcohol-benzene reagent were added. The remainder of the procedure was the same as described for Method N, above, beginning with the initial shaking of the tube.

Determination of Mitochondrial Protein

The amount of mitochondrial protein was determined by the method of Jacobs *et al.* (39, p. 153-164).

Verification

Results similar to those given in the tables and figures were commonly obtained in two or more experiments.

Materials

The ATP (disodium salt, from muscle), ADP (sodium salt, from muscle), hexokinase (type III, from yeast), Tris (Sigma 121), sodium pyruvate and crystalline bovine serum albumin were products of the Sigma Chemical Company.

The 2, 4-dinitrophenol, isobutyl alcohol, citric acid, sodium azide and succinic acid were obtained from Eastman Kodak Company. EDTA (practical grade) was obtained from Eastman Kodak Company, converted to the disodium salt and recrystallized twice by the method of Blaedel and Knight (4, p. 741-743). Myristic acid was obtained from Eastman Kodak Company and was of the white label quality.

Oligomycin was obtained as a gift from Dr. Bertram Pressman, and also purchased from Wisconsin Alumni Research Foundation.

The fumaric acid was obtained from Delta Chemical Works. The sucrose, magnesium chloride, ammonium molybdate and benzene were products of reagent grade from Baker Chemical Company. The P^{32} was obtained as inorganic orthophosphate, carrier-free, from Oak Ridge National Laboratory.

RESULTS AND DISCUSSION

Part I
Incubation of Mitochondria with Pi^{32} Followed by Addition of Trichloroacetic Acid

Conditions for Optimal Phosphorylation in Blowfly Mitochondria When Using Pi^{32}

Gregg *et al.* (33, p. 593-595) have reported preparation of active housefly mitochondria using standard homogenizing medium, as described under Method A in Methods and Materials, and a suspending medium containing no substrates. The medium used by Gregg *et al.* for the homogenization was also used in obtaining blowfly homogenates in Method A. However, in Method A the suspending medium also contained intermediates of the citric acid cycle, because this modification yielded mitochondria which were more active under the conditions employed in the P^{32} studies, as may be seen in Table I.

In the course of these studies, many methods were tested for the purpose of removing endogenous inorganic phosphate (Pi), and Pi absorbed from the standard homogenizing medium, since this interfered with the P^{32} studies. The results of these tests suggested that phosphate should be omitted from all media used in isolating the mitochondria, as was done in Methods B through E. Albumin was added to the final suspension of the mitochondria, in exploratory experiments, because Lehninger and Remmert (48, p. 2459-2464) reported that the

TABLE I. The Effect of Substrates in the Suspending Medium on the Activity of Blowfly Mitochondria

Expt. No. [1]	Added to Standard Medium[2]	Incubation Time (min.)	m μmoles of Phosphate Esterified: No Substrate in Suspending Medium	m μmoles of Phosphate Esterified: With Substrate in Suspending Medium
40	ADP-5	8	6.6	224.7
	ADP-0	3	18.6	1127.6
	Anti. A-0, ADP-5	8	1.5	9.8
	Anti. A-0, ADP-0	3	4.7	17.3
	Anti. A-5, ADP-7	10	2.2	12.7

[1] Two mitochondrial suspensions were prepared by Method A, except that only one of the preparations was made with substrates in the suspending medium. Each flask contained 30 μmoles pyruvate, 2 μmoles fumarate, 500 μmoles sucrose, 3 μmoles EDTA, 3.03 μmoles $MgCl_2$, 5 μmoles phosphate, 100 μmoles Tris, Pi^{32} and 6.1 mg. mitochondrial protein. Flasks containing mitochondria prepared with substrates in the suspending medium had 6 μmoles pyruvate, 6 μmoles succinate and 6 μmoles citrate, in addition to the substrates mentioned previously. ADP (2 μmoles) and antimycin A (2 μg.) were added where indicated. The reactions were stopped at the end of the incubation period by addition of 1.0 ml. of 0.8 M TCA. N.E. P^{32} was determined by Method N.

[2] The mitochondria were added to each flask at the beginning of the incubation (zero time); the number after each reagent indicates the minutes thereafter when that reagent was added.

swelling of mitochondria, caused by U factor, can be prevented by addition of bovine serum albumin. Addition of albumin was found to be beneficial in the blowfly preparations; therefore, this modification was included in Methods C through E. Steele and Remmert (67) have developed a new homogenizing medium, in which Pi is omitted and albumin is included. This medium (Steele homogenizing medium) yielded very active mitochondria for the P^{32} studies and was used as described in Method E for many preparations of the mitochondria.

Addition of ADP after a Period of Incubating Mitochondria with Pi^{32}

As shown in Figure 1, when ADP was added to the flasks in which mitochondria were incubating in the presence of Pi^{32}, a rapid increase of non-extractable P^{32} (N. E. P^{32}) was obtained during the first two minutes. This can be interpreted as an indication that before addition of ADP, some high-energy intermediate had accumulated. Antimycin A reduced the amount of N. E. P^{32} before the addition of ADP, and also caused a slower rate of increase when ADP was added. This result would suggest that accumulation of the intermediate depended upon electron transport.

Effects of Inhibitors

Oligomycin. The results presented in Figure 1 also indicate that oligomycin has several effects when added to the incubation medium,

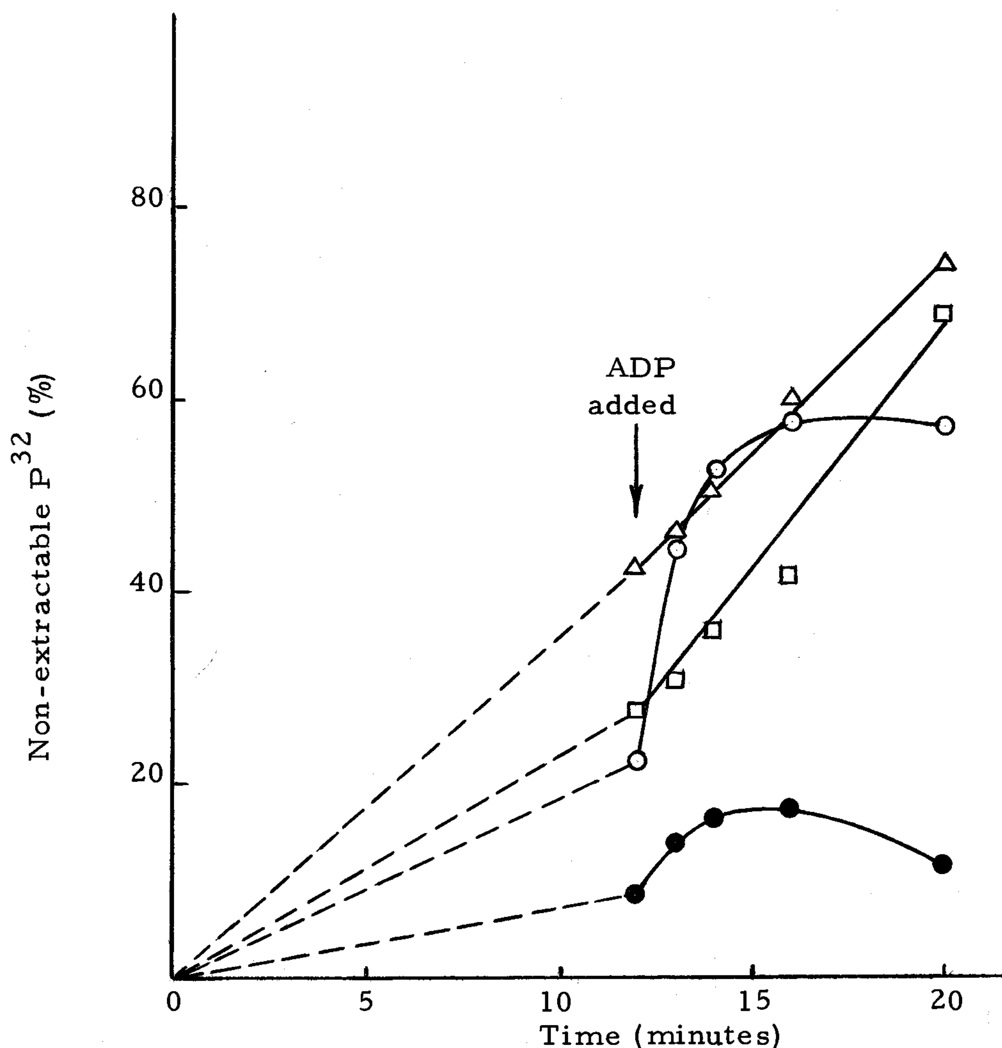


Figure 1. The effect of adding ADP after incubation of mitochondria with Pi^{32}. The mitochondrial preparation and the standard flask contents were the same as those given in Table II, with 10.4 mg. mitochondrial protein per flask and water to a final volume of 3.0 ml. ADP (2 μmoles) was added to each flask 12 minutes after the mitochondria; reaction was terminated at the points indicated by addition of 1.0 ml. of 0.8 M TCA. Incubated at 25°C. Control O; control + oligomycin (6 μg.) Δ ; control + oligomycin (6 μg.) + antimycin A (6 μg.) □ ; control + antimycin A (6 μg.) ●.

as will be discussed below.

(1) Oligomycin appeared to inhibit transfer of P^{32} to ADP, because the rate of increase of N. E. P^{32} was reduced during the first two minutes after adding ADP, in the presence of this inhibitor.

(2) Oligomycin appeared to stabilize, or to raise the steady state concentration of, acid-stable P^{32} esters (and possibly intermediates), since in its presence a larger amount of N. E. P^{32} was obtained before ADP addition. Wadkins (70, p. 411-415) reported two kinds of possible intermediates; one is acid-stable and the other is acid-labile. Because the reactions in these experiments were stopped with TCA, the N. E. P^{32} obtained is presumed to be in the acid-stable form.

(3) The results in Figure 1 suggest that oligomycin inhibited ATPase. When ADP was added to the control flask, N. E. P^{32} reached a steady state within four minutes. No steady state was obtained when oligomycin was present; N. E. P^{32} continued to increase, which may be interpreted to mean that the rate of ATP destruction was lowered. ATPase presumably caused hydrolysis of ATP in the control flask, so that a steady state was reached rather quickly.

It may be seen also in Figure 1 that a similar result was

obtained when oligomycin was present together with antimycin A. Since antimycin A is an electron transport inhibitor, a smaller amount of N. E. P^{32} would be expected and was obtained when this inhibitor was added alone (Figure 1). However, the results show that flasks with antimycin A together with oligomycin yielded higher amounts of N. E. P^{32} than those with antimycin A alone, and even more than the control flasks eventually. The results are consistent with the hypothesis that the effect of oligomycin was to stabilize an intermediate, as well as to inhibit ATPase, as discussed previously.

The effects of oligomycin are also shown in Figure 2. The amount of N. E. P^{32} obtained in the flask containing oligomycin and azide was not much different from the amount formed with azide alone, but was much lower than the amount with oligomycin alone (compare 1, 4 and 5). The results indicate that oligomycin has little effect in the presence of azide. This will be discussed in relation to the action of azide.

(4) As shown in Table II, oligomycin prevented the reduction of N. E. P^{32} observed when DNP was added after incubation with ADP; however, oligomycin did not prevent the reduction caused by DNP in the absence of ADP. These results suggest

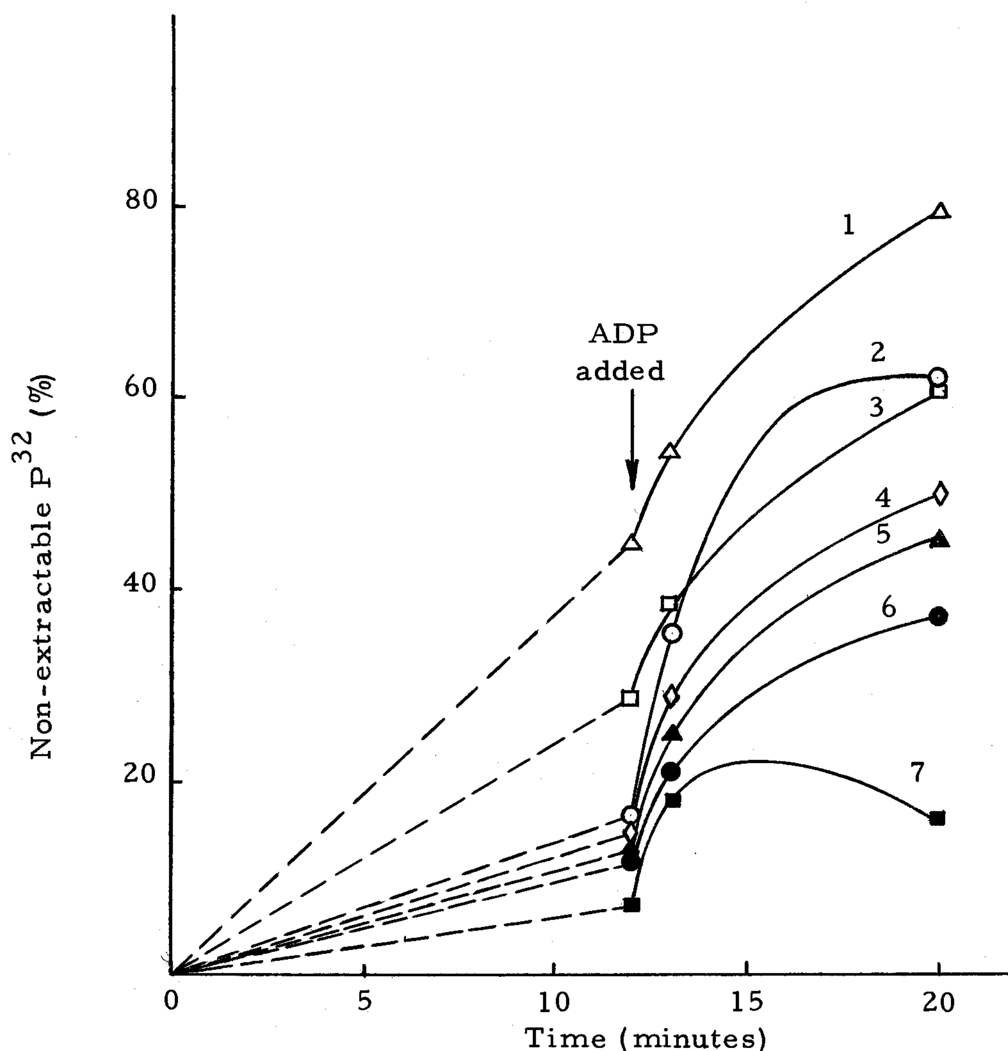


Figure 2. The effect of adding ADP after incubation of mitochondria with Pi^{32} and inhibitors. The mitochondrial preparation and the standard flask contents were the same as those given in Table II, with 7. 1 mg. mitochondrial protein per flask and water to a final volume of 3. 0 ml. ADP (2 μ moles) was added to each flask 12 minutes after the mitochondria; reaction was terminated at the points indicated by addition of 1. 0 ml. of 0. 8 M TCA. Incubated at 25°C. (1) Control + oligomycin, 6 μg. ; (2) Control; (3) Control + oligomycin, 6 μg. + antimycin A, 6 μg. ; (4) Control + oligomycin, 6 μg. + azide, 30 μmoles; (5) Control + azide, 30 μmoles; (6) Control + antimycin A, 6 μg. + azide, 30 μmoles; (7) Control + antimycin A, 6 μg.

TABLE II. The Effects of Oligomycin and Azide on DNP Reactions

Expt. No.[1]	Added to Standard Medium[2]	Incubation Time (min.)	% N. E. P^{32}
74	None	12	13.6
	None	20	14.2
	ADP-0	12	77.0
	ADP-0	20	73.0
	ADP-0, DNP-12	20	17.6
	Olig. -0	20	28.6
	Olig. -0, DNP-12	20	11.9
	ADP-0. olig. -0, DNP-12	20	75.0
76	None	12	6.4
	None	20	7.9
	DNP-12	20	4.2
	Olig. -0	20	17.2
	Olig. -0, DNP-12	20	8.5
	ADP-0	12	61.0
	Olig. -0, ADP-0	12	54.2
	ADP-0	20	54.0
	Olig. -0, ADP-0	20	68.0
	ADP-0, DNP-12	20	12.3
	ADP-0, olig. -0, DNP-12	20	56.6
	ADP-0, azide-12	20	45.8
	ADP-0, azide-12, DNP-12	20	46.8
82	ADP-0, olig. -0	20	72.8
	ADP-0, DNP-0, olig. -0	20	29.4
	ADP-0, DNP-6, olig. -0	20	51.0
	ADP-8, DNP-6, olig. -0	20	21.8

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[1] The mitochondria were prepared by Method A except that they were washed twice (20 ml. each) and suspended in suspending medium containing 1 mg. albumin per ml. Each flask contained 36 μmoles pyruvate, 2 μmoles fumarate, 6 μmoles citrate, 6 μmoles succinate, 500 μmoles sucrose, 3 μmoles EDTA, 3.03 μmoles $MgCl_2$, 100 μmoles Tris, 0.5 mg. albumin, Pi^{32} and approximately 7.5 mg. mitochondrial protein. ADP (2 μmoles), DNP (0.3 μmole), oligomycin (2 μg.) and azide (30 moles) were added where indicated. The reactions were stopped at the end of the incubation period by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

[2] The mitochondria were added to each flask at the beginning of the incubation (zero time); the number after each reagent indicates the minutes thereafter when that reagent was added.

that oligomycin inhibits the ATPase activity elicited by addition of DNP, but that it cannot prevent the destruction of an intermediate which results from DNP action.

Antimycin A. As shown in Figures 1 and 2, antimycin A inhibited phosphate esterification, though not completely. When ADP was added to the flask in which mitochondria had been incubated with antimycin A, an increase in N. E. P^{32} was obtained for four minutes, followed by a decrease. This may have been due to endogenous ATPase activity, becoming operative when ATP was formed, because oligomycin prevented this effect. More and more N. E. P^{32} was obtained upon incubation with ADP, antimycin A and oligomycin, which could result from a slow rate of electron transport (and phosphate esterification or exchange) and an even slower rate of ATP hydrolysis.

DNP. As shown in Tables II and III, DNP decreased the amount of N. E. P^{32} found in the presence or absence of ADP. It is commonly assumed that DNP causes hydrolysis of an intermediate and/or ATP; the results of this study suggest that the hydrolysis of ATP and the effect on an intermediate are distinguishable. As mentioned previously, oligomycin appears to inhibit DNP-stimulated hydrolysis of ATP, but failed to prevent loss of N. E. P^{32} caused by DNP in the absence of ADP. Thus, DNP appears to destroy an intermediate, or prevent its formation, even when ATPase is inhibited by oligomycin.

TABLE III. The Effects of DNP and Mg^{++} ion Concentration on Phosphate Esterification

Expt. No. [1]	Added to Standard Medium [2]	Incubation Time (min.)	% N. E. P^{32}
91	None	10	5.6
	$MgCl_2$(10)-0	10	9.7
	$MgCl_2$(20)-0	10	8.1
	DNP-0	10	6.1
	DNP-0, $MgCl_2$(10)-0	10	8.9
	DNP-0, $MgCl_2$(20)-0	10	9.0
	ADP-0	10	66.5
	ADP-0, $MgCl_2$(10)-0	10	77.5
	ADP-0, $MgCl_2$(20)-0	10	80.0
	ADP-0, DNP-0	10	19.5
	ADP-0, DNP-0, $MgCl_2$(10)-0	10	33.0
	ADP-0, DNP-0, $MgCl_2$(20)-0	10	42.1

[1] The preparation of the mitochondria and the standard flask contents were the same as given in Table II, except that each flask contained 4.7 mg. mitochondrial protein. ADP (2 μmoles), DNP (0.3 μmoles) and an additional ten (10) or twenty (20) μmoles of $MgCl_2$ were added where indicated. The reactions were stopped at the end of the incubation period by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

[2] The mitochondria were added to each flask at the beginning of the incubation (zero time); the number after each reagent indicates the minutes thereafter when that reagent was added.

Azide. The results shown in Figure 2 suggest that azide inhibits phosphate esterification, but not completely (compare 2 and 5), and that it also inhibits hydrolysis of phosphate esters (compare 6 and 7). Thus, azide has an effect similar to that of oligomycin, but appears to inhibit phosphate esterification or transfer to a greater extent, and ATPase to a lesser extent (compare 1 and 5, and 3 and 6). This could account for the reduced accumulation of N. E. P^{32}, which resulted when azide was added in the presence of oligomycin (compare 1, 4 and 5).

Azide can also inhibit the hydrolysis of ATP which occurs in the presence of myristic acid, as shown in Table IV. Addition of myristic acid after incubating with ADP for 12 minutes resulted in a large loss of N. E. P^{32}, presumed to be ATP^{32}, and azide prevented much of this loss. Similarly, azide prevented the hydrolysis of ATP caused by addition of DNP, as shown in Table II, experiment number 76.

Myristic Acid. Myristic acid may also cause hydrolysis of an intermediate, as suggested by the results in Table IV. By comparing set 1 and 2, it may be seen that in the absence of ADP myristic acid decreased the amount of N. E. P^{32} nearly 50%, even in the presence of azide. After incubating the mitochondria in the presence of ADP, addition of myristic acid stimulated ATPase and decreased esterified phosphate greatly; in this case, azide prevented most of the loss,

TABLE IV. Effect of Myristic Acid on Oxidative Phosphorylation

Expt. No.[1]	Added to Standard Medium[2]	Time of Adding			% N. E. P^{32}	
		Myristic Acid	Hexokinase System	TCA	Method N	Method H
100	None	After TCA	-	12	7.3	2.7
	Azide-0	After TCA	-	12	13.5	4.7
	Myristic acid-0	After TCA	-	12	0.28	0
	ADP-0	After TCA	-	12	46.6	13.6
	Azide-0, ADP-0	After TCA	-	12	34.8	10.4
	Myristic acid-0, ADP-0	After TCA	-	12	0.11	0
	None	12	-	16	4.5	2.2
	Azide-0	12	-	16	7.8	3.8
	Myristic acid-0	12	-	16	0.22	0.07
	ADP-0	12	-	16	16.2	8.85
	Azide-0, ADP-0	12	-	16	28.3	9.6
	Myristic acid-0, ADP-0	12	-	16	0	0
	None	12	16	26	3.3	2.1
	Azide-0	12	16	26	3.9	3.0
	Myristic acid-0	12	16	26	0.34	0.28
	ADP-0	12	16	26	13.9	10.5
	Azide-0, ADP-0	12	16	26	27.4	26.0
	Myristic acid-0, ADP-0	12	16	26	0.13	0.17

[1]The preparation of the mitochondria and the standard flask contents were the same as given in Table II, except that each flask contained 7.1 mg. mitochondrial protein. ADP (2 μmoles), azide (30 μmoles) and myristic acid (3 μmoles) were added where indicated. In Set 1, the reactions were stopped with TCA (1.0 ml. of 0.8 M) at 12 (minutes after addition of mitochondria), then myristic acid was added later. In Set 2, myristic acid was added at 12, then TCA at 16. In Set 3, myristic acid was added at 12, the hexokinase system (50 K.M. hexokinase and 50 μmoles glucose) at 16, and TCA at 26. Flasks which received myristic acid (3 μmoles) at zero time received a second addition (3 μmoles) at the times mentioned. N. E. P^{32} was determined by both Methods N and H.

[2]The mitochondria were added to each flask at the beginning of the incubation (zero time); the number after each reagent indicates the minutes thereafter when that reagent was added.

probably by inhibiting ATPase.

Myristic acid also inhibits phosphate esterification completely, as shown (Table IV) when both ADP and myristic acid were added at the beginning of the incubation.

Effect of the Mg^{++} ion Concentration in the Incubation Medium

In Table III, it is shown that a higher Mg^{++} ion concentration tends to reduce the effect of DNP. Presumably DNP hydrolyzes an intermediate and/or ATP and the higher Mg^{++} ion concentration permits an increased rate of synthesis.

Results in Table V show that N. E. P^{32} did not increase in the presence of azide when a higher Mg^{++} ion concentration was provided, while there was an increase upon adding more Mg^{++} ions in the presence of oligomycin. It may be postulated that the azide prevented the increased synthesis which a higher Mg^{++} ion concentration would have induced.

Effect of Omitting Substrate from the Incubation Medium

Data in Table VI show that when substrates were present in the incubation medium, a larger amount of esterified phosphate was obtained, than in the absence of substrate. This result indicates that phosphate esterification depended upon electron transport and oxidative phosphorylation. Without substrates added, the ADP effect

TABLE V. The Effects of Mg^{++} ion Concentration on Phosphate Esterification in the Presence of Oligomycin and Azide

Expt. No. [1]	Added to Standard Medium[2]	Incubation Time (min.)	% N. E. P^{32}
71	None	13	6.3
	Olig.-0	13	20.8
	Azide-0	13	7.7
	Olig.-0, azide-0	13	9.3
	None	20	7.8
	Olig.-0	20	22.2
	Azide-0	20	9.6
	Olig.-0, azide-0	20	10.4
	$MgCl_2$-0	20	15.7
	Olig.-0, $MgCl_2$-0	20	29.2
	Azide-0, $MgCl_2$-0	20	10.8
	Olig.-0, azide-0, $MgCl_2$-0	20	-
72	None	12	14.0
	Olig.-0	12	26.8
	Azide-0	12	11.0
	None	20	14.0
	Olig.-0	20	28.8
	Azide-0	20	11.5
	DNP-12	20	7.3
	Olig.-0, DNP-12	20	14.0
	Azide-0, DNP-12	20	8.4
	$MgCl_2$-12	20	-
	Olig.-0, $MgCl_2$-12	20	32.0
	Azide-0, $MgCl_2$-12	20	11.4

[1]The preparation of the mitochondria and the standard flask contents were the same as given in Table II, except that each flask contained approximately 7.5 mg. mitochondrial protein. Azide (30 μmoles), oligomycin (2 μg.), $MgCl_2$ (15 μmoles) and DNP (0.3 μmoles) were added where indicated. The reactions were stopped at the end of the incubation period by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

[2]The mitochondria were added to each flask at the beginning of the incubation (zero time); the number after each reagent indicates the minutes thereafter when that reagent was added.

TABLE VI. The Effect of Omitting Substrate from the Incubation Medium

Expt. No. [1]	Added to Standard Medium [2]	Incubation Time (min.)	% N. E. P^{32}	
			No Substrate	With Substrate
95	None	4	2. 7	6. 8
	Azide-0	4	6. 2	10. 2
	DNP-0	4	2. 4	4. 9
	ADP-0, hexokinase system-0	4	3. 9	20. 5
	Azide-0, ADP-0, hexo-kinzse system-0	4	4. 6	15. 2
	DNP-0, ADP-0, hexo-kinase system-0	4	2. 9	11. 7
	None	24	1. 7	12. 3
	Azide-0	24	6. 05	21. 6
	DNP-0	24	1. 0	6. 7
	ADP-0, hexokinase system-0	24	6. 2	36. 0
	Azide-0, ADP-0, hexo-kinase system-0	24	8. 5	49. 4

---

[1] The mitochondria were prepared by Method A, except that Pi and substrates were omitted from the homogenizing and suspending media, and albumin was included in the suspending medium. The standard flask contents were the same as given in Table II, except that substrates were omitted where indicated and each flask contained 7. 8 mg. mitochondrial protein. ADP (2 μmoles), DNP (0. 3 μmoles), azide (30 μmoles) and the hexokinase system (50 K. M. hexokinase and 50 μmoles glucose) were added where indicated. The reactions were stopped at the end of the incubation period by addition of 1. 0 ml. of 0. 8 M TCA. N. E. P^{32} was determined by Method N.

[2] The mitochondria were added to each flask at the beginning of the incubation (zero time); the number after each reagent indicates the minutes thereafter when that reagent was added.

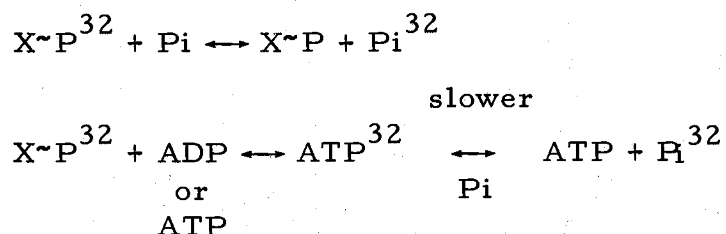
was very small, indicating that the large ADP effect obtained in the presence of substrates was not the result of phosphate exchange reactions.

Without substrates, there was no DNP effect. DNP decreased the amount of non-extractable P^{32} when substrate was present, with or without ADP, as would be expected if DNP acts upon oxidative phosphorylation.

In the absence of added ADP (Table VI), azide increased the amount of phosphate esterified either with or without substrate, but a greater effect was obtained when substrate was present. The increase caused by azide may have depended upon a small amount of endogenous ADP left in the mitochondria, and inhibition of ATPase by azide. When ADP was present, azide reduced the amount of N. E. P^{32} obtained after a short period of incubation, but more N. E. P^{32} was observed if azide and ADP were included in a longer incubation. This could depend upon the relative degree to which azide inhibited transfer of P^{32} to ATP and ATPase activity. In a short period, a smaller amount of ATP would form in the presence of azide, because azide would inhibit P^{32} transfer and ATP synthesis. On the other hand, in a longer period more ATP^{32} would be formed and inhibition of ATPase by the azide would be the important factor. The effect of azide was greater in the presence of substrates, as would be expected if the effect of azide was on oxidative phosphorylation.

Effect of Adding Unlabeled Phosphate

The results shown in Table VII suggest that an intermediate such as $X{\sim}P^{32}$ was formed before ATP. The addition of a large amount of Pi alone, and with ADP or ATP, gave different amounts of N. E. P^{32}. One possible reason for obtaining more N. E. P^{32} in the presence of ADP or ATP is that the $X{\sim}P^{32}$ reacted with ADP, or exchanged phosphate with ATP, very rapidly and faster than the exchange of $X{\sim}P^{32}$ with Pi. When Pi was allowed to react before ADP and ATP, smaller amounts of N. E. P^{32} were obtained as the time was increased, possibly because P^{32} in an intermediate ($X{\sim}P^{32}$) exchanged with Pi. No exchange was observed if ADP, ATP and Pi were added after the TCA, because the exchange enzyme was then inactivated. These reactions can be summarized as follows:



Effect of Preincubation of Mitochondria with ATP and Oligomycin, Followed by Addition of Pi^{32}

The results shown in Table VIII suggest that at 25°C. and in the presence of oligomycin and ATP, an intermediate becomes saturated with unlabeled phosphate; therefore, less Pi^{32} can be introduced in a subsequent four minute period. The unlabeled phosphate could be

TABLE VII. The Effects of Adding Phosphate, ADP and ATP After Incubation of Mitochondria with Pi^{32}

Expt. No.[1]	Added to Standard Medium[2]	Incubation Time (min.)	% N. E. P^{32}
136	Pi-20, ADP-20	23	10.4
	Pi-20, ADP-20, olig.-0	23	11.4
	Pi-20, ATP-20	23	11.2
	Pi-20, ATP-20, olig.-0	23	11.2
	Pi-20	23	3.3
	Pi-20, olig.-0	23	3.8
	Pi-19, ADP-20	23	7.8
	Pi-19, ADP-20, olig.-0	23	9.1
	Pi-19, ATP-20	23	7.8
	Pi-19, ATP-20, olig.-0	23	9.9
	Pi-19	23	2.45
	Pi-19, olig.-0	23	3.8
	Pi-17, ADP-20	23	4.7
	Pi-17, ADP-20, olig.-0	23	4.9
	Pi-17, ATP-20	23	4.0
	Pi-17, ATP-20, olig.-0	23	5.5
	Pi-17	23	2.1
	Pi-17, olig.-0	23	3.0
	Pi-26, ADP-26	23	13.6
	Pi-26, ADP-26, olig.-0	23	14.2
	Pi-26, ATP-26	23	12.4
	Pi-26, ATP-26, olig.-0	23	14.0
	Pi-26	23	11.0
	Pi-26, olig.-0	23	13.8

[1] The mitochondria were prepared by Method A, except that $MgCl_2$ was omitted from the standard homogenizing medium and the suspending medium; the mitochondria were washed twice (20 ml. each) and suspended in suspending medium containing 1 mg. albumin per ml. The standard flask contents were the same as given in Table II, except that each flask contained 600 μmoles sucrose and 6.8 mg. mitochondrial protein. ADP (2 μmoles), ATP (2 μmoles), Pi (40 μmoles) and oligomycin (2 μg.) were added where indicated. Incubated at 0°C. The reactions were stopped at the end of the incubation period by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

[2] The mitochondria were added to each flask at the beginning of the incubation (zero time); the number after each reagent indicates the minutes thereafter when that reagent was added.

TABLE VIII. The Effects of Incubating Mitochondria at Two Temperatures with Oligomycin and ATP Before Adding Pi^{32}

Expt. No.[1]	Added to Standard Medium[2]	Incubation Time (min.)	% N. E. P^{32} 25°C.	0°C.
135	ATP-0, Pi^{32}-0	4	57.0	7.5
	ATP-0, Pi^{32}-0, olig.-0	4	48.0	7.6
	ATP-0, Pi^{32}-2	6	49.6	8.6
	ATP-0, Pi^{32}-2, olig.-0	6	14.7	7.7
	ATP-0, Pi^{32}-12	16	37.8	7.0
	ATP-0, Pi^{32}-12, olig.-0	16	7.2	6.1
	ATP-0, Pi^{32}-0, ADP-0	4	77.3	10.0
	ATP-0, Pi^{32}-0, ADP-0, olig.-0	4	62.5	8.9
	ATP-0, Pi^{32}-2, ADP-2	6	48.6	11.9
	ATP-0, Pi^{32}-2, ADP-2, olig.-0	6	10.6	7.4
	ATP-0, Pi^{32}-12, ADP-12	16	39.5	13.8
	ATP-0, Pi^{32}-12, ADP-12, olig.-0	16	6.7	10.5

[1] The preparation of the mitochondria and the standard flask contents were as stated in Table VII, except that each flask contained 7.05 mg. mitochondrial protein. ATP (2 μmoles), Pi^{32}, ADP (2 μmoles) and oligomycin (2 μg.) were added where indicated. Incubated at 25°C. and 0°C. as noted. The reactions were stopped at the end of the incubation period by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

[2] The mitochondria were added to each flask at the beginning of the incubation (zero time); the number after each reagent indicates the minutes thereafter when the reagent was added.

introduced from ATP, even though previous evidence showed that oligomycin partially inhibits ATPase and transfer of P^{32} to ADP. It will be noted that more than six minutes was needed for the intermediate to become saturated with unlabeled phosphate. In the absence of oligomycin, more N. E. P^{32} was obtained, even after preincubation with ATP, presumably because ATPase action (inhibited by oligomycin) prevented accumulation of unlabeled phosphate intermediate. There was very little oligomycin effect at $0^{o}C.$, probably because oxidative phosphorylation was too slow at this temperature.

When ADP was added with the Pi^{32}, in the presence of ATP and oligomycin, P^{32} incorporation was faster than with Pi^{32} alone, only at the beginning. After incubation with ATP and oligomycin, the effect of the ADP disappeared. The results suggest strongly that an intermediate was saturated with unlabeled phosphate by incubation with ATP and oligomycin, thus preventing rapid P^{32} incorporation when Pi^{32} and ADP were added. It should be mentioned that observation of the ATP-oligomycin effect also depends upon inhibition by oligomycin of the transfer of phosphate from the intermediate to ADP or other acceptors.

The ATP-oligomycin effect does not occur if the mitochondria are incubated with ADP and oligomycin, as shown in Table IX. This is because ADP is primarily a phosphate acceptor and would tend to remove phosphate from an intermediate, rather than donating

TABLE IX. The Effects of Incubating Mitochondria with Oligomycin and ATP or ADP Before Adding Pi^{32}

Expt. No. [1]	Added to Standard Medium[2]	Incubation Time (min.)	% N. E. P^{32}
133	ATP-0, Pi^{32}-0	4	50.5
	ATP-0, Pi^{32}-0, olig.-0	4	48.8
	ADP-0, Pi^{32}-0	4	73.4
	ADP-0, Pi^{32}-0, olig.-0	4	79.0
	ATP-0, Pi^{32}-2	6	47.8
	ATP-0, Pi^{32}-2, olig.-0	6	28.8
	ADP-0, Pi^{32}-2	6	74.5
	ADP-0, Pi^{32}-2, olig.-0	6	79.1
	ATP-0, Pi^{32}-8	12	33.5
	ATP-0, Pi^{32}-8, olig.-0	12	9.5
	ADP-0, Pi^{32}-8	12	64.5
	ADP-0, Pi^{32}-8, olig.-0	12	70.7
	ATP-0, Pi^{32}-16	20	28.6
	ATP-0, Pi^{32}-16, olig.-0	20	7.6
	ADP-0, Pi^{32}-16	20	53.5
	ADP-0, Pi^{32}-16, olig.-0	20	68.4

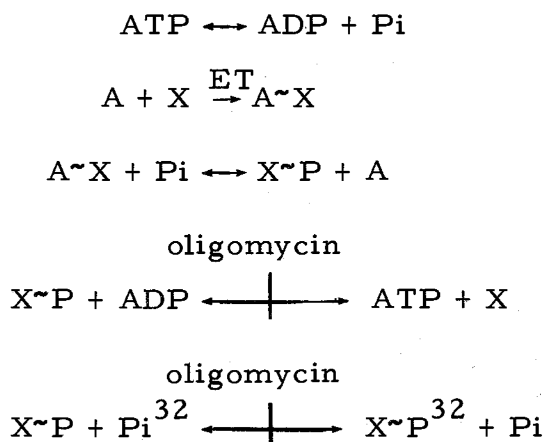
[1] The preparation of the mitochondria and the standard flask contents were as stated in Table VII, except that each flask contained 8.4 mg. mitochondrial protein. ATP (2 μmoles), ADP (2 μmoles), Pi^{32} and oligomycin (2 μg.) were added where indicated. The reactions were stopped at the end of the incubation period by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

[2] The mitochondria were added to each flask at the beginning of the incubation (zero time); the number after each reagent indicates the minutes thereafter when the reagent was added.

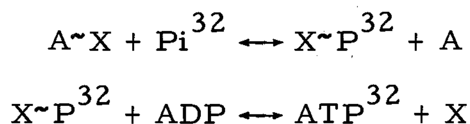
phosphate.

These results can be summarized as follows:

(1) When the mitochondria were preincubated with ATP and oligomycin:



(2) When ADP was added:



Part II
Incubation of Mitochondria with Pi^{32} Followed by Addition of Alcohol

Effectiveness of Various Concentrations of Alcohol in Preventing Oxidative Phosphorylation

Ethyl alcohol was studied in the hope that it might stop phosphorylation without destroying a preformed intermediate. Since Boyer (3, p. 1147-1153) has reported an intermediate which is acid-labile and

alkali-stable, it was recognized that the acid might destroy the intermediate when TCA was added. Various concentrations of alcohol were studied. In Table X, it may be seen that addition of one ml. of absolute alcohol to a three ml. incubation mixture, to give a final concentration of 25%, inhibited oxidative phosphorylation very well. However, 12 1/2% and 6 1/4% alcohol in the medium allowed oxidative phosphorylation to occur.

Alcohol Precipitate and Alcohol "Supernate"

Since alcohol (25%) could stop phosphorylation, and because it is neutral, further studies were made in an effort to get transphosphorylation from a phosphorylated intermediate, to form ATP. After the addition of alcohol, "alcohol precipitates" were removed by centrifugation and both the precipitate and supernatant fluid ("supernate") were studied. In experiments not tabulated in this thesis, it was found that upon incubation of ADP, hexokinase, Mg^{++} ions and glucose with both the alcohol precipitate and the alcohol supernate, more increase in N. E. P^{32} was obtained with the supernate than with the precipitate. However, it is uncertain that the small increase of N. E. P^{32} obtained came from the reaction of a preformed, phosphorylated intermediate and ADP, since no experiments were done to establish that phosphorylating fragments of mitochondria did not survive the alcohol treatment.

TABLE X. The Effects of Ethyl Alcohol on Oxidative Phosphorylation in Blowfly Mitochondria

Expt. No.[1]	Added to Standard Medium	Incubation Time (min.)	% N. E. P^{32}
126	25% alc-0, hexokinase system-13	26	1.5
	12 1/2% alc.-0, hexokinase system-13	26	30.2
	6 1/4% alc. -0, hexokinase system-13	26	73.8
128	25% alc. -0, hexokinase system-13	26	1.3
	12 1/2% alc. -0, hexokinase system-13	26	22.4

[1]The mitochondria were prepared by Method D. Each flask contained 36 μmoles pyruvate, 2 μmoles fumarate, 6 μmoles succinate, 6 μmoles citrate, 600 μmoles sucrose, 100 μmoles Tris, 3 μmoles $MgCl_2$, 3 μmoles EDTA, Pi^{32}, and 7.25 mg. (Expt. 126) or 7.35 mg. (Expt. 128) mitochondrial protein; the pH was 7.4. Ethyl alcohol (alc.) to give the final concentrations shown was added before the mitochondria. After the mitochondria were added, incubation continued at 25°C. to 7 minutes, at 0°C. from 7 to 16 minutes, and at 25°C. from 16 to 26 minutes. The hexokinase system (2 μmoles ADP, 3 additional μmoles $MgCl_2$, 50 μmoles glucose and 50 K. M. units hexokinase) was added at 13 minutes, when each flask was at 0°C. TCA was added at 26 minutes to terminate the incubation period. N. E. P^{32} was determined by Method N.

Molybdate Precipitate

In the determination of N. E. P^{32} in the alcohol "supernate" by Method A, as described in Methods and Materials, a white precipitate occurred when the molybdate reagent was added. A molybdate precipitate was also obtained by Griffiths (34, p. 1064-1070); he obtained a white insoluble NAD-molybdate complex on addition of acid molybdate reagent to solutions containing high concentrations of NAD. He interpreted this to be due to the formation of a phosphorylated derivative of NAD which is precipitated by acid molybdate.

Our "molybdate precipitate" was prepared by mixing a two ml. aliquot of alcohol supernate with one ml. of water and one ml. of molybdate reagent, as described in Table XI. After the precipitate was removed and washed twice, it was transferred to a planchet and counted.

The results in Table XI show that when ADP was present in the incubation medium for the mitochondria, a smaller amount of radioactivity was found in the molybdate precipitate. The molybdate precipitate obtained from the control flasks (no ADP), and from the flasks in which alcohol was added before the mitochondria, yielded about the same amount of radioactivity. It may be assumed that the precipitate with molybdate reagent was largely protein, because this precipitate was not obtained when the reactions were stopped with

TABLE XI. The Effect of ADP on the Amounts of P^{32} Found in Molybdate Precipitates

Expt. No. [1]	Added to Standard Medium	% P^{32} in Molybdate Precipitate	% N. E. P^{32} in Molybdate Supernate
116	Alcohol-12	21.2	1.5
	ADP-0, alcohol-12	10.6	4.1
	Alcohol-0	19.8	0.6
	ADP-0, alcohol-0	13.5	0.2
117	Alcohol-12	22.5	1.6
	ADP-0, alcohol-12	14.0	10.6
	Alcohol-0	25.5	0.3
	ADP-0, alcohol-0	21.7	0.2
119	Alcohol-12	30.3	2.2
	ADP-0, alcohol-12	12.2	31.1
	Alcohol-0	23.6	0.6
	ADP-0, alcohol-0	38.6	0.4

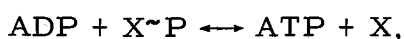
---

[1]The preparation of the mitochondria and the standard flask contents were as stated in Table VII, except that each flask contained 10.9 mg. (Expt. 116), 16.4 mg. (Expt. 117), or 6.8 mg. (Expt. 119) mitochondrial protein. ADP (2 μmoles) and ethyl alcohol (to a final concentration of 25%) were added at the time, in minutes, indicated by a number after each entry. Incubated at 25°C. until alcohol was added, then transferred to an ice bath. Precipitated protein was removed by centrifugation, then 2 ml. of the supernatant fluid was mixed with 1 ml. water and 1 ml. molybdate reagent. After standing 15 minutes at 0°C., the mixture was centrifuged to separate the "Molybdate Precipitate" and the "Molybdate Supernate" noted above. The molybdate precipitate was washed twice, by suspending in a mixture of 3 ml. water and 1 ml. molybdate reagent, followed by centrifugation; it was then transferred to a planchet and counted. N. E. P^{32} was determined on the molybdate supernate by Method N.

TCA; the molybdate reagent is acid and would be expected to cause precipitation of protein. Since every flask in a given experiment contained the same amount of protein, each molybdate precipitate would be expected to include the same amounts of protein, phosphate and molybdate.

The smaller amount of radioactivity obtained in the molybdate precipitate, when ADP was included in the incubation medium, suggests that P^{32} was lost by being transferred from protein to ADP. The protein involved could be part of an intermediate functioning in oxidative phosphorylation; it could be the "X" of "X~P."

Wadkins and Lehninger (72, p. 1589-1594) concluded that the ATP-ADP exchange reaction is a reflection of the terminal reaction of oxidative phosphorylation, by which ATP is formed from ADP and a "high-energy" phosphate donor:



$$ADP + X{\sim}P \longleftrightarrow ATP + X,$$

where X is an enzyme protein capable of undergoing phosphorylation.

When alcohol was present before the mitochondria were added (Table XI), the N. E. P^{32} obtained in the molybdate precipitate was about the same as in the control flasks. This may mean that a non-phosphorylated, high-energy intermediate was formed in the mitochondria even before their addition to the flasks and that the alcohol permitted this to react with Pi^{32}, to get $X{\sim}P^{32}$.

When both ADP and alcohol were added before the mitochondria, the amount of N. E. P^{32} obtained was variable, for reasons which are not yet understood.

In other experiments not tabulated in the thesis, ADP was added to the molybdate precipitate which was suspended in a solution containing 0.3 M sucrose and 0.1 M Tris. The suspension was incubated at 25°C. for 10 minutes, followed by addition of TCA. The N. E. P^{32} obtained in the TCA filtrate was just a little more than the background counts, indicating that no significant transfer of P^{32} to ADP had occurred. This was not surprising, because precipitation with the molybdate reagent probably caused extensive denaturation of the proteins present; in addition, the molybdate in the precipitate had not been removed before adding ADP.

N. E. P^{32} Found When the Incubation Was Terminated with Alcohol or TCA

In several experiments, additions of alcohol and of TCA were compared as means of terminating the incubation of mitochondria with Pi^{32} and other reagents. It was surprising to find that the smaller amount of N. E. P^{32} was observed after adding alcohol, as shown in Table XII. Although the question was not explored extensively, a likely explanation is that the alcohol failed to stop reactions leading to hydrolysis of an intermediate and/or ATP.

TABLE XII. N. E. P^{32} Found upon Terminating the Incubation with Alcohol or TCA

Expt. No.[1]	Added to Standard Medium	Added to Terminate Incubation	% N. E. P^{32}
115	None	TCA	2. 1
	None	Alcohol	0. 8
	Olig.	TCA	3. 4
	Olig.	Alcohol	1. 3
	ADP	TCA	14. 3
	ADP	Alcohol	8. 2
	ADP, olig.	TCA	10. 2
	ADP, olig.	Alcohol	6. 1
127	None	TCA	17. 7
	None	Alcohol	10. 5

[1]The preparation of the mitochondria and the standard flask contents were as stated in Table VII, except that each flask contained 6. 6 mg. (Expt. 115) or 9. 2 mg. (Expt. 127) mitochondrial protein. ADP (2 μmoles) and oligomycin (2 μg.) were added before the mitochondria, where indicated. In Expt. 115, the flasks were incubated at 25°C. for 12 minutes, when TCA (1 ml. of 0. 8 M) or ethyl alcohol (1 ml. absolute) was added. In Expt. 127, the flasks were incubated at 25°C. for 7 minutes, then at 0°C. for 5 minutes, when TCA or ethyl alcohol was added. After removal of precipitated protein, N. E. P^{32} was determined on the soluble material by Method N.

Part III
Incubation of Mitochondria with Pi^{32}, Followed by Removal from the Medium and Addition of ADP to the Mitochondria or to the Extract

Addition of ADP to Mitochondria

Results given in Table XIII show that there was not much increase in N. E. P^{32} when ADP was added to the mitochondria, except when oligomycin was present in the first incubation. These results suggest that oligomycin caused more Pi^{32} to be retained in the mitochondria. Therefore, when ADP was added to the second incubation, more N. E. P^{32} was obtained than in the absence of oligomycin. Oligomycin may have stabilized a phosphorylated intermediate, even though ADP was not added; however, it must be recognized that some oxidative phosphorylation probably occurred during the second incubation also.

Addition of ADP to "Supernate"

In the previous work using alcohol to stop the reaction, alcohol supernate gave some hope of a possible intermediate in the supernate, as mentioned already. However, there was some evidence that alcohol caused more destruction than TCA. Therefore, in the experiments reported in Table XIV the flasks were merely placed in the ice bath at the end of the initial incubation. The mitochondria were removed by centrifugation, and the supernatant fluid ("supernate")

TABLE XIII. N. E. P^{32} Formed in Mitochondria Incubated with Pi^{32} Followed by a Second Incubation

Expt. No. [1]	Added to Standard Medium		% N. E. P^{32}
	First Incubation	Second Incubation	
150	None	None	10. 1
	None	ADP	11. 4
	Oligomycin	None	12. 0
	Oligomycin	ADP	16. 7
153	None	None	13. 5
	None	ADP	13. 4
	Oligomycin	None	14. 5
	Oligomycin	ADP	21. 0

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[1] The mitochondria were prepared by Method E. In the first incubation, each flask contained 35. 5 μmoles pyruvate, 2 μmoles fumarate, 100 μmoles Tris, 3. 05 μmoles $MgCl_2$, 625 μmoles sucrose, 1. 5 μmoles EDTA, 0. 5 mg. albumin, Pi^{32} and 12. 5 mg. (Expt. 150) or 8. 8 mg. (Expt. 153) mitochondrial protein. Where indicated, oligomycin (6 μg.) was added before the mitochondria. After incubating 7 minutes at 25°C., the mitochondria were removed by centrifugation for 5 minutes at 0°C. and 6780 x g; they were then washed twice by suspension in 20 ml. Steele homogenizing medium, followed by centrifugation. The mitochondria were then suspended in 3 ml. of standard incubation medium (as described for the first incubation but <u>without</u> additional Pi^{32}), with ADP (2 μmoles) being included where indicated. After incubating 7 minutes at 25°C., reaction was terminated by addition of 1. 0 ml. of 0. 8 M TCA. N. E. P^{32} was determined by Method N.

TABLE XIV. The Effects of ADP and Oligomycin on the Amounts of N. E. P^{32} in the Supernatant Fluid from Mitochondria Incubated with Pi^{32}

Expt. No.[1]	Added in First Incubation	Second Incubation Added	Incubation Time (min.)	% N. E. P^{32} in Supernate
150	None	None	10	17.2
	None	ADP	10	23.8
	None	ADP, Hex. Syst.	10	23.1
	None	None	0	14.4
	Oligomycin	None	10	28.5
	Oligomycin	ADP	10	31.3
	Oligomycin	ADP, Hex. Syst.	10	32.0
	Oligomycin	None	0	31.6
153	None	None	10	13.6
	None	ADP	10	17.0
	None	ADP, Hex. Syst.	10	16.3
	None	None	0	15.2
	Oligomycin	None	10	31.4
	Oligomycin	ADP	10	37.2
	Oligomycin	ADP, Hex. Syst.	10	35.8
	Oligomycin	None	0	36.4
154	None	None	10	9.0
	None	ADP	10	16.0
	None	ADP, Hex. Syst.	10	16.5
	None	None	0	10.5
155	None	None	10	11.4
	None	ADP	10	22.5
	None	ADP, Hex. Syst.	10	22.5
	None	None	0	11.8

[1] The preparation of the mitochondria and the standard flask contents were the same as given in Table XIII, with 12.5 mg. (Expt. 150), 8.8 mg. (Expt. 153), or 7.95 mg. (Expt. 154 and 155) mitochondrial protein per flask. Where indicated, oligomycin (6 μg.) was added before the mitochondria. After incubating 7 minutes at 25°C., the flasks were chilled in ice and the mitochondria were removed by centrifuging at 6780 or 10,000 x g. A 2 ml. aliquot of each "Supernate" was then incubated at 25°C. in a 3 ml. system (second incubation) with ADP (2 μmoles) and the "Hex. Syst." (50 μmoles glucose and 50 K.M. units hexokinase) being added where indicated. The second incubation was terminated by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

was studied. The results given in Table XIV indicate that there is an intermediate of oxidative phosphorylation present in the supernate so obtained. When ADP was added to the supernate, N. E. P^{32} increased in comparison with flasks to which TCA was added before the supernate. No increase was obtained if ADP was not added. In the presence of hexokinase and ADP, the N. E. P^{32} obtained was about the same as with ADP alone. This suggests strongly that the complete process of oxidative phosphorylation did not occur during the second incubation, because if it had, more N. E. P^{32} should have been found in the presence of hexokinase. Also, if oxidative phosphorylation had occurred in the second incubation, then the N. E. P^{32} should have increased in the presence of ADP and oligomycin, as shown in Figures 1 and 2. Therefore, the increase in N. E. P^{32} when ADP was added in the second incubation is not from oxidative phosphorylation. In addition, because the supernate was clear, no oxidative phosphorylation would be expected. Other studies with hexokinase and ADP, and determination of N. E. P^{32} by both methods N and H, showed that glucose-6-phosphate was produced in the second incubation; this means that ATP was the product formed upon incubating ADP with the supernate.

These results indicate that a phosphorylated intermediate was present in these supernates. The intermediate, which reacted with ADP, was destroyed by TCA when ADP was not added in the second

incubation. The intermediate appears, therefore, to be an acid-labile one. The N. E. P^{32} which survives the addition of TCA without reaction with ADP is presumed to be the acid-stable form.

The Effect of Oligomycin in the First Incubation on the Amount of N. E. P^{32}

When oligomycin was present in the first incubation without ADP, the supernate yielded more N. E. P^{32} than when oligomycin was absent. However, addition of ADP to the supernate did not result in an increase in the amount of N. E. P^{32}, when compared to flasks with TCA added before the supernate (Table XIV). This would indicate that oligomycin stabilized the acid-stable form of intermediate. Upon incubation, the acid-stable form can be converted to the acid-labile form; Table XIV, and other experiments not tabulated, showed that when the supernate was incubated without ADP added, the amount of N. E. P^{32} surviving the addition of TCA decreased. This can be interpreted to mean that the acid-stable form changed to the acid-labile form, which was destroyed by TCA. However, when ADP and ADP+ the hexokinase system were added to the supernate, the acid-labile intermediate would presumably transfer P^{32} to the ADP. Therefore, the same amount of N. E. P^{32}, as the one with TCA before supernate, was obtained.

The Effect of Adding Oligomycin in the Second Incubation on the Amount of N. E. P^{32}

The results given in Table XV show that addition of oligomycin to the supernate did not increase the amount of N. E. P^{32} obtained in the absence of ADP. A smaller amount of N. E. P^{32} was obtained when oligomycin was present together with ADP, as compared with ADP alone. This suggests very definitely that oxidative phosphorylation did not occur in the supernate; otherwise larger amounts of N. E. P^{32} would be expected. In addition, oligomycin appeared to inhibit the transphosphorylation about 50% or more. This result is similar to Boyer's report (56, p. PC 1180-1182; 66, p. 781-784).

The Effects of Other Inhibitors and Inorganic Phosphate on the Amount of N. E. P^{32}

Dinitrophenol. It is well known that DNP is an uncoupling agent. Many investigators have reported that DNP stimulates ATPase (25, p. 476-477; 3, p. 65-67; 17, p. 369-377). Some investigators have postulated that DNP destroys a high-energy intermediate (5, p. 362-379), while another report (25, p. 476-477) suggests that DNP does not destroy an intermediate.

The results in Table XVI suggest that DNP does destroy an intermediate, or prevents its formation, because addition of DNP in the first incubation reduced the amount of N. E. P^{32}, and the ADP effect,

TABLE XV. The Effects of Adding Oligomycin to the Supernatant Fluid from Mitochondria Incubated with Pi^{32}

Expt. No.[1]	First Incubation Time (min.)	Added in Second Incubation	Second Incubation Time (min.)	% N. E. P^{32} in Supernate
161	7	None	10	19.5
		Olig.	10	20.2
		ADP	10	26.8
		ADP, olig.	10	23.4
		None	0	21.6
162	1	None	10	19.3
		Olig.	10	17.5
		ADP	10	26.5
		ADP, olig.	10	22.1
		None	0	19.5
	3	None	10	21.5
		Olig.	10	19.5
		ADP	10	28.4
		ADP, olig.	10	23.7
		None	0	21.2
	7	None	10	26.6
		Olig.	10	23.3
		ADP	10	34.6
		ADP, olig.	10	29.8
		None	0	26.3
	12	None	10	29.2
		Olig.	10	28.2
		ADP	10	37.4
		ADP, olig.	10	32.3
		None	0	31.6

[1]The preparation of the mitochondria and the standard flask contents for the first incubation were as stated in Table XIV, except that each flask contained 20 μmoles $MgCl_2$, 600 μmoles sucrose, and 7.65 mg. (Expt. 161) or 8.05 mg. (Expt. 162) mitochondrial protein. After the first incubation period (variable as noted), the "Supernate" was obtained and included in a second incubation, as described in Table XIV. Oligomycin (6 μg.) and ADP (2 μmoles) were included in the second incubation medium where indicated. The second incubation was terminated by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

TABLE XVI. N. E. P^{32} Found in the Supernatant Fluid after Inclusion of Inhibitors and Pi in the First or Second Incubation

Expt. No.[1]	Added in First Incubation	Added to Supernate	Added in Second Incubation	Second Incubation Time (min.)	% N. E. P^{32} in Supernate
158	-	-	None	10	22.0
	-	-	DNP (0.3 μm.)	10	10.6
	-	-	Myristic acid	10	14.5
	-	-	Azide, olig.	10	17.3
	-	-	Pi (3 μm.)	10	12.4
	-	-	None	0	18.4
	-	ADP	None	10	31.0
	-	ADP	DNP (0.3 μm.)	10	22.4
	-	ADP	Myristic acid	10	21.8
	-	ADP	Azide, olig.	10	23.2
	-	ADP	Pi (3 μm.)	10	25.3
	-	ADP	None	0	22.9
159	Pi (0.3 μm.)	-	None	15	1.69
	Pi (0.3 μm.)	-	ADP	15	1.73
	Pi (0.3 μm.)	-	Olig.	15	1.65
	Pi (0.3 μm.)	-	ADP, olig.	15	1.75
	Pi (0.3 μm.)	-	Pi (20 μm.)	15	1.63
	Pi (0.3 μm.)	-	ADP, Pi (20 μm.)	15	1.63
	Pi (0.3 μm.)	-	None	0	1.68
	Pi (0.3 μm.) DNP (1.5 μm.)	-	None	15	1.19
	Pi (0.3 μm.) DNP (1.5 μm.)	-	ADP	15	1.37
	Pi (0.3 μm.) DNP (1.5 μm.)	-	Olig.	15	1.18
	Pi (0.3 μm.) DNP (1.5 μm.)	-	ADP, olig.	15	1.33
	Pi (0.3 μm.) DNP (1.5 μm.)	-	Pi (20 μm.)	15	1.31
	Pi (0.3 μm.) DNP (1.5 μm.)	-	ADP, Pi (20 μm.)	15	1.25
	Pi (0.3 μm.) DNP (1.5 μm.)	-	None	0	1.25
163	-	-	None	10	30.4
	-	-	DNP (0.3 μm.)	10	25.8
	-	-	Olig.	10	30.1
	-	-	ADP, olig.	10	33.0
	-	-	None	0	30.3
	-	ADP	None	10	40.1

TABLE XVI. (cont.)

Expt. No.[1]	Added in First Incubation	Added to Supernate	Added in Second Incubation	Second Incubation Time (min.)	% N. E. P^{32} in Supernate
163	-	ADP	DNP (0.3 µm.)	10	34.6
	-	ADP	Olig.	10	35.6
	-	ADP	ADP, olig.	10	35.0
	-	ADP	None	0	34.4
	DNP (0.3 µm.)	-	None	10	11.5
	DNP (0.3 µm.)	-	DNP (0.3 µm.)	10	11.1
	DNP (0.3 µm.)	-	Olig.	10	11.2
	DNP (0.3 µm.)	-	ADP, olig.	10	14.3
	DNP (0.3 µm.)	-	None	0	11.6
	DNP (0.3 µm.)	ADP	None	10	15.2
	DNP (0.3 µm.)	ADP	DNP (0.3 µm.)	10	14.7
	DNP (0.3 µm.)	ADP	Olig.	10	14.3
	DNP (0.3 µm.)	ADP	ADP, olig.	10	14.8
	DNP (0.3 µm.)	ADP	None	0	13.0

[1] The preparation of the mitochondria and the standard flask contents were the same as given in Table XIII, except as noted. In the first incubation, each flask contained 5.8 mg. (Expt. 158), 4.8 mg. (Expt. 159), or 6.85 mg. (Expt. 163) mitochondrial protein. After the first incubation period, the "Supernate" was obtained and included in a second incubation, as described in Table XIV. Myristic acid (3 µmoles), azide (30 µmoles), oligomycin (6 µg.) and DNP and Pi (amounts stated above) were added in the first or second incubation as noted. ADP (2 µmoles) was added in the second incubation, or to the cold "Supernate" immediately after its separation from the mitochondria, as noted. The second incubation was terminated by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

in the supernate. Furthermore addition of DNP to the supernate, in the absence of ADP, caused a loss of N. E. P^{32} during the second incubation.

DNP did not stimulate ATPase in the supernate (Table XVI), since there was no decrease in N. E. P^{32} if DNP was added after a period of incubation of supernate with ADP. Thus, if the ADP was added to the cold supernate, followed by incubation with DNP, the amount of N. E. P^{32} obtained was about the same as the amount found without the second incubation.

It will be noted in Table XVI that addition of ADP to the cold supernate gave more N. E. P^{32} even at the beginning of the second incubation. This would suggest that some transfer occurred at 0°C., but at a slower rate, and that the rest of the intermediate reacted with ADP during incubation at 25°C. However, if DNP was present in the second incubation, the remaining intermediate was apparently destroyed before it could react with ADP, since there was no further increase during the second incubation.

Myristic Acid. Addition of myristic acid to the supernate (Table XVI) gave results very similar to those obtained with DNP. Myristic acid appeared to destroy a phosphorylated intermediate, since a decrease of N. E. P^{32} was obtained when myristic acid was incubated with the supernate in the absence of ADP. However, myristic

acid did not stimulate ATPase in the supernate, since it did not cause significant loss of N. E. P^{32} when added after ADP.

Azide and Oligomycin. Addition of both azide and oligomycin (Table XVI) to the supernate did not lead to destruction of an intermediate, since there was no appreciable loss of N. E. P^{32} during the subsequent incubation, in the presence or absence of ADP. However, the combination of azide and oligomycin apparently inhibited the transfer of P^{32} to ADP completely. As mentioned previously, oligomycin only partially inhibits this transfer in the supernates; therefore, azide may inhibit transfer to a greater extent than oligomycin.

Phosphate. In Table XVI, it may be seen that addition of Pi to the supernate, in the absence of ADP, caused a loss of N. E. P^{32} similar to that observed with DNP or myristic acid. In the presence of ADP, addition of Pi only reduced the amount of acid-stable N. E. P^{32} formed during the second incubation. These results suggest that Pi may exchange rapidly with P^{32} in an intermediate in the supernate.

When 0. 3 µmole of Pi was included in the first incubation, the amount of N. E. P^{32} found in the supernate was decreased by approximately 15 fold (Table XVI). This indicates that a very small amount of phosphate is bound in the intermediate detected in these supernates.

Part IV
Incubation of Mitochondria without P^{32}, Followed by Addition of Pi^{32} and ADP to the "Supernate"

The Effects of ADP, DNP and Pi on the Amount of N. E. P^{32} Formed in the Supernate

ADP. In Table XVII, it may be seen that the amount of N. E. P^{32} increased when Pi^{32} and ADP were added to the supernate. Since there was no P^{32} in the first incubation, preformed $X{\sim}P^{32}$ would not be expected. This result suggests that besides the phosphorylated intermediate, there is another high-energy intermediate (A~X) present in the supernate after the first incubation. This intermediate apparently reacted with Pi^{32} to form a phosphorylated intermediate, which then reacted with ADP to form ATP^{32}.

When Pi^{32} was added without ADP, an increase in N. E. P^{32} was also obtained, but this was smaller. A possible explanation is that acid-stable phosphate compounds formed in the absence of ADP, after the initial reaction of an intermediate with the added Pi^{32}.

DNP. When DNP was included in the first incubation, less N. E. P^{32} was formed upon incubation of the supernate with Pi^{32}, and the effect of adding ADP was largely eliminated (Table XVII). This result suggests that DNP destroyed most of the intermediate or prevented its formation.

Addition of DNP in the second incubation reduced the amount of

TABLE XVII. N. E. P^{32} Formed upon Adding Pi^{32} to the Supernatant Fluid from Incubated Mitochondria

Expt. No.[1]	Added in First Incubation	Added to Supernate	Added in Second Incubation	Second Incubation Time (min.)	% N. E. P^{32} in Supernate
155	-	-	Pi^{32}	10	4.1
	-	-	Pi^{32}, ADP	10	7.7
	-	-	Pi^{32}, ADP, Hex. Syst.	10	9.7
	-	-	Pi^{32}	0	0.6
	Pi^{32}	-	None	10	11.4
	Pi^{32}	-	ADP	10	22.5
	Pi^{32}	-	ADP, Hex. Syst.	10	22.5
	Pi^{32}	-	None	0	11.8
164	-	-	Pi^{32}	10	7.6
	-	-	Pi^{32}, DNP-start	10	1.8
	-	-	Pi^{32}, ADP, olig.	10	3.5
	-	-	Pi^{32}	0	1.0
	-	ADP	Pi^{32}	10	11.0
	-	ADP	Pi^{32}, DNP-start	10	3.0
	-	ADP	Pi^{32}, ADP, olig.	10	2.2
	-	ADP	Pi^{32}	0	1.0
	DNP	-	Pi^{32}	10	1.6
	DNP	-	Pi^{32}, DNP-start	10	1.5
	DNP	-	Pi^{32}, ADP, olig.	10	3.0
	DNP	-	Pi^{32}	0	0.9
	DNP	ADP	Pi^{32}	10	3.1
	DNP	ADP	Pi^{32}, DNP-start	10	2.8
	DNP	ADP	Pi^{32}, ADP, olig.	10	2.3
	DNP	ADP	Pi^{32}	0	1.0
166	-	Pi^{32}	None	10	3.4
	-	Pi^{32}	DNP-start	10	2.2
	-	Pi^{32}	DNP-10 min.	20	4.2
	-	Pi^{32}	None	0	0.97
	-	Pi^{32}, ADP	None	10	10.2
	-	Pi^{32}, ADP	DNP-start	10	7.8
	-	Pi^{32}, ADP	DNP-10 min.	20	11.5
	-	Pi^{32}, ADP	None	0	3.6
	-	Pi^{32}, Pi	None	10	0.34
	-	Pi^{32}, Pi	DNP-start	10	0.31
	-	Pi^{32}, Pi	DNP-10 min.	20	0.32
	-	Pi^{32}, Pi	None	0	0.37

TABLE XVII. (cont.)

Expt. No.[1]	Added in First Incubation	Added to Supernate	Added in Second Incubation	Second Incubation Time (min.)	% N. E. P^{32} in Supernate
166	-	Pi^{32}, Pi, ADP	None	10	0.81
	-	Pi^{32}, Pi, ADP	DNP-start	10	0.9
	-	Pi^{32}, Pi, ADP	DNP-10 min.	20	0.59
	-	Pi^{32}, Pi, ADP	None	0	0.5

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[1]The preparation of the mitochondria and the standard flask contents were the same as given in Table XIII, except that Pi^{32} was included only where indicated. In the first incubation, each flask contained 7.95 mg. (Expt. 155 and 164) or 7.35 mg. (Expt. 166) mitochondrial protein. After the first incubation period (3 to 7 minutes at 25°C.), the "Supernate") was obtained and included in a second incubation, as described in Table XIV. ADP (2 μmoles), the "Hex. Syst." (50 K. M. units hexokinase and 50 μmoles glucose), oligomycin (6 μg.), Pi (10 μmoles, not radioactive) and Pi^{32} were added in the first or second incubation, or to the cold "Supernate" immediately after its separation from the mitochondria, as noted. DNP (0.3 μmole) was added at the beginning of the first incubation where noted, or at the time indicated in the second incubation. The second incubation was terminated by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

N. E. P^{32} formed in the absence of ADP, suggesting destruction of an intermediate. However, when the supernate was incubated with ADP and Pi^{32} before the addition of DNP, this agent no longer caused hydrolysis. This result indicates a definite reaction with ADP to give acid-stable N. E. P^{32} (ATP^{32}). As mentioned previously, DNP did not stimulate ATPase in the supernate.

Phosphate. In Table XVII, experiment 166, it may be seen that addition of 10 µmoles Pi to the supernate, with the Pi^{32} or Pi^{32} plus ADP, reduced the amount of N. E. P^{32} obtained by ten fold or more. This result would be expected, since the large amount of Pi added would lower the specific activity of the phosphate available for reaction.

The Effect of Substrates on the Amount of N. E. P^{32} Formed in the Supernate

To further establish that phosphate esterification in the supernate depended upon a preformed intermediate, the effects of adding substrates in the first and second incubation were studied. In Table XVIII, the results show that omission of substrate during the first incubation abolished the effect of adding ADP to the supernate (with the Pi^{32}) almost completely. This indicates that formation of N. E. P^{32} in the supernate requires that electron transport had occurred during the first incubation, presumably to form a non-phosphorylated

TABLE XVIII. The Effects of Substrates on N. E. P^{32} Formed upon Adding Pi^{32} to the Supernatant Fluid

Expt. No.[1]	Added in First Incubation	Added in Second Incubation	Second Incubation Time (min.)	% N. E. P^{32} in Supernate
167	Substrates	Pi^{32}	10	1.88
	Substrates	Pi^{32}, ADP	10	6.35
	Substrates	Pi^{32}	0	0.78
	None	Pi^{32}	10	0.94
	None	Pi^{32}, ADP	10	1.55
	None	Pi^{32}	0	0.77
	None	Substrates, Pi^{32}	10	0.73
	None	Substrates, Pi^{32}, ADP	10	1.70
	None	Substrates, Pi^{32}	0	0.51
168	Substrates	Pi^{32}	10	2.04
	Substrates	Pi^{32}, ADP	10	3.96
	Substrates	Pi^{32}	0	0.90
	None	Pi^{32}	10	1.44
	None	Pi^{32}, ADP	10	1.77
	None	Pi^{32}	0	0.80
	None	Substrates, Pi^{32}	10	1.30
	None	Substrates, Pi^{32}, ADP	10	1.90
	None	Substrates, Pi^{32}	0	0.80

[1]The preparation of the mitochondria and the standard flask contents were the same as given in Table XIII, except that Pi^{32} and substrates were added only where indicated. In the first incubation, each flask contained 7.25 mg. (Expt. 167) or 5.5 mg. (Expt. 168) mitochondrial protein. After the first incubation period (3 minutes at 25°C.) the "Supernate" was obtained by centrifugation at 10,000 x g. and included in a second incubation, as described in Table XIV. ADP (2 μmoles), Pi^{32} and "substrates" (30 μmoles pyruvate and 2 μmoles fumarate) were added in the first or second incubation as noted. The second incubation was terminated by addition of 1.0 ml. of 0.8 M TCA. N. E. P^{32} was determined by Method N.

intermediate to react with Pi^{32} and ADP.

When substrates were omitted in the first incubation, their addition to the supernate gave no significant increase in the N. E. P^{32} formed when Pi^{32} was added, or when Pi^{32} and ADP were added. These results indicate clearly that the N. E. P^{32} formed in the supernate depended upon formation of an intermediate during the first incubation, and that formation of this intermediate depended upon electron transport.

SUMMARY

Mitochondria from blowflies, Phormia regina, were used in studies designed to demonstrate the existence of an intermediate which functions in oxidative phosphorylation. Evidence for accumulation of an intermediate, in the very small quantities which were anticipated, was obtained using radioactive orthophosphate (Pi^{32}) to measure phosphate esterification.

Non-extractable P^{32} (N. E. P^{32}) was found to form very rapidly when ADP was added to mitochondria previously incubated with Pi^{32}. This transient increase, which has been interpreted as evidence for an accumulated intermediate, was followed by a slower rate of N. E. P^{32} formation; it was inhibited by oligomycin, azide, antimycin A, DNP, and myristic acid. Rapid increase in N. E. P^{32} was observed even when unlabeled orthophosphate (Pi) was added to the mitochondria with the ADP, after incubation with Pi^{32}, suggesting that ADP reacted very rapidly with a preformed intermediate containing P^{32}. However, if unlabeled Pi was added before the ADP, the ADP-dependent formation of N. E. P^{32} soon became undemonstrable, indicating an exchange of Pi with the P^{32} of an intermediate.

The presence of detectable quantities of an intermediate was also suggested by the results of incubating mitochondria with oligomycin and ATP. When mitochondria were incubated with these two

compounds for six or more minutes at 25°C., followed by addition of Pi^{32}, a marked decrease in the subsequent rate of N. E. P^{32} formation was observed. The results indicated that in the presence of oligomycin an intermediate was slowly phosphorylated with unlabeled phosphate from ATP. Apparently oligomycin stabilized this intermediate, so that subsequent addition of Pi^{32} resulted in only a slow rate of P^{32} esterification.

Evidence was obtained for extraction of an intermediate when blowfly mitochondria were incubated with Pi^{32} in the absence of added ADP. After an initial incubation with the Pi^{32}, the mitochondria were removed by centrifugation. Addition of ADP to the supernatant fluid ("supernate"), followed by a second incubation, resulted in an increase in the amount of N. E. P^{32} which survived the addition of acid. The N. E. P^{32} resulting from incubation of ADP with the supernate was largely abolished by addition of DNP to the medium used for the initial incubation of the mitochondria. DNP also reduced the amount of N. E. P^{32} observed if it was added to the supernate before ADP; after the supernate was incubated with ADP, subsequent addition of DNP had no significant effect. The results mentioned, and others to be found in the thesis, indicate that under the incubation conditions employed, a phosphorylated intermediate capable of reacting with ADP is released into the incubation medium.

Other experiments indicated that a second, non-phosphorylated

intermediate may have been released into the medium in which blowfly mitochondria were incubated. After incubation of the mitochondria with substrate, but without Pi^{32}, removal of the mitochondria and addition of ADP and Pi^{32} to the supernate gave an increase of N. E. P^{32}. This increase depended upon addition of ADP with the Pi^{32}; it was abolished if substrates were omitted from the medium in which the mitochondria were incubated.

In summary, evidence was obtained for two types of intermediates in the medium in which blowfly mitochondria had been incubated. The nature of these two intermediates, and the possibility that they might be identical, can be determined only by additional research.

A number of inhibitors of oxidative phosphorylation were used and studied during the course of the investigation. Oligomycin was found to inhibit ATPase and the transfer of P^{32} from intermediate to ADP. Oligomycin increased the amount of acid-stable N. E. P^{32} formed during incubation of the mitochondria with Pi^{32}; however, the P^{32} esters formed in the presence of oligomycin did not appear to react with ADP.

Azide also inhibited ATPase, and appeared to inhibit transfer of P^{32} to ADP even more severely than did oligomycin.

As mentioned previously, DNP appeared to destroy both types of intermediates detected in the supernates. However, DNP did not stimulate hydrolysis of the ATP^{32} formed in the supernates, even

though it does stimulate ATPase in intact blowfly mitochondria.

Myristic acid was found to cause effects very similar to those obtained with DNP.

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