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Title OXIDATIVE PHOSPHORYLATION AND RELATED REACTIONS IN PARTICULATE FRACTIONS FROM INSECTS

Abstract approved [redacted] (Major Professor)

Oxidative phosphorylation and related reactions, particularly as affected by 2, 4-dinitrophenol (DNP), were studied with mitochondria and submitochondrial particles isolated from the flight muscle of the blowfly (Phormia regina) and housefly (Musca domestica). In the presence of a phosphate acceptor, the mitochondria oxidized pyruvate rapidly, and this was tightly coupled to phosphorylation. Added succinate and other citric acid cycle intermediates were not readily oxidized by the intact mitochondria. However, submitochondrial particles coupled succinate or NADH oxidation to phosphorylation, but did not utilize pyruvate. The substrate specificity of intact mitochondria appears to be related to a membrane permeability barrier.

Pyruvate oxidation was stimulated by DNP, but only in the presence of ATP (or ADP) and Pi. DNP inhibited the ATP-Pi exchange reaction and promoted ATP hydrolysis with no substrate present. However, with sufficient ATP and Pi-Pi^{32} added, little or no net ATP hydrolysis occurred when pyruvate oxidation was stimulated by DNP,

and ATP^{32} continued to be formed. The ATP (or ADP) and Pi requirements are due to their need in substrate-level phosphorylation because DNP still promoted respiration (in the presence of ATP, ADP, and Pi) after coupled phosphorylation and DNP-ATPase were completely inhibited by oligomycin. In the presence of oligomycin, DNP stimulated respiration, with ATP and Pi added, only when sufficient $MgCl_2$ (2 mM) was present to provide ADP for substrate-level phosphorylation. $MgCl_2$, however, did not promote respiration in the presence of oligomycin and in the absence of DNP, and $MgCl_2$ was not essential when ADP was present. These findings show that ATP (or ADP) and Pi are not obligatory in the basic mechanism by which DNP promotes electron transport in insect mitochondria; they also show that DNP can 'release' respiration at all three sites of coupled phosphorylation in the presence of oligomycin. However, at 0.1 to 0.15 mM DNP, maximal respiratory stimulation was obtained only in the absence of oligomycin, when DNP could promote ATP hydrolysis and uncouple phosphorylation. ATP^{32} formation from oxidative phosphorylation was demonstrated in experiments in which respiration was stimulated nearly maximally by 0.1 mM DNP in the presence of ATP and Pi-Pi^{32}. Other experiments, which utilized ADP, or ATP and hexokinase, as a phosphate acceptor, indicated that the equivalent of two phosphorylation sites were not completely uncoupled by 0.1 mM DNP, since P/O ratios significantly greater

than 1 were obtained with short incubation periods, even when the phosphate acceptor was not added until 10 minutes after the DNP. These results suggest that DNP does not 'release' respiration equally at each of the three sites of coupled phosphorylation.

In contrast to mitochondria, sonic or digitonin particles did not show ATP-Pi exchange or DNP-ATPase activity. Sonic particles coupled succinate or NADH oxidation to phosphorylation with P/O ratios between 0. 2 and 0. 8; the phosphorylation was inhibited by oligomycin and uncoupled by DNP. Therefore, DNP can uncouple respiration in one or more reactions that do not necessarily lead to ATP hydrolysis. Mg^{++}-ATPase was observed with both mitochondria and particle preparations.

At 0. 4 mM, DNP caused complete inhibition of pyruvate oxidation and coupled phosphorylation with mitochondria, but did not inhibit succinate or NADH oxidation with sonic particles, although it did uncouple phosphorylation completely.

OXIDATIVE PHOSPHORYLATION AND RELATED REACTIONS IN PARTICULATE FRACTIONS FROM INSECTS

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Typed by Marcia Ten Eyck

This thesis is affectionately dedicated to

my mother and stepfather, Sylvia E. and George Larson

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OXIDATIVE PHOSPHORYLATION AND RELATED REACTIONS IN PARTICULATE FRACTIONS FROM INSECTS

INTRODUCTION

"The voluntary muscles of almost all animals have an important feature in common: a single nerve impulse gives rise to a single response from a muscle. A significant exception is apparent in the flight of certain insects: their wings can beat many times faster than the most rapid rate at which a muscle responding to a series of nerve impulses can alternately contract and relax. Weight for weight the flight muscle of such insects generates more energy than any other tissue in the animal kingdom. . .

". . . flight muscles of this kind are found in insects of only four of the 30 or so recognized insect orders; the beetles (Coleoptera); the wasps and bees (Hymenoptera); the flies, mosquitos, and similar forms (Diptera); and certain true bugs (Hemiptera), including the aphids. Because such muscle contracts and relaxes at a much higher rate than the nerve signals it receives, it is called asynchronous muscle; the flight muscle of other insects contracts and relaxes in exact response to nerve signals (as do the skeletal muscles of vertebrates) and is therefore called synchronous" (61).

Adenosine triphosphate (ATP) is now known to be the compound used most commonly in transmitting cellular energy. In aerobic cells, most of the ATP is generated from adenosine diphosphate (ADP)

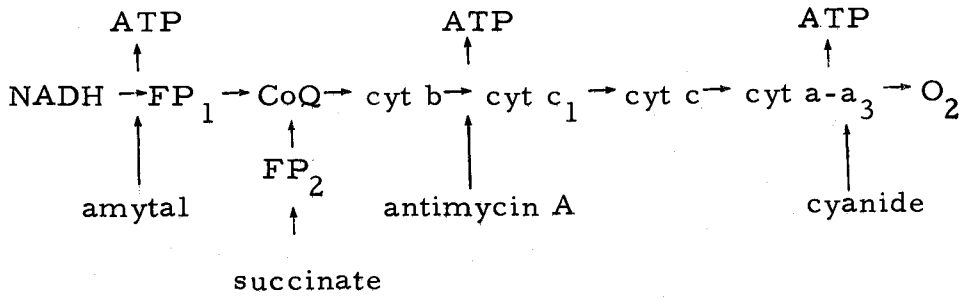
and inorganic phosphate (Pi), at the expense of the energy liberated during the catabolism of foodstuffs, by the process of mitochondrial oxidative phosphorylation. A molecule of ATP is formed in coupled reactions occurring at each of three points along the mitochondrial respiratory chain during the exergonic passage of a pair of electrons from substrate to molecular oxygen via the pyridine nucleotides, flavoprotein and cytochromes (43).

Chance and Sacktor (14) emphasized the role of α-glycerophosphate in supplying the energy for flight in the housefly since they found that this was the only substrate oxidized at a sufficient rate in their preparations to explain the respiratory activity in flight. Gregg, Heisler, and Remmert (31, 32), however, demonstrated rapid pyruvate oxidation in housefly mitochondria; they suggested that oxidation of this substrate was a more logical source of energy for muscular contraction than α-glycerophosphate, because pyruvate was found to be oxidized to carbon dioxide and water (30, p. 40) whereas the product of α-glycerophosphate oxidation (dihydroxyacetone) accumulated in mitochondria. Van den Bergh and Slater (62, 63) recently obtained respiratory activity with either pyruvate or α-glycerophosphate sufficient to account for the energy requirements in flight. Van den Bergh and Slater suggested that pyruvate oxidation is likely to be the more important substrate in insect flight muscle (five times on an oxygen basis and 7. 5 times on an energy basis). They concluded

that it no longer seemed necessary to assume that α-glycerophosphate is the only physiological substrate for flight muscle activity.

Therefore, it appears that insect mitochondria utilize essentially the same metabolic processes for energy production as are found in mammalian tissue. This is consistent with the recent results of Yamanaka, Tokuyama, and Okunuki (71), who found that housefly cytochrome c was very similar to mammalian cytochrome c. Also, Chance and Sacktor found spectroscopic evidence for the similarity of housefly and heart muscle respiratory chain components (14).

A schematic diagram of the respiratory chain of animal mitochondria, with the probable sites of coupled phosphorylation and sensitivities to inhibitors, may be shown as follows (47, p. 60):[1]



[1] The following abbreviations are used in this thesis: ADP and ATP adenosine di- and tri- phosphate, respectively; ATPase, adenosine triphosphatase; Pi, inorganic orthophosphate; Pi^{32}, radioactive Pi; ATP-Pi exchange, the exchange of the terminal phosphate group of ATP with Pi^{32}; ADP-ATP exchange, the reversible transfer of the terminal phosphate group of ATP to ADP; P^{32}, esterified or non-esterified Pi-Pi^{32} (form unspecified); ATP^{32}, ATP terminally labeled with P^{32}; P/O ratio, μmoles of Pi esterified per μgatom of O_2 consumed; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; FP, flavoprotein; cyt, cytochrome;

Much of the evidence for this sequence has come from the kinetic and spectroscopic studies of Chance and Williams (10, 11, 12). It is not yet certain that coenzyme Q is an obligatory member of the electron transport chain (47, p. 72).

It has already been indicated that three molecules of ATP are formed in the coupled reactions of oxidative phosphorylation for each pair of electrons passed through the respiratory chain from NAD -linked substrates such as pyruvate, malate, and β-hydroxybutyrate (P/O ratio = 3). Succinate, however, contributes electrons to FAD in the respiratory chain, and only two molecules of ATP are generated per pair of electrons (P/O ratio = 2). The oxidation of α-ketoglutarate to succinate in the citric acid cycle results in the formation of one molecule of ATP during the conversion of each molecule of succinyl CoA to succinate (substrate-level phosphorylation), and three ATP molecules are generated in the NAD -linked respiratory chain phosphorylation reactions (P/O ratio = 4). The complete oxidation of a molecule of pyruvate to CO_2 and H_2O in the citric acid cycle involves four NAD -linked oxidations (pyruvate, isocitrate,

CoQ, coenzyme Q; CoA, coenzyme A; pyr., pyruvate; fum., fumarate; succ., succinate; cit., citrate; mal., malate; BSA, crystalline bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylene-diaminetetraacetate; DNP, 2, 4-dinitrophenol; hex., hexokinase; KM, Kunitz-McDonald units (of hexokinase); gly-gly, glycylglycine; Mg^{++}, magnesium ion; TCA, trichloroacetic acid; olig., oligomycin; anti. A, antimycin A; μ, micro; μm., μmole(s); mM, millimolar; μM, micromolar; μg, microgram(s); μgatoms, microgram atoms; conc., concentration; min., minute(s); %, percent; No., number; expt., experiment; prep., preparation; g., gravity; inc., incubated.

α-ketoglutarate, and malate), one FP-linked oxidation (succinate), and one substrate-level phosphorylation via succinyl CoA. Thus, a total of 15 molecules of ATP are formed and five atoms of oxygen are consumed; the P/O ratio, then, is three.

Submitochondrial particles obtained from rat liver and beef heart mitochondria by treatment with digitonin show some retention of phosphorylation at each of the three coupling sites of the respiratory chain (24, 34, 36). This may also be the case with some rat liver particles obtained by the sonic oscillation treatment of Kielley and Bronk (41), but not those prepared by Gregg (33). The substrate-level phosphorylation complex is, however, lost from submitochondrial particle preparations.

The mitochondrial substrate-level phosphorylation step differs from the phosphorylation coupled to the respiratory chain in that it is insensitive to agents such as 2, 4-dinitrophenol (DNP) which inhibit respiratory chain phosphorylation (1, 4, 5). Also, Chappell and Greville (18) found that oligomycin, which blocks respiratory chain-linked phosphorylation at all three coupling sites (28, 53), did not inhibit substrate-level phosphorylation. However, Davis (23) has recently shown that oligomycin can also inhibit the substrate-level phosphorylation associated with α-ketoglutarate oxidation when mitochondria are preincubated in the absence of added $MgCl_2$.

The considerations given above are important in oxidative

phosphorylation studies with housefly or blowfly mitochondrial preparations with pyruvate as the substrate, since the oxidation of pyruvate through the citric acid cycle involves substrate-level and NAD - and FAD-linked phosphorylative reactions.

Cooper and Lehninger (21) formulated the following sequence to account for the coupling of phosphorylation to electron transport:

(1) $AH_2 + C \rightleftharpoons AH_2\text{-}C$

(2) $AH_2\text{-}C + B \rightleftharpoons A\sim C + BH_2$

(3) $A\sim C + Pi \rightleftharpoons A + C\sim Pi$

(4) $C\sim Pi + ADP \rightleftharpoons ATP + C$

As postulated, this sequence leads to the generation of an energy conserving high energy intermediate ($A\sim C$) at each of the three coupling sites along the respiratory chain, during the passage of a pair of electrons from pyridine nucleotide to molecular oxygen. Chance et al. (13) visualize a similar mechanism, except that the high energy compound is considered to involve the reduced form of the respiratory carrier (i. e., $AH_2\sim C$). The "high energy" bond between the components of the intermediate is presumed to form without the intervention of Pi or ADP. In the sequence shown above, it is apparent that components A and C must be regenerated for respiration to continue; it is this regeneration which requires the participation of ADP and Pi. When Pi is present in excess, ADP is limiting, and respiration is

found to be greatly stimulated in "tightly coupled" mitochondria upon the addition of ADP.

Reaction (4), and reactions (4) plus (3) are assumed to account for the adenosine triphosphate-adenosine diphosphate (ATP-ADP) and the adenosine triphosphate-inorganic orthophosphate (ATP-Pi) exchange reactions, respectively, of mitochondria and submitochondrial fragments. These reactions have been studied extensively in digitonin and sonic particles from rat liver mitochondria. Bronk and Kielley (7) and Cooper and Kulka (22) found that the ATP-ADP exchange reaction is always faster than the ATP-Pi exchange reaction when both are studied under the same conditions. This suggests that the ATP-ADP exchange (reaction 4) is the terminal reaction of the coupling sequence.

Freshly prepared digitonin particles from rat liver show ATP-Pi and ATP-ADP exchange activities, and couple phosphorylation to the oxidation of β-hydroxybutyrate and other substrates (45). While coupled phosphorylation was dependent upon electron transport, that is, aerobic conditions, the exchange reactions occurred anaerobically or in the presence of cyanide at the same rates as in air (21); they were not dependent upon electron transport. When digitonin particles were aged at 0°, the ATP-Pi exchange and coupled phosphorylation activities were lost, but ATP-ADP exchange activity was not (45). This showed that the ATP-ADP exchange did not depend on the

presence of the ATP-Pi exchange, or coupled phosphorylation, and provided evidence for reaction (4) above. Both exchange reactions and coupled phosphorylation were inhibited by 2, 4-dinitrophenol (DNP) in fresh preparations, but when coupled phosphorylation and ATP-Pi exchange activities were lost upon aging, DNP no longer inhibited the ATP-ADP exchange. Therefore, DNP did not act on the components of the ATP-ADP exchange reaction. That is, DNP inhibition of the ATP-ADP exchange appeared to depend on an effect on the ATP-Pi exchange. The two exchanges appeared to be related, perhaps by an intermediate common to both; both were required for coupled phosphorylation. Azide was found to inhibit the ATP-Pi exchange in fresh particles, but it did not inhibit ATP-ADP activity. When added to fresh particles, azide prevented DNP inhibition of the ATP-ADP exchange (65). Therefore, it was concluded that DNP acts at a point nearer to the respiratory chain than azide. Subsequently, Wadkins and Lehninger (67) were able to purify a factor which restored the sensitivity of the ATP-ADP exchange to DNP inhibition in aged particles. Racker (56), and Penefsky et al. (54) have also purified soluble protein factors, from submitochondrial particles, which are capable of reconstituting ATP-Pi exchange activity and coupled phosphorylation.

Neither ATP, ADP, nor inorganic phosphate (Pi) are required for electron transport in aged submitochondrial particles in which

coupling is inactivated (45), or in mammalian mitochondria when phosphorylation is uncoupled by DNP (5). Thus, the terminal coupling reactions of oxidative phosphorylation are not linked directly to the electron transport chain, and it is postulated that at least one additional energy transfer intermediate intervenes between the respiratory chain and the sites of Pi and ADP involvement as follows (15, 19):

$$\text{(5)}\quad \text{Carrier I} + \text{X} \underset{\text{(oxidative energy)}}{\overset{\text{electron transport}}{\rightleftharpoons}} \text{X}\sim\text{I} + \text{Carrier}$$

$$\text{(6)}\quad \text{X}\sim\text{I} + \text{Pi} \rightleftharpoons \text{X}\sim\text{Pi} + \text{I}$$

$$\text{(7)}\quad \text{X}\sim\text{Pi} + \text{ADP} \rightleftharpoons \text{ATP} + \text{X}$$

This sequence is similar to that given previously, but it emphasizes that conservation of energy for ATP formation is mediated by, and intimately linked with, one or more high energy intermediates which do not include respiratory chain components. As will be developed later, it is now thought that DNP and several other 'uncouplers' act by causing hydrolysis of the intermediate compound(s) (i. e., X~I of reaction 5, or A~C of reaction 2).

DNP has been shown to have at least five effects on freshly prepared intact animal mitochondria (45, 30, p. 6-7): it 'uncouples' phosphorylation from respiration, i. e., it inhibits phosphorylation while leaving the rate of respiration essentially unchanged; it stimulates hydrolysis of ATP to ADP and Pi; it inhibits the ATP-Pi and

ATP-ADP exchange reactions; it 'releases' respiration in the absence of a phosphate acceptor, that is, it greatly stimulates respiration as would the addition of ADP; and it inhibits respiration at high concentrations (16).

The first four of these effects can be explained with the aid of the previously given reaction sequence. Reaction (5) may be expanded as follows:

$$\text{(5a)}\quad \text{Carrier I} \overset{\text{electron transport}}{\rightleftharpoons} \text{Carrier}\sim\text{I}$$

$$\text{(5b)}\quad \text{Carrier}\sim\text{I} + \text{X} \rightleftharpoons \text{Carrier} + \text{X}\sim\text{I}$$

$$\text{SUM: (5)}\quad \text{Carrier I} + \text{X} \overset{\text{electron transport}}{\rightleftharpoons} \text{Carrier} + \text{X}\sim\text{I}$$

$$\text{(8)}\quad \text{X}\sim\text{I} + \text{DNP} \rightleftharpoons \text{X}\sim\text{I}\cdot\text{DNP}$$

$$\text{(9)}\quad \text{X}\sim\text{I}\cdot\text{DNP} \rightleftharpoons \text{X} + \text{I}\cdot\text{DNP}$$

$$\text{(10)}\quad \text{I}\cdot\text{DNP} \rightleftharpoons \text{I} + \text{DNP}$$

$$\text{SUM: (11)}\quad \text{X}\sim\text{I} \overset{\text{DNP}}{\rightleftharpoons} \text{X} + \text{I}$$

A reaction of DNP with Carrier~I, while possible, is not probable since Chance, Williams, and Hollunger (16) found no evidence for a shift of the absorbancy peaks of the carriers in the presence of DNP.

It is seen that the sum of reactions (8), (9), and (10), i. e., reaction (11), would uncouple phosphorylation since no ATP would be formed, and in the absence of phosphate acceptor (ADP), DNP would release respiration by regenerating X and I. The DNP-

stimulated ATPase activity would be represented by the following reactions:

$$(11)\quad X\sim I \xrightleftharpoons{DNP} X + I$$

$$(6)\quad X\sim I + Pi \rightleftharpoons I + X\sim Pi$$

$$(7)\quad X\sim Pi + ADP \rightleftharpoons X + ATP$$

The action of DNP in promoting reaction (11) to the right would stimulate ATPase and inhibit both exchange reactions. It has been suggested (16) that inhibition of electron transfer at a high DNP concentration may be due to the combination of more than one molecule of DNP with an intermediate of these equations to form a compound which dissociates slowly in a reaction similar to reaction (10).

Chappell (20) indicated that DNP acts at one site which is rate limiting, in the stimulation of respiration and ATP hydrolysis, since he observed that the K_{DNP} for both DNP-ATPase and DNP-stimulated malate and succinate oxidation, with both rat liver and pigeon heart mitochondria was in the range of 50-60 μM DNP at pH 7.2 and 25°. Bronk and Kielley (7) found that temperature changes gave parallel effects on DNP stimulation of ATPase and DNP inhibition of the ATP-Pi and ATP-ADP exchange reactions in sonic particles from rat liver; they also concluded that DNP acts at a single site.

As written, reactions (6 to 11) imply that the energy transfer

component X is not site specific since it does not form a complex with an electron carrier. However, reactant I is thought to be site specific since evidence has been obtained for specific inhibition of energy transfer components by amytal (15) and by galegine (19); both of these compounds appeared to inhibit the energy transfer process between NAD and cytochrome b.

Löw et al. (48) found that amytal, which is quite specific for inhibition of electron transport between NAD and FAD, also caused a progressive inhibition of the ATP-Pi and DNP-ATPase activities as the inhibitor concentration was increased. Since neither cyanide nor antimycin A, which are known to inhibit electron transport, showed comparable effects, they suggested that the first phosphorylation site was mainly responsible for DNP-ATPase and ATP-Pi exchange activities. However, it has also been observed that, at higher concentrations, amytal inhibits FAD-linked succinate oxidation in tightly coupled mitochondria (55). This could mean that the amytal inhibition of ATP-Pi exchange and DNP-ATPase activities also involves the second phosphorylation site. Hemker (39) found that inhibition of DNP-ATPase activity was influenced by the DNP concentration, since DNP-ATPase was partially inhibited by amytal at 1 mM DNP, and by amytal plus antimycin A at 0.25 mM DNP. His studies indicated, however, that the bulk of DNP-ATPase activity occurred at the first phosphorylation site.

On the basis of pH activity curves with increasing concentrations of DNP, Meyers and Slater (51) postulated the existence of three DNP-induced ATPases. However, these findings were later re-interpreted by Hemker and Hülsmann (38), who concluded that it is not possible to identify more than one DNP-stimulated ATPase by this method. Hemker later demonstrated two DNP-ATPases using increasing DNP concentrations at a fixed pH (39).

The evidence given above indicates that DNP acts on one or more intermediates in the coupling sequence of oxidative phosphorylation. Whether or not DNP acts on only one site of ATP formation, or on two or three sites, and in the same manner at each site, has not been established conclusively. Eisenhardt and Rosenthal (25) concluded that DNP does not prevent phosphorylation of preformed intermediates, but does interfere with their formation.

It has been suggested recently that uncoupling phenols may act by causing configurational changes in mitochondria, by a binding of the phenols to a protein moiety (69). This concept, while perhaps of importance, does not adequately explain the uncoupling phenomena, since no relation could be demonstrated between phenol binding capacity and the ability to uncouple.

Electron and energy transfer inhibitors have contributed greatly to increasing our understanding of oxidative phosphorylation, because they have permitted delineation of many of the partial reactions

involved. The antibiotic, antimycin A, has been shown to inhibit electron transport specifically at a site between cytochromes b and c_1 (59). Since DNP had no effect on the titration curve of antimycin A inhibition, it was concluded that antimycin A was not an inhibitor of phosphorylation (29). Thus, antimycin A is an inhibitor of oxidative rather than phosphorylative reactions.

The fish poison rotenone, like amytal, inhibits the aerobic oxidation of NAD-linked substrates, but does not affect DNP-ATPase or ATP-Pi exchange activities; it does not inhibit phosphorylation during succinate oxidation in intact mitochondria (26) This compound, then, is a more specific inhibitor of electron transfer, for oxidation of NAD-flavin-linked substrates, than is amytal.

The antibiotic, oligomycin, is a powerful inhibitor of coupled mitochondrial phosphorylation. Oligomycin appears to inhibit phosphorylation at all three coupling sites (27), but does not prevent stimulation of succinate oxidation by DNP (53). Oligomycin also inhibits the ATP-Pi exchange reaction and DNP-stimulated ATPase (67), as well as arsenate-stimulated succinate oxidation (28). Like azide, oligomycin prevents DNP inhibition of the ATP-ADP exchange reaction in digitonin particles (68). Since arsenate and Pi react competively at the site of Pi uptake in the coupling sequence, oligomycin must act nearer to the respiratory chain than Pi, or the ATP-ADP exchange; DNP must act between the respiratory chain and the

site of oligomycin inhibition (28).

Potassium atractylate also inhibits the ATP-Pi exchange and DNP-ATPase activities in mitochondria, but does not inhibit the ATP-ADP exchange (64). Atractylate inhibits β-hydroxybutyrate oxidation, coupled phosphorylation, and the ATP-Pi exchange to the same extent in intact mitochondria; surprisingly it inhibits only the ATP-Pi exchange in digitonin particles, without affecting β-hydroxybutyrate oxidation or coupled phosphorylation (64). However, atractyloside (atractylate) does not inhibit DNP-stimulated respiration (8). It has also been observed that the inhibitions produced by atractyloside can be competitively reversed with ADP (9). While this compound inhibits DNP-ATPase, it does not inhibit the Mg^{++}-stimulated ATPase of mitochondria treated with 1 mM deoxycholate (8). Oligomycin and atractylate, then, are energy transfer inhibitors which act at points further removed from the respiratory chain than the site at which DNP acts.

The work described in this thesis was concerned with extending the earlier observations of Gregg (30), who demonstrated rapid oxidation of the main products of glycolysis in housefly mitochondria. This study was designed to make a thorough study of the effects of DNP on oxidative phosphorylation, which were reported for these preparations (35), and to investigate DNP-ATPase, DNP inhibition of the ATP-Pi exchange reaction, and the ATP and Pi requirements

for DNP-stimulated respiration, using housefly and blowfly mitochondria and submitochondrial particles. A concurrent study by Meegungwan (50) was concerned with the demonstration of a 'high energy' intermediate of oxidative phosphorylation in blowfly mitochondria. Therefore no investigations were made in pursuit of these very 'elusive' compounds.

It was found during this study that 0. 1 mM DNP gave nearly maximal stimulation of pyruvate oxidation in blowfly mitochondria, but at this concentration it did not uncouple phosphorylation completely. In fact, evidence was obtained which indicates that two sites of coupled phosphorylation may be unaffected when respiration is stimulated by 0. 1 mM DNP.

It was also found that both ATP (or ADP) and Pi were required for maximal respiratory stimulation by DNP. These requirements can be partially, but not completely, explained on the basis of their participation in substrate-level phosphorylation reactions. Respiratory rates were obtained with pyruvate as substrate which, according to Van den Bergh and Slater (63), are sufficient to account for the energy requirements of flight.

Submitochondrial particles from blowflies were shown in these studies to catalyze coupled phosphorylation also. However, neither DNP-ATPase nor ATP-Pi exchange activities could be demonstrated, although DNP did uncouple phosphorylation. This suggests that one

coupling site, at least, does not participate in the DNP-ATPase or ATP-Pi exchange reactions, and that said site is functional in the submitochondrial particles.

MATERIALS

Fly Colonies

The blowflies, Phormia regina, and houseflies, Musca domestica, used in this study were from laboratory colonies maintained by the Department of Entomology at Oregon State University. The diet of the adult insects was the same as described previously (30, p. 15).

Reagents

The ATP, ADP, crystalline bovine serum albumin (BSA), hexokinase (Type II, III, or IV), Tris (Sigma 121), sodium pyruvate, NAD^+, alcohol dehydrogenase, glycylglycine, and antimycin A were obtained from Sigma Chemical Company.

Sucrose, EDTA, magnesium chloride, potassium phosphate, ammonium molybdate, and benzene were reagent grade products from Baker Chemical Company. The 2, 4-dinitrophenol, citric acid, sodium azide, succinic acid, and isobutyl alcohol were purchased from Eastman Organic Chemicals. Miscellaneous reagents were obtained as follows: fumaric acid, Delta Chemical Company; digitonin, Fischer Scientific Company; amytal (amobarbital), Eli Lilly and Co.; Pi^{32} (carrier free), Oak Ridge National Laboratory.

Oligomycin was generously supplied as a gift by Dr. Bertram Pressman, and also purchased from the Wisconsin Alumni Research

Foundation.

Other chemicals used were of reagent grade. Glass redistilled water was used in preparing all solutions used in the reaction vessels and enzyme preparation media.

METHODS

Preparation of Mitochondria

Adult flies, four to six days old, were anesthetized by chilling at 1° C for about one-half hour; then 25 to 100 ml of the whole flies were transferred to a tray standing on ice. This was covered by another tray containing ice. Subsequent steps in the isolation were done at room temperature, but all glassware, reagents, etc., were kept on ice, and centrifugations were made at 0° C with refrigerated equipment. The thoraces were removed by dissection and collected, then gently ground in a glass mortar with 150 ml of homogenizing medium. The homogenate was filtered through eight layers of cheese-cloth to remove pieces of chitin and other debris. Additional debris, muscle fibers, and whole cells were removed by centrifugation at 460 x g. for three minutes. The supernatant fluid was decanted through a glass wool plug, and centrifuged at 7,000 x g. for five minutes to sediment the mitochondria. The supernate was discarded, and the walls of the centrifuge tubes were carefully wiped with Kleenex tissue to remove fatty and incompletely sedimented materials streaked along the sides. The mitochondrial pellets were washed by suspension in 10 to 28 ml of washing medium, with the aid of a loose-fitting, 5 ml, glass, Potter-Elvehjem homogenizer, operated by hand. Mitochondria were again sedimented as described previously.

Finally, the mitochondrial pellets were suspended to the desired volume in the suspending medium.

The procedure given above was used throughout this study. However, the compositions of the homogenizing, washing, and suspending media were varied as outlined below. The media were always adjusted to pH 7.40 to 7.45, except where noted otherwise, or where buffer was not present. The pH adjustments were made with KOH or HCl.

Method A

The homogenizing medium contained: 0.25 M sucrose, 0.1 M Pi, 0.05 M Tris, 0.003 M EDTA, 0.003 M $MgCl_2$, and 0.006 M each of citrate, succinate, and pyruvate. The washing and suspending medium was of identical composition, except that Pi and substrates were omitted. Variations in the composition of washing and suspending media are listed below.

Method A-1. The washing and suspending medium contained 0.5 M sucrose plus 3×10^{-5} M $MgCl_2$.

Method A-2. The washing and suspending medium contained 0.3 M sucrose, 0.05 M tris, 0.003 M EDTA, and 1×10^{-4} $MgCl_2$.

Method A-3. The washing and suspending medium contained 0.5 M sucrose, 0.05 M tris, 0.003 M EDTA, and 3×10^{-5} M $MgCl_2$.

Method A-4. The washing and suspending medium contained 0.25 M sucrose plus 3×10^{-5} M $MgCl_2$.

Method A-5. The washing and suspending medium contained only 0.25 M sucrose.

Method A-6. The washing and suspension medium contained 0.25 M sucrose plus 3×10^{-4} M $MgCl_2$.

Method A-7. The washing and suspension medium contained 0.25 M sucrose, 0.05 M Tris, 3×10^{-5} M $MgCl_2$, 1.5×10^{-3} M pyruvate, 3×10^{-5} M fumarate, and 0.3 mg/ml BSA.

Method B

The homogenizing medium consisted of 0.3 M sucrose, 0.05 M Tris, 0.001 M EDTA, 1×10^{-4} M $MgCl_2$, 0.005 M pyruvate, 3×10^{-5} M fumarate, and 0.3 mg/ml BSA. The washing and suspending medium was of identical composition, except that the EDTA was 1×10^{-4} M.

Method B-1. This method was identical to Method B, except that pyruvate and fumarate were omitted from the washing and suspending medium.

Method B-2. This method was identical to Method B, except that the homogenizing, washing, and suspending medium contained 1.5×10^{-3} M pyruvate.

Preparation of Submitochondrial Particles

Digitonin Particles

Digitonin particles were prepared by a modification of the procedure of Devlin and Lehninger (24). The thoraces derived from approximately 100 ml of adult houseflies were treated by Method A-5. However, the washed mitochondria were suspended in 3 x 10^{-4} M $MgCl_2$ (1.5 ml); then, 0.66 volume of a cold two percent digitonin solution in 0.05 M sucrose was added to give a final digitonin concentration of 0.8 percent. The addition was made slowly with continuous stirring, and the suspension was kept at 0° C for 20 minutes with frequent stirring. Then, enough cold 0.25 M sucrose was added to make the final digitonin concentration 0.25 percent, and the mixture was centrifuged at 27,000 x g. for 25 minutes. The supernatant solution was decanted in such a manner as to also remove the upper, loosely packed, fluffy layer. The residue containing mitochondria and heavy particles was discarded. The mixed supernatant material was then centrifuged at 100,000 x g. for 25 minutes. The supernatant fluid was then discarded and the pellets were suspended to the desired volume in cold double distilled water. This suspension was used immediately for addition to the reaction vessels.

Sonic Particles

Sonic particles were isolated by modifications of the procedure of Kielley and Bronk (41). Normal practice was to suspend once-washed mitochondria, derived from the thoraces of 50 to 100 ml of blowflies, to 50 ml in the appropriate suspending medium. The suspension was then sonicated in the cavity of a 10 KC Raytheon sonic oscillator adjusted to produce maximum power output; the cavity was maintained at approximately 0° by a circulating ice water bath. Variations in the volume to be sonicated were occasionally made necessary when an experiment was done to study the effects of sonication time, reagent concentrations, etc. on the activities of sonic particles. When this was necessary, appropriate compensation was made so that all treatments within an experiment involved the same volumes and mitochondrial suspension concentrations. After sonication, intact mitochondria and heavy particles were removed by centrifuging at 14,000 x g. for 10 minutes. The supernatant fluid was decanted and then centrifuged at 100,000 x g. for 20 minutes to sediment the submitochondrial particles. The dark reddish-brown pellets were suspended in the appropriate medium with the aid of a hand operated, 5 ml, glass, Potter-Elvehjem homogenizer. The sonic particle suspension was either used immediately, or stored at -20° C for later use.

Variations in the composition of the media used for sonic particle preparations are listed below. These media were always adjusted to pH 7. 40 to 7. 45 except where noted otherwise, or where buffer was not present. The standard sonication time was 45 seconds. Variations in sonication times are listed in the appropriate tables.

Method C. Mitochondria were isolated from the homogenizing medium used in Method A, but were washed and sonicated in a medium consisting of 0. 25 M sucrose, 0. 05 M Tris, and 0. 003 M $MgCl_2$. The submitochondrial pellet was suspended in the latter medium.

Method D. Mitochondria were isolated from the homogenizing medium used in Method A, but were washed and sonicated in a medium consisting of 0. 25 M sucrose, 0. 03 M glycylglycine, and 0. 003 M $MgCl_2$. The submitochondrial pellet was suspended in the latter medium.

Incubation Procedures

All incubations were made at 25° C in double side arm Warburg vessels of approximately 15 ml capacity, or in 25 ml Erlenmeyer flasks. The total volume (including the enzyme preparation) was 3. 0 ml, except where noted otherwise. Incubations were made at pH 7. 40 to 7. 45 unless noted otherwise. Radioactive phosphorous (Pi^{32}) was added, when needed, in an amount to give approximately

5×10^5 counts per minute per flask. The range was from 1×10^5 to 8×10^6 counts per minute per flask.

Manometric Incubations

Manometric assays were made by conventional techniques with 0.2 ml of 20 percent KOH and a filter paper added to the center wells of the reaction vessels. Flasks were kept on ice until placed on the Warburg bath, immediately after adding the enzyme suspension. After a 10 minute thermal equilibration, the manometer stopcocks were closed, and the reactions were initiated by tipping the side arm contents into the main compartment. The reactions were stopped with 1.0 ml of 0.8 M TCA, and the precipitated protein was removed by filtration through Whatman No. 2 filter paper. Aliquots from the filtrates were used for Pi and P^{32} analyses.

Dubnoff Incubations

Studies of ATPase activity, of the ATP-Pi exchange, and of P^{32} incorporation, which did not involve measurement of oxygen uptake, were made in Erlenmeyer flasks incubated in a Dubnoff constant temperature bath. In this case, the reaction was initiated by the addition of the enzyme preparation, and incubations were made without the thermal equilibration period. The procedure for stopping the reactions, and filtering, was the same as described above.

Pi and P^{32} Assays

Pi Analyses

Inorganic phosphate (Pi) was determined by a modification of the method of Martin and Doty (49), which has been outlined in detail by Gregg (30, p. 17-19). The only additional modification was the use of isobutyl alcohol, saturated with water, for some analyses. This was done so that the volume of the aqueous phase was not changed, and permitted P^{32} and Pi determinations to be made on the same aliquot.

P^{32} Analyses

The esterification of radioactive phosphorous (P^{32}) was measured by the procedure of Nielsen and Lehninger (52) as modified to the requirements of this study. The details were as follows. Five-tenths ml aliquots of the TCA filtrates were transferred to glass-stoppered centrifuge tubes. Then 4 ml of isobutyl alcohol-benzene reagent (1:1, saturated with water), 2 ml of distilled water and 1 ml of ammonium molybdate reagent (50 g ammonium molybdate, dissolved in 400 ml of 10 N H_2SO_4 and diluted to 1 liter) were added to each tube. The tubes were stoppered and the contents mixed; they were then allowed to stand five minutes, and were then shaken vigorously for 30 seconds. After the aqueous and organic phases

had separated, the aqueous layers, which contained the esterified P^{32} compounds, were transferred to a second set of centrifuge tubes containing 4 ml of isobutyl alcohol-benzene reagent. The tubes were stoppered and again shaken vigorously for 30 seconds. The aqueous layers were filtered into clean culture tubes through Whatman No. 2 paper. The culture tubes were kept stoppered until the samples could be counted. P^{32} measurements were made on one ml aliquots of these samples.

Miscellaneous Methods

Mitochondrial Protein

Protein was estimated by the method of Jacobs et al. (40). Crystalline bovine serum albumin was used as the reference standard.

Preparation of the Alcohol Dehydrogenase-NADH System

The NADH substrate system was prepared as follows. Approximately 5 mg of alcohol dehydrogenase (3.5×10^4 units/mg) was dialyzed for 24 hours against four changes of 0.01 M Tris buffer (pH 7.4) to remove $(NH_4)_2SO_4$, and diluted with 0.01 M Tris to provide approximately 1×10^4 units/flask. The complete NADH generating system contained the alcohol dehydrogenase, 1.6 mg NAD^+, 0.1 ml absolute ethanol, and 200 μmoles semicarbazide.

Verification

Results similar to those given in the tables and figures have been obtained in two or more experiments. In addition, data presented in this thesis were commonly obtained as the averages of duplicate determinations.

RESULTS AND DISCUSSION

PART I

Oxidative Phosphorylation and Related Reactions in Insect Mitochondria

General Properties and Substrate Requirements of Insect Mitochondria

The insect mitochondria used in this study were isolated from either housefly or blowfly thoraces. Blowfly mitochondria were used for most of the experiments since it is much easier to obtain adequate yields from the thoraces of this insect, which is much larger than the housefly. Mitochondria from the two sources had the same qualitative characteristics for all of the reactions reported here: they oxidized pyruvate in a rapid and tightly coupled manner when the incubation medium was supplemented with ADP (or ATP), Pi, glucose, $MgCl_2$, and hexokinase; either Mg^{++} or DNP stimulated pyruvate oxidation in the presence of sufficient ATP and Pi; either Mg^{++} or DNP stimulated ATPase activity with substrates omitted.

Table I shows the substrate specificity of blowfly mitochondria. Oxidation was tightly coupled to phosphorylation only with pyruvate as substrate. The rate of pyruvate oxidation was six and one-half times that of succinate, and more than ten times that of any other citric acid cycle intermediate tested. β-hydroxybutyrate was oxidized

Table I. Substrate specificity of blowfly mitochondria.

Flask Variables				O_2	
Substrate	Conc. (mM)	Other	Min. Inc.	Uptake (μgatoms)	P/O
Pyruvate	10	hex.	30	27.7	2.6
Citrate	10	hex.	80	6.0	---
Malate	10	hex.	80	6.7	---
Fumarate	10	hex.	80	6.5	---
Succinate	10	hex.	80	11.5	0
β-hydroxybutyrate	3.3	hex.	80	2.4	---
Mixture[1]	3.3 ea.	hex.	30	24.2	2.2
Mixture[1]	3.3 ea.	None	30	14.1	

Mitochondria were prepared by Method A-6. Each flask contained the hexokinase system (150 KM hexokinase, 100 μm. glucose, and 20 μm. $MgCl_2$) in the side arm, where indicated; 250 μm. sucrose, 100 μm. Tris, 100 μm. Pi, 10 μm. ATP, 0.3 μm. $MgCl_2$, mitochondria (6.6 mg protein), and the indicated substrate in the main compartment. Fumarate (0.2 μm.) was also added to flasks containing pyruvate as the main substrate.

[1]The mixture contained citrate, malate, succinate, fumarate, and pyruvate.

very slowly. As is shown, succinate oxidation was completely uncoupled from phosphorylation. The substrate mixture was oxidized at nearly the same rate as pyruvate alone, which indicates that pyruvate was the only substrate oxidized appreciably.

The respiratory rate shown in Table I, in the absence of hexokinase, was quite high. In later experiments it was possible to reduce this nearly to zero by lowering the $MgCl_2$ concentration to 0.03 μm./3 ml. Further improvement was achieved by use of one of the procedures listed under Method B (see Methods) for isolation of the mitochondria, and also by including bovine serum albumin (BSA) in the incubation medium. Use of Method B and incubation with BSA generally yielded more stable and more active preparations, and also permitted a better demonstration of DNP-stimulated respiration.

The Effects of DNP on Insect Mitochondrial Reactions Under Respiratory and Non-respiratory Conditions

The effects of 0.1 mM DNP on several reactions of fly mitochondria under different incubation conditions are shown in Table II. It is seen that, with no substrate present, DNP stimulated ATPase

Table II. The effects of DNP on oxidation and phosphate transfer reactions in blowfly mitochondria.

Flask Variables		O_2	Pi	ATP^{32}	ATP^{32}
Substrate	DNP (mM)	Uptake (μgatoms)	Released (μmoles)	Formed (μmoles)	Specific Activity[1]
None	---	0	8.9	1.41	70
None	0.1	0	15.7	0	0
Pyr./fum.	---	2.8	0.9	5.30	135
Pyr./fum.	0.1	12.7	2.2	5.33	149
Succinate	---	1.9	9.4	2.15	120
Succinate	0.1	1.8	16.1	0.03	12

Housefly mitochondria were prepared by Method A-3. Each flask contained 19.2 μm. Pi-Pi^{32}, and DNP where indicated, in the side arm; 500 μm. sucrose, 100 μm. Tris, 16.9 μm. ATP, 3 μm. EDTA, 0.03 μm. $MgCl_2$, mitochondria (4.1 mg protein), and succinate (20 μm.), pyr./fum. (30 μm. pyruvate plus 0.2 μm. fumarate), or no substrate in the main compartment. Incubated 15 minutes.

[1]The ATP^{32} specific activity in this and subsequent tables was calculated on the basis of the c. p. m. in the sample counted. The values have, thus, been reduced by the dilution factor which was 28. The relation used in the calculations was:

$$ATP^{32} \text{ specific activity} = \frac{ATP^{32} \text{ c. p. m. counted}}{\mu\text{m. ATP/flask at end of incubation}}$$

activity and inhibited the ATP-Pi exchange reaction[2] (as shown by the lack of ATP^{32} formation) without stimulating respiration. Inhibition of the ATP-Pi exchange reaction occurred before all added ATP (16.9 μm.) was hydrolyzed by DNP-ATPase activity. Results similar to these have been obtained repeatedly with either housefly or blowfly mitochondria. In the absence of substrate, insect mitochondria show the same sensitivities to DNP as the animal mitochondria studied by other investigators (9, 45, 48).

When pyruvate was added as substrate, and with no DNP present, there was a slight respiration, and an increase in ATP^{32} formation and in its specific activity. There was also a decrease in net ATP hydrolysis, which may have been due to an actual resynthesis of ATP by means of oxidative phosphorylation. Azzone and Ernster (2) observed that Pi was reincorporated into ATP in rat liver mitochondria when mitochondrial ATPase was diminished by the addition of substrate.

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[2] The ATP-Pi exchange reaction of mitochondria is the exchange of the terminal phosphate group of ATP with Pi^{32} which occurs in the absence of electron transport. It can be demonstrated by incubating mitochondria with ATP and Pi^{32} with no substrate present. The formation of ATP^{32} in presence of substrate includes formation from the ATP-Pi exchange, but may also include ATP^{32} synthesis by oxidative phosphorylation. In this thesis, ATP^{32} formation in the absence of a substrate is referred to as the ATP-Pi exchange, while synthesis of ATP^{32} in the presence of substrate, and with hexokinase omitted, is referred to as ATP^{32} formation. The esterification of Pi or Pi^{32} through oxidative phosphorylation (i. e., in the presence of substrate and phosphate acceptor) is recorded in the tables as Pi or Pi^{32} uptake.

Pyruvate oxidation was stimulated when DNP was added, but net ATP hydrolysis was only slight (Table II). Under these conditions, it was observed that DNP did not inhibit ATP^{32} formation or decrease its specific activity. The retention of ATP^{32} formation when respiration is stimulated by DNP is not consistent with the concept that DNP acts by releasing respiration from phosphorylation. Furthermore, the absence of appreciable net ATP hydrolysis is indicative that ATP synthesis and hydrolysis occurred at nearly the same rates when respiration was stimulated by DNP.

As already mentioned, fly mitochondria prepared by the methods used in this study did not oxidize citric acid cycle intermediates at rapid rates. The bottom portion of Table II shows that succinate alone increased the ATP^{32} specific activity but did not prevent extensive ATP hydrolysis due to mitochondrial ATPase. It is to be noted that succinate was oxidized slightly with no DNP present; this respiration was equal to two-thirds the rate of unstimulated pyruvate oxidation. However, DNP did not stimulate additional succinate oxidation, but DNP did stimulate ATPase activity and inhibit ATP^{32} formation in the presence of succinate. The limiting factor for succinate oxidation by fly mitochondria appears to be the rate at which it can enter the mitochondria, since it is shown in Table XXII that submitochondrial particles will oxidize succinate readily. Also, Van den Bergh and Slater (63) found that damaged fly mitochondria will

oxidize succinate more rapidly than intact mitochondria. Therefore, the slight succinate oxidation shown in Table II may have been due to damaged mitochondria.

The failure of DNP to stimulate succinate oxidation cannot be completely explained. It is possible that DNP-stimulated ATP hydrolysis, and DNP inhibition of ATP^{32} formation occurred mostly in undamaged mitochondria where succinate may have had little or no effect. This possibility is supported by the similarity of the data with succinate added to that with no substrate present. Alternatively, succinate oxidation may have been so limited by the rate of entry into the mitochondria that a DNP stimulation could not be observed.

The data in Table II show that the effects of DNP on ATP hydrolysis and ATP^{32} formation, which were observed in the absence of a readily oxidized substrate, were almost completely eliminated in fly mitochondria when pyruvate oxidation was stimulated nearly maximally by 0. 1 mM DNP.

The Dependence of Respiratory Stimulation by DNP on pH

Table III shows the effects of DNP on respiration, ATPase, and the ATP-Pi exchange reaction at four different pH values. It is seen that the incorporation of Pi^{32} into ATP was rapid over a wide pH range either in the presence or absence of pyruvate when DNP was not added. The maximum incorporation was near pH 7. 4, and

Table III. The effects of pH on DNP-stimulated reactions of blowfly mitochondria.

pH	Substrate	DNP (mM)	O_2 Uptake (μgatoms)	Pi Released (μmoles)	ATP^{32} Formed (μmoles)	ATP^{32} Specific Activity
6.12	None	---	0	5.3	4.06	87
6.12	None	0.1	0	18.5	0	0
6.12	Pyr./fum.	---	0	7.0	5.36	122
6.12	Pyr./fum.	0.1	0	13.9	0	0
6.88	None	---	0	9.5	4.48	138
6.88	None	0.1	0	17.3	0	0
6.88	Pyr./fum.	---	0.6	-1.9	9.07	130
6.88	Pyr./fum.	0.1	0	17.9	0	0
7.45	None	---	0	6.9	5 24	132
7.45	None	0.1	0	11.8	0	1
7.45	Pyr./fum.	---	0.3	-1.8	9.46	133
7.45	Pyr./fum.	0.1	7.0	2.0	7.17	124
8.06	None	---	0	4.7	2.83	58
8.06	None	0.1	0	8.5	0	1
8.06	Pyr./fum.	---	0.4	6.9	6.06	147
8.06	Pyr./fum.	0.1	2.2	12.6	1.80	82

Mitochondria were prepared by Method A-1. Each flask contained Pi^{32}, and DNP where indicated, in the side arm; 500 μm. sucrose, 100 μm. Tris -100 μm. Pi buffer (adjusted to the indicated pH), 20 μm. ATP, 0.03 μm $MgCl_2$, mitochondria (7.9 mg protein), and pyr /fum. (30 μm. pyruvate plus 0.2 μm. fumarate) where indicated, in the main compartment. Incubated 15 minutes.

pyruvate increased the amount of ATP^{32} formed at each pH. Net mitochondrial ATPase activity was reduced by pyruvate at pH 6.88 and 7.45, and this resulted in a larger total formation of ATP^{32}, perhaps due to oxidative phosphorylation, but the specific activity was nearly the same as that due to the ATP-Pi exchange alone (i.e., with no substrate present). This shows that mitochondrial ATPase was not inhibitory to the ATP-Pi exchange within this pH range. The extensive mitochondrial ATPase activity was not reduced by pyruvate at pH 6.12 or 8.06.

DNP inhibited the ATP-Pi exchange reaction throughout the entire pH range; inhibition occurred before all added ATP had been hydrolyzed, which is in agreement with the data of Lehninger (45). DNP also inhibited ATP^{32} formation in the presence of pyruvate, except at pH 7.45 and 8.06 where DNP stimulated pyruvate oxidation. The maximal stimulation of respiration by DNP was at pH 7.45, where there was only a slight net hydrolysis of ATP, and DNP reduced the ATP^{32} specific activity by only seven percent. DNP stimulated respiration only slightly at pH 8.06 and, although ATP hydrolysis was extensive and exceeded the hydrolysis found with pyruvate omitted, ATP^{32} formation was not completely inhibited. This indicates that Pi^{32} was esterified to form ATP^{32} by means of oxidative phosphorylation, even with this slight respiratory stimulation, by utilization of the ADP formed as a result of ATPase activity.

Figure 1 presents a time study of changes in ATP^{32} specific activity and of ATPase activity. The data are typical of the effects produced by pyruvate and by pyruvate plus DNP, relative to the ATP^{32} specific activity, at pH 7.45. DNP caused an initial reduction of the ATP^{32} specific activity, but this was gradually eliminated during respiratory stimulation. Under the conditions of this experiment, there was little net hydrolysis of ATP at any time interval with either pyruvate or pyruvate plus DNP added. Therefore, the initial DNP reduction of the ATP^{32} specific activity may be attributed to the effect of this compound on the ATP-Pi exchange reaction; the gradual restoration of the ATP^{32} specific activity may have resulted from substrate- level phosphorylation, oxidative phosphorylation, or from a combination of these reactions during the DNP-stimulated respiration.

The Effects of Inhibitors of Respiration and Phosphorylation on ATP^{32} Formation and DNP-stimulated Reactions

Anaerobic Incubation. Anaerobiosis inhibits mitochondrial electron transport and coupled phosphorylation but does not markedly inhibit the ATP-Pi exchange reaction or DNP-ATPase (21). Table IV shows that electron transport was required for ATP^{32} synthesis in the presence of DNP and pyruvate, since DNP inhibited ATP^{32} formation and stimulated ATP hydrolysis when mitochondria were

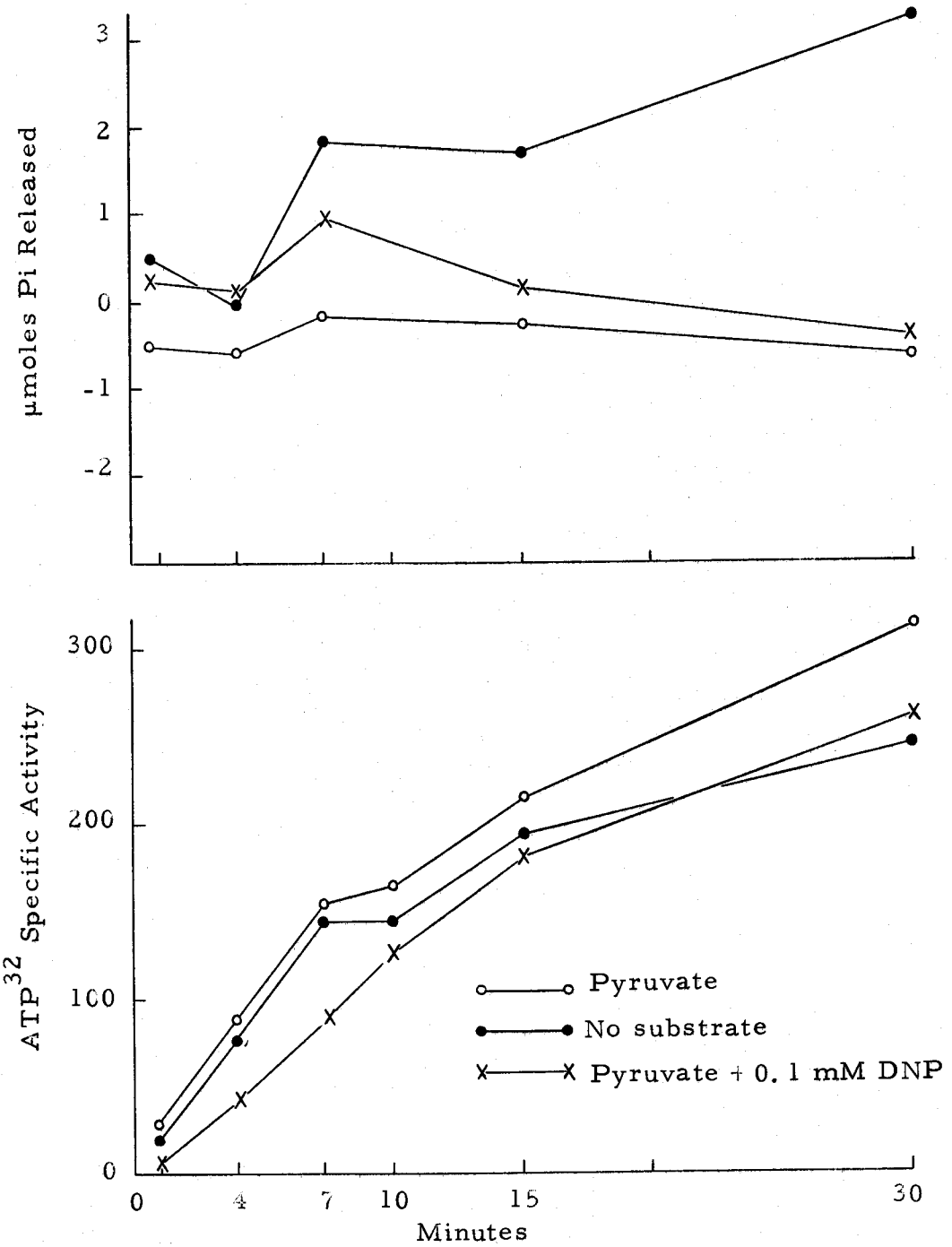

Figure 1. Time study of ATP^{32} specific activity and ATPase. Mitochondria were prepared by Method A, except that the washing and suspending medium was 0.3 M sucrose, 0.05 M Tris, 0.02 M Pi, and 3×10^{-5} M $MgCl_2$. Each flask contained Pi^{32} and DNP (where indicated) in the side arm; 300 μm. sucrose, 100 μm. Tris, 22.6 μm. Pi, 17.4 μm. ATP, 3 μm. EDTA, 0.03 μm. $MgCl_2$, mitochondria (4 mg protein), and pyruvate (30 μm. pyruvate + 0.2 μm. fumarate) where indicated, in the main compartment.

Table IV. The effects of DNP during aerobic and anaerobic incubations with blowfly mitochondria.

Incubation Conditions[1]	Substrate	DNP (mM)	O_2 Uptake (μgatoms)	Pi Released (μmoles)	ATP^{32} Formed (μmoles)	ATP^{32} Specific Activity
Aerobic,	None	---	0	1.4	6.30	1090
Aerobic,	None	0.1	0	16.6	0.01	63
Aerobic,	Pyr./fum.	---	0	-1.3	7.41	1125
Aerobic,	Pyr./fum.	0.1	13.9	2.2	6.13	1160
Anaerobic	None	---	---	0.7	4.67	778
Anaerobic	None	0.1	---	16.5	0.06	62
Anaerobic	Pyr./fum.	---	---	1.2	5.40	917
Anaerobic	Pyr./fum.	0.1	---	16.8	0.14	173
Aerobic-	None	---	0	2.5	5.17	952
following	None	0.1	0	18.5	0.03	49
anaerobic	Pyr./fum.	---	0	0.2	6.64	1077
treatment	Pyr./fum.	0.1	12.7	0.7	6.30	1048

Mitochondria were prepared according to Method A-2. Each flask contained Pi^{32}, 19.7 μm ATP, and DNP where indicated, in the side arm; 300 μm. sucrose, 100 μm. Tris, 22.1 μm. Pi, 3 μm. EDTA, 0.1 μm. $MgCl_2$, mitochondria (4.3 mg protein), and pyr./fum. (30 μm. pyruvate plus 0.2 μm. fumarate) where indicated, in the main compartment. Incubated 15 minutes.

[1] Aerobic incubations were as described for the standard procedure under Methods. Flasks incubated anaerobically were flushed with nitrogen for 10 minutes, equilibrated for 10 minutes at 25° C with the stopcocks closed, then incubated for 15 minutes after the side arm contents were tipped into the main compartment. Flasks incubated aerobically following the anaerobic treatment were flushed with nitrogen as described above, incubated 25 minutes without tipping the side arm contents into the main compartment, flushed with air for 10 minutes, equilibrated for five minutes at 25° C, and then incubated for 15 minutes after tipping the side arm contents into the main compartment.

incubated anaerobically. These effects of DNP were not due to mitochondrial damage resulting from the incubation conditions since DNP stimulated respiration normally, with retention of ATP^{32} formation, when the DNP was added to anaerobically treated flasks after restoration of aerobic conditions. Thus, ATP^{32} formation during DNP-induced pyruvate oxidation must have occurred by means of oxidative and/or substrate-level phosphorylation.

Antimycin A. The antibiotic, antimycin A, inhibits electron transport between cytochromes b and c_1 (47, p. 58), but has little or no effect on the ATP-Pi exchange reaction and does not inhibit mitochondrial or DNP-stimulated ATPase in mammalian mitochondria (48). The same results were observed when the insect mitochondria used in this study were incubated with antimycin A (Table V). It will be noted, however, that in the presence of pyruvate, antimycin A increased the net amount of ATP hydrolyzed and decreased the net amount of ATP^{32} formed. Presumably both of these effects depended upon inhibition of electron transport.

Amytal (amobarbital). Figure 2 shows that 1 mM amytal completely inhibited DNP-stimulated respiration, but caused only a 6 and 10 percent inhibition, respectively, of the DNP-ATPase and ATP-Pi exchange reactions. However, inhibition of each of the latter reactions was increased as the amytal concentration was raised to 5 mM. These results are in accord with the data of Löw et al. (48).

Table V. The effects of antimycin A on mitochondrial and DNP-stimulated reactions with blowfly mitochondria.

Substrate	DNP (mM)	Antimycin (μg. /flask)	O_2 Uptake (μgatoms)	Pi Released (μmoles)	ATP^{32} Formed (μmoles)	ATP^{32} Specific Activity
None	---	-	0	3. 7	10. 10	360
None	0. 1	-	0	16. 1	0. 06	18
None	---	1	0	4. 6	9. 84	361
None	0. 1	1	0	17. 8	0	0
Pyr. /fum.	---	-	0. 9	1. 5	11. 95	357
Pyr. /fum.	0. 1	-	7. 7	4. 5	8. 92	327
Pyr. /fum.	---	1	0	10. 0	4. 10	355
Pyr. /fum.	0. 1	1	0	17. 8	0. 10	0

Mitochondria were prepared by Method A-4. Each flask contained Pi^{32}, and DNP where indicated, in the side arm; 500 μm. sucrose, 100 μm. Tris, 17. 8 μm. ATP, 3 μm. EDTA, 0. 03 μm. $MgCl_2$, mitochondria (5. 8 mg protein), and antimycin A and pyr. /fum. (30 μm. pyruvate plus 0. 2 μm. fumarate) where indicated, in the main compartment. No Pi was added. Incubated 15 minutes.

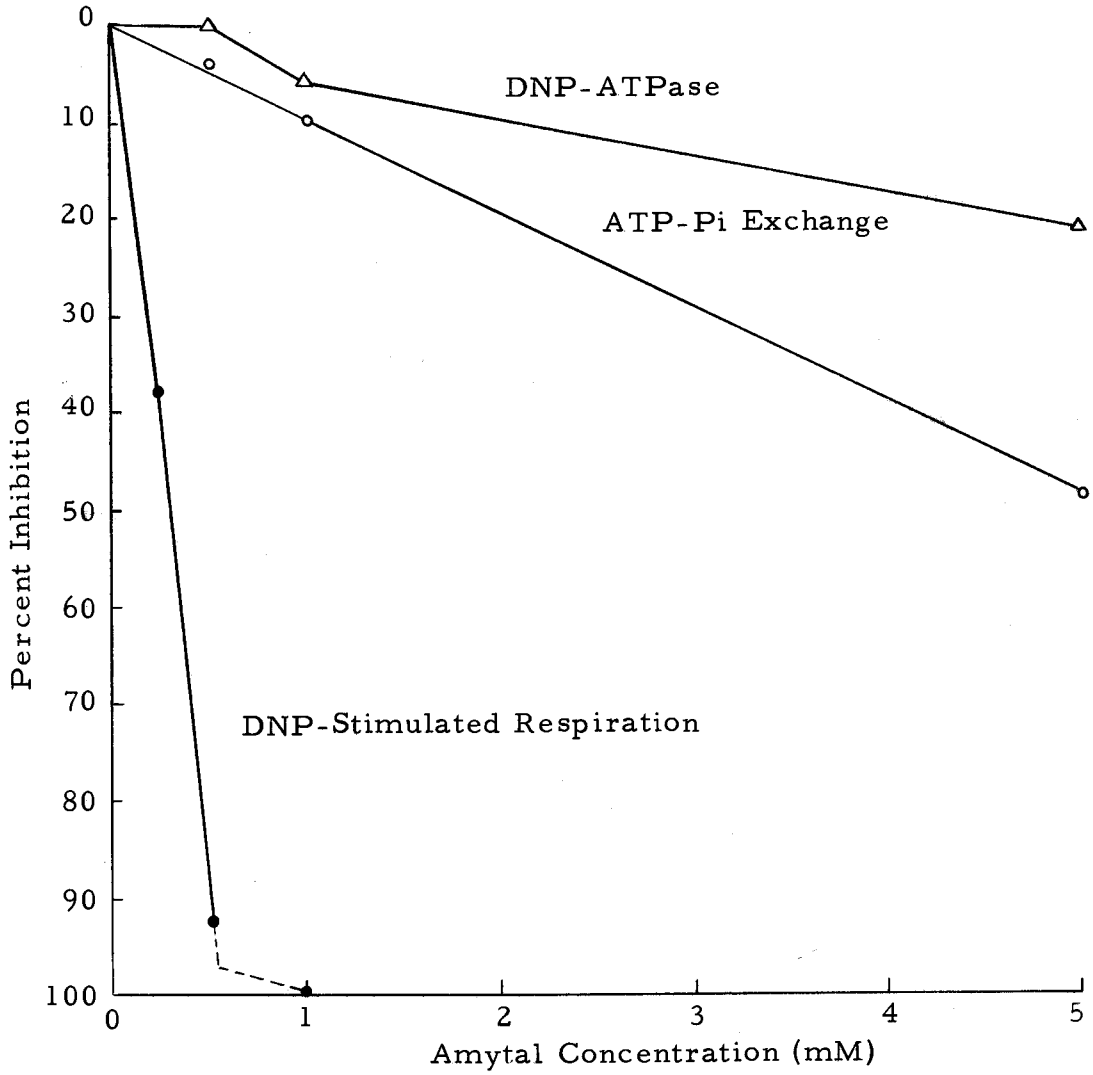

Figure 2. Inhibition of DNP-ATPase, the ATP-Pi exchange, and DNP-stimulated respiration by amytal. Mitochondria used in the respiratory study were prepared by Method B; those used in the study of the ATP-Pi exchange and DNP-ATPase were prepared by Method B-1. Each flask contained 600 μm. sucrose, 103 μm. Tris, 20 μm. Pi-Pi^{32}, 20 μm. ATP, 0.2 μm. each of EDTA and $MgCl_2$, 1.3 mg. BSA, mitochondria (2.2 mg protein), and amytal as indicated. DNP (0.3 μm.) was added to each flask in the ATPase and respiration studies; pyruvate (15 μm./flask) and fumarate (0.23 μm./flask) were added only in the respiration study. Incubated 15 minutes.

Löw et al. suggested that the first phosphorylation site may be primarily responsible for ATP-Pi exchange and DNP-ATPase activities because amytal, which was believed to be specific for inhibition between NAD and FAD, caused partial inhibition of these reactions with rat liver mitochondria and because the inhibition of each increased as the amytal concentration was raised, whereas antimycin A and cyanide did not show comparable effects. Such an interpretation may be subject to error, since it will be noted (Figure 2) that neither ATPase nor the exchange was inhibited completely by amytal at a concentration (5mM) which was five fold higher than that causing complete inhibition of electron transport. In addition, Pumphrey and Redfearn (55) found that succinate oxidation, which does not require electron transport between NAD and FAD, was also inhibited by amytal in tightly coupled mitochondria, but not in loosely coupled mitochondria. The data of Pumphrey and Redfearn may indicate that amytal inhibition is less specific than Low et al. assumed.

Azide. Table VI shows that 1 mM azide inhibited DNP-stimulated pyruvate oxidation by 44 percent. As is shown in Figure 3, this concentration of azide caused 45 and 35 percent inhibition of DNP-ATPase and ATP-Pi exchange activities, respectively. Thus, the effects of azide on the three activities correlated very well. This could indicate that DNP-stimulation of respiration is dependent upon the ability of DNP to promote ATP hydrolysis. DNP-stimulated

Table VI. The effect of azide on DNP-stimulated respiration with blowfly mitochondria.

Flask Variables	O_2 Uptake (μgatoms)
None	6.3
DNP	32.2
DNP + azide	18.2

Mitochondria were prepared by Method A-4. Each flask contained DNP (0.3μm.) and azide (3 μm.) as indicated, in the side arm; 250 μm. sucrose, 100 μm. Tris, 100 μm. Pi, 10 μm. ATP, 0.03 μm. $MgCl_2$, 30 μm. pyruvate, 0.2 μm. fumarate, and mitochondria (5.5 mg protein) in the main compartment. Incubated 30 minutes.

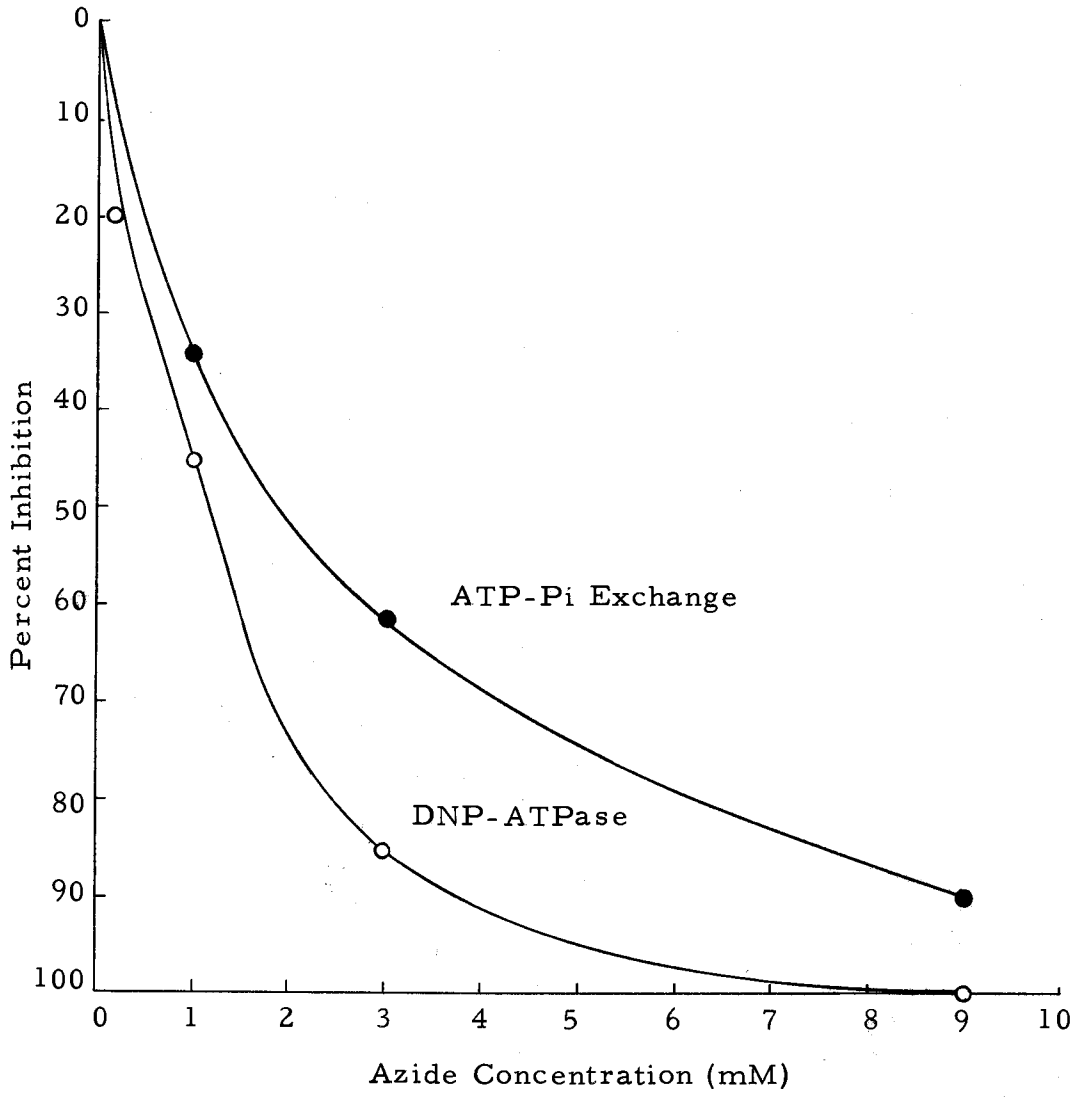

Figure 3. Inhibition of the ATP-Pi exchange and DNP-ATPase by azide. Blowfly mitochondria were prepared by Method B. Each flask contained: 600 μm. sucrose, 103 μm. Tris, 20.7 μm. Pi-Pi^{32}, 20 μm. ATP, 0.2 μm. each of EDTA and $MgCl_2$, 1.3 mg BSA, and mitochondria (1.6 mg protein). DNP (0.3 μm.) was added to each flask in the ATPase studies.

ATPase may, in turn, include all reactions which are involved in the ATP-Pi exchange. The sensitivities of these reactions to azide agree with the proposal of Gregg et al. (35), who suggested that DNP-stimulated pyruvate oxidation by housefly mitochondria might depend upon ADP produced by DNP-ATPase, since ATP was required for DNP to stimulate respiration. However, subsequent studies (Tables X, XI, and XII) have shown that DNP is capable of stimulating pyruvate oxidation, in the presence of ADP and Pi, after the ATP-Pi exchange, DNP-ATPase and coupled phosphorylation are inhibited by oligomycin. Therefore, it appears from the oligomycin studies that DNP-stimulated pyruvate oxidation in blowfly mitochondria is dependent upon retention of DNP-ATPase activity only under conditions in which this is needed to supply ADP for substrate-level phosphorylation.

The ATP and Pi Requirements for DNP-Stimulated Respiration

Remmert, Heisler, and Gregg (57) had reported previously that ATP was required for DNP-stimulated pyruvate oxidation with housefly mitochondria. The data in Table VII confirm the need for ATP and demonstrate that Pi is also essential for maximal respiratory stimulation. It is seen from the last line that these mitochondria, which were isolated in a phosphate-free medium, retained some nucleotide even after washing, because they still carried out tightly

Table VII. Phosphate and ATP requirements for DNP-stimulated pyruvate oxidation, and retention of nucleotide by blowfly mitochondria.

Flask Variables				O_2	Pi	
ATP (μm.)	Pi (μm.)	Other	Min. Inc.	Uptake (μgatoms)	Uptake (μmoles)	P/O
1	1	---	120	9.0		
0	0	DNP	120	8.4		
0	1	DNP	120	62.4		
1	0	DNP	120	90.4		
1	1	DNP	120	111.1		
0	20	hexokinase	40	6.5	17.4	2.68

Mitochondria were prepared by Method B, except that the homogenizing, washing, and suspending media contained 0.003 M EDTA. Each flask contained DNP (0.3 μm.) and the hexokinase system (150 KM hexokinase, 100 μm. glucose, and 20 μm. $MgCl_2$) as indicated, in the side arm; 600 μm. sucrose, 100 μm. Tris, 3 μm. EDTA, 0.1 μm. $MgCl_2$, 30 μm. pyruvate, 0.2 μm. fumarate, 3.3 mg BSA, mitochondria (8.3 mg protein), and ATP and Pi as indicated, in the main compartment.

coupled oxidative phosphorylation without any added nucleotide. The presence of mitochondrial nucleotide may explain why Pi alone permitted DNP to stimulate substrate oxidation. Addition of ATP (only) gave a larger respiratory stimulation in the presence of DNP than did Pi alone. This effect of added ATP could depend on the provision of both Pi and ADP through DNP-stimulated ATP hydrolysis. It is to be noted that maximum respiration was obtained only with both ATP and Pi added.

There are two obvious explanations which can account for the ATP (or ADP) and Pi requirements: The first is that both are involved in the mechanism by which DNP stimulates respiration in insect mitochondria; the second is that ATP (or ADP) and Pi are needed only for the substrate-level phosphorylation involved in the oxidation of α-ketoglutarate to succinate, during the complete degradation of pyruvate in the citric acid cycle. These possibilities should be distinguishable through studies with the antibiotic, oligomycin, as is discussed in the next section.

The Effects of Oligomycin on Mitochondrial Reactions in Relation to the ATP and Pi Requirements for DNP-Stimulated Respiration

Oligomycin inhibits the ATP-Pi exchange reaction, DNP-ATPase, and the phosphorylation which is coupled, at each of three sites, to electron transport in mammalian mitochondria (27). However, Chappell and Greville (18) found that this antibiotic did not inhibit

substrate-level phosphorylation. Studies by Azzone and Ernster (1), and Borst and Slater (4, 5) showed that Pi was not required for DNP-stimulated respiration with rat liver mitochondria, except with substrates which depended upon substrate-level phosphorylation for continued oxidation (e. g., α-ketoglutarate, glutamate, isocitrate). It has also been shown (53) that oligomycin inhibition of succinate oxidation with rat liver mitochondria, in the presence of Pi and ADP, could be relieved by DNP. This indicated that Pi and ADP (or ATP) were not involved in the mechanism by which DNP stimulated respiration with mitochondria from this tissue. However, Chappell (20) found that Pi was required for DNP to stimulate the single-step oxidation of malate and succinate with pigeon heart mitochondria. Since the single-step oxidation of malate and succinate does not involve substrate-level phosphorylation, Chappell suggested that the mechanism, by which DNP stimulates respiration, might actually be different in some tissues or species.

Initial studies of the effects of oligomycin on mitochondrial and DNP-stimulated reactions of blowfly mitochondria were made during 1961 and 1962, using oligomycin obtained from Dr. Bertram Pressman. The results of these studies are shown in Tables VIII and IX. It is seen (Table VIII) that 0. 6 micrograms of oligomycin per flask gave essentially complete inhibition of DNP-ATPase, and complete inhibition of the ATP-Pi exchange and DNP-stimulated pyruvate

Table VIII. The effect of oligomycin on mitochondrial and DNP-stimulated reactions with blowfly mitochondria incubated with Pi and ATP.

Substrate	DNP (mM)	Oligomycin (μg/flask)	O_2 Uptake (μgatoms)	Pi Released (μmoles)	ATP^{32} Formed (μmoles)	ATP^{32} Specific Activity
None	---	0	0	4.0	3.82	293
None	---	0.1	0	4.8	1.37	109
None	---	0.6	0	0.8	0	0
None	0.1	0	0	16.9	0	0
None	0.1	0.1	0	14.2	0	0
None	0.1	0.6	0	1.9	0	0
Pyr./fum.	---	0	0.6	0.8	7.30	468
Pyr./fum.	---	0.1	0.6	0.6	4.86	332
Pyr./fum.	---	0.6	0	0	0.01	3
Pyr./fum.	0.1	0	11.5	-2.7	6.02	417
Pyr./fum.	0.1	0.1	9.1	-1.2	5.01	350
Pyr./fum.	0.1	0.6	0	-0.7	0.01	0

Mitochondria were prepared by Method A-4. Each flask contained Pi^{32} and DNP as indicated, in the side arm; 500 μm. sucrose, 100 μm. Tris, 21.2 μm. Pi, 17.8 μm. ATP, 3 μm. EDTA, 0.03 μm. $MgCl_2$, mitochondria (3.5 mg protein), 0.02 ml of 50 percent ethanol, and oligomycin and pyr./fum. (30 μm. pyruvate plus 0.2 μm. fumarate) as indicated, in the main compartment. Incubated 15 minutes.

Table IX. Inhibition of substrate-level phosphorylation by oligomycin in the presence of ATP, ADP, Pi, and DNP.

Flask Variables				O_2 Uptake	ATP^{32} Formed
ATP (μm./flask)	ADP (μm./flask)	DNP (mM)	Olig. (μg./flask)	(μgatoms)	(μmoles)
8.9	--	---	-	1.7	4.75
8.9	--	0.1	-	14.0	4.29
8.9	--	---	2	0.3	0.17
8.9	--	0.1	2	0	0.01
8.9	10	---	-	3.0	8.19
8.9	10	0.1	-	8.8	7.09
8.9	10	---	2	1.7	0.54
8.9	10	0 1	2	0	0.06

Blowfly mitochondria were prepared by Method A-4. Each flask contained Pi^{32}, and DNP and ADP as indicated, in the side arm; 250 μm. sucrose, 100 μm. Tris, 21.2 μm. Pi, 0.03 μm. $MgCl_2$, ATP, 30 μm. pyruvate, 0.2 μm. fumarate, 0.02 ml. 50% ethanol, mitochondria (4.8 mg protein), and oligomycin as indicated, in the main compartment. Incubated 15 minutes.

oxidation (in the presence of ATP and Pi). It is also seen (Table IX) that the oligomycin inhibition of DNP-stimulated respiration was not relieved by ADP and Pi. This indicated that the DNP stimulation was dependent upon conditions which permitted DNP to hydrolyze ATP, and that the inhibition produced by oligomycin was due to a requirement of ADP and Pi for oxidative (coupled) phosphorylation, as well as for substrate-level phosphorylation. That is, it appeared that ATP (or ADP) and Pi were participants in phosphorylation reactions which continued to function during the DNP stimulation, and that oligomycin inhibited DNP-stimulated respiration by an effect on such reactions, in the same manner as it inhibited respiration induced by ADP. These results supported the previously mentioned findings of Chappell (20), and of Gregg et al. (35), which implicated Pi and ATP as direct participants in DNP-stimulated respiration with pigeon heart and housefly mitochondria.

Early in 1965, however, Davis (23) found that oligomycin could inhibit substrate-level phosphorylation in the presence of DNP, ADP, and Pi, unless the mitochondria were preincubated with at least 1 mM $MgCl_2$ prior to the DNP addition. Since the data given in Tables VIII and IX were obtained with only 0.01 mM $MgCl_2$ present, experiments were made to test Davis' observations, using our blowfly mitochondrial preparations. These results are shown in Tables X to XIII. The oligomycin used for these studies was obtained from the Wisconsin

Table X. Relief of the oligomycin inhibition of DNP-stimulated respiration by provision of ADP for substrate-level phosphorylation.

Flask Variables		O_2 Uptake	Pi Uptake	ATP^{32} Formed
Main Compartment	Side Arm	(μgatoms)	(μmoles)	(μmoles)
None	None	0.7	0.9	2.02
Olig.	None	0.5	1.0	0.12
None	DNP	15.2	0.7	1.70
Olig.	DNP	0.3	0.2	0
None	DNP + ADP	14.6	6.6	6.60
Olig.	DNP + ADP	10.1	2.9	1.52
$MgCl_2$	DNP + ADP	17.6	4.9	5.85
$MgCl_2$ + Olig.	DNP + ADP	10.2	1.9	1.45
$MgCl_2$ + Olig.	ADP	0.9	-0.3	0.13
$MgCl_2$ + Olig.	DNP	9.0	-0.5	0.47
Olig.	DNP + ADP + $MgCl_2$	9.9	1.6	0.83

Blowfly mitochondria were prepared by Method B-2. Each flask contained Pi^{32} and other reagents as indicated, in the side arm; 600 μm. sucrose, 103 μm. Tris, 21 μm. Pi, 10 μm. ATP, 0.2 μm. each of EDTA and $MgCl_2$, 11.5 μm. pyruvate, 0.23 μm. fumarate, 1.3 mg BSA, mitochondria (1.83 mg protein), and other reagents as indicated, in the main compartment. Where added, the amounts/flask were: DNP, 0.3 μm.; ADP, 10 μm.; $MgCl_2$, 6 μm.; oligomycin, 2 μg. Incubated 20 minutes.

Alumni Research Foundation because the original gift from Dr. Pressman had been exhausted.

In contrast to the previous results (Tables VIII and IX), it was now found that ADP largely relieved the oligomycin inhibition of DNP-stimulated pyruvate oxidation (Table X), but preincubation with $MgCl_2$ was not essential. It is also shown in Table X that DNP stimulated pyruvate oxidation, in the presence of oligomycin, without added ADP, when the incubation medium was supplemented with ATP and 2 mM $MgCl_2$. However, $MgCl_2$, without DNP, did not stimulate respiration in the presence of oligomycin, even with ADP added. These results indicate that DNP acts upon an intermediate of the coupling sequence, at each of the sites of phosphorylation, at a point between the respiratory chain and the reaction inhibited by oligomycin, as has been proposed by Estabrook (28), and others (4, 5). Presumably, the ADP (supplied as an addition or derived from Mg^{++}-ATPase) and Pi were needed in the presence of oligomycin only for substrate-level phosphorylation. It is to be noted that the substrate-level phosphorylation permitted a <u>net</u> ATP^{32} formation after the ATP-Pi exchange reaction, DNP-ATPase, and coupled phosphorylation had been inhibited by preincubating the mitochondria with oligomycin. The failure of DNP to stimulate respiration in the presence of oligomycin, ATP, and Pi (with no added ADP) can be explained by inhibition of DNP-ATPase (Tables VIII and X), which was required to

supply ADP for substrate-level phosphorylation.

It is to be noted that the failure of $MgCl_2$ to stimulate respiration in the presence of oligomycin (with DNP omitted) shows that oligomycin acts between the respiratory chain and the point at which $MgCl_2$ acts, if it is assumed that $MgCl_2$ causes decomposition of an intermediate in coupled phosphorylation. (Alternatively, $MgCl_2$ could stimulate an ATPase activity which does not involve reactions coupled to electron transport, even though it is inhibited by oligomycin.) It would appear that $MgCl_2$ permitted DNP to stimulate respiration in the presence of oligomycin, with ATP but without added ADP (Table X), by providing enough ADP for substrate-level phosphorylation (only). This is supported by other experiments which showed that oligomycin inhibited Mg^{++}-ATPase <u>almost</u> completely. Since addition of $MgCl_2$ and ADP, to the medium containing oligomycin but no DNP, failed to stimulate respiration (Table X), it is obvious that the Mg^{++} ion does not release respiration from phosphorylation as DNP does.

The major differences between the procedures used in obtaining the data of Tables VIII and IX and those of Table X were the sources of oligomycin and certain modifications in the media used in isolating the mitochondria. It was not possible to compare the two oligomycin samples in one experiment because the original supply obtained from Dr. Pressman was exhausted, but the isolation media were compared

(Table XI). The media differed mainly in that 0. 1 M Pi was included in the homogenizing medium in Method A-7, whereas no Pi was present in any of the media used in Method B-2. The data show that the previous failure of ADP to stimulate respiration in the presence of oligomycin, DNP, ATP and Pi (Table IX) was not due to the preparative media since this stimulation was obtained with both preparations. It is possible, then, that the previous anomally was due to the oligomycin used. That is, some oligomycin samples, for example, that used by Davis (23) and the gift obtained from Dr. Pressman and used in the earlier experiments reported here, may contain an inhibitor of substrate-level phosphorylation as a contaminant. The observation that it is not necessary to preincubate mitochondria with $MgCl_2$, in order to obtain DNP-stimulated respiration in the presence of oligomycin and ADP (Table X), shows that Davis' findings are not universal, and suggests the possibility that the oligomycin used in his experiments contained such an inhibitor.

From the data in Tables X and XI, it is seen that the addition of ADP to the oligomycin-inhibited reaction mixtures did not restore DNP-stimulated respiration to the maximum rate observed in the absence of oligomycin. Table XII shows that the absolute magnitude of respiration was controlled by the DNP concentration, either in the presence or absence of oligomycin, and that it was always less when coupled phosphorylation was inhibited by oligomycin, even though

Table XI. Comparison of blowfly mitochondrial preparation Methods A and B: Oligomycin inhibition of DNP-stimulated respiration and relief by provision of ADP for substrate-level phosphorylation.

Mitochondrial Preparation	Flask Variables: Main Compartment	Flask Variables: Side Arm	O_2 Uptake (μgatoms)	ATP^{32} Formed (μmoles)
B-2	None	None	1.0	2.92
"	Olig.	None	0.5	0.12
"	None	DNP	17.7	3.28
"	Olig.	DNP	0.2	0.02
"	None	DNP + ADP	16.9	9.06
"	Olig.	" "	9.9	1.89
"	Olig. + $MgCl_2$	" "	10.4	1.88
"	" "	DNP	9.8	1.03
"	" "	ADP	0.4	0.18
A-7	None	DNP	14.8	3.37
"	Olig.	DNP	2.0	0.22
"	None	DNP + ADP	12.8	8.19
"	Olig.	" "	7.5	1.47
"	Olig. + $MgCl_2$	" "	6.2	1.22

Each flask contained Pi^{32} and other reagents as indicated, in the side arm. With mitochondrial preparation B-2, the main compartment contained 600 μm. sucrose, 103 μm. Tris, 21 μm. Pi, 10 μm. ATP, 0.2 μm. each of EDTA and $MgCl_2$, 11.5 μm. pyruvate, 0.23 μm. fumarate, 1.3 mg BSA, 0.02 ml of 50% ethanol, mitochondria (1.77 mg protein), and other reagents as indicated. With mitochondrial preparation A-7, the main compartment contained 250 μm. sucrose, 103 μm. Tris, 21 μm. Pi, 10 μm. ATP, 0.03 μm. $MgCl_2$, 11.5 μm. pyruvate, 0.23 μm. fumarate, 0.3 mg BSA, 0.02 ml of 50% ethanol, mitochondria (1.55 mg protein), and other reagents as indicated. Where added, the amounts/flask were: DNP, 0.3 μm.; ADP, 10 μm.; $MgCl_2$, 6 μm.; oligomycin, 2 μg. Incubated 25 minutes.

Table XII. Influence of DNP concentration on the relief of oligomycin inhibition of respiration by ADP.

Flask Variables			O_2 Uptake (μgatoms)	Relative DNP Respiratory Stimulation (Percent)
Main Compartment	Side Arm			
	DNP (μM)	Other		
None	0	---	0.9	
Oligomycin	0	---	0.8	
Oligomycin	0	ADP	0.7	
None	100	---	13.3	100
Oligomycin	100	---	0.3	
Oligomycin	100	ADP	7.9	59.3
None	150	---	17.6	100
Oligomycin	150	---	0.3	
Oligomycin	150	ADP	9.9	56.2
None	200	---	13.3	100
Oligomycin	200	---	0.4	
Oligomycin	200	ADP	9.4	70.6
None	400	---	2.3	
Oligomycin	400	---	0.1	
Oligomycin	400	ADP	0.1	

Blowfly mitochondria were prepared by Method B-2. Each flask contained DNP and ADP (10 μm.) as indicated, in the side arm; 600 μm. sucrose, 103 μm. Tris, 21 μm. Pi, 10 μm. ATP, 0.2 μm. each of EDTA and $MgCl_2$, 11.5 μm. pyruvate, 0.23 μm. fumarate, 0.02 ml of 50% ethanol, 1.3 mg BSA, mitochondria (1.44 mg protein), and 2 μg oligomycin per flask where indicated, in the main compartment. Incubated 30 minutes.

adequate ADP and Pi were always present for substrate-level phosphorylation. This may indicate that DNP does not act equally at each of the coupling sites, or that DNP does not permit the maximum rate of electron transport through any or all of the coupling sites, without the intervention of ADP and Pi. Otherwise, DNP would be expected to stimulate respiration maximally, even in the presence of oligomycin.

The alternate possibility, that the oligomycin contained an inhibitor of electron transport, seems improbable because increasing concentrations of oligomycin did not alter the rate of DNP stimulation (Table XIII). The data from Tables XII and XIII, therefore, can be interpreted to mean that one or more of the coupling sites of the respiratory chain is not completely uncoupled by DNP, and that this site, or sites, may become rate-limiting under conditions which prevent decomposition of the high energy intermediate(s), that is, when oxidative phosphorylation and DNP-ATPase have been inhibited and the rate of respiration is entirely dependent upon the rate at which DNP causes uncoupling. Thus, the greater DNP-stimulation of pyruvate oxidation in the absence of oligomycin may indicate that one or more sites of phosphorylation continues to function mostly in a coupled manner when mitochondria are incubated with DNP, ATP and Pi. Under these conditions it is possible for DNP to stimulate ATP hydrolysis to form ADP and Pi. This could permit ATP

Table XIII. The effects of the DNP and oligomycin concentrations on pyruvate oxidation and relief of the oligomycin inhibition of respiration by ADP.

Flask Variables			O_2 Uptake (μgatoms)	ATP^{32} Formed (μmoles)
Main Compartment	Side Arm			
Oligomycin (micrograms)	DNP (μM)	Other		
0	0	---	1.2	3.65
1	0	---	1.3	0.05
2	0	---	1.3	0.03
3	0	---	1.3	0.02
1	0	ADP	1.4	0.02
3	0	ADP	1.1	0.01
0	100	---	18.1	4.05
1	100	---	0.5	0
1	100	ADP	13.6	2.09
2	100	ADP	13.5	2.05
3	100	ADP	13.5	1.99
0	150	---	24.6	3.57
1	150	---	0.8	0
1	150	ADP	17.8	2.83
2	150	ADP	18.0	2.81
3	150	ADP	17.8	2.86

Blowfly mitochondria were prepared by Method B-2. Flask contents were identical to those given in Table XII, except for the indicated variables and mitochondrial protein (1.71 mg per flask). Incubated 30 minutes.

synthesis to occur at the substrate-level and at one or more sites not readily attacked by DNP, and it would allow DNP to uncouple phosphorylation at one or more sites. The ADP provided by DNP-stimulated ATPase could also allow electron transport coupled with phosphorylation to supplement the electron transport induced by the uncoupling action of DNP, at one or all of the three sites. Further discussion of these possibilities will be given following subsequent sections of this thesis.

Table XII also shows that 400 micromolar DNP was almost completely inhibitory to respiration in the presence or absence of oligomycin and ADP. These results are in accord with the proposal of Chance, Williams, and Hollunger (16) which visualized respiratory inhibition by high concentrations of DNP as being due to the combination of more than one molecule of DNP with each molecule of the intermediate(s) of the coupling sequence, to form a complex which dissociates slowly (e. g., reaction (10) as given in the Introduction).

Oxidative Phosphorylation During DNP-Stimulated Respiration

The data of the preceding sections demonstrated that ATP was continuously formed by insect mitochondria when pyruvate oxidation was stimulated by DNP. It was found that substrate-level phosphorylation contributed to ATP formation, but the P/O ratio for ATP synthesis by this means was slightly less than the theoretical value of

0. 2 (Tables X and XI). It is difficult to determine whether the ATP-Pi exchange reaction has contributed to ATP^{32} formation when DNP has been allowed to stimulate respiration in the presence of ATP and Pi, because, although it was shown in Table II that DNP caused a rapid inhibition of the ATP-Pi exchange when mitochondria were incubated without substrate, this does not prove that DNP inactivated this exchange when pyruvate oxidation was stimulated. However, it has been possible to show that ATP was formed by means of oxidative phosphorylation when respiration was stimulated nearly maximally by 0. 10 to 0. 15 mM DNP, as is shown in the next sections.

Oxidative Phosphorylation in the Presence of DNP and ATP. The data in Table XIV show that maximal stimulation of pyruvate oxidation by 0. 1 mM DNP occurred when blowfly mitochondria were supplemented with 1 μmole (0. 33 mM) ATP and either 1 or 10 μmoles Pi; very little ATP^{32} was formed with this low level of ATP, and the ATP^{32} specific activity was decreased in comparison to flasks incubated without DNP. However, with 10, 30, or 60 μmoles of ATP added, DNP caused little or no reduction of the ATP^{32} specific activity. It was found in other experiments that the ATP^{32} specific activity, reduced by DNP with only 1 μmole of ATP present, was restored almost to the control value by a later addition of more ATP (10 μmoles) to the rapidly respiring mitochondrial suspension.

Table XIV. Synthesis of ATP^{32} through oxidative phosphorylation during DNP-stimulated pyruvate oxidation with blowfly mitochondria.

Flask Variables			O_2	Pi	ATP^{32}	ATP^{32}
Pi (μm./flask)	ATP (μm./flask)	DNP (mM)	Uptake (μgatoms)	Released (μmoles)	Formed (μmoles)	Specific Activity
1	1	---	0.4	0	0.33	7370
1	1	0.1	18.5	0.7	0.13	4346
1	10	---	1.0	-0.1	0.98	1328
1	10	0.1	14.3	6.0	2.09	1215
1	30	---	0.8	0	2.10	480
1	30	0.1	15.3	6.8	5.92	468
1	60	---	1.2	0.9	3.80	243
1	60	0.1	12.5	9.6	8.28	212
10	1	---	0.5	-1.1	0.69	1210
10	1	0.1	16.8	-0.1	0.04	320
10	10	---	0.5	0	4.03	723
10	10	0.1	17.1	-0.1	3.97	700
10	30	---	0.9	0.1	6.44	345
10	30	0.1	17.9	1.4	6.96	345
10	60	---	1.8	0.3	7.37	172
10	60	0.1	14.3	1.7	8.25	183

Mitochondria were prepared by Method B, except that the homogenizing, washing, and suspending media contained 0.003 M EDTA. Each flask contained Pi^{32} and DNP as indicated, in the side arm; 600 μm. sucrose, 110 μm. Tris, 3 μm. EDTA, 0.1 μm. $MgCl_2$, 35 μm. pyruvate, 0.2 μm. fumarate, 3.3 mg BSA, mitochondria (2.5 mg protein), and Pi and ATP as indicated, in the main compartment. Incubated 20 minutes.

The important result in Table XIV is the observation that with 30 or 60 μmoles of ATP, and 1 μm. Pi, more ATP^{32} was formed when pyruvate oxidation was stimulated by DNP than could have been formed by a combination of the ATP-Pi exchange reaction and substrate-level phosphorylation. With 30 or 60 μmoles of ATP and 1 μmole of Pi added, the net ATP^{32} synthesis during DNP-stimulated respiration exceeded ATP^{32} formation in the non-stimulated systems by 3. 82 and 4. 48 μmoles, respectively, while the corresponding oxygen uptake was 15. 3 and 12. 5 microgram atoms. Substrate-level phosphorylation accompanying this amount of respiration would form only 3. 1 and 2. 5 μmoles of ATP^{32}. The excess ATP^{32} (0. 7 and 1. 9 μmoles, respectively) must have been derived from oxidative phosphorylation during the DNP-stimulated respiration, by utilization of ADP which had been formed by DNP-ATPase activity.

It is also seen in Table XIV that the net amount of ATP hydrolyzed in the presence of DNP and substrate was markedly influenced by the amount of Pi added. The net ATP hydrolysis was less with 10 μmoles Pi added, than with a 1 μmole addition, for all ATP levels tested. This would be expected if phosphorylation was still functioning because a higher concentration of Pi would accelerate resynthesis of ATP from the ADP derived from DNP-ATPase activity.

The Effects of Albumin (BSA). The results given previously (Table XIV) were obtained in flasks supplemented with bovine serum albumin (BSA) which generally increased the stability and activity of mitochondrial preparations. BSA binds free fatty acids which uncouple oxidative phosphorylation (3, 6, 37, 44, 70), but does not prevent DNP-stimulated respiration. Gregg (30, p. 41-46) found that BSA increased P/O ratios but was not essential for oxidative phosphorylation with housefly mitochondria. This was also the case in the present study as shown in Table XV (expt. No. 108). This table also shows (expt. No. 107) that DNP-stimulated respiration was increased and unstimulated respiration was reduced when BSA was added at 1 mg/ml or less. BSA inhibited the DNP-stimulated respiration at higher levels, apparently by adsorbing the DNP. Because of its improvement of DNP-stimulated reactions, approximately 1 mg/ml of BSA was included in many of the studies reported here.

It is to be noted, however, that 1 mg/ml of BSA did not exert a measurable influence on the mitochondrial or DNP-stimulated phosphate transfer reactions. This is illustrated (Table XV, expt. No. 108) by the fact that the P/O ratios for the complete oxidative phosphorylation system, plus or minus 0.1 mM DNP, were nearly the same in the presence and absence of 1 mg/ml BSA. This shows that DNP was not adsorbed by BSA at this low level, although BSA

Table XV. The influence of BSA on mitochondrial and DNP-stimulated reactions.

Flask Variables							
Expt. No.	BSA (mg/flask)	DNP (mM)	Hexo-kinase System	Min. Inc.	O_2 Uptake (μgatoms)	Pi^{32} Uptake (μmoles)	P^{32}/O
107	0	---		60	12.7		
	0	0.1		60	32.6		
	0.3	---		60	11.4		
	0.3	0.1		60	39.2		
	0.9	---		60	6.9		
	0.9	0.1		60	46.1		
	3	---		60	3.8		
	3	0.1		60	50.9		
	9	---		60	3.0		
	9	0.1		60	26.9		
	30	---		60	3.4		
	30	0.1		60	8.4		
	60	---		60	3.3		
	60	0.1		60	5.1		
108	0	---	+	40	22.3	57.3	2.57
	0	0.1	+	40	16.0	26.1	1.63
	3	---	+	40	12.6	35.0	2.78
	3	0.1	+	40	7.4	9.0	1.22
	60	---	+	40	13.6	40.4	2.97
	60	0.1	+	40	13.5	36.6	2.71

Mitochondria were prepared by Method A-2. In experiment 107, each flask contained DNP as indicated, in the side arm; 300 μm. sucrose, 100 μm. Tris, 20 μm. Pi, 10 μm. ATP, 3 μm. EDTA, 0.1 μm. $MgCl_2$, 30 μm. pyruvate, 0.2 μm. fumarate, mitochondria (1.6 mg protein), and BSA as indicated, in the main compartment. The conditions were the same in experiment 108, except that Pi^{32} and the hexokinase system (150 KM hexokinase, 100 μm. glucose, and 20 μm. $MgCl_2$) were in the side arm, Pi (100 μm.) was in the main compartment, and each flask contained 2.2 mg of mitochondrial protein.

did adsorb DNP when present at 20 mg/ml. It was found in other experiments that 1 mg/ml of BSA did not prevent DNP inhibition of the ATP-Pi exchange reaction when pyruvate was omitted from the medium.

The data in Table XV also show a significant retention of oxidative phosphorylation when 0.1 mM DNP was added to the complete phosphorylation system. In fact, the results indicate that two sites of coupled phosphorylation may have been retained since the P/O ratios exceed 1.0 considerably; alternatively, all three sites may have continued to function with decreased efficiency in the presence of DNP.

While DNP inhibited respiration in the presence of the phosphate trapping system (hexokinase, glucose, $MgCl_2$) in experiment No. 108 (Table XV), it was possible to obtain mitochondrial preparations which did not show this effect. The latter preparations were obtained by Methods B or B-1 (see Methods), and, in general, did not give a large increase in respiration upon addition of Mg^{++}, in the presence of pyruvate and ATP, whereas preparations made by the various modifications of Method A (see Methods) usually gave a large respiratory response to addition of $MgCl_2$ (e.g., compare the relative DNP- and Mg^{++}-stimulated respiration of Tables XVIII and XXIX). This may mean that DNP inhibition of respiration in a complete oxidative phosphorylation system (Table XV) is partly due to instability,

or to structural changes of the mitochondria, when both DNP- and Mg^{++}-ATPase reactions are activated, since it has been found in other experiments that mitochondria prepared by Method B or B-1 also showed little Mg^{++}-stimulated ATPase activity.

As shown in a subsequent part of this thesis, studies with mitochondrial particles indicated that the Mg^{++}-ATPase is characteristic of damaged insect mitochondria, or of particles, whereas DNP-ATPase activity is most prominent with intact mitochondria. From this fact and the considerations given above, it might be predicted that retention of oxidative phosphorylation in the presence of 0. 1 mM DNP could be demonstrated more reproducibly in mitochondria prepared by Method B or B-1, than in those prepared by Method(s) A. This has been found to be the case, in general. These properties were not understood in early experiments, and many preparations obtained as described in Table XV (but with BSA omitted) showed complete inhibition of respiration and coupled phosphorylation by 0. 1 mM DNP, in the presence of Mg^{++} and the phosphate acceptor system.

The Efficiency of Oxidative Phosphorylation in the Presence of 0. 1 mM DNP

Tables XVI to XIX give data from several experiments which show retention of oxidative phosphorylation during respiratory

stimulation by 0.1 mM DNP. The results in these tables indicate that the equivalent of two phosphorylation sites of the respiratory chain may function in the presence of this concentration of DNP.

Table XVI shows that pyruvate oxidation was stimulated by DNP in the presence of Pi, and either ATP or ADP. In the presence of ADP, all ATP^{32} formation may be ascribed to oxidative and substrate-level phosphorylation, since ATP-Pi exchange after the initial phosphorylation of ADP would not increase the amount of ATP^{32} measured. The contribution to the P/O ratio from substrate-level phosphorylation is, theoretically, 0.2 (see also Table X). Consequently, the P/O ratio of 1.22 shows that the equivalent of at least one coupling site was retained; it is possible that partial uncoupling had occurred at all three sites.

Table XVII shows that the capacity to carry out coupled oxidative phosphorylation was retained even when the ADP was not added until DNP had stimulated respiration for 10 minutes. The data in Tables XVI and XVII were obtained without using a hexokinase system to 'trap' ATP as it was formed; therefore, the P/O ratios may have been reduced by DNP-ATPase activity. However, the data in both tables may suggest that only one phosphorylation site was uncoupled by 0.1 mM DNP. Furthermore, the results from Table XVII show that the prior respiratory stimulation by DNP did not impair the ability of the mitochondria to carry out coupled

Table XVI. The effect of DNP on respiration and oxidative phosphorylation in the presence of ATP and ADP.

Nucleotide (20 μm. /flask)	DNP (mM)	O_2 Uptake (μgatoms)	ATP^{32} Formed (μmoles)	P^{32}/O
ATP	---	0. 7	3. 85	
ATP	0. 1	4. 3	3. 42	
ADP	---	3. 7	8. 28	2. 23
ADP	0. 1	5. 0	6. 03	1. 22

Blowfly mitochondria were prepared by Method A-3. Each flask contained ATP, ADP, and DNP as indicated, in the side arm; 500 μm. sucrose, 100 μm. Tris, 57. 7 Pi-Pi^{32}, 3 μm. EDTA, 0. 03 μm. $MgCl_2$, 30 μm. pyruvate, 0. 2 μm. fumarate, and mitochondria (5 4 mg protein), in the main compartment. Incubated 15 minutes.

Table XVII. Oxidative phosphorylation by blowfly mitochondria after prior respiratory stimulation by DNP.

Flask Variables					Changes after Tipping SA No. 2			
SA No. 1*		O_2	SA No. 2		O_2	Pi^{32}		
DNP (mM)	Min. Inc.	Uptake (μgatoms)	ADP (μm./flask)	Min. Inc.	Uptake (μgatoms)	Uptake (μmoles)	P^{32}/O	P^{32}/O^1 Range
0.1	10	5.7	20	2.5	2.1	3.05	1.45	(1.45-1.78)
0.1	10	6.3	20	5	4.1	7.52	1.83	(1.35-1.85)
0.1	10	6.8	20	10	8.6	9.40	1.09	(1.09-1.44)
0.1	10	8.0	20	15	14.7	13.91	0.94	(0.89-1.18)
0.1	10	6.5	20	20	14.6	13.10	0.89	(0.76-1.05)
0.1	20	18.7	--					
---	20	0	---					

Mitochondria were prepared by Method B. Each flask contained the reagents indicated, in the side arms; 600 μm. sucrose, 111 μm. Tris, 40 μm. Pi-Pi^{32}, 1 μm. ATP, 6 μm. EDTA, 40 μm. pyruvate, 0.26 μm. fumarate, 3.3 mg BSA, and mitochondria (1.0 mg protein), in the main compartment. After temperature equilibration, DNP was tipped in and incubation was continued for the minutes indicated; then, ADP was tipped in and incubation was continued for the minutes indicated. The Pi^{32} uptake has been corrected for incorporation into ATP prior to the addition of the ADP.

*SA = side arm.

[1] The ranges indicate average results from three separate experiments identical to this one, except that 0.2 μm. $MgCl_2$ was also present in each main compartment.

phosphorylation. That is, it does not appear that DNP caused a configurational change within the mitochondria of the type proposed by Weinbach and Garbus (69).

In Table XVIII the efficiency of oxidative phosphorylation in the presence of 0.1 mM DNP was studied with hexokinase, glucose, and three $MgCl_2$ concentrations. It is to be noted that DNP did not inhibit respiration in the presence of the hexokinase system, and that $MgCl_2$ alone did not stimulate a substantial respiration. While the $MgCl_2$ concentration markedly influenced respiration and phosphorylation in the presence of hexokinase and glucose when DNP was omitted, it had only a moderate influence when DNP was added. The data indicate, therefore, that with the hexokinase system and DNP the respiratory rate was governed by DNP stimulation; this was especially true with only 0.4 mM of $MgCl_2$ present. However, the amount of phosphorylation (as judged by the P/O ratios) was more nearly proportional to the efficiency of the hexokinase system, which was related to the amount of $MgCl_2$ present. The P/O ratios in the presence of DNP were greater than one, even through a 30 minute incubation. DNP did not alter the oxidative phosphorylation efficiency increasingly with a longer incubation time, since a lowering of the P/O ratio during extended incubations (e.g. 30 vs. 15 minutes as in Table XVIII) as found in the presence of DNP, is also commonly observed in complete oxidative phosphorylation systems which do not

Table XVIII. The effects of DNP on respiration and oxidative phosphorylation with blowfly mitochondria at different concentrations of $MgCl_2$.

Flask Variables				O_2	Net Pi^{32}	ATP^{32}	
$MgCl_2$ (mM)	DNP (mM)	Hexokinase + Glucose	Min. Inc.	Uptake (μgatoms)	Uptake[1] (μmoles)	Formed (μmoles)	P^{32}/O
0.4	---		30	5.1		7.5	
"	0.1		30	26.7		8.1	
"	---	+	30	14.1	32.6		2.31
"	0.1	+	15	8.9	10.5		1.18
"	0.1	+	30	22.1	23.3		1.05
1.1	---		30	10.7		7.9	
"	0.1		30	22.9		6.3	
"	---	+	30	23.9	52.3		2.19
"	0.1	+	15	9.6	13.2		1.38
"	0.1	+	30	22.6	24.9		1.10
6.7	---		30	7.1		7.7	
"	0.1		30	30.0		7.8	
"	---	+	30	26.3	67.2		2.55
"	0.1	+	15	13.3	21.0		1.58
"	0.1	+	30	26.6	32.0		1.20

Mitochondria were prepared by Method B. Each flask contained DNP, hexokinase (150 KM), and glucose (100 μm.) as indicated, in the side arm; 600 μm sucrose, 111 μm. Tris, 100 μm. Pi-Pi^{32}, 10 μm. ATP, 0.2 μm. EDTA, 35 μm. pyruvate, 0.26 μm. fumarate, 3.3 mg BSA, mitochondria (2.0 mg protein), and $MgCl_2$ as indicated, in the main compartment.

[1]The Pi^{32} uptake in the presence of hexokinase and glucose has been corrected for ATP^{32} formed in their absence, since Pi-Pi^{32} and ATP were together in the main compartment prior to adding the hexokinase and glucose from the side arm.

contain DNP (58).

Table XIX shows that the hexokinase system increased the efficiency of oxidative phosphorylation in the presence of DNP as compared to the efficiency with ADP alone. This would be expected since DNP-ATPase activity could cause an appreciable hydrolysis of ATP^{32} when hexokinase is omitted. These results were, again, obtained after prior respiratory stimulation by DNP. Together with the results from Table XVIII, the data show that essentially the same P/O ratio was obtained when the phosphate acceptor system was added with the DNP, or after respiratory stimulation had been initiated by DNP.

The Effect of the DNP Concentration on Respiratory Stimulation and Oxidative Phosphorylation

Table XX shows the effects of increasing DNP concentrations on respiration and oxidative phosphorylation. The maximum respiratory stimulation occurred at 150 μM DNP; respiration was almost completely inhibited by 400 μM DNP. It is seen that both oxidation and phosphorylation were progressively inhibited by DNP in the presence of the phosphate acceptor system as the DNP concentration was increased. However, the P/O ratio of 0.65 in the presence of sufficient DNP (150 μM) to induce maximal respiratory stimulation may indicate that one phosphorylating site, at least, was not readily

Table XIX. Comparison of the efficiency of oxidative phosphorylation with ADP and with hexokinase after prior respiratory stimulation by DNP.

Flask Variables		Min. Incub. after adding		O_2 Uptake after adding		Pi^{32} Uptake	
Side Arm No. 1	Side Arm No. 2	SA #1	SA #2	SA #1 (μgatoms)	SA #2	(μmoles)	P^{32}/O
DNP	ADP	10	10	8.6	10.8	8.5	0.78
DNP	ADP, hex.	10	20	10.1	21.1	24.9	1.17
ADP, hex.	----	20		20.8		45.1	2.41

Mitochondria were prepared by Method B. Each flask contained the indicated reagents in side arm No. 1 or 2 (DNP, 0.3 μm.; hexokinase: 150 KM plus 100 μm. glucose; ADP, 20 μm.); 600 μm. sucrose, 111 μm. Tris, 100 μm. Pi-Pi^{32}, 1 μm. ATP, 0.2 μm. EDTA, 3.1 μm. $MgCl_2$, 35 μm. pyruvate, 0.26 μm. fumarate, 3.3 mg BSA, and mitochondria (3.1 mg protein), in the main compartment. After temperature equilibration, the reagents in side arm No. 1 were tipped in and incubation was continued for the minutes indicated; then, the reagents in side arm No. 2 were tipped in and incubation was continued for the minutes indicated.

The Pi^{32} uptake has been corrected for incorporation into ATP prior to adding the ADP, or ADP plus hexokinase and glucose.

Table XX. The effect of the DNP concentration on respiration and oxidative phosphorylation with blowfly mitochondria.

Flask Variables		O_2	Pi^{32}	ATP^{32}	
DNP (μM)	Hexokinase	Uptake (μgatoms)	Uptake (μmoles)	Formed (μmoles)	P^{32}/O
0		1.9		5.53	
0	+	15.1	40.15		2.67
25		5.3		5.35	
25	+	16.4	39.10		2.39
50		6.5		4.98	
50	+	15.7	32.70		2.08
100		12.6		5.10	
100	+	13.2	10.15		1.45
150		18.0		5.20	
150	+	10.0	6.46		0.65
200		13.9		3.00	
200	+	7.7	2.09		0.27
400		4.1		0.13	
400	+	1.7	0.14		----

Mitochondria were prepared by Method B-2. Each flask contained ATP (10 μm) and Pi^{32}, plus DNP and the hexokinase system (150 KM hexokinase, 100 μm. glucose, and 6 μm. $MgCl_2$) as indicated, in the side arm; 600 μm. sucrose, 103 μm. Tris, 100 μm. Pi, 0.2 μm. each of EDTA and $MgCl_2$, 15 μm. pyruvate, 0.23 μm. fumarate, 1.3 mg BSA, and mitochondria (0.73 mg protein), in the main compartment. Incubated 30 minutes.

uncoupled by this concentration of DNP. This is consistent with the previously mentioned studies with oligomycin (see Tables X to XIV and the related discussion).

The data in Table XX suggest that DNP may act upon more than one coupling site at DNP concentrations greater than 100 μM. It is, of course, also possible that all three coupling sites are partially inhibited by DNP, even at low DNP concentrations, as mentioned previously.

It is seen that the effects of DNP on respiration and coupled phosphorylation are dependent upon the DNP concentration rather than the amount; that is, DNP does not titrate a specific component of the coupling sequence such as has been observed with many other electron and energy transfer inhibitors (e.g., rotenone and oligomycin (26), or antimycin A (29)). This is consistent with the postulates developed in the introductory chapter to account for uncoupling of phosphorylation, as well as for respiratory stimulation and inhibition by DNP (reaction (11), also see reference (15)).

The Q_{O_2}[3] values which may be estimated from the data in Table XX are noteworthy. The Q_{O_2} for coupled phosphorylation with no DNP present was 465, while the maximum value for DNP-stimulated respiration with no phosphate acceptor present was 552.

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[3] Q_{O_2}: microliters O_2 consumed per hour per mg mitochondrial protein.

Both values approach the Q_{O_2} estimated to be required for sustained flight (14), and are near the maximum values obtained by Van den Bergh and Slater (63) for pyruvate oxidation in housefly mitochondria. In addition, the energy production, Q_{O_2} x P/O (30, p. 38), was also very high for the coupled system.

PART II

Oxidative Phosphorylation and Related Reactions in Submitochondrial Particles

General Properties of Sonic and Digitonin Particles

Insect submitochondrial particles were prepared during this study by both the digitonin and sonic oscillation procedures (see Methods). However, it was found that the sonic oscillation method gave higher yields and more active preparations than the digitonin treatment did. In addition, the digitonin method had the undesirable features that it was difficult to achieve a good separation of the particulate fraction from the 27,000 x g. pellet after the digitonin treatment, and that this treatment resulted in contamination with an unknown amount of digitonin in the final suspension. Qualitatively, however, particles obtained by the two methods showed the same characteristics (e. g., substrate specificity and ATPase activity). This similarity in activities indicates that identical particulate

fractions were obtained by the two procedures. Due to the above considerations, most of the data in this part of the thesis have been obtained from studies with sonic particles. As mentioned previously, blowflies were used most commonly, since an adequate yield of mitochondria could be obtained more easily from blowfly thoraces than from houseflies.

Substrate Requirements and Oxidative Phosphorylation. Table XXI shows the substrate specificity for phosphorylation by blowfly sonic particles. An identical specificity was found with housefly digitonin particles. Succinate was the only effective substrate of those tested in Table XXI; it is seen that more phosphorylation was obtained with the higher succinate concentration. The phosphorylation with a substrate mixture containing 3. 3 mM each of succinate, pyruvate, citrate, and malate was nearly the same as that found when only 3. 3 mM succinate was present. Albumin was required for coupled phosphorylation. It was included in all phosphorylation studies, since it had been reported that albumin binds free fatty acids which are capable of uncoupling phosphorylation (3).

It is significant that β-hydroxybutyrate was not an effective substrate. This was also observed in studies with digitonin particles, and differs from results reported for β-hydroxybutyrate oxidation with digitonin particles from rat liver and beef heart (24, 36). While only pyruvate was oxidized by intact blowfly mitochondria (Table I),

Table XXI. Substrate specificity of sonic particles from blowfly mitochondria.

Substrate	Substrate Conc. (mM)	BSA (mg/flask)	Pi^{32} Uptake/ mg protein/hr. (μmoles)
Succinate	3.3	6	3.5
Succinate	6.7	6	4.8
Succinate	6.7	-	0.6
Citrate	6.7	6	0.03
Pyruvate	6.7	6	0.11
Malate	6.7	6	0.09
β-hydroxybutyrate	1.7	6	0.05
Succinate, pyruvate, citrate, malate	3.3 ea.	6	3.0

Sonic particles were prepared by Method C except that Tris and $MgCl_2$ were omitted from the washing, sonicating, and suspending medium. The sonication time was 20 seconds. Each flask contained, in a total volume of 2.0 ml; 125 μm. sucrose, 100 μm. Tris, 40 μm. Pi-Pi^{32}, 10 μm. ATP, 10 μm. $MgCl_2$, 50 KM hexokinase, 50 μm. glucose, sonic particles (0.8 mg protein), and BSA and substrates as indicated. Incubated 60 minutes.

neither sonic nor digitonin particles catalyzed an appreciable phosphorylation with this substrate, or with NAD^+-linked intermediates of the citric acid cycle. However, it was found in later experiments that NADH (generated with alcohol dehydrogenase and ethanol in the presence of NAD^+) was an effective substrate with sonic particle preparations.

Results from several oxidative phosphorylation experiments with succinate and NADH as substrates are summarized in Table XXII. The P/O ratios in experiment 124 represent nearly the maximum values obtained. The P/O ratios have usually been between 0.4 and 0.8 with succinate, and have never exceeded 0.9; the ratios obtained with NADH were usually between 0.25 and 0.6. Experiment 129 shows that both respiration and phosphorylation increased as the succinate concentration was raised; the P/O ratios were nearly constant for the three succinate concentrations tested. The higher rates of oxidation obtained with NADH, as compared to succinate, may have been partially due to a more nearly optimal substrate concentration than that which was supplied with succinate as the substrate. Since the P/O ratio for NADH oxidation never exceeded the ratio obtained with succinate, it would appear that the phosphorylation site, between NAD and FAD, is absent in these preparations.

Experiments 122 and 125 show that sonic particles are very stable to freezing and may be stored while frozen with little loss of

Table XXII. Oxidative phosphorylation by sonic particles from blowfly mitochondria.

Expt. No.	Substrate	Subst. Conc. (mM)	Treatment Variables	O_2 Uptake (μ gatoms)	Pi^{32} Uptake (μ moles)	P^{32}/O
124	NADH			19.6	10.3	.52
	succinate	6.7		10.7	8.2	.77
129	none	---		0.6	0.07	---
	succinate	1.7		5.0	2.6	.53
	succinate	3.3		7.6	4.4	.58
	succinate	6.7		10.4	6.0	.58
122	NADH			33.4	10.8	.33
	NADH		frozen (1 d.)	28.4	8.9	.31
	succinate	6.7		13.9	5.9	.42
	succinate	6.7	frozen (1 d.)	13.1	5.7	.43
125	succinate	6.7		14.8	11.1	.75
	succinate	6.7	frozen (64 d.)	12.7	7.6	.60

Sonic particles were prepared by Method D, except that the final suspending medium contained 3×10^{-5} M $MgCl_2$. Each flask contained 40 μm. Pi-Pi^{32}, 150 KM hexokinase, 100 μm. glucose, and 20 μm. $MgCl_2$, in the side arm; 250 μm. sucrose, 130 μm. glycylglycine buffer, 10 μm. ATP, 0.03 μm. $MgCl_2$, 6 mg BSA, substrate as indicated, and sonic particles (2-4 mg protein), in the main compartment. NADH was generated as described under Methods. Frozen sonic particles were stored at -20° C. Incubated 60 minutes.

oxidative or phosphorylative activity. It was not convenient to take advantage of this stability for the preparation of large quantities of particles, in the present studies, because dissection to separate the thoracic flight muscles imposed a limitation on the amount of material used. It should be possible to obtain larger amounts by utilizing whole flies, since Gregg (30, p. 22) found that mitochondria from whole flies and thoraces had similar characteristics. Thus, it would be expected that particles derived from whole flies would have characteristics similar to those of particles from thoracic flight muscles.

The Effects of Sonication Time and the $MgCl_2$ Concentration in the Sonicating Medium. It was generally difficult to obtain uniform sonic particle preparations in different experiments. However, several experiments tended to indicate that the most suitable conditions were to use a sonicating time of from 45 to 90 seconds (Tables XXIII and XXIV), and a sonicating medium consisting of 0.25 to 0.3 M sucrose, 0.01 to 0.03 M Tris or glycylglycine buffer (pH 7.4), and 0.003 M $MgCl_2$. Sonication times in excess of one and one-half minutes resulted in decreased particle respiratory and phosphorylative capacity (Table XXIII). It is seen from the data in Table XXIV that succinate oxidation was more tightly coupled with 3 mM $MgCl_2$ in the sonicating medium than with 0.3 mM. The P/O ratio with NADH, however, was not greatly influenced by changes in the amount of $MgCl_2$ in the sonicating medium. It was found, in experiments

Table XXIII. The effect of sonication time on oxidative phosphorylation by blowfly particles.

Sonication Time (Minutes)	Substrate	Protein (mg/ flask)	O_2 Uptake (μgatoms)	Pi^{32} Uptake (μmoles)	P^{32}/O
1.5	NADH	2.2	13.0	6.50	0.50
1.5	succinate	2.2	5.2	4.34	0.83
3	NADH	2.2	8.0	4.01	0.50
3	succinate	2.2	3.3	3.02	0.91
10	NADH	*	0.9	0.12	0.15
10	succinate	*	0.2	0.14	0.57

Sonic particles were prepared by Method C, except that Tris was omitted from the washing, sonicating, and suspending medium. Each flask contained 34.6 μm. Pi-Pi^{32} in the side arm; 250 μm. sucrose, 100 μm. Tris, 10 μm. ATP, 23 μm. $MgCl_2$, 6 mg BSA, 150 KM hexokinase, 100 μm. glucose, sonic particles, and the indicated succinate (20 μm.) or NADH (described under Methods) in the main compartment. Incubated 60 minutes.

*Insufficient sample for protein determination.

Table XXIV. Effect of $MgCl_2$ concentration and sonication time on oxidative phosphorylation by blowfly particles.

Sonic. Time (sec.)	$MgCl_2$[1] (mM)	Substrate	Specific Activity: (μgatoms O_2/ hr./mg prot.)	O_2 Uptake (μgatoms)	Pi^{32} Uptake (μmoles)	P^{32}/O
45	0.3	NADH	3.57	22.2	10.00	0.45
45	0.3	succinate	2.60	16.2	7.84	0.48
90	0.3	NADH	3.55	24.1	8.42	0.35
90	0.3	succinate	2.47	16.7	6.78	0.41
45	3.0	NADH	3.66	19.1	10.27	0.54
45	3.0	succinate	2.00	10.7	8.22	0.77
90	3.0	NADH	3.60	22.0	10.22	0.46
90	3.0	succinate	2.18	13.4	9.13	0.88

The particles were obtained from mitochondria isolated by Method A. The washing, sonicating, and suspending medium consisted of 0.25 M sucrose, 0.03 M glycylglycine, and 0.003 or 0.0003 Mg $MgCl_2$. Each flask contained 37.6 μm. Pi-Pi^{32} in the side arm; 250 μm. sucrose, 110 μm. glycylglycine, 10 μm. ATP, 23.3 μm. $MgCl_2$, 150 KM hexokinase, 100 μm. glucose, 6 mg BSA, sonic particles, and succinate (20 μm.) or NADH (see Methods) as indicated, in the main compartment. Incubated 60 minutes.

[1] Concentration of $MgCl_2$ in the sonicating medium.

not tabulated in this thesis, that oxidation was even less tightly coupled to phosphorylation when $MgCl_2$ was omitted from the sonicating medium, and that increasing the $MgCl_2$ concentration to 30 mM did not improve coupling. Consequently, the usual practice during this study was to sonicate mitochondria for 45 to 60 seconds in a medium containing sucrose, Tris (or glycylglycine) and 3 mM $MgCl_2$.

Tris and Glycyl Glycine Buffer Effects. Table XXV shows the effects of including Tris and/or glycylglycine buffers in the sonicating and incubating media. It is seen that glycylglycine buffer gave improved coupling with succinate as substrate, but had a lesser effect on coupling with NADH. It is also to be noted that the major improvement was due to inclusion of the glycylglycine buffer in the incubation medium. The buffer employed in the sonicating medium was of less importance.

ATPase Activities of Sonic and Digitonin Particles

It has not been possible to demonstrate an appreciable DNP-stimulated ATPase activity with either digitonin or sonic particles (Tables XXVI and XXVII). In fact, digitonin strongly inhibited DNP-ATPase activity when added to intact mitochondria (Table XXVII), although it did not inhibit Mg^{++}-stimulated ATPase. It is seen from these tables that neither sonic nor digitonin particles showed extensive endogenous ATPase activity, but that both types of particles had

Table XXV. Comparison of Tris and glycylglycine buffers with sonic particles from blowfly mitochondria.

Prep. Buffer[1] (3.3 mM)	Incub. Buffer (33 mM)	Substrate	Protein (mg)	O_2 Uptake (μgatoms)	Pi^{32} Uptake (μmoles)	P^{32}/O
Tris	Tris	NADH	4.6	34.8	15.35	0.44
Tris	Tris	succ.	4.6	12.0	7.10	0.59
Tris	gly-gly	NADH	4.6	33.6	16.08	0.48
Tris	gly-gly	succ.	4.6	11.8	8.58	0.73
gly-gly	Tris	NADH	4.8	33.6	17.15	0.51
gly-gly	Tris	succ.	4.8	9.3	5.63	0.61
gly-gly	gly-gly	NADH	4.8	26.3	14.10	0.54
gly-gly	gly-gly	succ.	4.8	9.4	7.12	0.76

[1] The thoraces from 50 ml of blowflies were divided equally by weight. Mitochondria were isolated from one portion by Method A. The washing, sonicating (24 ml), and final suspending medium consisted of 0.25 M sucrose, 0.01 M Tris, and 0.003 M $MgCl_2$. The same procedure was used for the glycylglycine preparation, except that Tris buffer was replaced by glycylglycine in the homogenizing, washing, sonicating, and final suspending media. Each flask contained 38 μm. Pi-Pi^{32} in the side arm; 250 μm. sucrose, 10 μm. ATP, 23 μm. $MgCl_2$, 150 KM hexokinase, 100 μm. glucose, 6 mg BSA, and the indicated sonic particle preparation, buffer, succinate (20 μm.) and NADH (see Methods), in the main compartment. Incubated 50 minutes.

Table XXVI. ATPase activities of sonic particles from blowfly mitochondria.

Flask Variables		Pi Released (μmoles)	
$MgCl_2$ (mM)	DNP (mM)	15 Min.	30 Min.
0	0	0.4	0.2
0	0.1	0.4	0.3
0	0.4	0.3	0.3
1	0	13.3	17.9
6.7	0	13.7	18.4

Sonic particles were prepared by Method D, except that the sonication time was 1 minute, and $MgCl_2$ was omitted from the final suspending medium. Each flask contained 250 μm. sucrose, 100 μm. glycylglycine, 20 μm. ATP, sonic particles (1 mg protein), and $MgCl_2$ and CNP as indicated.

Table XXVII. The effect of digitonin on ATPase activities of mitochondria and particles from houseflies.

Enzyme Preparation	Flask Variables			Pi Released (μmoles)
	$MgCl_2$ (mM)	DNP (mM)	Digitonin (mg/flask)	
Mitochondria	0.01	---	-----	10.3
Mitochondria	0.01	0.1	-----	23.4
Mitochondria	0.01	0.1	0.005	23.8
Mitochondria	0.01	0.1	0.05	21.2
Mitochondria	0.01	0.1	0.5	11.6
Mitochondria	3.31	---	-----	28.2
Mitochondria	3.31	---	0.5	28.2
Digitonin particles	0.01	0.1	-----	0
Digitonin particles	3.31	---	-----	20.5

Mitochondria were prepared by Method A-5. Digitonin particles were prepared by the standard procedure. Each flask contained: 125 μm. sucrose, 100 μm. Tris, 30 μm. ATP, and $MgCl_2$ and mitochondria or sonic particles as indicated (5.6 and 1.6 mg protein, respectively). Incubated 30 minutes.

a very active Mg^{++}-ATPase. These observations differ from those reported by Cooper and Lehninger (21) for digitonin particles from rat liver, and the results obtained by Gregg (33) with sonic particles from rat liver, since both groups found appreciable endogenous as well as DNP- and Mg^{++}-stimulated ATPase activities in their preparations. The clear separation of Mg^{++}- and DNP-stimulated ATPase activities, as found in the present study, may mean that these are unrelated activities in intact mitochondria.

Lack of an ATP-Pi Exchange in Insect Particles

It was not possible to demonstrate ATP-Pi exchange activity in either blowfly sonic particles (Table XXVIII) or housefly digitonin particles (not shown). These results also differ from previously published data for mammalian submitochondrial particles (7, 21, 34) which show a measurable, but slight, ATP-Pi exchange activity. The data in Table XXVIII should be compared to the results for intact mitochondria (Table XXIX) which show the well known mitochondrial ATP-Pi exchange and its inhibition by DNP. It is noteworthy that $MgCl_2$ only partially inhibited the mitochondrial ATP-Pi exchange However, like DNP, $MgCl_2$ stimulated extensive mitochondrial ATPase in the absence of substrate, and caused increased respiration in the presence of substrate with little or no net hydrolysis of ATP (Table XXIX). Thus, the insect sonic particles had lost ATP-Pi

Table XXVIII. Respiration, ATPase, and ATP-Pi exchange activities of sonic particles from blowflies.

Succinate (mM)	Other	O_2 Uptake (μgatoms)	Pi Released (μmoles)	Pi^{32} Uptake (μmoles)	P^{32}/O	ATP^{32} Formed (μmoles)
6.7	None	13.1	0.9	0.03		
6.7	Hexokinase	9.3		6.4	.69	
6.7	DNP	13.5	0.6	0		
6.7	$MgCl_2$	12.3	17.5	0.1		
---	None	0	2.3			0
---	DNP	0	1.8			0
---	$MgCl_2$	0	19.5			0

Sonic particles were prepared by Method D, except that the final suspending medium contained 3×10^{-5} M $MgCl_2$. Each flask contained 20.5 μm. Pi-Pi^{32}, and DNP (0.3 μm.), $MgCl_2$ (20 μm.), or hexokinase (150 KM hexokinase, 100 μm. glucose, 20 μm. $MgCl_2$) as indicated, in the side arm; 250 μm. sucrose, 130 μm. glycylglycine, 19.5 μm. ATP, 0.03 μm. $MgCl_2$, 6 mg BSA, sonic particles (5.1 mg protein), and succinate as indicated, in the main compartment. Incubated 60 minutes.

Table XXIX. The effects of DNP and $MgCl_2$ on the ATP-Pi exchange, ATPase and ATP^{32} specific activity with blowfly mitochondria.

Flask Variables	O_2 Uptake (μgatoms)	Pi Released (μmoles)	ATP^{32} Formed (μmoles)	ATP^{32} Specific Activity
None	0	0	5.40	74.5
DNP	0	14.9	0.23	5.0
$MgCl_2$	0	11.3	1.82	51.0
Pyr./fum.	0.9	1.8	7.48	116.0
Pyr./fum. + DNP	7.3	1.1	7.05	108.0
Pyr./fum. + $MgCl_2$	8.5	0	10.51	148.5

Mitochondria were prepared by Method A-5. Each flask contained 100 μm. Pi-Pi^{32} and DNP (0.3 μm.) or $MgCl_2$ (20 μm.) as indicated, in the side arm; 250 μm. sucrose, 100 μm. Tris, 20 μm. ATP, mitochondria (5.2 mg protein), and pyr./fum. (30 μm. pyruvate plus 0.2 μm. fumarate) as indicated, in the main compartment. Incubated 15 minutes.

exchange activity and sensitivity to DNP-induced ATPase, but they retained the ability to couple oxidation to phosphorylation and to catalyze ATP hydrolysis in the presence of $MgCl_2$.

Low et al. (48), and Hemker (39) have suggested that the ATP-Pi exchange reaction and DNP-ATPase activity may involve primarily the phosphorylation site between NAD and FAD. The present findings with blowfly sonic particles are consistent with this view, because the ATP-Pi and DNP-ATPase activities were not detected, and because the observed oxidative phosphorylation did not appear to involve the NAD-FAD coupling site (Table XXII, and reference 34).

The Effects of DNP on Respiration and Oxidative Phosphorylation

The data in Table XXVIII showed that the maximal respiratory rate with sonic particles occurred when respiration was not coupled to phosphorylation by the hexokinase acceptor system; succinate oxidation was neither stimulated nor inhibited by either DNP or $MgCl_2$. The hexokinase system apparently reduced the respiratory rate by introducing a rate controlling step of coupled phosphorylation. It was found in other experiments that the hexokinase system did not reduce the respiratory rate when coupled phosphorylation was prevented by omitting ATP from the incubation medium. That is, the hexokinase-glucose-$MgCl_2$ mixture was not directly inhibitory to

respiration. The results of Table XXVIII show that $MgCl_2$ stimulated nearly complete hydrolysis of the added ATP, either in the presence or absence of substrate, which resulted in almost complete hydrolysis of added ATP. However, in the presence of substrate, respiration was not inhibited by the Mg^{++}-induced formation of ADP and Pi. It would appear, then, that the reduced respiratory rate observed when succinate oxidation was coupled to phosphorylation was due entirely to the introduction of a rate limiting step which was dependent upon the presence of Pi, ADP, $MgCl_2$, hexokinase and glucose. Lehninger and Gregg (46), and Gregg and Lehninger (34) have shown an inhibition of respiration by ADP in digitonin and sonic particles from rat liver, but this inhibition could be demonstrated only with ADP added and Pi omitted from the incubation medium. No inhibition was observed with both ADP and Pi present, such as was the case for the experiment shown in Table XXVIII.

According to current concepts, DNP acts on a high energy intermediate which is itself a part of the coupling sequence (16), and thus releases respiration from phosphorylation. If this is the case, DNP should restore the maximal respiratory rate, even in the presence of the hexokinase system, since DNP supposedly reacts with a rate limiting intermediate step of the coupling process, prior to the uptake of Pi, while hexokinase should affect only the terminal reaction -the transfer of the terminal phosphate group of ATP to glucose.

However, as shown in Table XXX, neither 0.1 nor 0.4 mM DNP restored the maximal respiratory rate in the presence of the hexokinase acceptor system, although DNP did inhibit phosphorylation. It is significant that 0.4 mM DNP was not inhibitory to sonic particle respiration either in the presence or absence of the hexokinase system (Table XXX), whereas this amount of DNP produced a nearly complete inhibition of respiration when added to intact mitochondria (Tables XII and XX).

The Effects of Inhibitors on Oxidation and Phosphorylation by Sonic Particles

Antimycin A. Antimycin A completely inhibited respiration with either succinate or NADH as substrates (Table XXXI). The complete inhibition of respiration with the very loosely coupled preparation (experiment number 2) shows that antimycin A reacted specifically with the electron transport chain, and was equally inhibitory to phosphorylating and non-phosphorylating particles.

Oligomycin in the Presence and Absence of DNP. The situation is different with oligomycin, which inhibits energy but not electron transfer reactions (Table XXXII). Oligomycin was only slightly inhibitory to either succinate or NADH oxidation when oxidation was not coupled to phosphorylation by the hexokinase trapping system. In the presence of hexokinase, however, oligomycin caused an additional,

Table XXX. The effect of DNP on oxidative phosphorylation and ATPase activity in sonic particles from blowflies.

Flask Variables	O_2 Uptake (μgatoms)	Pi Released (μmoles)	Pi^{32} Uptake (μmoles)	P^{32}/O
Hexokinase	6.3		4.4	.70
Hexokinase + 0.1 mM DNP	5.6		2.4	.43
Hexokinase + 0.4 mM DNP	5.5		0.04	
None	9.5	0		
0.1 mM DNP	9.6	0.5		
0.4 mM DNP	9.2	1.9		

Sonic particles were prepared by Method D, except that the final suspension was made in 0.25 M sucrose, 0.03 M Tris, and 3×10^{-5} M $MgCl_2$. Each flask contained 20 μm. Pi-Pi^{32}, and DNP and hexokinase (150 KM hexokinase, 100 μm. glucose, 20 μm. $MgCl_2$) as indicated, in the side arm; 250 μm. sucrose, 130 μm. glycylglycine, 20 μm. ATP, 0.03 μm. $MgCl_2$, 20 μm. succinate, 6 mg BSA, and sonic particles (4.5 mg protein), in the main compartment. Incubated 60 minutes.

Table XXXI. Inhibition of oxidative phosphorylation in blowfly sonic particles by antimycin A.

Expt. No.	Flask Variables: Substrate	Flask Variables: Antimycin A (μg. /flask)	O_2 Uptake (μgatoms)	Pi^{32} Uptake (μmoles)
1	NADH	-	16.0	6.40
	NADH	2	0.6	0.04
2	NADH	-	10.9	1.50
	NADH	2	0	0
	Succinate	-	2.6	0.16
	Succinate	2	0	0.02

Sonic particles were prepared by Method C, except that the washing, sonicating, and suspending medium consisted of 0.25 M sucrose and 1×10^{-4} M $MgCl_2$. In experiment number 1, each flask contained 39.1 μm. Pi-Pi^{32} in the side arm; 250 μm. sucrose, 100 μm. Tris, 10 μm. ATP, 6 mg BSA, 150 KM hexokinase, 100 μm. glucose, 20.1 μm. $MgCl_2$, sonic particles (3.2 mg protein), and antimycin A as indicated, in the main compartment. In experiment number 2, the flask contents were the same except that the amount of sonic particle protein was 2.8 mg. The substrate additions were: succinate, 20 μm. or NADH, as described under Methods. Incubated 30 minutes.

though incomplete, inhibition of respiration, and complete inhibition of phosphorylation. The failure of oligomycin to cause complete respiratory inhibition in the presence of the phosphate trapping system shows that the sonic particle preparations contained both phosphorylating and non-phosphorylating particles. It is possible, then, that the phosphorylating particles had retained more than one coupling site or that they consisted of a mixture with one or two phosphorylating sites intact. The possibility that more than one coupling site is functional is born out by the observation that the P/O ratio for succinate oxidation approached 1. However, this ratio apparently included oxidation by both electron transport and phosphorylating particles. Therefore, the contribution of the phosphorylating particles to the measured P/O ratio may have exceeded 1, even though the overall ratio did not. This point is worthy of further study, since it is shown in Table XXXII that DNP uncoupled phosphorylation without stimulating ATPase activity. It is, thus, possible that two phosphorylation sites can be uncoupled by DNP without permitting DNP-ATPase activity.

The most significant data in Table XXXII are the results shown for the separate and combined effects of oligomycin and DNP on oxidative phosphorylation. Experiment number 261 shows that DNP, alone, uncoupled phosphorylation, although it did not stimulate ATP hydrolysis (footnote, Table XXXII). In addition, DNP reversed some

Table XXXII. Inhibition of oxidative phosphorylation in sonic particles by oligomycin, azide, and DNP.

Expt. No.	Flask Variables: Substrate	Other	Min. Inc.	O_2 Uptake (μgatoms)	Pi^{32} Uptake (μmoles)
261[a]	succinate	none	40	12.1	0.28
	succinate	hex.	40	8.5	5.47
	succinate	hex. + DNP	40	8.1	1.10
	succinate	olig.	40	11.1	0.01
	succinate	olig. + hex.	40	5.9	0.01
	succinate	olig. +hex. +DNP	40	7.2	0.01
257	succinate	none	60	13.4	0.10
	succinate	olig.	60	10.9	0
	succinate	hex.	60	11.5	1.93
	succinate	olig. + hex.	60	7.8	0
	succinate	olig. +hex. +DNP	60	9.6	0
	succinate	azide	60	4.8	0.09
	succinate	azide + hex.	60	4.5	0.17
	succinate	azide +hex. +DNP	60	5.1	0.02
	NADH	none	60	28.0	0.35
	NADH	olig.	60	23.6	0.04
	NADH	hex.	60	27.0	3.79
	NADH	olig. + hex.	60	19.0	0.06
	NADH	olig. +hex. +DNP	60	24.6	0.06
260	NADH	none	30	19.1	0.25
	NADH	olig.	30	18.0	0.17
	NADH	hex.	30	17.6	5.16
	NADH	olig. + hex.	30	15.4	0.16
	NADH	olig. +hex. +DNP	30	17.8	0.16
	NADH	azide	30	5.8	0.16
	NADH	azide+hex.	30	3.8	0.31
	NADH	azide+hex. +DNP	30	3.8	0.16

Blowfly sonic particles were prepared by Method C, except that the washing, sonicating, and suspending medium consisted of 0.3 M sucrose, 0.05 M Tris, 1×10^{-4} M EDTA and 1×10^{-4}M $MgCl_2$. Each flask contained 40 μm. Pi-Pi^{32} and DNP (0.3 μm.) as indicated, in the side arm; 600 μm. sucrose, 110 μm. Tris, 20 μm. ATP, 0.2 μm. each of $MgCl_2$ and EDTA, 6 mg BSA, sonic particles (2.9, 2.4, and 1.3 mg protein in experiment numbers 257, 260, and 261, respectively), and succinate (60 μm. in expt. No. 261, 20 μm. in expt. No. 257), NADH (generated as described under Methods), oligomycin (2 μg), azide (9 μm.), and hexokinase system (150 KM hexokinase, 100 μm. glucose, and 20 μm. $MgCl_2$) as indicated, in the main compartment.

[a]A separate set of vessels was incubated according to conditions described in Table XXVI to test for DNP-ATPase activity. It was observed that 0.1 mM DNP did not stimulate any ATP hydrolysis during a 40 minute incubation period.

of the oligomycin-induced respiratory inhibition, with either succinate or NADH as substrate. That is, DNP actually stimulated respiration slightly under these conditions. Therefore, DNP appeared to act on an intermediate of the coupling sequence between the site of oligomycin inhibition and the respiratory chain (as was also concluded from studies with intact mitochondria, Tables X and XI). These results mean, then, that DNP uncouples phosphorylation at one or more of the coupling sites of the respiratory chain without stimulating ATP hydrolysis. Furthermore, DNP-ATPase activity cannot be adequately represented by reactions (11), (6), and (7) as given in the introductory chapter, if it is assumed that these are part of the coupling mechanism at each of the three coupling sites and that no other reaction is required for DNP-ATPase activity. In addition, the insensitivity of sonic particles to respiratory inhibition by 0.4 mM DNP (Table XXX) is not consistent with the suggestion of Chance, Williams, and Hollunger (16) that more than one molecule of DNP may combine with an intermediate of the coupling sequence to cause inhibition by forming a compound which dissociates slowly, since the results in Tables XXX and XXXII indicate that DNP does interact with an intermediate. It is possible, therefore, that respiratory inhibition by high concentrations of DNP is a property which is observed only in intact mitochondria, and that even this inhibition is not due to formation of a compound which dissociates slowly.

Azide. Azide was more inhibitory to respiration than oligomycin (Table XXXII), but was less effective than antimycin A (Table XXXI). Both oligomycin and azide caused essentially complete inhibition of phosphorylation. Therefore, it appears that azide inhibits both the energy and electron transfer reactions of sonic particles. It is to be noted that 0.1 mM DNP did not relieve the respiratory inhibition caused by azide, although it did abolish P^{32} incorporation. The latter point shows that DNP did uncouple the very slight phosphorylation occurring in the presence of azide and the phosphate acceptor system.

SUMMARY AND CONCLUSIONS

The present study has shown that mitochondria and submitochondrial particles from blowfly and housefly flight muscle have certain metabolic properties that are common to mammalian preparations. The mitochondria oxidized pyruvate rapidly, and this was tightly coupled to phosphorylation. ADP and Pi were required for pyruvate oxidation in the absence of an uncoupling agent. In the presence of DNP, ADP and Pi were required for substrate-level phosphorylation, which showed that the enzymes for α-ketoglutarate oxidation were present. Although added succinate and other citric acid cycle intermediates were not readily oxidized by the mitochondria, submitochondrial particles coupled the oxidation of succinate and NADH to phosphorylation, but did not oxidize pyruvate. Therefore, the inability of intact insect mitochondria to utilize such substrates as malate, succinate, and citrate which are oxidized by mammalian mitochondria, appears to depend entirely upon a membrane permeability barrier. Van den Bergh and Slater (63) had arrived at a similar conclusion from studies with intact and damaged housefly mitochondria.

Pyruvate oxidation in the fly mitochondria was stimulated by DNP, but only in the presence of ATP (or ADP) and Pi. DNP inhibited the mitochondrial ATP-Pi exchange reaction and promoted

ATP hydrolysis when no substrate was present. However, with sufficient ATP and Pi-Pi^{32} added, little or no net ATP hydrolysis occurred when pyruvate oxidation was stimulated by DNP; ATP^{32} continued to be formed with nearly the same specific activity as that which occurred in unstimulated systems, i. e., in the absence of DNP. ADP and Pi were required, in the presence of DNP, only for substrate-level phosphorylation, because DNP still stimulated respiration (in the presence of Pi and ATP or ADP) after coupled phosphorylation and DNP-ATPase were completely inhibited by oligomycin. In the presence of oligomycin, DNP stimulated respiration, with ATP and Pi added, only if sufficient $MgCl_2$ (2 mM) was present to provide ADP for substrate-level phosphorylation. However, $MgCl_2$ did not promote respiration, in the presence of oligomycin and in the absence of DNP, and $MgCl_2$ was not essential for respiratory stimulation by DNP when ADP was present. These results show that ATP (or ADP) and Pi are not obligatory in the basic mechanism by which DNP promotes electron transport in insect mitochondria. Therefore, it is no longer necessary to postulate a special process for the initiation of respiration by DNP in insect tissues, to account for previous reports (35. 37) that ATP is required for DNP-stimulated respiration in housefly mitochondria.

It was demonstrated that DNP promoted respiration, with both intact mitochondria and sonic particles, after coupled phosphorylation

was completely inhibited by oligomycin. Thus, DNP must react with the energy conserving sequence at a point between the respiratory chain and the site(s) of oligomycin inhibition. The results obtained with intact mitochondria, with pyruvate as the substrate, indicate that DNP can uncouple ('release') respiration at all three coupling sites in the presence of oligomycin. In contrast to its effects on mitochondria, DNP did not promote ATP hydrolysis with sonic or digitonin particles. Therefore, the ability of DNP to uncouple phosphorylation and its action in promoting ATPase may represent separate effects of DNP; another possibility is that DNP-ATPase requires at least one additional reaction which is not necessarily involved in its uncoupling action. These results suggest that DNP may not promote ATP hydrolysis at each coupling site, but that it does uncouple phosphorylation and release electron transport at all three sites. It is possible that the coupling sequence was damaged in the particle preparations so that DNP-ATPase could not be demonstrated. This possibility is supported by the fact that, with sonic or digitonin particles, albumin was required in order to couple substrate oxidation to phosphorylation. The parallel observations that Mg^{++}-stimulated ATPase was more pronounced in particles, while DNP-ATPase was most prominent in intact mitochondria, make it plausible to assume that DNP-ATPase activity occurs primarily in association with the intact mitochondrial membrane.

The possibility that DNP promotes ATP hydrolysis principally or entirely at the NAD-FAD level of the respiratory chain is supported by the finding that both DNP-ATPase and the ATP-Pi exchange reactions were progressively inhibited by increasing concentrations of amytal, which inhibits oxidation of NAD-linked substrates. These results are in accord with previous reports for mammalian mitochondria (48, 39). However, it has also been shown that amytal is not a specific inhibitor of the first phosphorylation site in 'tightly coupled' mitochondria (55). DNP was found to have distinguishable effects on the ATP-Pi exchange and ATP hydrolysis; it inhibited the ATP-Pi exchange very rapidly, while continuing to promote ATP hydrolysis. In contrast, it was found that sonic particles did not show either DNP-ATPase or ATP-Pi exchange activity. Therefore, the relationships between the reaction of DNP with the respiratory chain coupling sites, its ability to promote ATP hydrolysis, and its inhibition of the ATP-Pi exchange reaction, require additional study.

It was shown in several experiments that ATP^{32} was formed by means of oxidative phosphorylation when pyruvate oxidation was stimulated by DNP in the presence of ATP and $Pi\text{-}Pi^{32}$. Extensive ATP^{32} formation by oxidative phosphorylation was also demonstrated in the presence of DNP and a phosphate acceptor (ADP, or ATP, hexokinase, glucose, and $MgCl_2$). Studies of the efficiency of phosphorylation in the presence of DNP demonstrated that DNP did not

uncouple oxidative phosphorylation completely unless the DNP concentration was raised to a level at which it began to inhibit respiration, i. e., at 0. 2 to 0. 4 mM. Phosphate/oxygen ratios significantly greater than 1 were obtained in the presence of 0. 1 mM DNP and a phosphate acceptor, even when the phosphate acceptor was not added until 10 minutes after respiration was initiated by DNP. This suggests that the equivalent of two coupling sites were not readily uncoupled by 0. 1 mM DNP; it also demonstrates that DNP does not cause extensive configurational changes within mitochondria when it stimulates respiration, nor does it cause irreversible changes in the coupling sequence under these conditions.

As mentioned previously, DNP was capable of stimulating respiration in mitochondria after coupled phosphorylation and DNP-ATPase were inhibited by oligomycin. However, it was found that, at a given DNP concentration, maximal respiration occurred only in the absence of oligomycin, that is, under conditions which permitted DNP to stimulate ATP hydrolysis, in addition to uncoupling phosphorylation. Under these conditions, ADP (from DNP-ATPase) was phosphorylated in the substrate-level reaction, and at one or more sites which, perhaps, are not readily uncoupled by DNP. These data are consistent in some respects with the previously mentioned studies (39, 48) which suggested that DNP may not react equally at each of the three coupling sites. It is, of course, also possible that all three sites are

partially uncoupled by DNP.

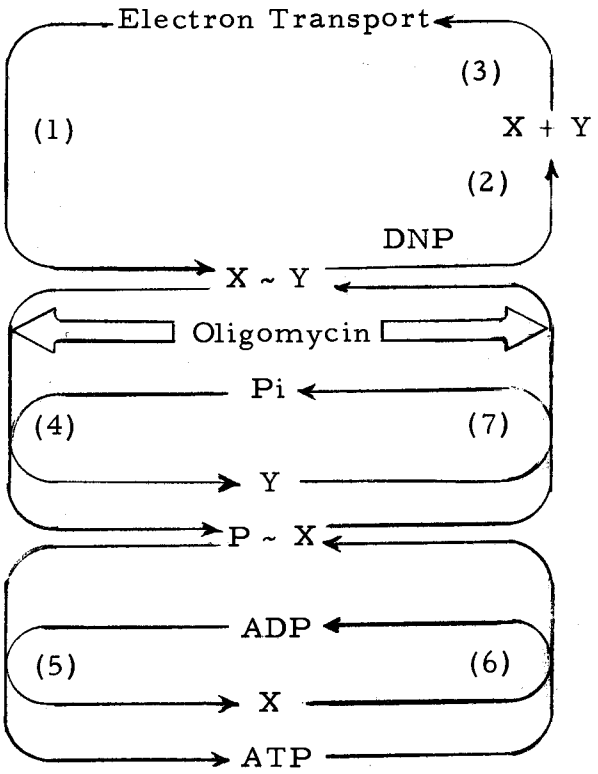
Little or no net ATP hydrolysis occurred when DNP promoted mitochondrial respiration with 6. 7 mM each of ATP and Pi-Pi^{32} added. However, it was possible to demonstrate that Pi^{32} was incorporated into ATP by means of oxidative phosphorylation when DNP promoted respiration in the presence of 10 or 20 mM ATP and 0. 33 mM Pi-Pi^{32}. This shows that ATP hydrolysis and synthesis took place concurrently. ATP^{32} formation was not as rapid in the absence of DNP as in its presence, and it can be concluded that DNP-ATPase activity was prominent when respiration was stimulated by DNP. That is, DNP-ATPase activity provided ADP and Pi for oxidative phosphorylation, as well as for substrate-level phosphorylation, and this contributed to the overall process by which DNP promoted maximum respiration. ATP^{32} formation, in the presence of DNP, ATP, Pi, and substrate, was inhibited by anaerobic incubation and by the same reagents that inhibit electron transport.

Although not emphasized in the Results and Discussion section of this thesis, it was frequently observed that DNP-stimulated respiration was inhibited partially by addition of a phosphate acceptor, i. e. , by ADP or ATP plus hexokinase (see Tables IX and XX, for example). The combination of DNP and the acceptor may, in fact, completely inhibit respiration in some preparations, but it is usually observed that respiration proceeds at a rate near to the maximum rate

attainable for coupled phosphorylation in the absence of DNP. Therefore, it appears that a high concentration of ADP reduces the amount of respiration stimulated by DNP, by competing for the intermediates which are affected by DNP.

At a level of 0.4 mM, DNP caused essentially complete inhibition of respiration in intact mitochondria, although it still stimulated ATPase. This concentration of DNP had little or no effect on respiration but did not promote ATPase, in sonic particles. Since it was found that 0.4 mM DNP uncoupled phosphorylation completely with either intact mitochondria or sonic particle preparations, it appears that the abilities of DNP to promote ATPase and to inhibit respiration, when present at a high concentration, are not equivalent and not common to all three coupling sites, even though DNP uncouples phosphorylation at all three sites.

The effects of DNP on respiration and phosphorylation in blowfly mitochondria may be summarized with the aid of the following diagram.



X and Y represent reactants which form intermediates of the coupling sequence, similar to those developed in the introductory chapter. It is assumed that an X (or X_1, X_2, or X_3) and a Y (or Y_1, Y_2, or Y_3) participate at each site of respiratory chain phosphorylation.

For respiration by intact mitochondria, in the presence of DNP (in all cases), the following points are compatible with the experimental data.

A. In the presence of oligomycin:

1. Reaction (4), required for phosphorylation, and reaction (7), required for DNP-ATPase, are prevented.

2. Respiration depends on reactions (1), (2), and (3) and on the provision of ADP and Pi for substrate-level phosphorylation. The respiratory rate is then limited only by the rate at which DNP is capable of causing decomposition of X~Y by reaction (2).

B. In the absence of oligomycin:

1. With enough ADP and Pi, reactions (4) and (5) compete with reaction (2), giving electron transport and phosphorylation from electron transport.
2. When Pi^{32} and ATP are added, ADP and Pi from reactions (6) and (7), and Pi^{32}, would compete with reaction (2) and be used in reactions (4) and (5) to form ATP^{32}; little or no net ATP formation or hydrolysis would be observed. Respiration could proceed at a rapid rate.
3. Without ATP (or ADP) and Pi, respiration would be prevented by the need for ADP and Pi in substrate-level phosphorylation.
4. With too little ATP (or ADP) and Pi, respiration would become limited because the concentration of ADP and/or Pi would be kept too low for substrate-level phosphorylation, by reactions (4) and (5), and by substrate-level phosphorylation itself.

5. With DNP and a high concentration of ADP (and Pi), or with too high a concentration of DNP, respiration could be inhibited by structural changes resulting from too low a concentration of X~Y.

If DNP stimulates respiration by "splitting" an intermediate, as indicated in the previous diagram, it must do so at all three coupling sites. Otherwise, DNP could not stimulate respiration in the presence of oligomycin because electron transport could not occur at the site where DNP did not act, even with ADP and Pi present for substrate-level phosphorylation, since oligomycin prevents coupled phosphorylation (and, therefore, electron transport) at all three sites.

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