EFFECTS OF HERBICIDES ON OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM CABBAGE, *BRASSICA OLERACEA*

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PRABHAKAR DATTARAM LOTLIKAR

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APPROVED:

[redacted]

Professor of Chemistry

In Charge of Major

[redacted]

Chairman of Department of Chemistry

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

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Typed by Miriam Schubert

Dedicated

to

My Parents

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INTRODUCTION

Lehninger has defined oxidative phosphorylation as the process in which the energy, liberated as electron equivalents pass from substrate to molecular oxygen via the respiratory carriers, is harnessed to drive the coupled synthesis of adenosine triphosphate (ATP)[1] from adenosine diphosphate (ADP) and orthophosphate (42, p. 176).

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[1] The following abbreviations are used in this thesis: AMP, adenosine 5-phosphate; ADP and ATP, adenosine di- and triphosphate, respectively; ATPase, adenosine triphosphatase; DPN and TPN, di- and tri-phosphopyridine nucleotide, respectively; FP, flavoprotein; GSH, reduced glutathione; TPP, thiamine pyrophosphate; CoA, coenzyme A; Tris, tris-(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; cyt., cytochrome; DNP, 2,4-dinitrophenol; PCP, pentachlorophenol; Pi, inorganic orthophosphate; $Q_{O_2}(N)$, microliters of oxygen consumed per hour per mg. of mitochondrial nitrogen; P:O, micromoles of inorganic orthophosphate esterified per microgram atom of oxygen consumed; ug. atom/hr/mg N, microgram atom per hour per mg. of mitochondrial nitrogen; umoles/hr/mg N, micromoles per hour per mg. of mitochondrial nitrogen; IPC, isopropyl-N-phenyl carbamate; CIPC, isopropyl-N-(3-chlorophenyl)carbamate; EPTC, ethyl-N,N-di-n-propylthiol carbamate; CMU, p-chlorophenyl 1,1-dimethyl urea; CDAA, 2-chloro-N,N-diallyl acetamide; ATA, 3-amino-1,2,4-triazole; simazin, 2-chloro-4,6-bis-(ethylamino)-s-triazine; mylone, 3,5-dimethyltetrahydro-1,3,5,2H-thiadiazine-2-thione; DCPA, 2,2-dichloropropionic acid; NPA, N-napthylpthalamic acid; 2,3,6-TBA, 2,3,6-trichlorobenzoic acid; MH, maleic hydrazide; TCP, 2,2,3-trichlorosodium propionate; 2-CPA and 4-CPA, 2- and 4-chlorophenoxyacetic acid, respectively; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4,5-T and 2,4,6-T, 2,4,5- and 2,4,6-trichlorophenoxyacetic acid, respectively; MCPA, 2-methyl-4-chloro-phenoxyacetic acid; 2,4-DB, γ-(2,4-dichlorophenoxy)-butyric acid; MCPB, γ-(2-methyl-4-chlorophenoxy)-butyric acid; TCA, trichloroacetic acid; conc., concentration; K. M., Kunitz-McDonald; u, micro; b, beta.

Using P^{32}, it was shown qualitatively that oxidative phosphorylation was associated with fresh mitochondria isolated from rat liver (35, p. 957-972). Since then, mitochondrial preparations isolated from several mammalian tissues such as heart (62, p. 28-40) and brain (8, p. 685-696) were shown to have oxidative phosphorylative activity. It was necessary to add only the oxidizable substrate (a citric acid cycle intermediate, or b-hydroxybutyrate, or glutamate, etc.), Mg^{++}, orthophosphate, ADP and sufficient sucrose or KCl (or certain other solutes to maintain a minimum level of osmolarity) for the demonstration of oxidative phosphorylation in suspensions of isolated rat liver mitochondria (35, p. 957-972). The hexokinase system (hexokinase, glucose, Mg^{++} and ATP) was used effectively as a trapping agent for the phosphate incorporated into ATP. Under these conditions, only catalytic amounts of ATP or ADP were necessary and the glucose-6-phosphate, formed in large amounts, was not readily attacked by the phosphatase of the mitochondria.

The differentiation of mitochondria from morphologically similar forms of plastids in plant cells was first shown by Sorokin (63, p. 28-33). Millerd et al. (49, p. 855-862) and Bonner and Millerd (7, p. 135-148) first showed that a mitochondrial preparation obtained from the hypocotyls of etiolated seedlings of the mung bean Phaseolus aureus was

capable of carrying out phosphorylation upon the oxidation of citric acid cycle intermediates. They observed that the adenylate required for oxidative phosphorylation could be satisfied by either ADP or AMP.

Oxidative phosphorylation was demonstrated with mitochondrial preparations from lupine (17, p. 23-32), cauliflower buds (41, p. 557-575; 28, p. 247-256), skunk cabbage (30, p. 27-32), sweet potato (45, p. 420-424), avocado fruit (5, p. 48-63) and the endosperms of germinating castor beans (1, p. 115-118). Ohmura (55, p. 187-194) and Howard and Yamaguchi (32, p. 424-428) reported oxidative phosphorylation in particulate preparations isolated from spinach and pepper fruit, respectively. Freebairn and Remmert were the first to show oxidative phosphorylation in subcellular particles from cabbage, Brassica oleracea, with P:O ratios as high as 3.8 with α-ketoglutarate as the substrate (27, p. 374-376).

Forti and Tognoli (23, p. 170-180) investigated various factors, such as the requirements for cofactors, pH values and the ADP concentration, while studying oxidative phosphorylation in mitochondria isolated from etiolated pea seedlings. Switzer showed esterification of inorganic phosphate coupled with oxidation of succinate, with isolated mitochondria from etiolated soybean hypocotyls (65, p. 42-44). These investigators used exogenous adenylate (AMP,

ADP or ATP) as a phosphate acceptor, the hexokinase system as a trapping agent and in a few cases, sodium fluoride for inhibiting ATPase, to obtain maximum P:O ratios.

These findings left no doubt that plant systems could be as efficient as animal systems in trapping the energy released during the oxidation of intermediates of the citric acid cycle.

The following respiratory chain and probable sites of phosphorylation in animal mitochondria were postulated by Lehninger et al. (43, p. 450-456) who used digitonin fragments of rat liver mitochondria:

$$\text{Substrate} \rightarrow \text{DPN} \overset{\text{ATP}}{\uparrow} \rightarrow \text{FP} \rightarrow \text{Cyt.b} \overset{\text{ATP}}{\uparrow} \rightarrow \text{Cyt.c} \rightarrow \text{Cyt.a} \overset{\text{ATP}}{\uparrow} \rightarrow \text{Cyt.a}_3 \rightarrow \text{O}_2$$

Chance and Williams indicated the same sequence of electron transport in animal mitochondria as a result of their kinetic studies with the spectrophotometer (13, p. 81). These authors studied the nature of the spectroscopic changes related to the components of the respiratory chain. The spectroscopic changes induced by the addition of certain inhibitors indicated three points of inhibition upon depletion of the ADP in the incubation medium. These three points of inhibition were regarded as the probable sites of phosphorylation. On the basis of these studies, Chance and Williams (13, p. 65-134) suggested that the oxidation of DPNH, of cytochrome b, and of cytochrome c were the probable sites of phosphorylation. Thus, one of the sites of

phosphorylation indicated by Chance and Williams was different from one suggested by Lehninger et al. (43, p. 450-456).

Recently Chance and Hackett studied the electron transfer system of skunk cabbage mitochondria (15, p. 33-49). The respiratory chain corresponds roughly to that shown in animal systems. However, very little has been done to determine the probable sites of phosphorylation in plant mitochondria. Fritz and Naylor (28, p. 247-256) found one phosphorylation step to be between cytochrome c and oxygen, by using mitochondria isolated from cauliflower buds and mung bean seedlings. These authors also showed another phosphorylation step to be between succinate and cytochrome c, by using ferricyanide as the final electron acceptor and employing mitochondria isolated from mung bean seedlings. Preliminary kinetic studies by Chance and Hackett (15, p. 33-49) suggest that the sites of phosphorylation in skunk cabbage mitochondria might be similar to those in mammalian mitochondria. By means of kinetic studies of the effects of respiratory inhibitors on the absorption of orthophosphate, Hagen et al. (31, p. 496-506) showed that cytochrome b and DPNH were the components of the respiratory chain involved in the rate limiting steps of orthophosphate uptake by excised barley roots. However, definite conclusive evidence is lacking for determining the phosphorylation sites

during electron transport in plant systems.

Many studies of herbicides have had the main purpose of evaluating herbicidal potency. However, some investigations have been made to determine the mechanism by which herbicides act. The widely used auxin herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), was reported by Akers and Fang (2, p. 34-37) to inhibit the rate of CO_2 assimilation during photosynthesis. In addition, these authors observed increased amino acid synthesis, and increased catabolism or oxidation of glutamic acid and aspartic acid, when bean plants were treated with 2,4-D in a sealed system containing $C^{14}O_2$. Humphreys and Dugger (33, p. 136-140) investigated the effect of 2,4-D on the amount of glucose catabolized by different pathways in root tips of pea, corn and oat. In all plants tested, 2,4-D caused an increase in the amount of glucose catabolized via the pentose phosphate pathway. Humphreys and Dugger (34, p. 530-536) also reported that etiolated pea seedlings treated with 2,4-D maintained a higher rate of respiration than did untreated seedlings. These authors postulated from these results that 2,4-D increases respiration by causing more glucose to be catabolized via the pentose phosphate pathway.

Reports of the effects of herbicides on oxidative

phosphorylation in plant particulate preparations are indeed scarce. Switzer (65, p. 42-44) showed that 2,4-D at 5×10^{-4} M and higher concentrations inhibited both oxidation and phosphorylation appreciably, with phosphorylation showing a greater effect, in soybean mitochondria with succinate as the substrate.

The effects of 2,4-D and other plant growth substances on oxidative phosphorylation in rat liver mitochondria were investigated by Brody (9, p. 533-536). Brody reported that 2,4-D at 1×10^{-3} M had very little effect on respiration but that it lowered the P:O ratio to 20% of the control. Uncoupling effects were seen at 2,4-D concentrations as low as 5×10^{-5} M. In a low phosphate (5×10^{-4} M) system, it was found that increasing the concentration of 2,4-D (1×10^{-4} M to 1×10^{-3} M) resulted in an increase in the respiratory rate. 2,4-D at 1×10^{-3} M doubled the respiratory rate in the low phosphate system, causing it to equal or exceed the rate in the high phosphate (2×10^{-2} M) control system.

The inhibition of oxidative phosphorylation by herbicides might be due to an effect on either electron transport or the transfer reactions in the coupling sequence, or both. Studies of these reactions have been performed using mammalian mitochondrial systems, and the following scheme has been postulated for the mechanism of oxidative

phosphorylation (19, p. 574):[2]

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

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

(3) $A \sim C + Pi \longleftrightarrow A + P \sim C$

(4) $P \sim C + ADP \longleftrightarrow ATP + C$

The two reactions (3) and (4) are combined into one, in the sequence postulated by Chance et al. (12, p. 439-451). According to these authors, the high energy compound must be in the reduced state, $AH_2 \sim C$, instead of the oxidized state, $A \sim C$. Wadkins and Lehninger (69, p. 681-687), however, have obtained further evidence indicating that the carrier is in the oxidized state when involved in the 'high energy' compound.

Reactions (1) to (4) of the hypothesis would explain the dependence of the respiratory rate on the availability of a phosphate acceptor (ADP) and Pi. ATPase activity could result from the reversal of reaction (4), followed by an enzymatic or spontaneous hydrolysis of $P \sim C$ or $A \sim C$. Reactions (3) and (4) would represent the exchange reaction between orthophosphate and the terminal phosphate of ATP and this is designated as the ATP-P^{32} exchange. Reaction (4) itself represents the exchange between ADP and ATP, and this is designated as the ATP-ADP exchange.

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[2] A and B, AH_2 and BH_2 are respiratory chain carriers in the oxidized and reduced states, respectively; C is an enzyme involved, and $\sim$ represents a 'high energy' bond.

Cooper and Lehninger (18, p. 547-560; 19, p. 561-578) provided evidence for the involvement of the ATPase activity and the ATP-P^{32} exchange reaction in the mechanism of oxidative phosphorylation in digitonin extracts of rat liver mitochondria. These processes showed absolute specificity for the adenine nucleotides and were sensitive to the same inhibitors, such as 2,4-dinitrophenol (DNP), gramicidin, pentachlorophenol (PCP) and dicumarol. These inhibitors uncoupled oxidative phosphorylation, stimulated the ATPase activity and inhibited the ATP-P^{32} exchange in rat liver mitochondrial preparations. All three activities were lost at the same rate when the enzyme complex was aged at 32° C (19, p. 561-578).

Wadkins and Lehninger (68, p. 1589-1597) have partially purified the enzyme which catalyzes the ATP-ADP exchange. These authors reported that this enzyme was free of the ATPase and ATP-P^{32} exchange activity and that it required the presence of Mg^{++} or Mn^{++} ions for its optimum activity.

Recently several investigators collected further evidence on the mode of action of DNP in uncoupling oxidative phosphorylation (10, p. 369-377; 21, p. 1574-1577). Bronk and Kielley (10, p. 369-377) reported that the mode of action of DNP was through activation of the breakdown of a non-phosphate-containing intermediate state of the enzyme complex. The point of action of DNP proposed by

Bronk and Kielley is essentially the same as that proposed by Myers and Slater (52, p. 363-364; 53, p. 572-579). Therefore, the DNP-stimulated ATPase would represent reversal of reactions (4) and (3), followed by the decomposition of A$\sim$C. The evidence reported so far indicates that the DNP-stimulated ATPase, the ATP-P^{32} exchange and the ATP-ADP exchange reactions are part of the process of oxidative phosphorylation. There is no evidence available to indicate whether or not the Mg^{++}-stimulated ATPase activity is a part of the coupling mechanism.

It has been assumed that there are two distinct types of ATPases: Mg^{++}-stimulated ATPase and DNP-stimulated ATPase. Cooper (20, p. 484-491) studied the ATPase activity in sub-mitochondrial particles of rat liver and found that the properties attributable to a DNP-stimulated ATPase could be reconciled mainly with an effect by DNP on Mg^{++}-stimulated ATPase. He suggested that the two ATPases could be considered as one. He also suggested that magnesium, either bound or free, is required for the demonstration of ATPase activity under all conditions.

Recently Beyer (4, p. 588-589) obtained evidence in support of two ATPase pathways in rat liver mitochondria. He indicated that Mg^{++} was not a limiting factor in the DNP-stimulated ATPase and that this ATPase was readily inactivated by far ultraviolet light while, after

deoxycholate treatment, Mg^{++}-stimulated ATPase was resistant to such inactivation.

If Mg^{++}-stimulated ATPase is considered to be a part of oxidative phosphorylation, then the hypothesis shown above may need some modifications. An additional step between reactions (3) and (4) would seem to be necessary. A part of the modified hypothesis may be indicated as:

(3) $A \sim C + Pi \longleftrightarrow A + P \sim C$

(3') $P \sim C + Y \longleftrightarrow C + P \sim Y$

(4') $P \sim Y + ADP \longleftrightarrow Y + ATP$

where Y is another enzyme involved in the process.

As mentioned earlier, Wadkins and Lehninger (68, p. 1589-1597) found that the partially purified enzyme which catalyzes the ATP-ADP exchange requires Mg^{++} ions for its optimum activity. These authors also reported that this enzyme was free of the ATPase and ATP-P^{32} exchange activities. These observations and the assumption that the Mg^{++}-stimulated ATPase is a part of oxidative phosphorylation may require the introduction of an additional step in the hypothesis. The Mg^{++}-stimulated ATPase could be visualized as a reversal of reactions (4') and (3'), followed by decomposition of $P \sim C$. Similarly, according to this modified hypothesis, DNP-stimulated ATPase would represent the reversal of reactions (4'), (3') and (3), followed by the decomposition of $A \sim C$.

DNP has been shown to have the following five effects on freshly prepared mammalian mitochondrial systems (10, p. 369-377):

1. Complete uncoupling of phosphorylation from respiration.
2. Stimulation of ATPase.
3. Inhibition of the ATP-P^{32} exchange.
4. Inhibition of the ATP-ADP exchange.
5. Release of respiration in the absence of a phosphate acceptor.

DNP has been shown to uncouple oxidative phosphorylation in plant mitochondrial preparations from skunk cabbage (30, p. 27-32), mung bean (7, p. 135-148), soybean (64, p. 89), sweet potato (45, p. 420-424) and castor bean (1, p. 115-118).

There have been only a few reports on the effects of DNP on the ATPase in plant particulate preparations. Baltscheffsky (3, p. 393-394) found that isolated spinach chloroplasts exhibited a Mg^{++}-dependent ATPase activity; DNP, however, had no effect on the ATPase with or without added Mg^{++}. Laties (40, p. 199-214) reported that DNP did not stimulate the ATPase in cauliflower mitochondria. Forti (22, p. 898-909) found that DNP stimulated the ATPase in pea mitochondria, but only in the presence of low concentrations of phosphate (0.001 M). Forti also obtained

evidence that only the terminal phosphate group of ATP is hydrolyzed in this reaction; ADP was found to be a powerful inhibitor of the ATPase activity.

Laties (40, p. 199-214) found that DNP did not increase respiration in cauliflower mitochondria in the absence of a phosphate acceptor, but that a DNP stimulation could be obtained in the presence of ATP. There have been no reports on the ATP-P^{32} and the ATP-ADP exchange reactions in plant mitochondria.

In order to elucidate the mode of action of 2,4-D and other herbicides on oxidative phosphorylation, studies on systems such as ATPase, ATP-P^{32} exchange, ATP-ADP exchange and released respiration should be carried out. A review of the literature indicates that no work has been reported on the effects of herbicides on the four systems mentioned above.

The work reported in this thesis was carried out with mitochondria isolated from cabbage. Cabbage is available at all times during the year, and mitochondria isolated from cabbage have high respiratory activity, comparable to the rates found in mammalian mitochondria (25, p. 264). These two important factors were considered in selecting cabbage as the source of mitochondria. The mitochondria were isolated from cabbage by modifications of the procedure used by Freebairn and Remmert (27, p. 374-376). The

effects of 2,4-D and 21 other herbicides on oxidative phosphorylation in the cabbage mitochondria are presented in the earlier part of the thesis. Various 'classical' uncoupling agents, such as DNP, pentachlorophenol, dicumarol and gramicidin, have been studied for their effects on oxidative phosphorylation, ATPase and release of respiration. Free fatty acids, such as oleic acid, myristic acid and stearic acid were used also in such studies. The effects of 2,4-D on released respiration, and on the ATPase and the ATP-P^{32} exchange reactions have permitted speculation on the probable site at which 2,4-D acts on oxidative phosphorylation. Some of the results to be reported appear to be contradictory and may indicate that the mechanism of oxidative phosphorylation in cabbage mitochondria differs somewhat from that involved in mammalian mitochondria.

METHODS AND MATERIALS

Isolation of Cabbage Mitochondria

The mitochondria were isolated from cabbage, Brassica oleracea var. capitata L., by modifications of the procedure used by Freebairn and Remmert (27, p. 374-376). Fresh cabbages were obtained from a local wholesale market. They were stored in a cold room held at 0° to 5° C, and were used within a week. Inner portions of young cabbage heads were chopped into pieces smaller than one cubic centimeter. Each of four 110 gram portions of chopped cabbage was blended in a Waring blendor during 5 short periods (1-1-1-5-2 seconds) with 45 ml. of homogenizing medium.[3] The blendor was operated at full speed at all times. During homogenization, the blendor motor and bowl were rocked as a unit to facilitate contact of the cabbage tissues with the blades. After blending, each of the four homogenates was strained through 8 layers of cheesecloth into a 1 liter beaker which was previously chilled. Homogenization was carried out in a cold room held at 0° to 5° C. The pH of the homogenate was between

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[3] The homogenizing medium contained 1.0 M sucrose, 0.1 M potassium phosphate, and 0.01 M ethylenediaminetetraacetate (EDTA). The pH of this medium was 7.4. Freebairn and Remmert (26, p. 20-28) used the same medium, but the pH was 7.2.

6.7 and 6.9. The homogenate was centrifuged at 3,000 x g for 10 minutes to sediment cells, chloroplasts and debris. The supernatant fraction of the first spin was centrifuged at 12,000 x g for 10 minutes to sediment the mitochondria. The mitochondria were washed twice, first with 80 ml. and then with 40 ml. of washing medium,[4] by suspending and recentrifuging at 12,000 x g. The centrifugation was carried out in a Servall refrigerated centrifuge kept between 0° and 5° C throughout the operations. The washed mitochondrial pellet was finally suspended in a sufficient volume of suspension medium[5] by the method described by Freebairn (24, p. 44-45). In this method, a 20 ml. Gooch crucible was placed in a crucible holder and the whole assembly was fitted into a 125 ml. filter flask which contained chopped ice and a 25 ml. cylinder. A mat of fine pyrex glass wool was placed in the bottom of the Gooch crucible. The mitochondrial pellet was suspended partially and transferred to the glass mat, globules were broken up by gentle stirring, and the suspension was drawn through the mat by partial evacuation of the filter flask. The entire suspension was passed through the glass mat a

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[4] The washing medium contained 0.375 M sucrose, 0.1 M potassium phosphate, and 0.001 M EDTA. The pH of this medium was 7.4.

[5] The suspension medium contained 0.375 M sucrose, 0.1 M potassium phosphate, pH 7.4.

second time. Uniform mitochondrial suspensions were obtained routinely by use of this procedure.

The Warburg Manometric Studies

Warburg constant volume respirometers with air as the gas phase were used for studies of oxygen consumption at 30° C by conventional manometric techniques (67, p. 1-17). The incubation medium contained (unless indicated otherwise) ATP, 3.3×10^{-3} M; $MgCl_2$, 5×10^{-3} M; DPN, 4×10^{-4} M; TPN, 8×10^{-5} M; TPP, 5.6×10^{-6} M; CoA, 4.2×10^{-5} M; GSH-Na, 2.5×10^{-3} M; hexokinase, 50 Kunitz-McDonald (K.M.) units; sucrose, 0.39 M; glucose, 0.2 M; potassium phosphate buffer, pH 7.4, 6.66×10^{-2} M; mitochondrial preparation, 0.3 - 0.6 mg N/ml; substrate, pH 7.4, 0.0133 M; other additions as indicated and glass distilled water to a final volume of 3.0 ml. Two-tenths ml of 20% KOH was added to the center well of each Warburg flask, along with a small piece of filter paper. Fresh solutions of DPN, TPN, TPP, CoA, ATP, GSH and hexokinase were prepared for each experiment. All of the other solutions were kept frozen when not in use. The Warburg flasks were kept cold, over a tray filled with chopped ice, until the mitochondrial preparation was added. The flasks were equilibrated for 10 minutes at 30° C; the manometers were then closed and hexokinase, glucose and $MgCl_2$ were tipped in from the

side arm. The flasks were incubated for 90 minutes at 30° C for measurements of oxygen uptake and phosphate esterification.

After incubation, the enzymatic reaction was stopped by pipetting a 1 ml. aliquot from each flask into a 100 ml. volumetric flask containing 1 ml. of 1 N HCl. The volume was made up to 100 ml. with glass distilled water. The volumetric flasks were shaken thoroughly for 10-15 seconds. The diluted solutions, which had pH values between 1.8 and 2.0, were used for determinations of inorganic phosphate. The initial inorganic phosphate level was determined by taking aliquots from certain flasks at zero time.

A modification of the method of Martin and Doty (3, p. 965-967) was used for the determinations of inorganic phosphate. The procedure was as follows: 5 ml. of isobutyl alcohol, 1 ml. of the ammonium molybdate reagent and 3 ml. of sample (containing 5 to 75 micrograms of phosphorus) were added to a 6 inch culture tube. A reagent blank and standards, containing 30, 60, and 90 micrograms of inorganic phosphate (dilute standard phosphorus solution) in 3.0 ml., were carried through the entire procedure. The tubes were corked tightly and shaken vigorously for 10 seconds. The phases were allowed to separate for at least 15 minutes; during this period the tubes were

tapped gently a few times to disperse bubbles clinging to the walls of the tubes. After the phases had separated, 2.0 ml. of the isobutyl alcoholic phase were transferred into a second culture tube. Finally, 10 ml. of acidified ethanol reagent and 0.4 ml. of Fiske-Subbarow reagent were added. The tubes were corked and shaken immediately. After one hour, absorbancy readings were taken in a Coleman Spectrophotometer at 650 millimicrons.

The ammonium molybdate reagent was prepared as described by Martin and Doty (46, p. 965-67); the preparation of the standard phosphorus solution was as described by Pons, Stansbury, and Hoffpauir (56, pp. 492-503). The acidified ethanol reagent was prepared by combining the following: 940 ml. of 95% ethanol, 60 ml. of 10 N sulfuric acid, and one liter of distilled water. The Fiske-Subbarow reagent was prepared as described by Umbreit, et al. (67, p. 273).

The nitrogen contents of the mitochondrial preparations were determined using the micro-Kjeldahl method and modification of the Kjeldahl-Gunning-Dyer method (16, p. 987-988). Aliquots of 0.5 to 1.0 ml. of the mitochondrial preparations were taken in duplicate for the nitrogen determinations. The digestion mixture contained 500 mg. K_2SO_4, 40 mg. HgO and 100 mg. of sucrose per flask. One and five-tenths ml. of concentrated H_2SO_4 were added to each flask and the digestion carried out for about 60-90

minutes, when the mixture turned pale yellow to colorless. After cooling, the digest was transferred to the distillation unit and 20 ml. of 10% NaOH with 5% sodium thiosulfate were added. The ammonia was steam distilled and absorbed into a mixture of 5 ml. of 4% boric acid solution and 4 drops of mixed indicator (bromocresol green and methyl red) in a 50 ml. Erlenmeyer flask. The ammonia was then titrated with 0.01 N potassium biniodate. A reagent blank was carried through the entire procedure with each group of samples. The concentration of mitochondrial nitrogen was between 0.3 and 0.6 mg. per ml.

The ATPase and ATP-P^{32} Exchange Studies

The procedure described previously was used for the isolation of mitochondria. The mitochondrial pellet was washed twice with 0.25 M sucrose and then suspended in 0.25 M sucrose for additions to the reaction vessels. The standard incubation medium contained: Tris-maleate buffer pH 7.4, 0.1 M; ATP, pH 7.4, 1×10^{-2} M; mitochondrial suspension, 0.2-0.6 mg. N/ml.; sucrose, 0.083 M; special additions and glass distilled water to a final volume of 3.0 ml. The flasks were incubated for 30 minutes at 30° C. The enzymatic reaction was stopped by adding 3.0 ml. of ice cold 0.5 M trichloroacetic acid (TCA). The flask contents were transferred to 15 ml. tubes and centrifuged for

10 minutes at 1200 revolutions per minute. The supernatant fraction was then filtered and the necessary aliquot from the filtrate was used for determining inorganic phosphate as described above. The ATPase activity was estimated as the amount of inorganic phosphate formed during the incubation period.

For the ATP-P^{32} exchange studies, the incubation medium was as described for measuring ATPase activity, except that P^{32} equivalent to about 1×10^6 counts/minute/flask was also added. The period of incubation was for 30 minutes at 30° C. The enzymatic reaction was stopped by adding 3.0 ml. of ice cold 0.5 M TCA. The protein precipitate was removed completely by centrifugation and filtration as described previously.

The amount of P^{32} incorporated into ATP was determined by the method of Nielson and Lehninger (54, p. 555-570). A 1.0 ml. aliquot of each TCA filtrate was transferred into a separate glass stoppered centrifuge tube containing the following: 4 ml. isobutyl alcohol-benzene reagent, 1 ml. ammonium molybdate reagent and 2 ml. of glass distilled water. The centrifuge tubes were shaken vigorously for 30 seconds; they were then centrifuged in an International centrifuge for 4 minutes in order to obtain complete separation of the two phases. Each aqueous layer was filtered into a second centrifuge tube containing

4.0 ml. of isobutyl alcohol-benzene reagent. The tubes were shaken vigorously for 30 seconds and then centrifuged as above. The aqueous layer was filtered and the final filtrate was used for radioactive measurements. The total counts added to each incubation mixture was also determined using a diluted sample of the TCA filtrate.

The isobutyl alcohol-benzene reagent was prepared by mixing 200 ml. isobutyl alcohol, 200 ml. benzene and 40 ml. of distilled water, and shaking the mixture for 30 minutes. The ammonium molybdate reagent was prepared as described by Martin and Doty (46, p. 965-67).

Materials

The ATP-disodium salt (from muscle), ADP-sodium salt (from muscle), AMP (from muscle), CoA (from yeast), DPN (from yeast), TPN-monosodium salt (from yeast), hexokinase (from yeast), and sodium pyruvate were products of the Sigma Chemical Company. Glutathione (monosodium salt) was obtained from Schwarz Laboratories, Inc. TPP was the product of Nutritional Biochemicals Corporation. 2,4-dinitrophenol, pentachlorophenol, isobutyl alcohol, citric acid, L-malic acid and succinic acid were obtained from Eastman Kodak Company. Dicumarol, gramicidin and L-thyroxine sodium pentahydrate were obtained from Mann Research Laboratories. EDTA (practical grade) was obtained from

Eastman Kodak Company, converted to the disodium salt and recrystallized twice by the method of Blaedel and Knight (6, p. 741-743). Myristic acid and stearic acid were obtained from Eastman Kodak Company and were of the white label quality. Oleic acid (U.S.P. grade) was the product of Matheson, Coleman and Bell. P^{32} was obtained as $H_3P^{32}O_4$ from the Oak Ridge National Laboratory.

The herbicides were supplied by Dr. Virgil H. Freed of the Department of Agricultural Chemistry. They were obtained by him from various sources. The following is the list of the herbicides and their sources: IPC and CIPC, Columbia Southern Chemical Corporation; EPTC, Stauffer Chemical Company; CMU, duPont & Company; CDAA, 2,4,5-T and MCPA, Monsanto Chemical Company; ATA and TCP, American Cyanamid Company; simazin, Geigy Chemical Corporation; mylone, Union Carbide Chemical Company; DCPA, The Dow Chemical Company; NPA and MH, Naugatuck Chemical Division, U. S. Rubber Company; 2,3,6-TBA, Hooker Electrochemical Company; sodium chlorate, Chipman Chemical Company; 2,4-DB and MCBA, May & Baker Company; 2,4,6-T, AmChem Products, Inc.; 2,4-D, Eastman Kodak Company; 2-CPA and 4-CPA, prepared and crystallized by Dr. Freed.

Other chemicals used were of reagent grade.

RESULTS AND DISCUSSION

Part I

Conditions for Optimal Respiration and Phosphorylation in Cabbage Mitochondria

The cabbage mitochondria employed in these studies were isolated by using the procedure of Freebairn and Remmert (27, p. 374-376), with some modifications. The procedure used is described in "Methods and Materials."

Cofactors: Freebairn and Remmert (27, p. 374-376) in their studies, used ATP and the hexokinase system, and DPN, TPN, glutathione (GSH), cytochrome c, and Mg^{++} and Mn^{++} ions as cofactors in order to obtain high substrate induced oxygen and Pi uptakes in cabbage mitochondria. These authors stated that added cytochrome c appeared to stimulate oxygen and Pi uptakes only in the case of preparations characterized by low P:O ratios, and suggested that cytochrome c might have been lost during the isolation procedure. In the present studies, DPN, TPN, TPP, CoA and GSH were added as cofactors to obtain oxidative phosphorylation in cabbage mitochondria.

The energy liberated during oxidative phosphorylation is conserved in the form of ATP. ADP and orthophosphate are necessary for trapping the liberated energy. This process may be shown in the form of a simple reaction as

follows:

(1) ADP + Pi $\rightleftharpoons$ ATP

The terminal phosphate group of ATP is very labile to hydrolysis either by an enzyme or by an acid. Mitochondrial preparations commonly contain an enzyme (ATPase) or enzymes which may hydrolyze ATP and give low P:O ratios. The hexokinase system may be used as an effective trapping agent for the terminal phosphate of the ATP formed during phosphorylation studies. Under these conditions, only catalytic amounts of ATP or ADP are necessary for oxidative phosphorylation to proceed with maximum efficiency and the effect of an ATPase is much reduced. Mg^{++} plays a very important role in this process, because it is a necessary cofactor in the hexokinase reaction, which is:

(2) ATP + Glucose $\xrightarrow[Mg^{++}]{\text{hexokinase}}$ glucose-6-phosphate + ADP

The results in Table I show that the endogenous Mg^{++} was not sufficient to obtain optimal oxidative phosphorylation, since when Mg^{++} was added at 5×10^{-3} M, phosphorylation was increased five fold. This increase in phosphorylation could be explained easily on the basis of the Mg^{++} requirement for reaction (2); in the presence of an adequate concentration of Mg^{++}, the hexokinase system would maintain a continuous supply of ADP to serve as phosphate acceptor. With Mg^{++} at 5×10^{-3} M, a high rate

Table I

The Magnesium Requirement for Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were: citrate, 0.0125 M; ATP, 3.1×10^{-3} M; DPN, 3.8×10^{-4} M; TPN, 7.5×10^{-5} M; TPP, 5.3×10^{-4} M; CoA, 3.9×10^{-5} M; GSH-Na, 2.3×10^{-3} M; hexokinase, 50 K.M. units; NaF, 0.02 M; Sucrose, 3.67×10^{-2} M; glucose, 0.2 M; phosphate buffer, pH 7.4, 0.0625 M; $MgCl_2$, as indicated; mitochondrial preparation, 0.33 mg.N/ml; glass distilled water added to a final volume of 3.2 ml. Incubated 90 minutes at 30° C. The values are averages from duplicate flasks.

Concentration of Added $MgCl_2$	O_2 uptake (microgram atoms)	Pi esterified (micromoles)	P:O
none	14.9	23.7	1.59
5×10^{-4} M	47.9	108.9	2.27
5×10^{-3} M	51.9	125.9	2.43

of respiration and a P:O ratio as high as 2.43 were obtained, with citrate as the substrate.

In these studies ATP or AMP served equally well as a phosphate acceptor, when glucose and hexokinase were used as the trapping system. AMP has been used as a phosphate acceptor in many plant mitochondrial preparations, such as mung bean (7, p. 135-148), soybean (65, p. 42-44) and avocado fruit (5, p. 48-63). It is possible that the role of AMP as a phosphate acceptor can be explained on the basis of the presence of adenylic kinase, which has been reported in many plant preparations (47, p. 37-43). Adenylic kinase, in the presence of small amounts of ATP in these preparations, would convert AMP to ADP which then would be the primary phosphate acceptor. However, ATP and the hexokinase system were used to provide the phosphate acceptor (ADP) in the present studies with cabbage mitochondrial preparations.

Table II shows a study of the concentration of ATP required for efficient phosphorylation in the cabbage mitochondria. These results indicate that there was not sufficient endogenous adenylate (adenosine phosphate) in the preparations to conserve the energy released during the respiration. The oxygen uptake and phosphorylation were increased by approximately 25% and 100%, respectively, when the ATP concentration was increased from 0.1 umole to

Table II

The ATP Requirement for Oxidative Phosphorylation with Citrate as Substrate

The flask contents were: Citrate, 0.0133 M; $MgCl_2$, 5×10^{-3} M; DPN, 4×10^{-4} M; TPN, 8×10^{-5} M; TPP, 5.6×10^{-4} M; CoA, 4.2×10^{-5} M; GSH-Na, 2.5×10^{-3} M; Hexokinase 50 K.M. units; Sucrose, 0.39 M; glucose, 0.2 M; phosphate buffer, pH 7.4, 6.66×10^{-2} M; mitochondrial preparation, 0.5 mg. N/ml; additions as indicated and glass distilled water to a final volume of 3.0 ml. The values are averages from duplicate flasks.

Concentration of ATP (in micromoles)	O_2 uptake (microgram atoms)	Pi esterified (micromoles)	P:O
0.1	43.8	61.9	1.41
1.0	52.3	98.7	1.89
10.	58.4	117.1	2.01

10 umoles per flask. The presence of 10 umoles of ATP gave a high respiratory rate with citrate and a P:O ratio of 2.0.

Hexokinase: Studies were made to determine whether or not the hexokinase concentration was adequate to obtain maximal P:O ratios. Three hexokinase concentrations, namely 50, 100, and 150 K.M. units per flask, were used. The glucose concentration was the same in all flasks (0.2 M). It was found that there was no difference in the respiratory rate or the P:O ratio with the hexokinase concentrations tested. These results indicate that in presence of 0.2 M glucose and 10 umoles of ATP, 50 K.M. units of hexokinase per flask are sufficient for maintaining an adequate supply of ADP in the system used.

On the basis of these results, 10 umoles of ATP, 15 umoles of Mg^{++}, and 50 K.M. units of hexokinase were added routinely for the oxidative phosphorylation studies.

Fluoride: Many investigators (27, p. 374-376; 7, p. 135-148) have used sodium fluoride to inhibit ATPase activity during their studies on oxidative phosphorylation in plant mitochondrial preparations. These workers obtained higher P:O ratios in the presence of fluoride. In the present studies, however, the presence of fluoride (0.02 M) decreased oxygen uptake and phosphorylation to the same degree, without any appreciable change in the P:O

ratio. Therefore, fluoride was not added in routine studies of oxidative phosphorylation in the cabbage mitochondria.

Substrate: In these studies it was found that all of the citric acid cycle intermediates tested, such as citrate, ∝-ketoglutarate, oxaloacetate, succinate, and malate, were oxidized with concomitant phosphorylation. Oxidation of pyruvate required the presence of one of the intermediates of the citric acid cycle. In routine work, 40 umoles of citrate or a mixture of 10 umoles each of citrate, malate, succinate and pyruvate were found to give an average P:O ratio of 2.0. In these studies, an incubation period of 90 minutes was employed, to obtain more Pi uptake and greater accuracy in the Pi determination; it is probable that higher P:O ratios would have been obtained if a shorter incubation period had been used.

Part II

The Effects of Herbicides on Respiration and Phosphorylation in Cabbage Mitochondria

Oxidative phosphorylation is an important process in which a large amount of energy is made available to a living organism by the oxidation of intermediates of the citric acid cycle. The energy liberated at various steps during the transfer of electrons from substrate to oxygen

is conserved in the form of ATP.

If a herbicide could uncouple phosphorylation from respiration in a plant, the plant would not be able to survive due to lack of sufficient useful energy. It was thought that the mode of action of herbicides might involve a major effect on this important process of oxidative phosphorylation. Therefore, the effects of 2,4-D and 21 other herbicides on oxidative phosphorylation in the cabbage mitochondria were investigated.

Herbicides have been classified in the following four groups:

1. Carbamates
2. Phenoxyacetic acids
3. Phenoxybutyric acids
4. Miscellaneous

1. Carbamates: IPC, CIPC, and EPTC were used for the study. These herbicides are specifically more active on graminaceous plants than on dicotyledonous plants. IPC and EPTC are used as selective grass killers. CIPC is found to be effective against crab grass and other grasses tolerant to IPC.

The effects of carbamate herbicides on oxidative phosphorylation are summarized in Table III. It was observed that ethyl alcohol, in the amounts added with the herbicides, did not have any appreciable effect on oxygen uptake and phosphorylation. IPC at 5×10^{-4} M inhibited oxygen uptake and phosphorylation by 13% and 53%,

Table III

The Effects of Carbamate Herbicides on
Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were: Substrate, 0.0133 M; ATP, 3.3×10^{-3} M; $MgCl_2$, 5×10^{-3} M; DPN, 4×10^{-4} M; TPN, 8×10^{-5} M; TPP, 5.6×10^{-4} M; CoA, 4.2×10^{-5} M; GSH-Na, 2.5×10^{-3} M; Hexokinase 50 K.M. units; Sucrose, 0.39 M; glucose, 0.2 M; phosphate buffer, pH 7.4, 6.66×10^{-2} M; mitochondrial preparation, 0.3-0.6 mg.N/ml; additions as indicated and glass distilled water to a final volume of 3.0 ml. The herbicides were dissolved in ethyl alcohol. Incubated 90 minutes at 30° C. The values are averages from duplicate flasks.

Herbicide	Substrate	Herbicide Concentration tested	% Inhibition of O_2 uptake	% Inhibition of Pi esterified	P:O Ratio	Conc. for 50% Decrease of P:O	Conc. for 100% Decrease of P:O
Isopropyl-N-phenyl carbamate (IPC)	Citrate	0	0	0	1.90	5×10^{-4} M	3×10^{-3} M
		5×10^{-4} M	13	53	1.03		
		1×10^{-3} M	35	93	0.21		
		3×10^{-3} M	72	100	0		
		5×10^{-3} M	73	100	0		
Isopropyl-N-(3-chlorophenyl) carbamate (CIPC)	Mixture[a]	0	0	0	1.74	10^{-3} M-10^{-2} M	
		1×10^{-3} M	85	91	1.03		
		1×10^{-2} M	90	97	0.50		
Ethyl-N, N-di-n-propyl thiol carbamate (EPTC)	Mixture	0	0	0	1.74	10^{-3} M-10^{-2} M	
		1×10^{-3} M	77	100	0		
		1×10^{-2} M	85	94	0.96		

[a] The substrate mixture consisted of 10 micromoles each of citrate, succinate, malate and pyruvate.

respectively. Phosphorylation was inhibited more or less completely with IPC at 1 x 10^{-3} M; oxygen uptake also was reduced by 35% at that concentration. CIPC and EPTC at either 1 x 10^{-3} M or 1 x 10^{-2} M inhibited oxidative phosphorylation severely, inhibition of phosphorylation being slightly greater than that of respiration. Thus, all three herbicides tested were very effective in inhibiting oxidative phosphorylation in the cabbage mitochondria. These results suggest that carbamate herbicides could act *in vivo* by uncoupling phosphorylation from respiration in grasses.

2. Phenoxyacetic acids: 2,4-D has been used widely for the weeding of cereals and other crops; its phytotoxic action is more pronounced in broad-leaved plants than in cereals. Switzer reported that 2,4-D at 5 x 10^{-4} M and higher concentrations inhibited both oxygen uptake and phosphorylation in soybean mitochondria (65, p. 42-44). He found a greater effect on phosphorylation than on oxygen uptake; this gave a decrease of the P:O ratio at those levels of 2,4-D treatment. Brody (9, p. 533-536) found that 2,4-D at 1 x 10^{-3} M had very little effect on respiration in rat liver mitochondria, but lowered the P:O ratio to 20% of the control. In addition, Brody reported uncoupling effects by 2,4-D at concentrations as low as 5 x 10^{-5} M.

Table IV shows the effects of phenoxyacetic acids on oxidative phosphorylation in the cabbage mitochondria. 2,4-D at 2.5×10^{-3} M inhibited phosphorylation completely, and lowered respiration in the cabbage mitochondria by about 65%. A marked inhibition of phosphorylation was found with 2,4-D at a concentration as low as 5×10^{-4} M. These results are similar to those reported by Switzer (65, p. 42-44) and Brody (9, p. 533-536).

Phenoxyacetic acid herbicides are more effective on broad-leaved plants whereas carbamate herbicides are more effective on monocotyledonous plants such as cereals and grasses. Since both types of herbicides had an appreciable effect on oxidative phosphorylation in the cabbage mitochondria (Tables III and IV), their specificity cannot be explained by these in vitro studies. It is conceivable that the specificity of these herbicides in vivo may be due to differences in their concentrations at the site of action.

2,4,5-T is generally less phytotoxic than 2,4-D; it is, however, more effective on specific weeds, particularly on woody species such as mesquite. 2,4,6-T is herbicidally inactive. However, the effects of 2,4,5-T and 2,4,6-T on oxidative phosphorylation in the cabbage mitochondria were about equal (Table IV). 2,4,5-T and 2,4,6-T at the highest concentration tested

Table IV

The Effects of Phenoxyacetic Acids on Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were as described in Table III. All values are averages from duplicate flasks.

Herbicide	Substrate	Herbicide Concentration tested	% Inhibition of O_2 uptake	% Inhibition of Pi esterified	P:O Ratio	Conc. for 50% Decrease of P:O	Conc. for 100% Decrease of P:O
2-chlorophenoxyacetic acid (2-CPA)	Citrate	0	0	0	1.92	$5x10^{-3}$ M	
		$1x10^{-3}$ M	17	25	1.75		
		$5x10^{-3}$ M	48	80	1.81		
4-chlorophenoxyacetic acid (4-CPA)	Citrate	0	0	0	1.92	10^{-3} M-$5x10^{-3}$ M	$5x10^{-3}$ M
		$1x10^{-3}$ M	18	36	1.49		
		$5x10^{-3}$ M	64	100	0		
2,4-dichlorophenoxyacetic acid (2,4-D)	Citrate	0	0	0	1.92	$1x10^{-3}$ M	$5x10^{-3}$ M
		$1x10^{-3}$ M	54	83	0.71		
		$5x10^{-3}$ M	63	100	0		
2,4-dichlorophenoxyacetic acid (2,4-D)	Mixture	0	0	0	1.81	$5x10^{-4}$ M-$2.5x10^{-3}$ M	$2.5x10^{-3}$ M
		$1x10^{-4}$ M	6	11	1.73		
		$5x10^{-4}$ M	27	55	1.12		
		$2.5x10^{-3}$ M	68	100	0		
2-methyl-4-chlorophenoxyacetic acid (MCPA)	Mixture	0	0	0	1.81	$2.5x10^{-3}$ M	
		$1x10^{-4}$ M	6	6	1.81		
		$5x10^{-4}$ M	12	12	1.80		
		$2.5x10^{-3}$ M	36	70	0.86		

Table IV, Continued

Herbicide	Substrate	Herbicide Concentration tested	% Inhibition of O_2 uptake	% Inhibition of Pi esterified	P:O Ratio	Conc. for 50% Decrease of P:O	Conc. for 100% Decrease of P:O
2,4,5-trichlorophenoxyacetic acid (2,4,5-T)[a]	Mixture	0	0	0	1.29		
		$1x10^{-4}$ M	9	5	1.35		
		$5x10^{-4}$ M	17	53	0.73		
2,4,6-trichlorophenoxyacetic acid (2,4,6-T)[a]	Mixture	0	0	0	1.29		
		$1x10^{-4}$ M	10	21	1.13		
		$5x10^{-4}$ M	15	47	0.81		

[a] The herbicides were dissolved in ethyl alcohol. The other herbicides were added as potassium salts in water solutions.

(5×10^{-4} M) inhibited respiration and phosphorylation by about 15% and 50%, respectively.

4-CPA at 1×10^{-3} M did not inhibit oxidative phosphorylation to the same degree as 2,4-D at 1×10^{-3} M. However, both herbicides at 5×10^{-3} M inhibited phosphorylation and respiration in the cabbage mitochondria by 100% and 65%, respectively.

Some relationship may be seen between the effects of certain of the phenoxyacetic acids (2,4-D, 2-CPA, 4-CPA and MCPA) on oxidative phosphorylation (Table IV) and their structural components. Thus, a chlorine (electrophilic halogen group) atom at the 4 position (para) in the ring is common to three of the herbicides, namely 2,4-D, 4-CPA and MCPA, and these herbicides inhibited oxidative phosphorylation appreciably. Similarly, compounds with a chlorine atom at the 2 position (ortho), such as in 2,4-D and 2-CPA, gave inhibition. However, compounds with a chlorine atom at both the 2 and 4 positions in the ring, as in 2,4-D, 2,4,5-T and 2,4,6-T, were more inhibitory than compounds having only one chlorine atom in the ring (such as 2-CPA and 4-CPA). Substitution of one of the two chlorine atoms in the ring (as in 2,4-D) by a methyl group (nucleophilic group), as in MCPA, decreased the inhibitory effect. Muir et al. (51, p. 359-366) investigated some of these phenoxyacetic acids and their effects on cell elongation of Avena coleoptile sections.

These authors related the growth activity to chemical structure in a manner similar to that mentioned above.

3. Phenoxybutyric acids: In this group, two herbicides (2,4-DB and MCPB) were studied. There is a major difference between these herbicides and the phenoxyacetic acids, in their effects on plants. For example, it has been found that 2,4-DB is ineffective until it is selectively broken down by b-oxidation to form 2,4-D. 2,4-DB and MCPB are used for selective broadleaf weed control in legume crops.

2,4-DB was found to be a slightly better uncoupling agent than MCPB (Table V). 2,4-DB at 2.5×10^{-3} M inhibited phosphorylation and respiration by 100% and 48%, respectively. MCPB at the same concentration (2.5×10^{-3} M) inhibited phosphorylation and respiration by 94% and 73%, respectively.

4. Miscellaneous: DCPA, known as Dalapon, and TCP belong to a group of herbicides which are more toxic to monocotyledonous than to dicotyledonous plants. They are used mainly for the control of graminaceous weeds in crops such as beet.

Redemann and Meikle (60, p. 106-112) reported effects of DCPA on pyruvate oxidase and carboxylase enzyme systems. The results of these authors did not indicate that the blocking of pyruvic acid metabolism was the primary cause of the herbicidal action of DCPA.

Table V

The Effects of γ-phenoxybutyric Acids on Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were as described in Table III. The herbicides were added as potassium salts. All values are averages from duplicate flasks.

Herbicide	Substrate	Herbicide Concentration tested	% Inhibition of O_2 uptake	% Inhibition of Pi esterified	P:O Ratio	Conc. for 50% Decrease of P:O	Conc. for 100% Decrease of P:O
γ-(2-methyl-4-chlorophenoxy)-butyric acid (MCPB)	Mixture	0	0	0	1.93	$0-5x10^{-4}$ M	
		$5x10^{-4}$ M	40	81	0.60		
		$2.5x10^{-3}$ M	73	94	0.43		
γ-(2,4-dichlorophenoxy)-butyric acid (2,4-DB)	Mixture	0	0	0	1.93	$0-5x10^{-4}$ M	$2.5x10^{-3}$ M
		$5x10^{-4}$ M	49	88	0.44		
		$2.5x10^{-3}$ M	48	100	0		

DCPA and TCP, at concentrations of 1×10^{-3} M to 1×10^{-2} M, did not have an appreciable effect on oxidative phosphorylation in the cabbage mitochondria (Table VI). These results suggest that the herbicidal action of DCPA and TCP is not due to inhibition of oxidative phosphorylation.

Table VI, in addition, shows the effects of NPA and 2,3,6-TBA on respiration and phosphorylation in the cabbage mitochondria. NPA is used as a pre-emergence herbicide for the control of germination of seeds of grass and broad-leaved weeds in cucurbitaceous crops. NPA at 1×10^{-3} M inhibited phosphorylation and respiration by 32% and 20%, respectively. At higher concentrations (6×10^{-3} M), NPA inhibited phosphorylation completely and lowered respiration by 80% or more. 2,3,6-TBA is used for the control of dicotyledonous weeds, particularly in corn. At 6×10^{-3} M, 2,3,6-TBA was not as effective as NPA in inhibiting oxidative phosphorylation in the cabbage mitochondria.

Maleic hydrazide (MH) is a selective herbicide effective against grasses. Sodium chlorate is, however, strongly phytotoxic to all green plants. MH and sodium chlorate at 1×10^{-2} M did not have an appreciable effect on oxidative phosphorylation (Table VII). Sodium chlorate at very high concentrations (0.1 M) inhibited both respiration and phosphorylation appreciably; this inhibition

Table VI

The Effects of Miscellaneous Acids on Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were as described in Table III. The 2,3,6-TBA was dissolved in ethyl alcohol. The other herbicides were dissolved in water. All values are averages from duplicate flasks.

Herbicide	Substrate	Herbicide Concentration tested	% Inhibition of O_2 uptake	% Inhibition of Pi esterified	P:O Ratio	Conc. for 50% Decrease of P:O	Conc. for 100% Decrease of P:O
2,2-dichloropropionic acid (DCPA)	citrate	0	0	0	1.91		
		$1x10^{-3}$ M	3	0	1.94		
		$3x10^{-3}$ M	11	2	2.16		
		$6x10^{-3}$ M	9	14	1.81		
		$9x10^{-3}$ M	8	11	1.84		
		$1.2x10^{-2}$ M	14	15	1.90		
2,2,3-trichlorosodium propionate (TCP)	Mixture	0	0	0	1.74		
		$1x10^{-3}$ M	1	1	1.75		
		$1x10^{-2}$ M	11	20	1.58		
N-napthylphthalamic acid (NPA)	Citrate	0	0	0	2.04	10^{-3} M-$3x10^{-3}$ M	$9x10^{-3}$ M
		$1x10^{-3}$ M	20	32	1.74		
		$3x10^{-3}$ M	52	80	0.84		
		$6x10^{-3}$ M	77	97	0.29		
		$9x10^{-3}$ M	87	100	0		
		$1.2x10^{-2}$ M	92	100	0		
2,3,6-trichlorobenzoic acid (2,3,6-TBA)	Citrate	0	0	0	2.57	$6x10^{-3}$ M	$1.2x10^{-2}$ M
		$1x10^{-3}$ M	16	21	2.44		
		$3x10^{-3}$ M	44	47	2.43		
		$6x10^{-3}$ M	53	77	1.27		
		$9x10^{-3}$ M	69	91	0.72		
		$1.2x10^{-2}$ M	82	100	0		

Table VII

The Effects of Miscellaneous Herbicides on Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were as described in Table III. The CMU was dissolved in ethyl alcohol. The other herbicides were added in aqueous solutions. The values are averages from duplicate flasks.

Herbicides	Substrate	Herbicide Concentration tested	% Inhibition of O_2 uptake	% Inhibition of Pi esterified	P:O Ratio	Conc. for 50% Decrease of P:O
p-chlorophenyl 1, 1-dimethyl urea (CMU)	Citrate	0	0	0	1.91	3×10^{-3} M
		5×10^{-4} M	20	57	1.03	
		1×10^{-3} M	15	48	1.19	
		3×10^{-3} M	25	63	0.96	
Maleic hydrazide (MH)	Citrate	0	0	0	1.68	
		1×10^{-3} M	1	5	1.61	
		3×10^{-3} M	1	29	1.20	
		6×10^{-3} M	7	16	1.50	
		9×10^{-3} M	2	21	1.34	
		1.2×10^{-2} M	18	29	1.45	
2-chloro-N,N-diallyl acetamide (CDAA)	Citrate	0	0	0	2.0	9×10^{-3} M
		1×10^{-3} M	10	21	1.78	
		3×10^{-3} M	23	40	1.58	
		6×10^{-3} M	33	59	1.24	
		9×10^{-3} M	59	78	1.07	
Sodium chlorate	Citrate	0	0	0	2.14	
		1×10^{-5} M	2	9	1.99	
		1×10^{-4} M	1	4	2.08	
		1×10^{-3} M	5	11	2.01	
		1×10^{-2} M	9	12	2.09	
		1×10^{-1} M	45	64	1.38	

might have been due to an osmotic effect. Switzer reported a large inhibitory effect on oxygen uptake in soybean mitochondria, with a high concentration of sodium chlorate (65, p. 42-44). Switzer also suggested that the inhibition might be due to an osmotic effect.

CDAA is a selective pre-emergence herbicide, affecting certain grasses. CMU was more effective than CDAA in uncoupling oxidative phosphorylation in the cabbage mitochondria (Table VII); CMU and CDAA at 3×10^{-3} M inhibited phosphorylation by 63% and 40%, respectively. At this concentration, both herbicides inhibited oxygen uptake by about 25%. CMU at 3×10^{-3} M and CDAA at 9×10^{-3} M decreased the P:O ratio by 50%.

The effects of mylone, ATA and simazin on oxidative phosphorylation in the cabbage mitochondria are summarized in Table VIII. Mylone was very effective in inhibiting oxidative phosphorylation. At 6×10^{-3} M, it inhibited oxygen uptake and phosphorylation by 52% and 82%, respectively.

Simazin, at very low concentrations, has been used as a pre-emergence herbicide in corn, tomatoes, grapes, and asparagus. Simazin at the highest concentration tested (6×10^{-5} M), inhibited phosphorylation and respiration in the cabbage mitochondria by 21% and 6%, respectively. Higher concentrations of simazin were not used because of

Table VIII

The Effects of Miscellaneous Herbicides on Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were as described in Table III. The ATA was dissolved in water. The other two herbicides were dissolved in acetone. All values are averages from duplicate flasks.

Herbicide	Substrate	Herbicide Concentration tested	% Inhibition of O_2 uptake	% Inhibition of Pi esterified	P:O Ratio	Conc. for 50% Decrease of P:O
3,5-dimethyl tetrahydro-1,3,5,2H-thiadiazine-2-thione (mylone)	Citrate	0	0	0	2.17	3×10^{-3} M- 6×10^{-3} M
		1×10^{-3} M	26	43	1.69	
		3×10^{-3} M	53	62	1.75	
		6×10^{-3} M	52	82	0.81	
		9×10^{-3} M	58	93	0.39	
3-amino-1,2,4-triazole (ATA)	Citrate	0	0	0	1.93	
		1×10^{-3} M	2	0	2.00	
		3×10^{-3} M	5	0	2.11	
		6×10^{-3} M	4	8	1.83	
		9×10^{-3} M	3	7	1.86	
		1.2×10^{-2} M	4	4	1.92	
2,chloro-4,6-bis (ethylamino)-s-triazine (simazin)	Citrate	0	0	0	2.30	
		1×10^{-5} M	10	16	2.13	
		3×10^{-5} M	8	13	2.14	
		6×10^{-5} M	6	21	1.94	

its low solubility. Moreland et al. (50, p. 432-435) found that simazin at 4.6×10^{-6} M inhibited ferricyanide reduction in isolated barley chloroplasts by 50%. These authors also reported that in in vivo experiments the inhibitory effects of simazin were overcome by supplying glucose to treated barley roots. It appears that simazin is more inhibitory to the Hill reaction than to oxidative phosphorylation.

It has been suggested that ATA interferes with chlorophyll synthesis by blocking porphyrin formation, but there has been no experimental evidence to support this contention. ATA at the highest concentration tested (1.2×10^{-2} M) did not have any effect on oxidative phosphorylation in the cabbage mitochondria (Table VIII). The herbicidal action of ATA must, therefore, be due to an effect on some process other than oxidative phosphorylation.

To summarize, these results indicate that some herbicides, such as IPC, 2,4-D, 2,4-DB, NPA and mylone, may act on plants by their uncoupling properties. Herbicides such as ATA, simazin, MH, $NaClO_3$, DCPA and TCP may act by affecting some processes other than oxidative phosphorylation in plants.

Part III

Release of Respiration

Acceptor Ratios for Cabbage Mitochondria: It has been shown that the rate of respiration in intact mitochondria is dependent on the concentration or availability of a phosphate acceptor (ADP) (11, p. 383-393; 14, p. 283-295; 35, p. 215-224). If the rate of respiration of a mitochondrial preparation is increased several fold upon the addition of ADP, then that preparation is said to exhibit "respiratory control". This may be expressed as an acceptor ratio, which is defined as the ratio of respiration in the presence and in the absence of a phosphate acceptor. An acceptor ratio between 4 and 10, when associated with a high P:O ratio, is considered to be good evidence to indicate that respiration is "tightly coupled" with phosphorylation (61, p. 1-5; 11, p. 383-393).

A number of experiments were carried out to determine whether or not respiration was tightly coupled with phosphorylation in the cabbage mitochondria (Table IX). In these experiments ATP and the hexokinase system were used to provide ADP; in certain other flasks, one or more components of the hexokinase system were omitted to obtain the respiratory rate in the absence of a phosphate acceptor. It was found that when ATP alone was omitted from

the system, appreciable respiration occurred, and comparison to a complete system gave a low acceptor ratio (about 2.0). However, it was also found that with ATP omitted, and the other components of the hexokinase system present, about 20% as much phosphorylation occurred as was found in a complete system. These results indicated that endogenous adenylate was not removed completely even by two washings of the mitochondrial preparations. Under these circumstances a low acceptor ratio (as given in Table IX) would be expected by comparison of respiratory rates with and without the added ATP.

Plant mitochondria have been shown to contain hexokinase which is firmly bound to them. Mitochondria also possess invertase activity and hence it is obvious that plant mitochondria suspended in sucrose may make some glucose available for use in the hexokinase reaction. Bonner and Millerd demonstrated these characteristics in mung bean mitochondria (7, p. 135-148). In the present studies, low acceptor ratios (2 to 4) were obtained when either glucose and hexokinase or glucose, hexokinase and ATP were omitted and the respiratory rates were compared to those obtained in a complete system. These results are consistent with the idea that the cabbage mitochondrial preparations contained hexokinase and invertase, as well as the traces of endogenous adenylate mentioned previously.

Table IX

The Activity and Acceptor Ratios for Cabbage Mitochondria

The flask contents were as described in Table III, except that in some flasks components of the hexokinase system were omitted to obtain $Q_{O_2}(N)$ values in the absence of a phosphate acceptor. Citrate was used as the substrate. The values given are averages with the range in parentheses.

Number of Experiments	$Q_{O_2}(N)$ with acceptor system	$Q_{O_2}(N)$ without complete acceptor system	P:O	Acceptor Ratio
1	771	392[a]	2.16	1.97
8	893 (720-1160)	324[b] (262-382)	2.03 (1.77-2.37)	2.72 (2.38-3.23)
7	998 (771-1174)	314[c] (248-390)	2.16 (1.77-2.43)	3.21 (2.54-3.95)
4	1029 (773-1174)	147[d] (84-178)	2.05 (1.77-2.43)	7.30 (6.60-9.20)
3	774 (720-830)	111[e] (90-122)	2.01 (1.77-2.16)	7.18 (5.90-9.22)

[a] $Q_{O_2}(N)$ values obtained in the absence of ATP.

[b] $Q_{O_2}(N)$ values obtained in the absence of glucose and hexokinase.

[c] $Q_{O_2}(N)$ values obtained in the absence of glucose, hexokinase and ATP.

[d] $Q_{O_2}(N)$ values obtained in the absence of glucose, hexokinase, ATP and Mg^{++}.

[e] $Q_{O_2}(N)$ values obtained in the absence of glucose, hexokinase and Mg^{++}.

Since Mg^{++} is a cofactor for the hexokinase system it was used as a variant with glucose, hexokinase and ATP. Acceptor ratios as high as 9.0 were obtained when glucose, hexokinase, ATP and Mg^{++} were omitted and the respiratory rate was compared to that in a complete system. Similar ratios were obtained by omitting glucose, hexokinase, and Mg^{++} but including ATP in the system (Table IX).

To summarize, acceptor ratios between 2.0 and 9.0 were obtained with the cabbage mitochondria by comparing the rates found with, and without, various components of the acceptor system. These results, therefore, would indicate that respiration was tightly coupled with phosphorylation in these mitochondria. The cabbage mitochondria also showed high respiratory activity as reported previously by Freebairn and Remmert (25, p. 259-266).

Release of Respiration by 2,4-D: The 'classical' uncoupling agent, DNP, has been shown to have five different effects on fresh mammalian mitochondria, as mentioned in the "Introduction" of this thesis. One of these effects is that DNP 'releases' respiration in mammalian mitochondria. 'Release' of respiration is the property of permitting the respiration to proceed without the presence or participation of a phosphate acceptor (ADP). It

is believed that the release of respiration by DNP is due to activation of the breakdown of a non-phosphate containing intermediate in the sequence of reactions involved in the synthesis of ATP (61, p. 1-5).

As shown in Part II, 2,4-D and many other herbicides inhibit oxidative phosphorylation in the cabbage mitochondria. Because 2,4-D has been used extensively as a herbicide, many studies were made to elucidate the mode of its action on oxidative phosphorylation. If the effect of 2,4-D in the cabbage mitochondria was similar to the uncoupling effect of DNP in mammalian mitochondria, then 2,4-D should be able to release respiration in the cabbage preparations. However, 2,4-D at the concentrations tested (5×10^{-4} M to 1×10^{-2} M) did not release respiration in the absence of an acceptor system (glucose, hexokinase and ATP). In fact, 2,4-D did not release respiration in such a system even in the presence of added ATP. These results suggested that the effect of 2,4-D in the cabbage mitochondria might be different from the uncoupling effect of DNP in mammalian mitochondria.

If DNP would release respiration in the cabbage mitochondria, then it would be interesting to find the effects of 2,4-D on this uncoupled respiration. Studies of this type might help to determine the site of action of 2,4-D on oxidative phosphorylation. It was very important,

therefore, to study DNP and other 'classical' uncoupling agents, such as PCP and gramicidin, for their effects on oxidative phosphorylation and for their ability to release respiration in the cabbage mitochondria.

The Effects of 'Classical' Uncoupling Agents on Oxidative Phosphorylation in the Cabbage Mitochondria: DNP has been shown to uncouple oxidative phosphorylation in plant particulate preparations from skunk cabbage (30, p. 27-32), mung beans (7, p. 135-148), soybeans (64, p. 89), sweet potatoes (45, p. 420-424) and castor beans (1, p. 115-118).

PCP, which has been used as a selective herbicide in certain crops, has been shown to uncouple oxidative phosphorylation in mammalian mitochondrial systems (70, p. 393-397). Its effects on plant mitochondrial systems have not been tested previously.

The effects of DNP and PCP on oxidative phosphorylation in the cabbage mitochondria are summarized in Table X. DNP at 1×10^{-4} M decreased the P:O ratio by 44%; at higher concentrations, such as 1×10^{-3} M, DNP inhibited phosphorylation and respiration by 100% and 60%, respectively. PCP at 3×10^{-5} M inhibited phosphorylation completely, with some lowering of respiration. It may be seen that the concentration required to inhibit phosphorylation completely was much lower for PCP (3×10^{-5} M) than for DNP

Table X

The Effects of "Classical Uncouplers" on Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were as described in Table III, with citrate as the substrate. DNP was dissolved in water and adjusted to pH 7.4. PCP was dissolved in water at pH 9.4. The solution was slightly turbid. The values are averages from duplicate flasks.

Chemical	Concentration tested	O_2 uptake ug.atom/hr./mg.N	Pi esterified umole/hr./mg.N	P:O	Percent of Control O_2 uptake	Percent of Control Pi esterified	Percent of Control P:O
2,4-dinitrophenol (DNP)	0	70.2	127.7	1.82	100	100	100
	$1x10^{-5}$ M	66.6	121.5	1.82	95	95	100
	$1x10^{-4}$ M	60.2	60.6	1.01	86	48	56
	$1x10^{-3}$ M	28.8	0	0	41	0	0
	$1.7x10^{-3}$ M	20.4	0	0	29	0	0
Pentachlorophenol (PCP)	0	63.9	88.2	1.38	100	100	100
	$3x10^{-6}$ M	64.9	61.8	0.95	102	70	69
	$1x10^{-5}$ M	64.6	28.4	0.44	101	32	32
	$3x10^{-5}$ M	54.0	0	0	85	0	0
	$1x10^{-4}$ M	26.0	0	0	41	0	0
	$3x10^{-4}$ M	8.4	0	0	13	0	0
	$1x10^{-3}$ M	3.9	0	0	6	0	0
	$3x10^{-3}$ M	2.10	0	0	3	0	0

(1×10^{-3} M).

Gramicidin and thyroxine inhibit oxidative phosphorylation in mammalian mitochondria (54, p. 555-570; 66, p. 341-349). The effects of these chemicals on plant particulate preparations have not been reported previously. Gramicidin and L-thyroxine inhibited oxidative phosphorylation in the cabbage mitochondria (Table XI). Gramicidin at 1×10^{-4} M inhibited phosphorylation and oxygen uptake by 100% and 54%, respectively. L-Thyroxine at that concentration (1×10^{-4} M) inhibited oxygen uptake and phosphorylation by 50% and 85%, respectively.

Thus, in the cabbage preparations all of the 'classical' uncoupling agents tested had a greater inhibitory effect on phosphorylation than on respiration.

Release of Respiration by 'Classical' Uncoupling Agents: The classical uncoupling agents were studied for their ability to release respiration in the absence of a phosphate acceptor system. DNP did not release respiration in a system from which glucose, hexokinase, and ATP were omitted. ATP was found to be necessary in such a system for release of respiration by DNP, as shown in Table XII. It was found that small quantities of ATP, such as 0.1 and 1.0 umole, were not sufficient to permit release of respiration by DNP. However, in presence of 10 umoles ATP, DNP at 5×10^{-4} M gave a large increase in

Table XI

The Effects of L-Thyroxine and Gramicidin on Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were as described in Table III, with citrate as the substrate. L-Thyroxine was dissolved in dilute alkali. Gramicidin was dissolved in ethyl alcohol. The values are averages from duplicate flasks.

Chemical	Concentration tested	O_2 uptake ug.atom/hr./mg.N	Pi esterified umole/hr./mg.N	P:O	Percent of Control		
					O_2 uptake	Pi esterified	P:O
L-Thyroxine	0	65.9	123.2	1.87	100	100	100
	$1x10^{-6}$ M	64.7	129.2	2.00	98	105	107
	$1x10^{-5}$ M	63.3	117.3	1.85	96	95	99
	$5x10^{-5}$ M	54.5	86.3	1.58	83	70	85
	$1x10^{-4}$ M	32.8	18.8	0.57	50	15	31
	$1.66x10^{-4}$ M	16.7	8.1	0.49	25	7	26
Gramicidin	0	83.3	154.1	1.85	100	100	100
	$1x10^{-5}$ M	37.3	29.7	0.80	45	19	43
	$5x10^{-5}$ M	41.9	16.3	0.39	50	11	21
	$1x10^{-4}$ M	38.4	0	0	46	0	0

Table XII

The ATP Requirement for Release of Respiration by DNP

The flask contents were as described in Table III except that glucose and hexokinase were omitted and the ATP concentration was varied. The values are averages from duplicate flasks.

Concentration of ATP (in micromoles)	Presence of DNP (5×10^{-4} M)	O_2 uptake (microgram atoms)
0.1	-	21.5
0.1	+	17.6
1.0	-	21.5
1.0	+	22.5
10	-	21.2
10	+	44.1

respiration in a system without glucose and hexokinase. The amount of respiration, however, was not equal to that obtained in a complete system; the respiration induced by DNP was only 60% that found with the complete system. The requirement for ATP, for release of respiration by DNP, was also observed in cauliflower mitochondria (40, p. 199-214) and in insect mitochondria (29, p. 58). It may be that ATP is required to maintain the mitochondrial integrity (40, p. 199-214). In the case of cabbage mitochondria, however, the large amounts of ATP (10 umoles), required for release of respiration by DNP, suggested that the increase in respiration might be due to stimulation of ATPase activity. This would, of course, provide ADP to serve as a phosphate acceptor, and the release would then be only an apparent one.

Further evidence for actual release of respiration by DNP was gained through the use of NaF. Table XIII shows that DNP at 1×10^{-4} M increased respiration even in the presence of 0.02 M NaF. The increase of respiration by DNP was 80% that given by the hexokinase system in the presence of 0.02 M NaF. Laties (40, p. 199-214) has shown that NaF at 0.03 M inhibits ATPase activity in cauliflower mitochondria by 80%. These results would tend to reject the hypothesis that stimulation of ATPase activity by DNP explains the 'release' of respiration in cabbage

Table XIII

The Effect of DNP on Respiration in the Presence of Fluoride

The flask contents were as described in Table III, except that glucose and hexokinase were omitted. The values are averages from duplicate flasks.

Additions	Presence of 0.02 M NaF	O_2 uptake (microgram atoms)
none	-	18.1
none	+	14.7
DNP, 1×10^{-4} M	+	22.4
DNP, 3×10^{-4} M	+	14.8

mitochondria. It has not been possible to explain why large amounts of ATP, such as 10 μmoles, were required for the stimulation of respiration by DNP.

It may be emphasized that Mg^{++} also was required for release of respiration by DNP (Table XIV). In addition, it was found that the concentration of DNP was very critical; DNP at 1×10^{-5} M caused a slight release of respiration and a concentration of 1×10^{-4} M was required for a large increase. Respiration was inhibited by DNP at 1×10^{-3} M (Table XIV).

PCP was studied for its ability to release respiration in the cabbage mitochondria. Table XV shows that PCP at 1×10^{-5} M gave almost total release of respiration in a system from which glucose and hexokinase were omitted. At higher concentrations of PCP, the respiration was inhibited.

It has been suggested that in animal mitochondria the respiration released by DNP in the absence of a phosphate acceptor system is related to the ability to carry on oxidative phosphorylation (61, p. 1-5). Further investigations were made, therefore, to determine the effect of DNP on oxidative phosphorylation and in releasing respiration in fresh and aged cabbage mitochondria. The results are summarized in Table XVI. It was found that DNP uncoupled phosphorylation in fresh cabbage mitochondria; in

Table XIV

The Effect of DNP on Respiration in the Presence and Absence of Mg^{++}

The flask contents were as described in Table III except that glucose, hexokinase and Mg^{++} were omitted unless indicated. The values are averages from duplicate flasks.

Additions	Presence of Mg^{++} ($5x10^{-3}$ M)	O_2 uptake (microgram atoms)
none	-	5.2
DNP, $1x10^{-5}$ M	-	5.9
DNP, $1x10^{-4}$ M	-	5.8
DNP, $1x10^{-3}$ M	-	2.2
none	+	19.2
DNP, $1x10^{-5}$ M	+	21.8
DNP, $1x10^{-4}$ M	+	30.6
DNP, $1x10^{-3}$ M	+	9.9

Table XV

The Effect of Pentachlorophenol on Release of Respiration

The flask contents were as described in Table III, except glucose and hexokinase which were omitted unless indicated. The values are averages from duplicate flasks.

Additions	Concentration of Pentachlorophenol	O_2 uptake (microgram atoms)
glucose + hexokinase	-	43.8
none	-	18.4
none	1×10^{-6} M	19.7
none	3×10^{-6} M	25.6
none	1×10^{-5} M	35.9
none	3×10^{-5} M	26.6
none	1×10^{-4} M	9.0
none	3×10^{-4} M	4.3

Table XVI

The Effect of DNP on Oxidative Phosphorylation and Release of Respiration with Fresh and Aged Mitochondrial Preparations

The flask contents were as described in Table III, except that glucose and hexokinase were omitted except as indicated. The fresh mitochondrial preparation was kept in the refrigerator for 24 hours and was used as the aged mitochondrial preparation. The values are averages from duplicate flasks.

Additions	Concentration of DNP	O_2 uptake microgram atoms		P:O	
		Fresh	Aged	Fresh	Aged
glucose + hexokinase	-	39.2	15.8	1.97	0.16
glucose + hexokinase	3×10^{-5} M	40.6	14.5	1.53	0.10
glucose + hexokinase	1×10^{-4} M	37.1	12.9	1.18	0.03
none	-	16.3	4.4		
none	3×10^{-5} M	19.8	4.0		
none	1×10^{-4} M	25.8	4.3		

addition, DNP at 1 x 10^{-4} M released respiration in a system from which glucose and hexokinase were omitted. After aging for 24 hours, the cabbage mitochondria had lost, almost completely, their ability to catalyze oxidative phosphorylation. DNP did not release respiration in the aged preparation. These results may indicate a requirement of structural integrity for release of respiration in the cabbage mitochondria.

The Effect of 2,4-D on Released Respiration: If the respiration released by DNP is related to the process of oxidative phosphorylation in cabbage mitochondria, it would be appropriate to find the effect of 2,4-D on this released respiration. Such studies might enable us to locate the site of action of 2,4-D. The results given in Table XVII indicate that 2,4-D at 5 x 10^{-4} M did not have any effect on the respiration released by DNP at 1 x 10^{-4} M. However, 2,4-D at 5 x 10^{-3} M and at 1 x 10^{-2} M completely inhibited the respiration released by DNP. 2,4-D at 5 x 10^{-3} M showed similar inhibition of the respiration released by dicumarol (Table XVIII). These results may indicate that 2,4-D interferes with energy transfer reactions at a site preceding the point of action of DNP and dicumarol, or that 2,4-D combines with some component of the coupled reactions and prevents it from functioning normally.

Table XVII

The Effect of 2,4-D on the Release of Respiration by DNP

The flask contents were as described in Table III except that glucose and hexokinase were omitted. The values are averages from duplicate flasks.

Experiment Number	Additions	Presence of DNP ($1x10^{-4}$ M)	O_2 uptake (microgram atoms)
1	none	-	15.5
	none	+	23.5
	+ 2,4-D-$5x10^{-4}$ M	+	24.0
	+ 2,4-D-$5x10^{-3}$ M	+	4.2
2	none	-	14.0
	+ 2,4-D $1x10^{-2}$ M	-	6.4
	none	+	22.1
	+ 2,4-D $1x10^{-2}$ M	+	5.2

Table XVIII

The Effect of 2,4-D on Release of Respiration by Dicumarol

The flask contents were as described in Table III, except that glucose and hexokinase were omitted. The values are averages from duplicate flasks.

Concentration of Dicumarol	Presence of 2,4-D $5x10^{-3}$ M	O_2 uptake (microgram atoms)
-	-	19.6
-	+	17.3
$5x10^{-6}$ M	-	24.2
$5x10^{-6}$ M	+	18.7
$1x10^{-5}$ M	-	29.2
$1x10^{-5}$ M	+	17.2
$1x10^{-4}$ M	-	18.8
$1x10^{-4}$ M	+	11.0

Part IV

ATPase Activity and ATP-P^{32} Exchange

To extend further our knowledge of the mechanism of action of 2,4-D on oxidative phosphorylation, its effects on the ATPase reaction and ATP-P^{32} exchange were investigated.

ATPase Activity: DNP and other uncoupling agents such as PCP, dicumarol and gramicidin stimulate the ATPase activity in digitonin extracts of rat liver mitochondria (18, p. 547-560). As shown earlier, DNP, PCP and dicumarol uncoupled oxidative phosphorylation and released respiration in the cabbage mitochondria. It has been suggested that in mammalian mitochondria the stimulation of ATPase by DNP is due to the reversal of reactions (4) and (3), followed by breakdown of the intermediate $A \sim C$ as presented in the Introduction. Therefore, this DNP-stimulated ATPase has been considered to represent a part of the energy transfer reactions in oxidative phosphorylation. Hence, study of the effect of 2,4-D on the DNP-stimulated ATPase might throw some light on our knowledge of the mechanism by which 2,4-D inhibits oxidative phosphorylation.

It should be mentioned that in early studies the effects of DNP on cabbage mitochondrial ATPase were investigated at two pH values, namely 6.5 and 7.4. It was found

that DNP (3×10^{-6} M to 1×10^{-3} M) did not stimulate the ATPase activity at either of these pH values. In later experiments, the ATPase studies were made generally at pH 7.4, since this was the pH of the medium used for measuring oxidative phosphorylation.

Earlier observations on oxidative phosphorylation and release of respiration in the cabbage mitochondria indicated that these preparations had a low content of endogenous Mg^{++}. Hence concentration studies were made in relation to the effects of Mg^{++} and DNP on the ATPase activity in the cabbage mitochondria (Tables XIX and XX). It was found that cabbage mitochondrial ATPase activity was stimulated by increasing the concentration of Mg^{++}. DNP (3×10^{-6} M to 1×10^{-4} M) did not stimulate ATPase activity either in the presence or in the absence of added Mg^{++}. On the other hand, DNP at 1×10^{-4} M inhibited the Mg^{++}-stimulated ATPase activity to some extent (Table XIX). All attempts to demonstrate ATPase stimulation by DNP, in cabbage mitochondria, failed.

Laties (40, p. 199-214) also was not able to show the ATPase stimulation by DNP, in cauliflower mitochondria. There is only one report that DNP stimulates ATPase in plant mitochondrial preparations. Forti (22, p. 898-909) showed that DNP at 3×10^{-5} M in the presence of Mg^{++} at 9×10^{-3} M and phosphate at 1×10^{-3} M stimulated the

Table XIX

The Effect of Mg^{++} and DNP
on the ATPase of Cabbage Mitochondria

Each flask contained 30 micromoles ATP, pH 6.5; 25 micromoles Tris-maleate buffer, pH 6.5; 1.0 ml. of mitochondrial preparation in 0.25 M sucrose; additions as indicated and glass distilled water to a final volume of 3.0 ml. Incubated 30 minutes at 30° C. The values are averages from duplicate flasks.

Concentration of Mg^{++}	Presence of DNP ($1x10^{-4}$ M)	Pi released micromoles
-	-	2.27
-	+	1.95
$1x10^{-6}$ M	-	2.22
$1x10^{-6}$ M	+	1.94
$1x10^{-5}$ M	-	2.24
$1x10^{-5}$ M	+	1.90
$1x10^{-4}$ M	-	2.60
$1x10^{-4}$ M	+	1.99
$1x10^{-3}$ M	-	4.84
$1x10^{-3}$ M	+	3.09

Table XX

Concentration Study of Mg^{++} and DNP for Their Effect on the ATPase of Cabbage Mitochondria

The flask contents were as described in Table XIX. The values are averages from duplicate flasks.

Concentration of Mg^{++}	Concentration of DNP	Pi released micromoles
-	-	0.55
$2x10^{-4}$ M	-	0.72
$2x10^{-4}$ M	$3x10^{-6}$ M	0.68
$2x10^{-4}$ M	$1x10^{-5}$ M	0.67
$2x10^{-4}$ M	$3x10^{-5}$ M	0.68
$2x10^{-4}$ M	$1x10^{-4}$ M	0.67
$1x10^{-2}$ M	-	1.98
$1x10^{-2}$ M	$1x10^{-4}$ M	1.93

ATPase activity in pea mitochondria. Forti suggested that the ATPase activity in pea mitochondria was similar to the ATPase in rat liver mitochondria. However, the ATPase stimulation by DNP, observed with pea mitochondria, was not appreciable (90%). In rat liver mitochondria, the ATPase stimulation by DNP has been shown to be many fold (39, p. 357-370; 57, p. 893-908).

Other uncoupling agents such as PCP, dicumarol and gramicidin were studied for their effects on ATPase in the cabbage mitochondria. These uncoupling agents did not stimulate cabbage mitochondrial ATPase at all, even in the presence of added Mg^{++}. These results may or may not indicate that the mechanism of oxidative phosphorylation is somewhat different in cabbage than it is in mammalian mitochondria.

Pressman and Lardy have reported that saturated free fatty acids such as myristic acid, palmitic acid and stearic acid stimulate the ATPase in rat liver mitochondria (59, p. 458-466). These authors also found that cis-isomers of unsaturated fatty acids enhance the ATPase activity, whereas trans-isomers have no effect.

Oleic acid has been studied extensively in mammalian mitochondrial systems. It has been found to uncouple oxidative phosphorylation, stimulate the ATPase activity, inhibit the ATP-P^{32} exchange reaction and release

respiration in rat liver mitochondria (44, p. 2459-2464). As previously mentioned, DNP also exhibits the same effects in mammalian mitochondrial preparations. On the basis of these results, it may be speculated that DNP and oleic acid have the same point of action on oxidative phosphorylation in mammalian mitochondria. Recently oleic acid has been shown to be a potent inhibitor of both the Hill reaction (36, p. 421-430) and oxidative photosynthetic phosphorylation (37, p. 2205-2210). It was considered pertinent, therefore, to determine whether or not oleic acid would stimulate the ATPase and uncouple oxidative phosphorylation in the cabbage mitochondria.

Table XXI shows the effects of oleic acid on oxidative phosphorylation in the cabbage mitochondria. Oleic acid at 3×10^{-4} M was found to inhibit phosphorylation and oxygen uptake by 100% and 33%, respectively.

In the cabbage mitochondria, oleic acid did not release respiration in a system containing ATP but no added glucose or hexokinase. In this respect, oleic acid differed from DNP which, in the presence of ATP, did stimulate respiration (see Table XII).

Stimulation of the ATPase by Oleic Acid: It was found that oleic acid stimulated the cabbage mitochondrial ATPase only in the presence of added Mg^{++}. The results of a typical experiment are presented in Table XXII. In the

Table XXI

The Effect of Oleic Acid on Respiration and Oxidative Phosphorylation in Cabbage Mitochondria

The flask contents were as described in Table III, except that the phosphate concentration was .0313 M. Citrate was used as the substrate. The oleic acid was 50% neutralized before addition. The incubation period was 45 minutes at 30° C. The concentration of mitochondrial nitrogen was 0.37 mg./ml. The values are averages from duplicate flasks.

Concentration of Oleic Acid	O_2 uptake ug.atoms/hr./mg.N	Pi esterified umole/hr./mg.N	P:O	Percent of Control		
				O_2 uptake	Pi esterified	P:O
0	117.1	219.8	1.88	100	100	100
3×10^{-6} M	125.7	248.2	1.98	107	113	105
1×10^{-5} M	128.3	249.3	1.94	110	113	103
3×10^{-5} M	119.6	234.9	1.96	102	107	104
1×10^{-4} M	108.1	139.1	1.29	92	63	69
3×10^{-4} M	78.2	0	0	67	0	0
1×10^{-3} M	18.4	0	0	16	0	0
3×10^{-3} M	4.4	0	0	4	0	0

Table XXII

The Effects of Mg^{++} and Oleic Acid on the ATPase of Cabbage Mitochondria

Each flask contained Tris-maleate Buffer, pH 7.4, 0.1 M; ATP, pH 7.4, 1 x 10^{-2} M; 1.0 ml. of mitochondrial preparation in 0.25 M Sucrose; additions as indicated and glass distilled water to a final volume of 3.0 ml. Incubated 30 minutes at 30° C. The values are averages from duplicate flasks.

Concentration of Oleic Acid	Presence of Mg^{++} ($1x10^{-3}$ M)	Pi released micromoles
-	-	0.67
$1x10^{-3}$ M	-	0.80
$3x10^{-3}$ M	-	0.70
-	+	1.04
$1x10^{-3}$ M	+	3.09
$3x10^{-3}$ M	+	3.86

presence of Mg^{++}, oleic acid at 1×10^{-3} M stimulated the ATPase activity almost threefold. The stimulation was increased to fourfold with 3×10^{-3} M oleic acid.

There was no appreciable change in the pH of the incubation medium when oleic acid was added in the ATPase studies; it is not likely, therefore, that the stimulation by oleic acid was due to a change in the pH of the incubation medium. This may be substantiated further by the results of experiments concerning the effects of Mg^{++} and oleic acid on cabbage mitochondrial ATPase, at pH values ranging between 5.0 and 8.0. Figure 1 shows that in the presence of Mg^{++} at 5×10^{-3} M, the ATPase activity increased gradually as the pH was increased from 5.0 to 8.0. In the presence of Mg^{++}, the oleate-stimulated ATPase showed a sharp increase as the pH was increased between 7.0 and 8.0. Most of the work done on the ATPase activity was carried out at pH 7.4. At this pH, oleic acid stimulated the ATPase activity threefold or more.

Mazelis (48, p. 153-158) reported apyrase activity (an enzyme which hydrolyzes ATP to form AMP and orthophosphate) in cytoplasmic preparations from cabbage. For these studies, Mazelis used a preparation which consisted of a mixture of mitochondria and microsomes. Using the technique of paper chromatography, he found that his preparations converted ATP to AMP and products of further hydrolysis. Mazelis concluded that his results could not be explained by the combined action of an ATPase and adenylic kinase, because

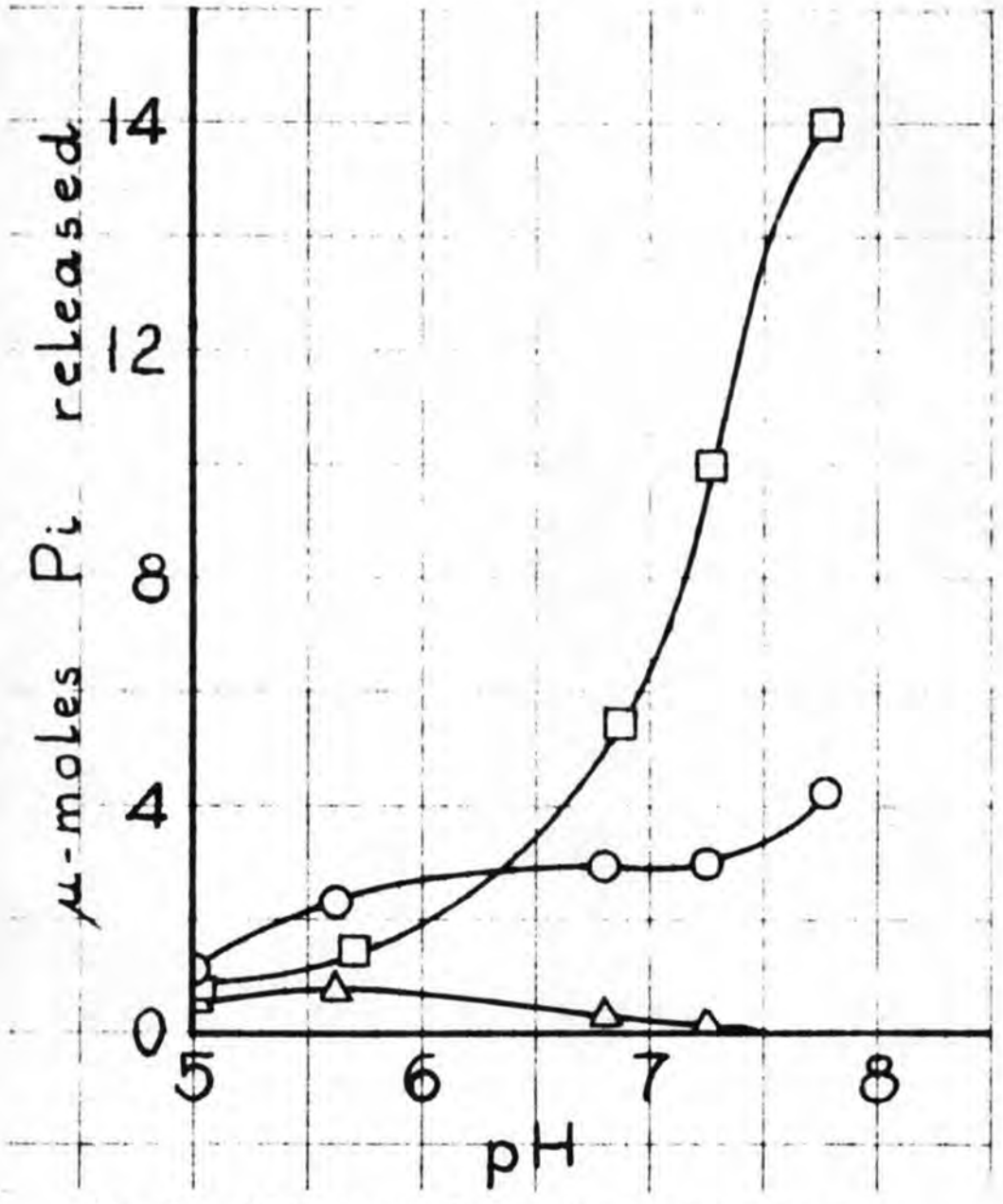

Fig. 1. Effect of pH on the ATPase activity in cabbage mitochondria. Each flask contained 0.01M ATP, 0.1M tris-acetate buffer at pH indicated, 0.083M sucrose, 0.12 mg. of mitochondrial nitrogen, other additions as indicated and glass-distilled water to a final volume of 3.0 ml. Incubated 30 minutes at 30° C. Control, △—△; control + Mg^{++} (5×10^{-3} M), O—O; control + Mg^{++} (5×10^{-3} M) + oleic acid (3×10^{-3} M), □—□.

he was unable to demonstrate ATP on a chromatogram after using ADP as the substrate. However, it is possible that the adenylic kinase reaction rate was slow as compared to the ATPase activity. If this were the case, with ADP as substrate ATP might not accumulate even in the presence of adenylic kinase. As soon as ATP and AMP were formed by adenylic kinase, the ATP might have been converted to ADP by the ATPase system. Therefore, the failure to demonstrate the presence of ATP on a chromatogram after using ADP as the substrate does not exclude the possibility of the presence of adenylic kinase in cabbage preparations.

The author is not aware of any specific inhibitor which would block either ATPase or adenylic kinase activity without appreciably affecting the other. Hence, detailed studies on this question were not carried out with the cabbage mitochondrial preparations. However, investigations were made of the cabbage mitochondrial phosphatase activity with AMP, ADP and ATP as substrates. Table XXIII shows that oleic acid had an appreciably greater effect on the hydralysis of ATP than on the hydrolysis of the other two adenylates. These results indicate, therefore, that the major effect of oleic acid was to stimulate ATPase activity.

It was demonstrated also that the ATPase of cabbage mitochondria could be stimulated by saturated fatty acids such as myristic acid and stearic acid (Table XXIV). It

Table XXIII

The Effects of Mg^{++} and Oleic Acid on Mitochondrial Phosphatase Activity with ATP, ADP, and AMP as Substrates

The flask contents were as described in Table XXII except that ATP was replaced by AMP or ADP where indicated.

Substrate	Presence of Mg^{++} $5x10^{-3}$ M	Presence of Oleic Acid $5x10^{-3}$ M	Pi released micromoles
AMP	-	-	0.46
AMP	+	-	0.57
AMP	+	+	0.56
ADP	-	-	1.08
ADP	+	-	1.93
ADP	+	+	3.27
ATP	-	-	0.69
ATP	+	-	3.07
ATP	+	+	8.30

Table XXIV

The Effects of Saturated Fatty Acids on the ATPase of Cabbage Mitochondria

Each flask contained Tris buffer, pH 7.4, 0.066 M; ATP, pH 7.4, 0.01 M; $MgCl_2$, 1 x 10^{-3} M; sucrose, 0.39 M; mitochondrial preparation, 0.56 mg.N/ml; additions as indicated and glass distilled water to a final volume of 3.0 ml. Incubated 90 minutes at 30° C. The values are averages from duplicate flasks.

Concentration of Stearic Acid	Concentration of Myristic Acid	Pi released micromoles
-	-	3.00
$1x10^{-5}$ M	-	3.00
$1x10^{-4}$ M	-	3.58
$3x10^{-4}$ M	-	4.50
$1x10^{-3}$ M	-	5.86
$3x10^{-3}$ M	-	8.69
-	$1x10^{-5}$ M	3.15
-	$1x10^{-4}$ M	4.16
-	$3x10^{-4}$ M	7.78
-	$1x10^{-3}$ M	19.19
-	$3x10^{-3}$ M	20.36

was found that myristic acid stimulated the ATPase activity much more than did stearic acid at the same concentration (1×10^{-3} M). Similar results were obtained with ATPase activity in rat liver mitochondria (59, p. 458-466).

As mentioned earlier, oleic acid and DNP show similar effects on oxidative phosphorylation in rat liver mitochondria (44, p. 2459-2464; 10, p. 369-377). According to Lehninger's hypothesis of the energy transfer mechanism in oxidative phosphorylation, DNP-stimulated ATPase represents a part of the process of oxidative phosphorylation in mammalian mitochondria (19, p. 561-578). Hence, it is assumed tentatively that the oleate-stimulated ATPase also involves reactions which are part of phosphorylation. Since DNP did not stimulate ATPase in the cabbage preparations, the effects of 2,4-D on the oleate-stimulated ATPase were investigated, to extend further our knowledge on the mode of action of 2,4-D.

Tables XXV and XXVI show that 2,4-D, at any concentrations studied (1×10^{-4} M to 1×10^{-2} M), did not have any effect on Mg^{++}-stimulated ATPase in the cabbage mitochondria. 2,4-D at 1×10^{-2} M inhibited oleate-stimulated ATPase to a large extent. These results probably indicate that 2,4-D interferes with the energy transfer mechanism in oxidative phosphorylation in the cabbage mitochondria.

<u>ATP-P^{32} Exchange</u>: As mentioned in the Introduction

Table XXV

The Effect of 2,4-D on the ATPase Stimulated by Mg^{++} and Oleic Acid

The flask contents were as described in Table XXII. The 2,4-D was dissolved in Tris-maleate Buffer, pH 7.4. The values are averages from duplicate flasks.

Concentration of Mg^{++}	Presence of 2,4-D ($1x10^{-2}$ M)	Presence of Oleic Acid ($3x10^{-3}$ M)	Pi released micromoles
-	-	-	0.81
-	+	-	0.84
$1x10^{-3}$ M	-	-	1.80
$1x10^{-3}$ M	+	-	1.86
$1x10^{-3}$ M	-	+	5.42
$1x10^{-3}$ M	+	+	2.30
$1x10^{-2}$ M	-	-	3.78
$1x10^{-2}$ M	+	-	4.02
$1x10^{-2}$ M	-	+	10.29
$1x10^{-2}$ M	+	+	5.30

Table XXVI

The Effect of 2,4-D on ATPase Stimulated by Mg^{++} and Oleic Acid

The flask contents were as described in Table XXII. The values are averages from duplicate flasks.

Concentration of 2,4-D	Presence of Mg^{++} ($5x10^{-3}$ M)	Presence of Oleic Acid ($3x10^{-3}$ M)	Pi released micromoles
-	-	-	1.20
-	-	+	1.11
-	+	-	4.21
-	+	+	13.02
$1x10^{-4}$ M	+	-	4.15
$1x10^{-4}$ M	+	+	10.28
$1x10^{-3}$ M	+	-	4.06
$1x10^{-3}$ M	+	+	10.08
$1x10^{-2}$ M	+	-	3.92
$1x10^{-2}$ M	+	+	6.22

of this thesis, it has been suggested that the ATP-P^{32} exchange also represents a part of the process of oxidative phosphorylation in mammalian mitochondria. There are no reports available on this exchange reaction in plant mitochondrial preparations.

Preliminary studies indicated that Mg^{++} was required for the ATP-P^{32} exchange reaction in the cabbage mitochondria (Table XXVII). The exchange rate was increased many fold in the presence of Mg^{++} (1×10^{-3} M to 1×10^{-2} M).

In order to acquire further information on the mode of action of 2,4-D, its effects on the ATP-P^{32} exchange were investigated. The results of a typical experiment are given in Table XXVIII. It was found that 2,4-D at low concentrations, such as 1×10^{-5} M and 1×10^{-4} M, increased the exchange almost two fold. 2,4-D at 1×10^{-2} M inhibited the exchange almost completely.

The stimulation of the ATP-P^{32} exchange at low concentrations of 2,4-D may be related to the stimulation of respiration in plant tissues when they are treated with low concentrations of 2,4-D (1×10^{-5} M to 1×10^{-6} M). However, it was not possible in the present studies to demonstrate a stimulation of respiration in cabbage mitochondrial preparations by the addition of low concentrations of 2,4-D. This failure to obtain the stimulation of respiration may have been due to the many differences

Table XXVII

The Dependence of the ATP-P^{32} Exchange on Magnesium Concentration

The flask contents were as described in Table XXII except that P^{32} equivalent to about 10^6 counts per minute was added. Incubated 30 minutes at 30° C. The values are averages from duplicate flasks.

Concentration of Mg^{++}	ATP-P^{32} Exchange millimicromoles
-	1.6
1×10^{-5} M	2.2
1×10^{-4} M	2.8
1×10^{-3} M	57.7
1×10^{-2} M	185.0

Table XXVIII

The Effect of 2,4-D on the ATP-P^{32} Exchange

The flask contents were as described in Table XXVII except that all the flasks contained $MgCl_2$, 5 x 10^{-3} M. The values are averages from duplicate flasks.

Concentration of 2,4-D	ATP-P^{32} Exchange millimicromoles
-	72.6
$1x10^{-5}$ M	138.0
$1x10^{-4}$ M	124.9
$1x10^{-3}$ M	46.5
$1x10^{-2}$ M	2.5

between the composition of the incubation medium and that of the cytoplasm of plant cells.

DNP and other uncoupling agents such as PCP, dicumarol, and gramicidin have been shown to inhibit the ATP-P^{32} exchange in mammalian mitochondrial systems (19, p. 561-578). Oleic acid also has been reported to inhibit this exchange in mammalian mitochondria (44, p. 2459-2464). In the present studies, it was found that oleic acid and DNP inhibited the ATP-P^{32} exchange in the cabbage mitochondria (Table XXIX).

The ATP-ADP exchange also has been suggested to be a part of the process of oxidative phosphorylation (68, p. 1589-1597). This exchange, however, was not studied in the cabbage mitochondria.

In the present studies, some of the effects shown in the cabbage mitochondria by oleic acid and DNP are similar to those found in mammalian mitochondria. Thus, oleic acid and DNP inhibit oxidative phosphorylation and the ATP-P^{32} exchange in both cabbage and mammalian mitochondria. In addition, the stimulation of the ATPase by oleic acid is similar in cabbage and mammalian mitochondria. DNP, however, did not stimulate ATPase in the cabbage mitochondria. The effects of DNP and oleic acid on respiration in the cabbage mitochondria are different from those observed in mammalian systems. Thus, neither DNP nor oleic acid

Table XXIX

The Inhibition of the ATP-P^{32} Exchange by DNP and Oleic Acid

The flask contents were as described in Table XXVII. The values are averages from duplicate flasks.

Conditions	ATP-P^{32} Exchange millimicromoles
Control	72.6
control + Oleic Acid, $1x10^{-3}$ M	3.3
control + DNP, $1x10^{-3}$ M	1.6

showed all of the four effects, in the cabbage mitochondria, which have been observed in animal mitochondria.

On the basis of the results with mammalian mitochondria, it may be assumed tentatively that DNP and oleic acid act at the same point in the phosphorylation process. This would mean that oleic acid may stimulate the breakdown of the intermediate $A \sim C$, formed during the energy transfer process presented in the Introduction. This point of action of oleic acid would account for its inhibition of oxidative phosphorylation and the ATP-P^{32} exchange, and its stimulation of ATPase in the cabbage mitochondria.

The observed effects of Mg^{++}, DNP, oleic acid and 2,4-D may be related to the schematic mechanism for the coupling of phosphorylation to electron transport presented in the Introduction, as follows:

(1) $AH_2 + C \longleftrightarrow AH_2 - C$

(2) $AH_2 - C + B \longleftrightarrow A \sim C + BH_2$

(3) $A \sim C + Pi \overset{2,4\text{-D}}{\longleftrightarrow} A + P \sim C$

(3') $P \sim C + Y \longleftrightarrow P \sim Y + C$

(4') $P \sim Y + ADP \underset{Mg^{++}}{\longleftrightarrow} ATP + Y$

(5) $A \sim C \xrightarrow[\text{Oleic acid}]{\text{DNP}} A + C$

(6) $P \sim C \xrightarrow{Mg^{++}} P + C$

It may be speculated that 2,4-D appears to inhibit reaction (3) of the sequence shown above. This point of action of 2,4-D would account for its inhibition of oxidative phosphorylation, of the oleate-stimulated ATPase and of the ATP-P^{32} exchange in cabbage mitochondria. It is possible that at very low concentrations of 2,4-D (1×10^{-6} M to 1×10^{-5} M), there may be stimulation instead of inhibition of reaction (3). This would explain the stimulation of the exchange observed with very low concentrations of 2,4-D. Inhibition of reaction (3) only, by higher concentrations of 2,4-D, would be consistent with the insensitivity of the Mg^{++}-stimulated ATPase, if this ATPase were as depicted above. However, 2,4-D inhibition of the respiration released by DNP in the presence of ATP cannot be explained by inhibition of reaction (3).

In the present studies, some of the effects of oleic acid and DNP cannot be explained satisfactorily. Thus, the failure of DNP to release respiration in the absence of a phosphate acceptor in cabbage mitochondria is contrary to the mode of action postulated above. If DNP stimulates the breakdown of the intermediate $A \sim C$, then it is obvious that it should release respiration in the absence of a phosphate acceptor. However, it was found that DNP stimulates respiration only in the presence of ATP. In addition, the scheme given above fails to account for the inability

of DNP to stimulate ATPase activity in cabbage mitochondria. In a similar manner, the failure of oleic acid to release respiration, in the presence or absence of a phosphate acceptor, cannot be rationalized at present with its stimulation of ATPase and its inhibition of the ATP-P^{32} exchange. These results, which appear to be contradictory, may indicate that the mechanism of oxidative phosphorylation in cabbage mitochondria differs somewhat from that involved in mammalian mitochondria.

The effects of DNP and oleic acid should be reinvestigated using submitochondrial fragments, because the mitochondrial membrane might have been involved in some of the contradictory results obtained in this study. Use of submitochondrial fragments might reveal more consistent information about the coupling mechanism in plant mitochondria.

Studies on the ATP-ADP exchange and the effects of 2,4-D on it would enable us to learn more about the mode of action of 2,4-D. The effects of other herbicides may be studied in a similar manner. These investigations may help us to explain the action of herbicides on oxidative phosphorylation.

In the present studies, the results concerning the effects of 2,4-D <u>in vitro</u> are not by any means complete. It would be premature, therefore, to speculate about any

relationship between the effects of 2,4-D *in vitro* and *in vivo*. Oxidative phosphorylation is a vital process in the economy of plant cells because a large portion of the energy is liberated by this process during the catabolism of foodstuffs. It is possible that 2,4-D may exert its toxic effects in plants primarily by interfering with this vital process, and this possibility should be investigated further.

SUMMARY

The data presented indicate that ADP and Mg^{++} are required for optimal respiration and phosphorylation in cabbage mitochondria. Acceptor ratios between 2.0 and 9.0 were obtained by comparison of respiratory rates in the presence and in the absence of individual components of the system used to provide ADP. These results indicate that respiration in cabbage mitochondria is controlled by the availability of ADP, which serves as the phosphate acceptor.

A number of herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D), γ-(2,4-dichlorophenoxybutyric acid), isopropyl-N-phenyl carbamate and N-napthylphthalamic acid, cause greater inhibition of phosphorylation than of respiration, suggesting that these compounds may interfere with reactions which couple respiration and phosphorylation. Some herbicides, such as 3-amino-1,2,4-triazole, maleic hydrazide and sodium chlorate, do not have any appreciable effect on oxidative phosphorylation.

Using cabbage mitochondria, the uncoupling agent, 2,4-dinitrophenol (DNP), was found to cause some but not all of the effects which this agent produces in mitochondria from mammalian tissues. DNP has a greater inhibitory effect on phosphorylation than on respiration. It does

not increase respiration in the cabbage mitochondria in the absence of a phosphate acceptor; however, a DNP stimulation can be obtained in the presence of ATP. DNP does not stimulate ATPase activity in the cabbage mitochondria; it does, however, inhibit the ATP-P^{32} exchange reaction. Similar results are obtained when other uncoupling agents, such as pentachlorophenol, dicumarol and gramicidin, are added to the cabbage mitochondria.

Oleic acid was found to uncouple oxidative phosphorylation in cabbage mitochondria as it does in mammalian mitochondria. However, oleic acid does not increase respiration in cabbage mitochondria, either in the presence or in the absence of ATP. Oleic acid does stimulate the ATPase activity and inhibit the ATP-P^{32} exchange reaction in cabbage mitochondria.

2,4-D was found to inhibit oxidative phosphorylation, the oleate-stimulated ATPase and the ATP-P^{32} exchange reaction in cabbage mitochondria. However, 2,4-D does not affect the Mg^{++}-stimulated ATPase. These results have permitted speculation on the probable site at which 2,4-D acts on oxidative phosphorylation. However, an effect at the site proposed would not explain satisfactorily the fact that 2,4-D inhibits the respiration which DNP stimulates in these preparations.

Some of the results reported appear to be contradictory and may indicate that the mechanism of oxidative phosphorylation in cabbage mitochondria differs somewhat from that involved in mammalian mitochondria.

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