OXIDATIVE PHOSPHORYLATION IN COTYLEDONS
OF PISUM SATIVUM, VAR. ALASKA

DISSERTATION
Presented in Partial Fulfillment of the Requirements
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Graduate School of The Ohio State
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By

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The Ohio State University
1957

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Adviser
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I INTRODUCTION

In the metabolism of the germinating seed, synthetic processes are primarily associated with the development of the embryo. Just as with any other growing tissue, synthetic, along with many degradative processes take place in the growing embryo. The storage tissue on the other hand has until recently been considered merely a reserve of nutrients for the seedling, in which only degradation occurs during germination. Such reactions as hydrolyses of the reserve proteins, fats and starch have long been accepted as part of the germination process associated with the storage tissue. Synthesis was hardly considered to occur until very recently.

Synthetic processes, particularly those concerned with the formation of larger or more complex molecules, require energy. If synthesis is to take place in the storage tissue of germinating seeds, then the question of energy source or availability arises. Does it rely on the sprouting embryo or is the storage tissue a self sustaining tissue?

Metabolic energy utilized by biological systems, according to present day knowledge, comes either directly or indirectly from ATP (adenosinetriphosphate).
While a small amount is generated through glycolysis, the principal source of ATP in aerobic organisms is oxidative phosphorylation in mitochondria. Mitochondrial oxidative phosphorylation in the storage tissue of seeds is therefore the subject of this investigation. The seed of *Pisum sativum* var. Alaska was selected as the source of material. The effect of germination on the mitochondrial activity of the cotyledon was studied and the cotyledon cultured alone was compared with the cotyledon of the germinated seed.

The proteolytic and other hydrolytic action taking place in the storage tissue of seeds cultured alone or as part of the seedling naturally results in a gradual deterioration of the storage tissue. Any active mitochondria present must therefore also lose their functional capacity. This loss of mitochondrial activity, particularly in the initial stages, was also investigated.
II REVIEW OF LITERATURE

The dependency of phosphorylation on a respiring system was perhaps first observed by Engelhardt (15) in 1930, when he showed that regeneration of organic pyrophosphate compounds in pigeon erythrocytes is dependent upon oxygen consumption. In 1939 Kalckar (24) and Belitzer et al. (5) separately reported that addition of various Kreb's cycle acids simultaneously increased oxygen uptake and phosphate esterification by kidney extracts and minced heart muscle respectively. The concept that phosphorylation is associated with electron transfer as well as with substrate oxidation was established by Ochoa (38) and by Belitzer et al. (5). This represented a major step in helping to understand the nature of coupled oxidation and phosphorylation. However, it was not until 1949 that Cross et al. (12), Friedkin et al. (17), Lehninger (30), and Hunter (19) demonstrated that phosphorylation coupled to electron transport is confined to an insoluble cellular particle, the mitochondrion. The heretofore mentioned references point out only the highlights in the development of the work on mitochondrial oxidative phosphorylation, which was until recently confined to animal tissue. The complete list of references
on the early developments are too numerous to mention in this dissertation, but are amply covered in various reviews (20, 26, 33, 48).

Although cytologists have been aware of the existence of cytoplasmic particles in plant tissue since 1904, it was not until 1938 that Sorokin (45) developed the staining method for distinguishing the mitochondria from other morphologically similar plastids. Various investigators subsequently isolated insoluble particulates which had the ability to oxidize certain Kreb's cycle intermediates, but it was not until 1951 that Millerd et al. (35) first demonstrated the organized respiratory activity by particles isolated from the mung bean. Drummond et al. (8) subsequently confirmed the operation of the Kreb's cycle in plant mitochondria by incubating the mitochondria with CH$_3$-C$^{14}$-COOH and then isolating each acid of the cycle. C$^{14}$ had been incorporated into each acid.

The observation that phosphorylation is coupled to oxidation in a plant mitochondrial preparation was first reported by Millerd et al. (35). This was demonstrated by isolating P$^{32}$ - labeled ATP after incubating the mitochondria with P$^{32}$ - labeled inorganic phosphate and an oxidizable substrate. Not until 1953 though were any P/O
ratios reported for plant mitochondria. In that year Bonner and Millerd (6) isolated a preparation from mung bean seedlings, while Millerd et al. (36) isolated mitochondria from avocado. In each instance maximum P/O ratios obtained were approximately 1.0. In the same year however, Laties (27) reported the isolation of cauliflower bud mitochondria which yielded P/O ratios as high as 1.4 for succinate and 2.4 for α-ketoglutarate. Since 1953 other investigators (3, 4, 13, 18, 39) have isolated mitochondria from various kinds of plant tissue.

The demonstration of oxidation and phosphorylation by mitochondria from the endosperm of seeds has been reported since this investigation was undertaken. Stanley (46) isolated mitochondria from the endosperm of germinated sugar pine seeds which have the capacity to oxidize all of the Kreb's cycle intermediates, but with no demonstrated phosphorylating ability. Smillie (44) isolated mitochondria from various tissues of the pea plant including fresh and germinated seeds. The preparations yielded P/O ratios as high as 1.5 for succinate and 3.0 for α-ketoglutarate as substrates. Akazawa et al. (1) studied the respiratory and phosphorylative activity of the castor bean endosperm mitochondria during the second to fifth days of germination after which it rapidly decreased. During
the maximum activity his preparations yielded P/O ratios up to 3.0 with $\Delta$-ketoglutarate as substrate. Akazawa also noted that mitochondrial nitrogen of the endosperm increased during the first five days of germination, after which it rapidly decreased. The increasing mitochondrial nitrogen during the first five days may however have been the result of more efficient isolation. Morphological changes in the mitochondria during aging of the endosperm may possibly enhance their isolation resulting in an apparent increase in mitochondrial nitrogen.

Optimum phosphorylating activity of mitochondrial preparations have generally been found to require the addition of one or more cofactors depending upon the source of the preparation. An adenine nucleotide invariably has been found to be a requirement since it functions as a phosphate acceptor. Although this cofactor has been shown to be present in mitochondria by Siekevitz et al. (43), its concentration may not be high enough for maximum phosphorylation, or it may be bound to the mitochondria in such a way to prevent it from functioning properly. Even in the presence of a secondary acceptor system such as glucose and hexokinase, an adenine nucleotide increases phosphorylation. Likewise $\text{Mg}^{++}$ usually enhances phosphorylation in the intact mitochondria, but Baltscheffsky (2)
reported a preparation which did not yield decreased P/O ratios in the absence of added Mg++. Of course it is quite possible that the intramitochondrial Mg++ concentration may have been high enough to warrant no effect from adding Mg++. 

Some preparations require the addition of cofactors associated with the oxidation of the appropriate substrate. Ohmura (39) observed that TPN (triphosphopyridine nucleotide) stimulated the oxidation of citrate by mitochondria isolated from green leaves. Beaudreau et al. (3) noted that mitochondria isolated from bean seedlings required cytochrome C, glutathione, TPP (Thiamine pyrophosphate), Mn++, and DPN (diphosphopyridine nucleotide) or TPN in addition to ATP and Mg++ for maximum respiratory activity. Beevers (4) found that germinating castor bean mitochondria required ATP, DPN, TPP and Co A (coenzyme A) to oxidize Kreb’s cycle acids. On the other hand, Bonner et al. (5), Laties (27) and Smillie (44) among others found no effect from the addition of cofactors other than an adenine nucleotide and Mg++ to their preparations. More recently Pressman et al. (41) have obtained a stimulation in respiration and phosphorylation from the addition of K+, while Martius et al. (34) have obtained an increase in P/O ratio as much as sixty per cent from the addition of
vitamin K to the incubating medium.

Isolation medium as well as addition of cofactors can influence the activity of mitochondria. While various cofactors influence either phosphorylation or respiration, the isolation medium has been found to affect only the phosphorylating ability of the mitochondria. Lattes (27) was able to isolate phosphorylating mitochondria from cauliflower buds using only 0.5M sucrose. Bonner et al. (6) however reported that phosphate must be present in the isolation medium to obtain phosphorylating mitochondria from mung bean. The phosphate may be functioning simply as a buffer though. Smillie (44) found no effect from phosphate in the isolation medium but versene increased respiration and phosphorylation of pea mitochondria two-fold to threefold. Lieberman et al. (32) also found that 10^{-2} M versene in the isolation medium increased respiratory activity of broccoli bud mitochondria threefold to fourfold. If the versene was omitted from the isolation medium, but added to the incubation medium, it was without effect. Versene apparently functions as a chelating agent in removing deleterious ions. Its effect is well demonstrated by the work of Jacobs et al. (22), who found that oxidative phosphorylation uncoupled by a Cd^{+} concentration as low as 5 \times 10^{-6} M was completely reversed by 10^{-3} M versene.
Beevers et al. (4) obtained optimum respiratory activity from their preparation by adding Mg$^{++}$ in addition to phosphate to the isolation medium. Mg$^{++}$ has been frequently demonstrated to increase P/O ratios when added to the incubation medium, but its function here is quite indefinite. In contrast to Bonner's findings, Ohmura (39) observed that phosphate in the isolation medium decreased oxidative and phosphorylative activity of his preparation, but versene and citrate enhanced it.

One very common procedure in isolating plant mitochondria is the use of 0.5 M sucrose or sucrose plus other solutes totaling approximately 0.5 molarity. The high tonicity of the medium is necessary to prevent swelling of the mitochondria, a phenomenon known to uncouple phosphorylation from oxidation. Laties (27) reported that washing a preparation of mitochondria once in water completely and irreversibly uncoupled oxidative phosphorylation. Tedeschi et al. (50) studied the effect of hypotonic solutions on mitochondria and found that they undergo reversible volume changes when the osmotic pressure of the solution is changed. He expressed the opinion that the mitochondria have a semi-permeable membrane which is high in lipid and similar to that of a cell. Palade (40) offers further support for the existence of a mitochondrial membrane with electron micrographs.
Tonicity however is not the only condition of the medium affecting swelling of mitochondria. Witter et al. (51) observed that pH of the medium greatly influenced swelling. Hunter et al. (21) reported the complete loss of phosphorylation of mitochondria preincubated in 0.5 M sucrose with added phosphate, Ca$$^{++}$$, or various other ions. Swelling accompanied the uncoupling of oxidative phosphorylation. Tapley (49) also reported the uncoupling of oxidative phosphorylation accompanied by swelling of mitochondria preincubated in isotonic solution with thyroxine or Ca$$^{++}$$. These effects of various ions and certain other solutes on the swelling of mitochondria and uncoupling of oxidative phosphorylation were observed only in the absence of conditions favoring respiration. In the presence of actively respiring mitochondria, the effects were either greatly diminished or nonexistant.

The uncoupling of oxidative phosphorylation by preincubating mitochondria under the previously described conditions is in contrast to uncoupling by DNP (2, 4-dinitrophenol). Where DNP causes immediate, irreversible uncoupling; uncoupling by the other agents (hereafter referred to as "mild uncouplers") is slower and frequently reversible by simultaneously preincubating with other compounds. Witter et al. (51) reported that swelling of mitochondria
at pH 8.0 can be prevented by including ATP, ADP (adenosindiphosphate), Mg\(^{++}\), or versene in the medium. Hunter et al. (21) reported that uncoupling of oxidative phosphorylation by preincubation with phosphate, Ca\(^{++}\) and other ions can be prevented by including versene, Mg\(^{++}\), Mn\(^{++}\), and to a lesser extent citrate, AMP (adenosinemonophosphate), ADP, ATP or DPN in the preincubation medium. Similarly Fronesu et al. (16) noted that AMP, Mn\(^{++}\) or versene prevented mitochondrial swelling under certain conditions. DNP also differs from the "mild uncouplers" by not causing swelling of the mitochondria. Witter et al. (51) reported that it even has some tendency to lessen swelling in hypotonic solutions.

The difference between the "mild uncouplers" and DNP indicates that the former have an indirect effect on oxidative phosphorylation in contrast to direct uncoupling by DNP. Preincubating mitochondria in the presence of the "mild uncouplers" may cause the loss of certain components necessary for the functioning of the mitochondria as a unit. The work of Jacobs et al. (23) certainly helps to support this theory. They have demonstrated that oxidative phosphorylation, completely uncoupled by preincubating mitochondria in phosphate, can be recovered up to fifty per cent of the control by the addition of Mn\(^{++}\) to
the incubation medium. The inhibition of uncoupling by including ATP, ADP, etc., as mentioned previously (16, 21, 51) in the preincubation medium may similarly be due to a replacement of factors lost by seepage or a prevention of their loss. Witter (51) however feels that preincubating mitochondria with "mild uncouplers" stimulates mitochondrial enzyme activity which eventually causes the destruction of their own coupling capacity.
III EXPERIMENTAL PROCEDURE

Preparation of Material and Isolation of Mitochondria

Before germination peas were soaked in 5 per cent chlorox solution for five minutes, and rinsed at least three times with distilled water. The seeds were then allowed to germinate at 20-25°C. in culture plates using about 30 per cent more water than the weight of the peas. After germination the seed coat and seedling were removed and mitochondria isolated from the cotyledons.

To prepare cotyledons from non-germinated seeds, the cotyledons were dissected from the dry seed and then cultured in the same manner as the germinated peas except that washing with chlorox was omitted.

The method of isolating the mitochondria was similar to the procedure of Millerd et al. (6). Ten grams of cotyledons were chilled to 0-5°C in a mortar with an equal weight of sand. All subsequent manipulations were carried out at 0-5°C. The cotyledons were ground in enough isolation medium to form a thick slurry, the isolation medium consisting of 0.4 M sucrose and 0.1 M phosphate, pH 7.1. After suspending the slurry in a total volume of 40 ml. of isolation medium, it was filtered through cheesecloth and the filtrate centrifuged at 1,500
x g for five minutes to remove cell walls, starch and nuclei. The supernatant fraction was poured into another set of tubes and centrifuged at 15,000 x g for fifteen minutes to sediment the mitochondria. After pouring off the supernatant fraction the sediment was resuspended in one-half the original volume of the above isolation medium and centrifuged again at 15,000 x g for fifteen minutes. The sediment was then taken up in five ml. of 0.5 M sucrose. One ml. of this mitochondrial suspension contains the mitochondria from two grams of wet weight cotyledons.

**Methods of Measuring Mitochondrial Respiration and Phosphorylation**

Respiratory activity of the mitochondria was measured in the conventional Warburg respirometer, using flanks with one side arm and a center wall. The incubation medium was set up in the Warburg flask prior to isolation of the mitochondria to minimize time delays in handling the mitochondria. All components of the incubation medium except mitochondria and substrate were introduced into the main compartment of the flask. Substrate solution was introduced into the side arm and 0.25 ml. of 20 per cent of NaOH in the center well. Immediately after isolating the mitochondria, 1 ml. of suspension was introduced into the incubation medium. The flasks were
allowed to equilibrate for five minutes on the monometer in the constant temperature water bath. The water bath was maintained at 30° C. throughout all the experiments. After equilibration the substrate was tipped into the main compartment, at which time measuring of oxygen uptake was commenced. Substrate was omitted from one flask to serve as a control for oxygen uptake. Unless otherwise indicated all preparations were incubated for one hour.

At the end of the incubation time 1.5 ml. of fifteen per cent trichloroacetic acid was added to the incubation mixture and the suspension filtered through Whatman #1 filter paper. Inorganic phosphate was measured on an aliquot of filtrate by the method of Sumner (47). The flask used as a control for oxygen uptake also served as a control for phosphate determination. The difference in inorganic phosphate concentration between the control and sample was taken as a measure of phosphate esterification.

Preparation of Cotyledon Extract

Seeds were allowed to germinate for several days after which the seed coats and seedling were removed. The cotyledons were ground in an equal weight of 0.02 M phosphate solution, pH 7.1 with a mortar and pestle. A little sand was added to facilitate grinding. The slurry was then filtered through cheesecloth and the filtrate centrifuged
at 15,000 x g for fifteen minutes. The supernatant was
dialed at 0-5°C. for 24 hours against an equal volume
of 0.02 M phosphate, pH 7.1. The fraction dialyzing out,
referred to as the "dialysate," was used in subsequent
experiments.

**Measuring Mitochondrial ATP-ase Activity**

Essentially the same incubation medium was used as
for measuring phosphate esterification, except that phos-
phate, substrate and hexokinase were omitted and 0.01 M
ATP added. Increase in inorganic phosphate was used as a
measure of ATP-ase activity.
IV RESULTS

Respiration and Phosphorylation by the Pea Cotyledon Mitochondria

The respiratory and phosphorylative activity towards various substrates of mitochondria isolated from two-day-old germinated pea cotyledons is shown in Table I. The complete incubation medium consisted of the following:

- 0.02 M substrate, pH 7.1
- 0.2 M sucrose
- 0.1 M glucose
- 0.01 M phosphate, pH 7.1
- 0.01 M NaF
- $10^{-3}$ M ADP
- $4 \times 10^{-3}$ M MgCl$_2$
- 0.1 mg. hexokinase
- 1 ml. of mitochondrial suspension in a total volume of 2.5 ml.

The results here and throughout this dissertation are based on mitochondria isolated from two grams of pea cotyledons rather than on the basis of mitochondrial nitrogen. One ml. of the incubation medium contained 0.8-1.2 mg. of mitochondrial nitrogen unless stated otherwise. DPNH (reduced DPN) and succinate are readily oxidized while $\alpha$-ketoglutarate is rather slowly oxidized. No known soluble cofactors are associated with the oxidation of the first two substrates in Table I, so the mitochondrion is apparently a complete unit for their oxidation. $\alpha$-Ketoglutarate however requires several soluble cofactors for its oxidation which
may be deficient in the preparation resulting in its comparatively slow rate of oxidation.

Table I

Respiratory and Phosphorylative Activity of Mitochondria Isolated from Two-Day-Old Germinated Pea Cotyledons

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ul O₂ Uptake/Hr.</th>
<th>P/O Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>176ᵃ</td>
<td>1.5-1.9ᵇ</td>
</tr>
<tr>
<td>DPNH</td>
<td>212ᵃ</td>
<td>1.5-1.9ᵇ</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>54ᵃ</td>
<td>2.6-3.0ᵇ</td>
</tr>
<tr>
<td>β-hydroxybutyrate</td>
<td>0</td>
<td>--------</td>
</tr>
</tbody>
</table>

(a) Mean value of four or more determinations.
(b) Extreme values of four or more determinations.

As seen in Table I, d1-B-hydroxybutyrate was tested as a potential substrate, but in contrast to results with animal mitochondria (7, 31), where it served as an excellent substrate, this preparation demonstrated no oxidation of the compound. According to Lehninger et al. (31), the L-isomer of B-hydroxybutyrate requires DPN and ATP for its oxidation by mitochondria, while the D-isomer requires
only DPN for its oxidation. Since the preparation was capable of oxidizing \( \alpha \)-ketoglutarate, a substrate which also requires DPN for its oxidation, but not capable of oxidizing B-hydroxybutyrate, it appears that the mitochondria lack the enzyme necessary for the oxidation of the latter compound.

Phosphorylation accompanying the oxidation of succinate yielded very nearly the theoretical maximum P/O ratio of 2.0. While P/O ratios as high as 1.9 coupled to the oxidation of succinate have been reported for animal mitochondria, the value is 0.4-0.5 units higher than previously reported for plant mitochondria (27, 44). The P/O ratios for DPNH and \( \alpha \)-ketoglutarate oxidation are each approximately one unit below the theoretical values of 3.0 and 4.0 respectively.

In Table II is shown the effect of omitting various components from the incubation medium on the respiration and P/O ratio of mitochondria isolated from the cotyledons of two-day-old germinated peas. The incubation medium is same as previously described for the experiments of Table I with succinate serving as the substrate. The oxygen uptake in the absence of added substrate is most likely due to intra-mitochondrial substrate.
Table II
Requirements for Oxidation of Succinate and Coupled Phosphorylation by Mitochondria from Two-Day-Old Germinated Pea Catyledons

<table>
<thead>
<tr>
<th>System</th>
<th>$\text{ul O}_2\text{ Uptake/ hr.}$</th>
<th>P/O Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>$140^a$</td>
<td>$1.7^a$</td>
</tr>
<tr>
<td>Complete - succinate</td>
<td>$21^a$</td>
<td>---</td>
</tr>
<tr>
<td>Complete - ADP</td>
<td>$147^a$</td>
<td>$0.2^a$</td>
</tr>
<tr>
<td>Complete - Mg$^{++}$</td>
<td>$158^a$</td>
<td>$1.3^a$</td>
</tr>
<tr>
<td>Complete - hexokinase</td>
<td>$153^a$</td>
<td>$0.9^a$</td>
</tr>
<tr>
<td>Complete $\cdot$ DNP</td>
<td>$122^a$</td>
<td>$0^a$</td>
</tr>
</tbody>
</table>

(a) Average of two determinations.

The effect of omitting ADP is a drop in P/O ratio to nearly zero with no simultaneous inhibition of respiration. This is in contrast to results reported by Chance et al. (10), who observed that in the absence of added ADP respiration was inhibited. Subsequent addition of ADP to his preparation restored respiration to the normal level until all the ADP was phosphorylated.

It appears that some intermediate attached to the electron transfer chain is phosphorylated first, from
which the phosphate is transferred to ADP. The generated ATP can then transfer its terminal phosphate to glucose in the presence of hexokinase, regenerating the ADP. In the absence of available ADP the phosphate is released from the intermediate acceptor and returned to solution as inorganic phosphate, permitting respiration to proceed at the same rate as in the presence of ADP. This fact is further supported by the effect of DNP on the complete system which is noted in Table II. While the DNP uncouples oxidative phosphorylation, it does not, as has been observed by other investigators, enhance respiration.

The effect of hexokinase, by regenerating ADP, is similar to adding ADP. In these experiments its effect is not as marked as from the addition of ADP because of the good transphosphorylating capacity of the mitochondria. Mitochondrial ADP however is apparently very low or unavailable, though some must be present, accounting for the small amount of phosphorylation in the absence of added ADP.

The result of adding Mg** is quite representative of its effect observed on mitochondrial phosphorylation in general; namely increasing the P/O ratio approximately 50 per cent above the value in its absence. Its function however is not quite as clear. In contrast to its effect on the intact mitochondria, Cooper et al. (11) showed that Mg** does not enhance phosphorylation when added to a phosphorylating particle extracted from rat liver mitochondria.
Effect of Germination and Cotyledon Age on Mitochondrial Activity

To determine the effect of germination on mitochondrial respiration and phosphorylation cotyledons were cultured alone and as part of the germinating seed as described under procedure. Mitochondria were isolated and tested for respiratory and phosphorylative activities every twelve hours until the third day. After the third day both activities were checked daily until the tenth day in the "germinated cotyledon mitochondria" and until the fourth day in the "dissected cotyledon mitochondria." "Germinated cotyledon mitochondria" refers to the mitochondria isolated from cotyledons of germinated peas. "Dissected cotyledon mitochondria" refers to the mitochondria isolated from cotyledons which were cultured after their dissection from the seed. The respiratory activity is represented in Table III and Fig. 1, while the phosphorylative activity is represented in Table IV and Fig. 2.

Initially germination shows no effect on either respiration or phosphorylation by mitochondria of the pea cotyledons. Mitochondria are undoubtely present in the dry cotyledon as evidenced by the rapid appearance of activity. Formation of new mitochondria probably could not occur rapidly enough to account for the sharp rise in activity. It is more likely that existing mitochondria become active soon after their hydration. By the time that imbibition is
Table III
Respiratory Activity of Mitochondria from Pea Cotyledons after Various Periods of Culturing the Cotyledons

<table>
<thead>
<tr>
<th>Cotyledon Age (days)</th>
<th>ul O₂ Uptake /Hr.</th>
<th>Germinated Cotyledon</th>
<th>Dissected Cotyledon</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0ᵇ</td>
<td>0ᵇ</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>46ᵇ</td>
<td>73ᵇ</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>152ᵃ</td>
<td>159ᵃ</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>178ᵃ</td>
<td>177ᵃ</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>169ᵃ</td>
<td>172ᵃ</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>174ᵃ</td>
<td>165ᵃ</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>183ᵇ</td>
<td>140ᵇ</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>178ᵇ</td>
<td>88ᵇ</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>160ᵇ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>171ᵇ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>150ᵇ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>134ᵇ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>116ᵇ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>97ᵇ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Mean value of four or more determinations.
(b) Average of two determinations.
### Table IV

**Phosphorylative Activity of Mitochondria from Pea Cotyledons after Various Periods of Culturing the Cotyledons**

<table>
<thead>
<tr>
<th>Cotyledon Age (days)</th>
<th>P/O Ratio</th>
<th>Germinated Cotyledon</th>
<th>Dissected Cotyledon</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0(^b)</td>
<td>0(^b)</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.5(^b)</td>
<td>0.7(^b)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.6(^a)</td>
<td>1.7(^a)</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>1.7(^a)</td>
<td>1.4(^a)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.7(^a)</td>
<td>0.2(^a)</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>1.7(^a)</td>
<td>0(^a)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.7(^b)</td>
<td>0(^b)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.8(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.8(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.8(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.4(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.1(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.7(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.5(^b)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Mean value of four or more determinations.

(b) Average of two determinations.
Fig. 1

Respiration of Mitochondria from Pea Cotyledons as a Function of Time of Culturing the Cotyledons
Fig. 2
Phosphorylation by Mitochondria from Pea Cotyledons as a Function of Time of Culturing the Cotyledons
complete, which is within ten to twelve hours, both respiration and phosphorylation are nearly 50 per cent of the maximum values eventually attained. The P/O ratio attains its maximum value shortly before twenty-four hours while respiration rate attains its maximum value shortly after twenty-four hours. Up to this point the cotyledon mitochondria were capable of functioning without the influence of the sprouting embryo. After the first day however, the mitochondria of the dissected cotyledons begin to lose their phosphorylating capacity. The drop in P/O ratio is quite rapid until it reaches zero shortly after the second day. When the phosphorylating ability disappears, respiration rate of the dissected cotyledon mitochondria begins to decrease. A general deterioration of the cotyledon follows, the cotyledon becoming quite mushy after the third or fourth day.

The presence of the growing seedling attached to the cotyledon has a preservative effect on the mitochondria and the cotyledon in general. As seen in Fig. 2, the P/O ratio of the germinated cotyledon mitochondria maintains a maximum value until about the fourth or fifth day, then proceeds to decrease slowly. Even after the tenth day a significant amount of phosphorylating activity remains. Respiration rate begins to decrease after the fifth day, but about half of the decrease is due to a decrease in mitochondrial nitrogen or to a decrease in mitochondria. In contrast, the
drop in respiration rate of the dissected cotyledon mitochondria between the second and fourth days is entirely due to a decrease in efficiency of the mitochondria. No significant decrease in mitochondrial nitrogen occurred during this period. On a mitochondrial nitrogen basis then, respiration rate of the germinated cotyledon mitochondria on the tenth day of germination is still over 75 per cent of the maximum originally attained. Some of these cotyledons begin to show signs of deterioration on the tenth day, but most of them still appeared to be solid and intact.

Restoration of Mitochondrial Activity with Cotyledon Extract

The difference in phosphorylating activity between the germinated and dissected cotyledon mitochondria led to an investigation of the influence of germination. The dialyzable fraction of an extract of two-day-old germinated pea cotyledons was prepared as described under Methods. The effect of this dialysate on the phosphorylating activity of dissected cotyledon mitochondria was determined by adding 1 ml. of it to the incubating mitochondria. The incubation medium was the same as previously described (page 18) with the dialysate replacing an equal volume of water. The effect of the dialysate on the P/O ratio of mitochondria isolated from dissected cotyledons, using succinate as substrate, is shown in Fig. 3 and Table V.
Table V

Effect of Extract from Two-Day-Old Germinated Peas on Phosphorylation by Mitochondria from Dissected Cotyledons

<table>
<thead>
<tr>
<th>Cotyledon Age (Hrs.)</th>
<th>P/O Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Dialysate</td>
</tr>
<tr>
<td>24</td>
<td>1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>36</td>
<td>1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>42</td>
<td>0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>48</td>
<td>0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>48</td>
<td>0.22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>51</td>
<td>0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(a) Mean value of four or more determinations.

The dialysate had little effect on the phosphorylating activity of the mitochondria prior to the first day and is thus not shown in Fig. 3 or Table V. While phosphorylating activity is at a maximum, the dialysate is without effect on it, but once the P/O ratio begins to drop the dialysate is capable of restoring part of the phosphorylating activity which was lost, but never all of it. As the P/O ratio continues to drop a greater proportional increase is observed; that is, the increase in relation to the P/O
Fig. 3

Effect of Extract from Two-Day-Old Germinated Pea Cotyledons in Restoring Phosphorylation Lost by Mitochondria of Dissected Cotyledons

![Graph showing the effect of extract on P/O ratio over time.](image-url)
ratio is greater as the P/O ratio decreases. As the P/O ratio approaches zero, the effect of the dialysate was found to be quite variable. However, fifty-one-hour-old dissected cotyledon generally yielded mitochondria with a P/O ratio of 0.1 - 0.2 which was increased twofold to sixfold by the addition of the dialysate to the incubation medium. Due to the inaccuracy of detecting small changes in phosphate concentration, it is difficult to say whether the dialysate is capable of restoring oxidative phosphorylation to mitochondria once it is completely uncoupled. In several experiments when there seemed to be no apparent phosphorylation in the absence of added dialysate, a P/O ratio as high as 0.5 or greater was obtained from the addition of the dialysate to the incubating mitochondria.

The dialysate had little effect on the oxidation of succinate, increasing the respiration rate of the mitochondria 0 - 20 per cent above the figure in its absence. When the dialysate was added to incubating mitochondria (isolated from fifty-one-hour-old cotyledons) in the absence of added substrate, approximately half the volume of O₂ was taken up as in the presence of succinate. Under the same conditions no phosphorylation was ever observed.

Table VI shows the effect of the dialysate on the mitochondria of fifty-one-hour-old dissected cotyledons with α-ketoglutarate as substrate. In contrast to succinate, α-ketoglutarate oxidation is increased fourfold to
fivefold. Phosphorylation is increased by at least a similar amount. The error in determining phosphate does not permit an accurate measurement of the small amount of phosphate uptake in the absence of added dialysate. Phosphorylation is increased from some value less than 1.0 to a P/O ratio of approximately 1.1.

Table VI

The Effect of the Extract on the Oxidation of α-Keto-glutarate and the P/O Ratio by Mitochondria from Fifty-One-Hour-Old Dissected Cotyledons

<table>
<thead>
<tr>
<th>Without Dialysate</th>
<th>Dialysate Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>ul O₂ Uptake /Hr.</td>
<td>32</td>
</tr>
<tr>
<td>P/O Ratio</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Although the dialysate used in these experiments was prepared from germinated pea cotyledons, the dissected cotyledons yielded a dialysate with equal activity if extracted from the cotyledons while the mitochondria contained high phosphorylating activity. The dialysate was not prepared from dissected cotyledons after the phosphorylating activity had disappeared, so it is not known whether the dialysate must be prepared from a source containing mitochondria which are capable of coupling phosphorylation
to oxidation.

The dialysate, when used to effect the restoration of the phosphorylating capacity lost by the mitochondria of the germinated pea cotyledon, was completely without effect. It was used on the mitochondria isolated on the tenth day of germination, using succinate as a substrate. The P/O ratio at this stage was approximately 0.4 to 0.6. In comparison, when the P/O ratio of the non-germinated cotyledon mitochondria dropped to a similar level, the dialysate generally increased it twofold or more.

In an effort to localize the effect of the dialysate, isolation of a phosphorylating particle from the mitochondria was attempted, using the method of Cooper et al. (11). The preparation isolated from mitochondria of two-day-old germinated pea cotyledons did not demonstrate any phosphorylation. However, it cannot be said whether the mitochondria lack such a particle or if some other means of isolating it must be employed to retain its activity.

Since an increase in mitochondrial ATP-ase activity has frequently been demonstrated to accompany the uncoupling of oxidative phosphorylation, the influence of the dialysate on the ATP-ase activity was determined. First the ATP-ase activity of mitochondria from both the germinated and dissected cotyledon was measured as described under methods. The results are represented in Fig. 4 and Table VII. Although fluoride inhibits ATP-ase, the activ-
ity was measured in its presence in order to maintain conditions as similar as possible to the conditions used in measuring phosphorylation. The ATP-ase of the mitochondria from germinated pea cotyledons remains nearly zero during the first 2$\frac{1}{2}$ days; that of the dissected cotyledons begins to rise shortly after one day, about the time the P/O ratio begins to drop (Fig. 2). At 2$\frac{1}{2}$ days, when oxidative phosphorylation is completely uncoupled, ATP-ase activity is high enough to account for an increase of 0.36 mg. phosphorous as inorganic phosphorus in one hour of incubation. On the assumption that all this phosphate is derived from the terminal phosphate of ATP, 53 per cent of the terminal ATP phosphate has been liberated. When 1 ml. of dialysate was substituted for an equal volume of water, it was found to be without any effect on the ATP-ase activity of the dissected cotyledon mitochondria.

Various cofactors were added to the incubation medium of mitochondria isolated from 51-hour-old dissected cotyledons (just prior to the complete loss of their phosphorylating ability) in an effort to duplicate the effect of the dialysate. None were found to restore either wholly or in part the activity restored by the dialysate. Among the factors added individually were:
### Table VII

**ATP-ase Activity of Mitochondria from Dissected Cotyledons at Various Ages**

<table>
<thead>
<tr>
<th>Cotyledon Age (Hrs.)</th>
<th>Phosphate Hydrolyzed/Hr. Incubation</th>
<th>Per Cent Terminal P of ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg. P</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>36</td>
<td>0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12</td>
</tr>
<tr>
<td>48</td>
<td>0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>60</td>
<td>0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53</td>
</tr>
</tbody>
</table>

(a) Average of two determinations,

TPN, DPN, Mn<sup>2+</sup>, Fe<sup>3+</sup>, thiamine, riboflavin, cytochrome C, ascorbic acid, glutamine, glutamic acid, and glutathione. Ascorbic acid, glutamic acid, and glutamine were also added in substrate concentrations but yielded no better P/O ratios than did succinate. While glutamic acid and glutamine were readily oxidized, ascorbic acid was not an oxidizable substrate. Similarly both versene and indole-acetic acid produced negative results when added to either the isolation medium or the incubation medium.
Fig. 4

ATP-ase Activity of Mitochondria from Germinated and Dissected Pea Cotyledons

Mg P Hydrolyzed

Dissected Cotyledon

Germinated Pea Cotyledon

Cotyledon Age (Hours)
The dialysate was submitted to various chemical treatments in an effort to characterize the active components. The results are summarized on page 38.

When a hot solution of an active dialysate was treated with activated charcoal and filtered, the filtrate exhibited activity equal to the original dialysate. Concentrating the charcoal-treated dialysate on a steam bath to one-third of the original volume in either 0.1 N HCl or 0.1 N NaOH, readjusting the pH to 7.1 and rediluting to the original volume resulted in no loss of activity by either treatment. In the presence of the added HCl the pH was lower than 2 while in the presence of the added NaOH the pH was above 12.

The charcoal-treated dialysate was passed through a strong acid cation exchange resin which had first been washed with 4 N HCl followed by distilled water. After adjusting the effluent of the dialysate to pH 7.1, it was found to be equal in activity to the original dialysate. Similarly the charcoal-treated dialysate was passed through a strong base anion exchange resin which had first been washed with 4 N NaOH followed by distilled water. Again the effluent of the dialysate was found to have 100 per cent of the original activity. For comparison, the anion exchange resin removed only 90-95 per cent of the inorganic phosphate indicating that it did not remove all the anions
Original Dialysate (active)

Treat Hot Solution with Activated Charcoal and Filter
(Filtrate active)

Concentrate on Steam Bath
Pass Through Resin

to 1/3 of the Original Volume

Anion Exchanger
Cation Exchanger

in 0.1 N HCl in 0.1 N NaOH

(active) (active)

Concentrate on Steam Bath to 1/3 Original Volume

in 0.1 N HCl

(inactive)

in 0.1 N NaOH

(active)
or potential anions. The resin was washed with distilled water after saturating it with acid or base until the pH of the effluent water was within 3 units of pH 7.0.

The dialysate that had been passed through the anion exchange resin, when concentrated on a steam bath to one-third of its original volume in the presence of 0.1 N HCl, followed by adjusting the pH to 7.1 and dilution to the original volume, was found to be completely inactive. In contrast, similar treatment with HCl prior to passing through the resin did not render the dialysate inactive. Similar treatment of the anion exchange resin effluent with 0.1 N NaOH did not destroy any of the activity. Drying the dialysate at 105°C or ashing it destroyed all the activity.
V DISCUSSION

The particles isolated from the pea cotyledons are quite representative of mitochondria in general, though some differences exist as compared with preparations previously reported. The preparations have demonstrated the oxidation of several Kreb's cycle substrates and DPNH and of the coupling of phosphorylation to their oxidation. The mitochondria were found to be comparatively complete units for carrying out oxidative phosphorylation. Aside from components taking part in the overall reaction, the only factors added were ADP, Mg\(^{++}\), and hexokinase. Of these only ADP had a pronounced effect on oxidative phosphorylation, increasing the P/O ratio approximately tenfold. The lack of inhibition of respiration in the absence of added ADP, however, is in direct contrast to findings reported by Chance et al. (10), Laties (29) and others. Chance demonstrated a stimulation in respiration rate of as much as fivefold resulting from the addition of ADP to the incubating mitochondria. The effect of added ADP points out one major difference in this preparation from others previously reported.

Mitochondria of the type which have reduced respiration rates in the absence of added ADP may be described as
being "tightly coupled." Chance et al. (9) have proposed a mechanism for the coupling of phosphorylation of ADP to the oxidation of a substrate which is compatible with their own results and others reported. Using DPNH as the substrate being oxidized, they proposed the following sequence of reactions involved in the phosphorylation of ADP (\(~\) represents a high energy bond, while I and X are unknown intermediates):

\[
\begin{align*}
\text{DPNH} + \text{I} & \rightarrow \text{DPN} \sim \text{I} \\
\text{DPN} \sim \text{I} + \text{X} & \rightarrow \text{DPN} + \text{X} \sim \text{I} \\
\text{X} \sim \text{I} + \text{PI} + \text{ADP} & \rightarrow \text{ATP} + \text{X} + \text{I}.
\end{align*}
\]

Provided that the above scheme is an actual representation of the phosphorylation of ADP in a "tightly coupled" preparation, the absence of ADP will obviously inhibit respiration by preventing the liberation of the free intermediate, I, which is necessary for combining with DPN as DPNH is being oxidized.

The mitochondria which were isolated from the pea cotyledon however may be described as being "loosely coupled." In the presence of appropriate requirements phosphorylation is coupled to oxidation, but in the absence of the phosphate acceptor, ADP, no phosphorylation takes place, although at the same time respiration is not inhibited. The scheme for
phosphorylation of ADP proposed by Chance could not apply to this type of preparation unless the mitochondria have another means for regenerating the intermediates, I and X in their free form. This would be possible only with respiration as the limiting factor in the mitochondria. In the absence of added ADP the generated \( X \sim I \) as a result of oxidation of the substrate would be broken by some process in the mitochondria, regenerating the free I and X. The addition of ADP and Pi would act as a drain on the generated \( X \sim I \) to produce ATP, but at the same time would not enhance respiration due to some other factor limiting the latter process.

An alternate explanation for the behavior of the pea cotyledon mitochondria is that some acceptor other than ADP would couple phosphorylation to oxidation by accepting the inorganic phosphate. In the absence of added ADP the phosphate would be released as inorganic phosphate through the action of some enzyme associated with the mitochondria. When ADP was present however it would preferentially accept the phosphate from the primary acceptor, but would not result in any effect on respiration. This latter theory could readily be tested by incubating the mitochondria in the absence of phosphate. If an unknown factor were phosphorylated prior to transferring the phosphate to ADP, then in the absence of phosphate, respiration would be inhibited.
The yield of mitochondria with the phosphorylating ability shown in Fig. 2 and Table IV demonstrate the ability of the pea cotyledon to furnish a plentiful supply of its own available energy which may be required by many processes associated with life. Furthermore it is capable of doing this without the influence of the growing seedling. With this capacity the cotyledon meets the first requirement to function independently to the same extent as other organs of the plant. Whether the cotyledon is able to carry out various synthetic processes depends upon the presence of the necessary enzyme systems.

The cotyledon cultured alone functions quite well independently during the early stages of culturing, but soon loses its organized state and begins to deteriorate rapidly. This however occurs with many other organs if detached from the organism, though perhaps not nearly as rapidly. While the cotyledon functions independently from the start, the growing seedling must be attached to extend the original activity of the cotyledon beyond two days. With the germinated pea the cotyledon is firmly attached to the growing portion of the embryo so that it is an integral part of the plant and therefore subject to its influence. The nature of this preservative effect by the growing portion of the embryo is unknown, but one of several causes seems likely. Either the growing portion of the embryo may synthesize some hormone or
vitamin which can diffuse into the cotyledon, thereby exerting its influence, or an accumulation of end products in the dissected cotyledon may be toxic. With the germinated pea the sprout can utilize end products of reactions in the cotyledon for synthesis of its own tissue, removing potentially toxic compounds from the cotyledon. The preservative effect of the growing sprout on the cotyledon appears to be initially on the oxidative phosphorylation process of the mitochondria. While the dissected cotyledon still appears to be intact and healthy and respiration of its mitochondria proceeds unimpaired, the uncoupling of phosphorylation from oxidation begins and proceeds rapidly until the P/O ratio drops to zero. With the loss of a source of energy for the dissected cotyledon, the means of maintaining the healthy state of the cells disappears. Respiration rate of mitochondria then begins to decrease and a general deterioration of the cotyledon rapidly ensues.

The disappearance of phosphorylating activity from the mitochondria of the dissected cotyledon is quite analogous to the reports on uncoupling of oxidative phosphorylation by mitochondria subjected to ageing (25), preincubation in the absence of substrate (21), or other treatments that may result in destruction of the structural integrity of the mitochondria. Millerd et al. (36) similarly observed an uncoupling of oxidative phosphorylation by mitochondria of
ripe avocado fruit. The mitochondria of the dissected cotyledon are being subjected to conditions similar in many ways to ageing mitochondria or mitochondria of ripening fruit; that is, a state of inactivity. Destruction of the structural integrity of the mitochondria could be a cause for uncoupling, though Cooper et al. (11) reported oxidative phosphorylation by a fractional particle of the mitochondria. However disruption of the mitochondria by Cooper's method may result in maintaining certain structural features which are destroyed by milder, more natural treatments. Besides destruction of structural integrity, uncoupling may result from loss or release of necessary cofactors, appearance of inhibitors or uncouplers, or a combination of several factors. The uncoupling may result from loss of soluble requirements from within the mitochondria or release of components which were bound to the insoluble fraction of the mitochondria, the latter of which may be brought about by enzymatic reactions.

Uncoupling of oxidative phosphorylation induced by ageing or other treatments resulting in destruction of structural integrity has generally been observed to be accompanied or preceded by swelling of the mitochondria. Whether the swelling is either a cause of uncoupling or a result of it is not known. However the uncoupled mitochondria isolated from the dissected cotyledons appeared to
have undergone swelling, though this could definitely be established only by electron micrographs or by optical density measurements.

Restoration of part of the phosphorylative activity lost by mitochondria of dissected cotyledons with an extract as shown in Fig. 3 at first suggests that some factor required for oxidative phosphorylation is leaking out of the mitochondria. Table IV however shows that the extract also increases the oxidation rate of $\alpha$-ketoglutarate, a substrate whose oxidation rate is quite low compared to the other substrates used. While the increased oxidation rate could be the result of enhanced phosphorylation, it does not seem likely in view of the fact that uncoupling oxidative phosphorylation with DNP did not increase respiration rate. The extract thus appears to improve upon processes in the mitochondria which have been impaired. In view of these results and other results previously reported (14, 42), it is suggested that the extract exerts its effect on the mitochondria in a general or indirect way rather than participating directly in the oxidative phosphorylation process. It could, for instance, aid in maintaining the structural integrity of the mitochondria by inhibiting destructive enzymes, or possibly combine with an inhibitor being released by the mitochondria. Pullman et al. (42)
reported partial restoration of phosphorylating activity by the addition of serum albumin to the incubating mitochondria. Decreased P/O ratios had been induced in the mitochondria by preincubation in the absence of an oxidizable substrate for thirty to sixty minutes. Such a compound as serum albumin does not likely participate in oxidative phosphorylation, but rather may exert its effect on oxidative phosphorylation by improving the structural integrity of the mitochondria. Dianzani (14) however supports the view that some factor involved in oxidative phosphorylation leaks out of mitochondria during ageing. He prepared an extract from mitochondria with optimal phosphorylating activity, which, when added to aged mitochondria, restored part of the lost phosphorylating activity. The extract worked equally well when added to artificially reduced P/O ratios by preincubation in absence of a substrate, or when added to mitochondria with reduced P/O ratios isolated from pathological organs.

Although the ATP-ase activity of mitochondria increases with uncoupling of oxidative phosphorylation, the enhanced phosphorylation resulting from the addition of the extract was found not to be due to inhibition of ATP-ase. This evidence is in contrast to the view proposed by Myers and Slater (37) concerning the action of ATP-ase and its relation to the phosphorylation of ADP. In finding three
ATP-ases in mitochondria stimulated by DNP, they suggested that the three enzymes are associated with the three steps of oxidative phosphorylation in the respiratory chain, catalyzing the reversible reaction in phosphorylation of ADP. However if this were so, then by enhancing phosphorylation, the extract should simultaneously inhibit ATP-ase, which was not found to be true.

Results of the various treatments of the extract indicate that the active component is a very stable compound and a rather small molecule, probably much smaller than the maximum size which can pass through the dialysis membrane. Most of the known vitamins and cofactors as mentioned in the results were tested for their ability to replace the extract without success. Among the more likely ones not tested are a nucleotide other than an adenine nucleotide, coenzyme A, lipoic acid, and vitamin K. A nucleotide or coenzyme A would have been absorbed on charcoal, while lipoic acid would have been removed by the anion exchange resin, leaving only vitamin K from among the more likely untested, known factors as being the possible active component of the extract.

Since the activity of the extract was not decreased by passing through either a strong acid or strong base exchange resin, the active component is neither ionic nor potentially ionized by a strong acid or a strong base;
unless its concentration is too low to be appreciably absorbed by the resin. After passing through the anion exchange resin however, concentrating on the steam bath to 1/3 of the original volume in 0.1 N HCl destroys all the activity of the extract; while neither of the two treatments alone destroys any activity. Such results imply that either: (1) there are two active components in the extract, either of which functions equally well, one of which is rendered inactive by HCl; or (2) the resin removes a complexing agent permitting the HCl treatment to render the extract inactive. The loss of activity from the HCl treatment is a result of lability of the compound towards acid or its distillation from an acid solution. The latter possibility could be readily determined by collecting the distillate and adding back to the undistilled portion, the recombined extract then tested for activity in restoring phosphorylative activity lost by the mitochondria of the dissected cotyledon.

Characterization of the activity in the extract may lead to a better knowledge of the components readily lost by the mitochondria which are required to maintain the mitochondrial activities at their optimum, and possibly to a better understanding of the processes associated with the mitochondria.
VI. SUMMARY

1. Mitochondria were isolated from pea cotyledons which had the ability to oxidize several Kreb's cycle acids and yielded P/O ratios up to 1.9 with succinate and up to 3.0 with α-ketoglutarate. No factors were found to influence respiration of the mitochondria, but ADP was required for phosphorylation with Mg and hexokinase lesser requirements.

2. The respiratory and phosphorylative activity was found to be absent in the dry cotyledon, but both activities attained a maximum after about one day of culturing. After the first day, presence of the growing sprout attached to the cotyledon (germinated pea) resulted in preservation of the mitochondrial respiration and phosphorylation at the maximum until the fifth to sixth day after which both decreased slowly. In contrast, cotyledons cultured alone yielded mitochondria whose phosphorylation and respiration started to decrease soon after the first day, the former reaching zero shortly after the second day.

3. Part of the phosphorylative activity lost by the mitochondria of dissected cotyledons was capable of being restored with an extract prepared from cotyledons containing mitochondria with high P/O ratios. The active
portion of the extract was found to be dialyzable, stable to heat, not absorbed on charcoal or a strong acid or strong base exchange resin, and stable in hot 0.01 N acid or base. However concentrating the extract in 0.01 N HCl to 1/3 of the original volume on a steam bath after passing through the anion exchange resin resulted in complete loss of the original activity.

4. Action of the extract on the mitochondria in restoring phosphorylation is discussed in the light of results obtained and of results reported on the subject.
VII. LITERATURE CITED


50. Tedeschi, H. and D. L. Harris. The Osmotic Behavior and Permeability to Non-electrolytes of Mitochondria.

I, Steve Vanecko, was born in St. Clairsville, Ohio on October 15, 1926, where I also received my elementary education. I received my secondary education at Martin's Ferry, Ohio, graduating in June, 1944.

I enrolled at The Ohio State University in September, 1948, after spending two years in the Army and two years working on several jobs. In August, 1951, I completed my undergraduate studies and received the degree Bachelor of Science in Agriculture from The Ohio State University.

I enrolled in the Graduate School at The Ohio State University in September, 1951, at which time I received an appointment as Research Fellow in the Department of Agricultural Biochemistry, a position which I held until receiving the degree Master of Science in Agricultural Biochemistry from the Ohio State University in March, 1953. From March, 1953 until September, 1953, I was employed by the Ohio Agricultural Experiment Station as Research Assistant, after which I enrolled again in the Graduate School of The Ohio State University. I was Assistant in the Department of Agricultural Biochemistry from September,
1953, until June, 1954, when I received an appointment as Research Fellow in July, 1954, in the same department which I held until June, 1956. Since July, 1956, I have been employed by Chemical Abstracts of the American Chemical Society while completing requirements for the degree Doctor of Philosophy.