It has been known since 1921 that the respiration rate of leaves declines during aging (7). Smillie (19, 20, 22) has shown that the amounts of some soluble respiratory enzymes of pea leaves decrease as they grow older and that young leaves are the best source of active mitochondria with high phosphorylating capacity. In addition, adverse effects of relatively high growing temperatures on the yield of active mitochondria have been reported (17) and the effects of exposure of normal tissues to high temperature may be due to a selective inhibition of the Krebs cycle (1, 14).

In this work pea plants were grown at 10, 20 (optimal for growth) and at 30°. At selected stages of growth, respiration rates of leaf tissue and the biochemical activities of mitochondria isolated from them were measured. The ability of leaf tissue to utilize acetate-C\(^{14}\) and the activities of some mitochondrial enzymes were also determined. Declining respiratory rates during aging were correlated with striking decreases in mitochondrial activity. These in turn appeared to be related to increased disorganization of the internal structure of the mitochondria.

**Materials and Methods**

*Plant Material and Sampling Methods.* Pea seeds (*Pisum sativum* L. var. Alaska) were soaked in deionized water for 4 to 8 hours, surface sterilized with either 0.001 M HgCl\(_2\) or Spergon, and then sown in sterilized soil or vermiculite. Seeds sown in sterilized soil were watered, as needed, with deionized water while those sown in vermiculite were watered with Withrow A solution (23).

Pea plants used to study both temperature and aging effects were grown in 3 controlled climate rooms. The temperature in each room was maintained continuously at a different level, namely 10, 20, and 30°. These temperatures are respectively suboptimal, close to optimal and supraoptimal for growth (fig 1). Photoperiod (16 hours), light intensity (400-600 ft-c) and average relative humidity (60 %) were the same in each room. The plants grown at different temperatures were generally uniform in internode length and leaf size but plants grown at 30° were considerably lighter green in color. Some plants used to study aging effects only were grown in a greenhouse in early spring.

Leaves were sampled in 2 ways: A) Plants at each temperature were grown to predetermined heights of 3, 10, and 23 cm and all of the leaves were removed. Figure 1 describes the growth of pea plants at the 3 temperatures employed and the stages of development at which leaves were sampled are indicated by arrows. For convenience the 3 stages are designated as developmental ages 1, 2, and 3. B) The second sampling method consisted of growing plants to a height of 23 cm at each temperature and detaching leaves at 3 levels. The third plant shown...
in figure 2 illustrates this method and the levels at which leaves were detached are indicated by arrows. The 3 levels are designated as age groups 1, 2, and 3 starting from the shoot apex. Experiments were conducted on the leaves as soon as possible after detachment.

Leaf Respiration. Respiration of pea leaves was determined manometrically by measuring O₂ uptake of samples (0.2 g) in the dark at 30⁰C. No bathing solutions were added.

Mitochondrial Respiration. Mitochondrial preparations were isolated from leaf samples (10 g) as described by Smillie (18). Final resuspension was done so that 1 ml of mitochondrial preparation represented 2.5 g fresh weight of tissue. Oxidative capacity was determined manometrically at 30⁰C. The Warburg flasks contained the following reaction mixture: 1 ml mitochondrial preparation, 0.75 ml cofactor solution, and 0.25 ml substrate, giving a final volume of 2.0 ml. The final concentration of the following components was obtained: sucrose 0.5 M, phosphate 0.01 M, DPN 0.001 M, ADP 0.001 M, TPP 0.0005 M, CoA 0.001 M, and MgSO₄ 0.001 M. Final substrate concentrations were 0.02 M (0.001 M for malate sparker). All substrate and cofactor solutions were adjusted to pH 7.0.

Reactions were started by tipping in substrates from the side arm after a period of equilibration. The flasks contained 0.2 ml of 20% KOH in the center wells. The reaction period was 1 hour. Final pH of the reaction mixture was 6.8 to 7.0. All rates given are corrected for endogenous O₂ uptake.

For measurement of oxidative phosphorylation O₂ uptake was determined manometrically as described above except that NaF was added to a final concentration of 0.01 M. In addition, glucose at a final concentration of 0.02 M and 0.2 mg hexokinase per flask were added as a trapping system. Phosphorylation was determined by the disappearance of Pi (9) from the vessels during the period of O₂ consumption. One vessel of each treatment was removed when the stop cocks were closed for initial phosphate determination.

Enzyme Assays. DPNH oxidase, DPNH-cytochrome c reductase, and diaphorase activities were determined spectrophotometrically as described by Crane (3).

DPNH Oxidase. DPNH (0.24 μM) was added to 0.1 to 0.2 ml of mitochondrial preparation in 120 μmoles of Tris chloride, pH 7.4. Total volume was 3 ml and the assays were run at room temperature (25⁰C). A slow rate of oxidation occurred in the presence of 10 μmoles KCN and the oxidation rate in the absence of KCN was corrected for this cyanide resistant oxidation. The influence of exogenous cytochrome c was determined by adding 0.1 ml of a 1% solution of Sigma cytochrome c.

DPNH-Cytochrome c Reductase. DPNH (0.24 μM) was added to a reaction mixture containing 0.1 to 0.2 ml mitochondrial preparation, 100 μmoles potassium phosphate pH 7.4, 0.1 ml of 1% cytochrome c (Sigma Chemical Co.) and 10 μmoles KCN in a total volume of 3 ml. Assays were run at room temperature and the rate of oxidation of DPNH by cytochrome c was determined by following reduction of cytochrome c by increase in absorbance at 550 μM.

Diaphorase. DPNH (0.24 μM) was added to a reaction mixture containing the same components as were used in the cytochrome c reductase assay except that 0.1 ml of 0.2% 2,6-dichlorophenolindophenol (DCIP) was substituted for cytochrome c. Reduction of DCIP was determined by following decrease in absorbance at 600 μM. In both the diaphorase and cytochrome c reductase assays reduction of DCIP and cytochrome c without addition of DPNH was followed and found to be small, and all rates given are corrected for this endogenous rate.

Cytochrome oxidase was determined as described by Mackler and Green (11). The reaction was started by adding 0.1 to 0.2 ml of mitochondrial preparation to a reaction mixture containing 0.2 M potassium phosphate pH 7.5, 0.3 ml 1% reduced cytochrome c and 0.3 ml of 10% bovine serum albumin. Total volume was 3 ml and the assays were run at room temperature.

Protein Nitrogen. Aliquots of mitochondrial preparations (0.3–0.5 ml) were subjected to Kjeldahl digestion and protein nitrogen was determined by nesslerization.

Acetate-1-C¹⁴ Feeding Experiments. Acetate-1-C¹⁴ (specific activity 64 mc/mmoles) was obtained from Nichem Inc. The evolution of total CO₂ and C¹⁴O₂ from leaf samples (1 g) incubated with acetate-1-C¹⁴ (2 μmoles) in 20 ml of 0.05 M potassium phosphate pH 5.0 was determined in the dark in an apparatus described by Harley and Beevers (6). Aliquots of 0.2 ml were taken before and after the CO₂ absorption period to determine the initial and
final C\(^{14}\) content of the incubating solutions. The BaCO\(_3\) collected after 24 hours was plated on micro-porous porcelain planchets, weighed and the C\(^{14}\) counted under thin window continuous gas flow GM tubes. Corrections were made for self absorption.

**Electron Microscopy.** Leaves were cut into small sections approximately 1 mm square, placed in a vacuum flask containing deionized water and evacuated until they sank. The leaf sections were then cooled to 0\(^\circ\)C, the water decanted off and the sections fixed for 45 minutes with 1% buffered OsO\(_4\), pH 7.4 at 0\(^\circ\)C (equal parts veronal acetate buffer and 2%...
O₃O₄). Dehydration, infiltration, embedding, sectioning, and photography was accomplished as described by Nadakavukaren (13).

**Results**

Preliminary experiments indicated that exposure of plants to growing temperatures above optimum probably did not induce a preferential inhibition of the Krebs cycle since RQ values considerably greater than 1.0 were not observed. It was realized, however, that inhibition of the Krebs cycle need not invariably be attended by high RQ values. The experiments did indicate that high growing temperatures had the effect of lowering respiration and that decreased respiratory rates of aging leaves may be paralleled by decreasing mitochondrial activity. Therefore, respiratory rates and mitochondrial activity of leaves grown at 10, 20, and 30° throughout the period of growth described previously were surveyed.

**Leaf Respiration.** Figure 3 shows the decreasing respiratory rates of leaves taken from plants of increasing age grown at the 3 different temperatures. With this method of sampling decreases in Qₒ₂ between the youngest and oldest leaves range from about 40 to 60% (taking youngest age as 100%) for the 3 temperatures employed. Similarly, the decrease in Qₒ₂ between the lowest and highest temperature ranges from 50 to 60% (taking that at the lowest temperature as 100%) for the 3 developmental ages. If leaves were taken from different levels of the same plants the same respiratory pattern with respect to aging and temperature held true. The decrease in Qₒ₂ between 10 and 20° is considerably greater than that between 20 and 30° for the first and second ages.

**Mitochondrial Activity.** Several Krebs cycle intermediates were used as substrates in order to determine if there was a general decrease with age of the ability of mitochondria to carry out Krebs cycle oxidations or if there were some specific sites of inactivation. The rates of oxidation of these substrates by mitochondrial preparations from leaves of plants of different ages grown at 10, 20, and 30° are shown in figures 4 through 10. The decreases in Qₒ₂(N) between the mitochondria from leaves at the youngest and oldest ages range between 60 to 100% with most values greater than 70%. It is clear that the oxidation of each of the substrates by the mitochondria is strongly impaired as the growing temperature is increased or during aging of the leaves at any temperature. The decreases in activity between the mitochondria from leaves from the lowest and highest temperatures range from about 30 to 100%. Again, as with leaf respiration, the decrease between 10 and 20° is greater than that between 20 and 30°. Mitochondrial preparations isolated from leaves taken from different levels of the same plants showed the same pattern of activity with respect to age.

Table I shows that good P/O ratios for succinate oxidation are obtained only with mitochondrial preparations from young leaves at all growing temperatures. Phosphorylative capacity was considerably reduced at the second developmental age and none could be detected at the oldest age at any growth temperature.

Comparison of O₂ uptake in the presence and absence of ADP may give an indication of the degree of coupling of the phosphorylative system. With tightly coupled mitochondria omission of the phosphate acceptor, ADP, should result in a decrease in O₂ uptake. Conversely, with loosely coupled or uncoupled mitochondria the absence of ADP should have little or no effect on O₂ uptake. Table II shows that O₂ uptake was measured for 1 hour.

<table>
<thead>
<tr>
<th>Table I. Oxidative Phosphorylation of Mitochondrial Preparations from Leaves of Plants of Different Ages</th>
</tr>
</thead>
<tbody>
<tr>
<td>The substrate was 0.02 m succinate. O₂ uptake was measured for 1 hour.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Developmental age</th>
<th>Growing temp (°C)</th>
<th>O₂-uptake (µl)</th>
<th>µ atoms oxygen</th>
<th>Pi esterified</th>
<th>P/O ratio</th>
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<tr>
<td>2</td>
<td>10</td>
<td>151</td>
<td>13.50</td>
<td>10.50</td>
<td>0.81</td>
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<td>1</td>
<td>0.08</td>
<td>0</td>
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</tbody>
</table>

Table II. Effect of ADP on Oxidative Phosphorylation of Mitochondrial Preparations

The substrate was 0.02 m succinate. O₂ uptake was measured for 1 hour.

<table>
<thead>
<tr>
<th>Age group</th>
<th>O₂-uptake (µl)</th>
<th>µ atoms oxygen</th>
<th>Pi esterified</th>
<th>P/O ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ ADP</td>
<td>1</td>
<td>191</td>
<td>17.08</td>
<td>30.74</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>21</td>
<td>1.88</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>0.27</td>
<td>0</td>
</tr>
<tr>
<td>− ADP</td>
<td>1</td>
<td>119</td>
<td>10.64</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23</td>
<td>2.06</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
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uptake was reduced about 40% when ADP was absent from the reaction mixture with mitochondrial preparations from young leaves. With mitochondrial preparations from leaves of the second and third age groups, however, the presence or absence of ADP had no effect on O₂ uptake. The system even at age 1 appears to be loosely coupled since O₂ utilization decreased less than 2-fold without ADP, and O₂ uptake was too low at ages 2 and 3 to make a judgment concerning the coupling of phosphorylation.

Acetate-1-C¹⁴ Utilization. It is possible that the decreased catalytic activities of isolated mitochondria with increasing age and higher temperature may not be a true reflection of their ability to function in vivo but may reflect only a greater susceptibility to physical or enzymic damage during isolation (4, 8). The recent work of MacLennan et al. (12) shows that there is no doubt that the major pathway of acetate dissimilation in higher-plant cells is the Krebs cycle. Therefore the ability of the various tissues to utilize added acetate was examined in order to assess mitochondrial activity in vivo. Acetate-1-C¹⁴ was incubated in the dark with intact leaf tissues of the 3 age groups from plants grown at 10, 20, and 30°C. 

C¹⁴O₂ evolution was determined at intervals from 3 to 24 hours. Figure 11 plots per cent of total acetate-1-C¹⁴ taken up by the tissues and evolved as C¹⁴O₂ during the 24 hour experimental period against age of tissue. The same general pattern was found at 3, 6, 9, and 12 hours. Less acetate-1-C¹⁴ was converted

![Graph](image_url)

**Fig. 11.** Per cent of total acetate-1-C¹⁴ taken up and evolved as C¹⁴O₂ in 24 hours by pea leaves from plants grown at different temperatures. 10°C (*--*), 20°C (*--*), 30°C (*--*).

**Fig. 12–16.** Specific activity (µeq/mg protein N/min). Electron transport enzyme activities by mitochondrial preparations isolated from leaves from plants grown in a greenhouse. Rates are expressed on the basis of a 2 electron transfer; the observed rate of oxidation or reduction of cytochrome c in µmoles is 2 times that given above.
to $^{14}$O$_2$ as the tissues aged. Similarly when they were grown at the higher temperatures less acetate carbon was recovered as CO$_2$. The decrease in acetate conversion to CO$_2$ during aging ranged from 40 to 70% for the 3 growth temperatures employed and about 50 to 80% with respect to temperature for the 3 age groups. This correlates well with the trend found for leaf respiration (fig 3) and for oxidation of Krebs cycle intermediates by mitochondrial preparations (fig 4-10).

**Electron Transport Enzyme Activities.** It was of interest to determine whether individual enzymes of the electron transport system of isolated mitochondria showed the same trend of decreasing activity during aging as was exhibited when Krebs cycle substrates were oxidized. Figures 12 through 16 show the pattern of activity with increasing age of tissue for DPNH oxidase with and without added cytochrome c, DPNH-cytochrome c reductase, diaphorase, and cytochrome oxidase. It is clear that as the tissues aged the yield of each of the enzymes was reduced. However the maximum decreases observed (50-60%) were less striking than the almost complete loss of the ability of mitochondria from the corresponding tissues to oxidize intermediates of the Krebs cycle (fig 4-10).

**Electron Microscopy.** Electron micrographs of sections of pea leaf tissues grown at 10, 20, and 30° for the 3 age groups were prepared. No marked effect of the growing temperature on mitochondrial structure was observed. Changes in structure as a result of aging were readily discernible, however. Micrographs of young leaves showed mitochondria densely packed with microvilli or mitochondria with microvilli concentrated around the periphery of the organelles and giving the appearance of being doughnut shaped (fig 17). Tissues of age groups 2 and 3 contained mitochondria with the tubular ingrowths scattered less densely throughout their interior. In some micrographs only the scattering was marked, whether or not there was a decrease in the number of microvilli was not easily recognized. In others, however, a decrease in the number of microvilli was definitely indicated (fig 18).

**Discussion**

The data presented show that as pea leaves age there is a decrease in their respiratory activity which is paralleled by a decreasing ability of isolated mitochondria to oxidize Krebs cycle intermediates and carry out oxidative phosphorylation. Smillie (22) has also studied the relationship between the changes in respiratory rates accompanying leaf maturation and the activities of some soluble respiratory enzymes and found a strong correlation between them. Although he did not undertake a systematic study of mitochondrial activity with respect to leaf age, Smillie (19, 20) also noted that the best yields of biochemically active mitochondria were obtained from young leaves of rapidly growing pea plants and that younger leaves showed higher abilities for oxidative phosphorylation (21). Thus, as pea leaves mature there is a general demise in the entire respiratory system.
machinery which manifests itself readily whether tissue respiration or isolated enzymes are studied. As far as the oxidative component of respiration is concerned (the mitochondria) the decreased ability to function is apparently not restricted to specific sites since Krebs cycle oxidation, oxidative phosphorylation, and electron transport activities all are diminished. It is important to note too that the progressive disability of the mitochondria during aging does not result in aerobic fermentation; respiratory quotients of leaves were shown to be close to 1.0 regardless of age or growing temperature. Thus glycolysis is also correspondingly curtailed in older leaves. Smillie's (22) observations on the level of the glycolytic enzyme enolase suggest that this lowered activity represents something more than the control of glycolytic rate by reactions in the mitochondria.

The decline in biochemical activity of the mitochondria could be due to a decreased number of mitochondria or to decreased biochemical efficiency of the mitochondria or both. If the almost complete loss of mitochondrial activity were due solely to decreased number of mitochondria, then mitochondrial protein nitrogen should decrease to the same extent. Since the amount of protein nitrogen in the mitochondrial preparations remained relatively constant throughout the ranges of age employed, it is concluded that a decrease in the number of mitochondria was not the basic cause for the reduced enzyme activities.

From the results presented here the general trend of decreasing respiratory and mitochondrial activities with aging is clear. It seems that the only effect of exposure to growing temperatures above and below the optimum is that of either accelerating or decelerating the demise of the system which occurs normally with increasing age at the optimal temperature for growth. The important factor determining decreased respiratory activity is apparently the degree of maturity; high growing temperatures accelerate the onset of this condition, low growing temperatures decelerate it. A comparison of the growth made by plants at the various temperatures (fig 1) with respiratory activity of leaves (fig 3) and mitochondrial preparations (fig 4–10) illustrates this point.

The pattern of acetate-1-C14 conversion to C14O2 (fig 11) with respect to aging shows that the decreased mitochondrial activities exhibited in vitro are not due solely to injurious effects of the physical environment (8) or to damage of an enzymic nature (4) during isolation. Decreased ability of mitochondria to function apparently occurs in the intact tissue. These results also support what has been said above concerning the effect of growing temperature on respiratory activity.

The changes in degree of structural organization of the mitochondria observed in situ may be correlated with the biochemical evidence presented. Thus disorganization of internal structure and decreased oxidative capacity occur with increased age of tissue. The structural changes observed with aging are rather gross, and very possibly more subtle structural changes may occur which influence the biochemical capacity of the mitochondria. The doughnut shape observed in mitochondria from young leaves may represent the first stages of disorganization. The correlation between disorganization of mitochondrial structure and decreased biochemical capacity extends to green leaves what has been found in other plant tissues (2, 5, 10, 15, 16).

Summary

During growth of pea leaves at the optimal temperature of 20°, the oxidative and phosphorylation abilities of the isolated mitochondria decline even more sharply than the respiration rate of the intact tissue.

Low temperature (10°) slowed down, and high temperature (30°) accelerated, the changes which occurred at 20°.

The pattern of conversion of acetate-1-C14 to C14O2 was the same as that observed for leaf respiration and isolated mitochondrial activity and shows that the decreases observed with age were not due solely to physical and/or enzymic damage to the mitochondria during isolation.

Electron micrographs showed that there was a disorganization of internal mitochondrial structure in situ with leaf maturation.

Literature Cited

Steric Specificity in Synthesis of Heptuloses by Plants 1, 2, 3

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Department of Soils and Plant Nutrition, University of California, Davis

Of the possible stereoisomers of heptuloses, only one, D-altro-heptulose (sedoheptulose), has been assigned a specific role in plant metabolism (1, 3). Enzymic studies have revealed that phosphate esters of D-altro-heptulose can be produced in vitro in the presence of enzymes which are known to occur in plant tissues (5). That the heptulose can be metabolized in plant leaves also has been shown (2, 26).

Heptuloses other than D-altro-heptulose have been found in plants. D-manno-heptulose has been identified in a variety of species (11, 15, 17, 22), and talo-heptulose tentatively has been identified in avocado fruits (6). Heptuloses have been induced to accumulate in plant tissues by introducing into the sugars which presumably serve as precursors. When the pentose D-ribose was fed to plant tissues D-altro-

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1 Received Feb. 4, 1964.
2 Presented in part at the meetings of the Western Section of the American Society of Plant Physiologists, Stanford Univ., June 18, 1963.
3 Supported in part by a grant (GM07714) from the National Institutes of Health, Bethesda, Maryland.