saturated; approximately 75% of the fatty acid fraction is composed of acids of the 18-carbon series. Linolenic acid is the predominant unsaturated fatty acid present and palmitic acid is the predominant saturated acid. About 11% of the total fatty acids is present as an unsaturated component tentatively identified as a 16-carbon trienoic acid. Analytical results after hydrogenation of the samples are in good agreement with the data presented.

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2. Chibnall, A. C. 1939. Protein Metabolism in the Plant. Yale Univ. Press.

Condensing Enzyme from Higher Plants

A. J. Hiatt

Department of Agronomy, University of Kentucky, Lexington

Condensing enzyme, which couples acetate and oxaloacetate to form citrate, has been extracted from a variety of animal tissues, yeast, and bacteria (12). The enzyme was obtained in crystalline form from pig heart by Ochoa et al. (10) in 1951. The presence of condensing enzyme in the tissues of higher plants, however, has not been directly demonstrated.

Brummond and Burris (1) demonstrated that the rate at which mitochondrial preparations from cotyledons of lupine seedlings oxidized malate and pyruvate in combination was greater than the sum of their rates singly. They also reported that when pyruvate-2-C14 was oxidized in the presence of L-malate, the specific activity of the citrate produced is almost identical to that of the pyruvate used. Walker and Beevers (15) reported that a particular fraction from castor bean endosperm oxidized neither oxaloacetate nor pyruvate alone and that oxygen uptake in the presence of a combination of these substrates proceeds at its maximum rate from the onset only when CoA, cocarboxylase, ATP, and DPN are present. Vickery and Zelitch (14) supplied pyruvate-2-C14 to cultures of tobacco leaves and found that the citrate formed possessed the same specific activity as the pyruvate used.

The available evidence strongly suggests, therefore, that the synthesis of citrate in plant tissues proceeds by the reaction of an enzyme system analogous to the condensing enzyme of Stern and Ochoa (13). Although Brummond and Burris (2) demonstrated the presence of most of the enzymes of the citric acid cycle in the green leaves of young lupine plants, the evidence for the presence of condensing enzyme was inconclusive. In this paper we describe the preparation from various plant tissues of enzyme extracts which possess condensing enzyme activity. The en-

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2 Contribution of the Agronomy Department, Kentucky Agr. Exp. Sta., Lexington, Ky., and published with the permission of the Experiment Station director.
zyme from tobacco leaves was partially purified and some of its properties are reported.

**Materials & Methods**

**Preparation of Condensing Enzyme Extracts.**

The condensing enzyme extracts used in most of our experiments were obtained from acetone powders of *tobacco leaf particles which precipitated between 1,000 and 10,000 × g*. This procedure is similar to the procedure used to obtain soluble succinic dehydrogenase from plants (3). Seeds of *Nicotiana tabacum* L. (var. Burley 21) were germinated and grown for 4 weeks in vermiculite treated with nutrient solution. The plants were then transplanted into containers of aerated Hoagland’s solution and grown for 3 additional weeks. After this the leaves were harvested, the midribs removed, and mitochondrial preparations were obtained by the procedure described by Pierpoint (11). The mitochondrial pellet was transferred to a Waring blender containing 20 to 30 volumes of cold (−20 °C) acetone and blended for 45 seconds. The suspension was filtered through filter paper in a small Buchner funnel and the filter cake was blended again with approximately 200 ml of cold acetone. After filtering, the powder was dried in a suction flask by evaporation for 2 hours at 0 to 4 °C. The acetone powder could be stored for months at −15 °C without loss of condensing enzyme activity.

All extraction and purification procedures were carried out at 0 to 4 °C. Crude extracts of condensing enzyme were obtained by stirring a 2% suspension of acetone powder for 20 minutes in 0.02 M potassium phosphate buffer at pH 7.4. The suspension was centrifuged at 25,000 × g for 30 minutes and the precipitate was discarded. Centrifugation at 30,000 × g for periods up to 2 hours failed to precipitate the enzyme.

Extracts from acetone powders of mitochondria from tissues of other species were prepared for experiments concerning the distribution of condensing enzyme in higher plants. Spinach (*Spinacea oleracea* L.) was obtained from local markets. Beans (*Phaseolus vulgaris* L., var. Black Valentine), peas (*Pisum sativum* L., var. Laxton Progress) and soybeans (*Glycine max* Merr., var. Clark) were grown in vermiculite for 10 to 15 days in the greenhouse. Acetone powders of leaf mitochondria were prepared in the manner described for tobacco. The roots were washed with cold distilled water containing 0.001 M EDTA (ethylene-diaminetetraacetate). Mitochondria were isolated from roots by the procedure described by Withrow and Wolff (16) and acetone powders of root mitochondria were prepared.

Crude extracts of condensing enzyme contained a small amount of DPNH oxidase which was removed by purification. The crude extract was adjusted to pH 5.5 by slowly adding, with stirring, 0.2 M acetic acid. Calcium phosphate gel was added (0.3 mg gel solids per mg protein) and the suspension was stirred for 3 hours. The suspension was centrifuged for 10 minutes at 10,000 × g and the supernatant solution was discarded. Condensing enzyme was eluted from the gel by resuspending it in 15 ml of 0.1 M potassium phosphate buffer, pH 7.4, stirring for 20 minutes, and centrifuging for 10 minutes at 10,000 × g. This procedure was repeated five times and the eluates were combined. Ammonium sulfate (243 mg/ml) was added slowly, with stirring, to the combined eluates. The suspension was centrifuged for 10 minutes at 15,000 × g and ammonium sulfate was added to the supernatant solution at the rate of 205 mg/ml. After centrifugation the precipitate was redissolved in 5 to 10 ml of 0.02 M potassium phosphate buffer at pH 7.4. This procedure resulted in a 10- to 12-fold purification of the tobacco leaf enzyme. This extract will hereafter be referred to as purified extract.

**Other Materials.** Oxaloacetate and Coenzyme A (CoA, 75% purity) were obtained from Nutritional Biochemicals Corp. Oxidized diphosphopyridine nucleotide (DPN, 98% purity) was obtained from the Sigma Chemical Co. All other reagents were commercial preparations of high purity.

Acetyl CoA was prepared from acetic anhydride and CoA according to the procedure outlined by Ochoa (9).

**Assay Procedures.** The standard assay procedure used in these studies was based on conversion of acetyl CoA and oxaloacetate to citrate and CoA with subsequent determination of citrate formed. The standard reaction mixture contained the following constituents in a volume of 1.0 ml: 0.02 M tris-HCl buffer, pH 8.6, 5 × 10−3 M potassium oxaloacetate, 10−3 M acetyl CoA, and 0.3 to 0.5 units of condensing enzyme. A control tube without acetyl CoA was included. After the reactants were added to the reaction tubes in an ice bath, the mixture was incubated for 10 minutes in a water bath at 30 °C. The reaction was stopped by adding 1.0 ml of 15% trichloroacetic acid, and denatured protein was removed by centrifugation. A 1.0 ml aliquot was removed for citrate determination by the method of Natelson et al. (8). None of the following substances interferes with the method: aconitic acid, isocitric acid, pyruvic acid, lactic acid, fumaric acid, succinic acid, malic acid, maleic acid, tartaric acid, α-ketoglutaric acid, β-keto acids, β-hydroxy acids, saturated and unsaturated fatty acids, acetaldehyde, ethanol, glucose. In experiments where acetyl CoA concentration was determined, a 0.5 ml aliquot was taken from the reaction vessel for acetyl CoA determination before adding trichloroacetic acid.

The reaction rate was linear with enzyme concentrations to at least 0.5 unit per ml and for periods of incubation of at least 20 minutes. Boiling the enzyme...
for 3 minutes destroyed activity. One unit of condensing enzyme activity is defined as that amount which, under the condition of the above assay, causes the synthesis of 1.0 micromole of citrate in 10 minutes at 30 C.

In experiments concerning the effect of concentration of acetyl CoA upon enzyme activity an optical assay procedure was used. This procedure was based on the measurement of DPN reduction when malic dehydrogenase was coupled with condensing enzyme. In the optical assay procedure the experimental cuvette contained the following constituents in a volume of 2.5 ml: 0.02 M tris-HCl buffer, pH 8.6, 8 x 10^-3 M potassium malate, 2 x 10^-4 M acetyl CoA, 4 x 10^-4 M DPN, and sufficient condensing enzyme to give an optical density increase of 0.040 to 0.080 per minute. The reaction, carried out at room temperature (24 C), was initiated by adding acetyl CoA to the assay mixture after the malic dehydrogenase reaction had reached equilibrium. The reaction rate is expressed as the change in optical density at a wavelength of 340 nm during the time interval between 30 and 60 seconds after the reaction was started. Both crude enzyme preparations and purified enzyme preparations contained a very active malic dehydrogenase. The concentration of malic dehydrogenase in the reaction vessel was eight to tenfold greater than the amount required to assure that condensing enzyme was rate limiting. Under the above conditions the reaction rate was linear for at least 2 minutes. Crude preparations of condensing enzyme from plants could not be assayed by the optical procedure because of the presence of DPNH oxidase.

Acetyl CoA concentration was determined by the hydroxamic acid method described by Lipmann and Tuttle (5). Protein was determined by the Folin-phenol method of Lowry et al. (6), using crystalline bovine albumin as the standard.

Results

Evidence for Reaction. When assayed by the standard procedure, presence of both acetyl CoA and oxaloacetate was essential for catalysis of citrate synthesis by purified extracts of acetone powder of tobacco leaf mitochondria. Acetyl CoA disappearance was approximately equal to citrate synthesis under conditions of the standard assay procedure.

Distribution of Condensing Enzyme in Plants. Condensing enzyme was found in every plant tissue which was surveyed for its presence. The data in table I were obtained with crude extracts of acetone powders of mitochondria from the indicated tissues. Extracts of acetone powders of whole leaves and whole roots had a specific activity of approximately one-tenth the specific activity of extracts of acetone powders of mitochondria from the same tissue.

Experiments were conducted to determine the distribution of condensing enzyme in the cell. The recovery of condensing enzyme in various fractions of tobacco leaf homogenates is shown in table II. The procedure of Pierpoint (11) was used for preparation of mitochondria with the exception that citrate was omitted from the extraction medium. The 10,000 x g precipitate, which contained a relatively large amount of chloroplast fragments, is the fraction referred to as mitochondria throughout this paper. More than half of the total activity was recovered in the 10,000 x g precipitate and the specific activity of this fraction was more than threefold greater than that of the homogenate. The specific activity of the chloroplast fraction (1,000 x g precipitate) was less than that of the homogenate which suggests that the major portion of condensing enzyme activity in the 10,000 x g precipitate was associated with the mitochondria. Further centrifugation of the 10,000 x g supernatant solution for 1 hour at 30,000 x g precipitated no appreciable amount of condensing enzyme.

Optimum pH. The effect of pH of the assay medium upon the activity of purified condensing enzyme from tobacco leaves is shown in figure 1. These curves were obtained with tris-HCl and

Table I

<table>
<thead>
<tr>
<th>Plant tissue</th>
<th>Units/ml enzyme</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco leaves</td>
<td>9.4</td>
<td>7.8</td>
<td>1.20</td>
</tr>
<tr>
<td>Tobacco roots</td>
<td>3.4</td>
<td>3.6</td>
<td>0.95</td>
</tr>
<tr>
<td>Bean leaves</td>
<td>5.7</td>
<td>7.1</td>
<td>0.81</td>
</tr>
<tr>
<td>Bean roots</td>
<td>5.1</td>
<td>4.8</td>
<td>1.05</td>
</tr>
<tr>
<td>Spinach leaves</td>
<td>4.4</td>
<td>6.3</td>
<td>0.70</td>
</tr>
<tr>
<td>Pea seeds</td>
<td>4.6</td>
<td>22.0</td>
<td>0.21</td>
</tr>
<tr>
<td>Soybean leaves</td>
<td>7.2</td>
<td>7.0</td>
<td>1.03</td>
</tr>
<tr>
<td>Soybean roots</td>
<td>5.4</td>
<td>3.5</td>
<td>1.55</td>
</tr>
<tr>
<td>Pea roots</td>
<td>4.7</td>
<td>2.8</td>
<td>1.67</td>
</tr>
</tbody>
</table>

* All extracts were crude extracts prepared from acetone powders of mitochondria as described under Materials and Methods and were assayed by the standard assay procedure.

Table II

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Specific activity (units/mg protein)</th>
<th>Activity recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>0.19</td>
<td>100</td>
</tr>
<tr>
<td>1,000 x g precipitate</td>
<td>0.14</td>
<td>19</td>
</tr>
<tr>
<td>10,000 x g precipitate</td>
<td>0.62</td>
<td>54</td>
</tr>
<tr>
<td>10,000 x g supernatant</td>
<td>0.09</td>
<td>28</td>
</tr>
</tbody>
</table>

* The leaves were disrupted in extraction medium lacking citrate, the suspension was squeezed through two thicknesses of cheesecloth, and fractions sedimented at 1,000 x g for 7 minutes and 10,000 x g for 30 minutes. The precipitates were resuspended in one-tenth the original volume of extraction medium. The standard assay procedure was used.
glycine-NaOH buffers. When assayed by the standard assay procedure, condensing enzyme exhibited a rather broad pH optimum. Maximum activity was observed at pH levels of 8.2 to 8.8 when tris-HCl buffer was used. Enzyme activity in the presence of glycine-NaOH was considerably less than activity in the presence of tris-HCl.

Effect of Acetyl CoA Concentration. In order to obtain initial reaction rates, experiments concerning the effect of acetyl CoA concentration upon activity were assayed by the optical assay procedure. Figure 2 shows the effect of acetyl CoA concentration upon activity of purified extracts of condensing enzyme from tobacco leaves. Saturation of the enzyme by acetyl CoA was attained at a concentration of approximately $10^{-4} \text{M}$. The data from figure 2 were plotted according to the method of Lineweaver and Burk (4) and the Michaelis constant ($K_M$) for acetyl CoA was calculated to be $1.8 \times 10^{-5} \text{M}$ at pH 8.6 and 24°C.

Effect of Oxaloacetate Concentration. Figure 3 shows the effect of oxaloacetate concentration upon activity of purified extracts of condensing enzyme from tobacco leaves. Saturation of the enzyme by oxaloacetate was attained at a concentration of approximately $4 \times 10^{-4} \text{M}$. No effort was made to determine the $K_M$ for oxaloacetate because initial reaction rates with low concentrations of oxaloacetate could not be obtained by the standard assay procedure.

**Discussion**

Condensing enzyme has been identified in animal tissues and microorganisms and the enzyme has been obtained in crystalline form from pig heart (10). Evidence has been obtained by a series of workers that plant mitochondria can oxidize all of the acids in the citric acid cycle and that pyruvate is oxidized only when an additional acid is present. Although there

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Fig. 1. pH Optimum of tobacco leaf condensing enzyme. The standard assay procedure was used with purified condensing enzyme (0.02 mg protein) from tobacco leaves.

Fig. 2. Effect of acetyl CoA concentration on enzyme activity. The optical assay procedure was used with purified condensing enzyme (0.08 mg protein) from tobacco leaves.

Fig. 3. Effect of oxaloacetate concentration on enzyme activity. The standard assay procedure was used with purified condensing enzyme (0.025 mg protein) from tobacco leaves.
has been no direct evidence that condensing enzyme occurs in plants it has generally been assumed to be present. Most of the evidence for occurrence of condensing enzyme in plants has been obtained by determining the specific activity of citrate produced after pyruvate-2-C\(^4\) was oxidized by whole plant tissues or by mitochondrial preparations.

The degree to which the citric acid cycle is the main pathway of pyruvate oxidation in higher plants has been uncertain. Lack of convincing evidence that condensing enzyme is present contributed much to this uncertainty. The acetate activating enzyme, acetic thiokinase, is apparently widely distributed in plants (7). Both crude and purified condensing enzyme extracts from tobacco leaves contained an active acetic thiokinase. The demonstration of the presence of an active condensing enzyme in tissues of higher plants reported in this paper provides evidence that higher plants possess the enzymes which catalyze the entry of acetate into the citric acid cycle.

Condensing enzyme in plants is apparently associated with mitochondria. The recovery of approximately one-fourth of the enzyme activity in the supernatant solution after high speed centrifugation may be due to fragments of mitochondria or to dissolution of some of the enzyme.

**Summary**

Condensing enzyme was isolated from tissues of higher plants. The enzyme was present in every plant which was surveyed for its presence. It appears to be associated with the plant mitochondria and occurs in both leaves and roots. Extracts of acetone powders of mitochondrial preparations contained condensing enzyme in a soluble form.

The enzyme from *Nicotiana tabacum* L. (var. Burley 21) was partially purified and some of its properties were studied. Condensing enzyme from tobacco exhibited a broad pH optimum. Maximum activity was produced with tris-HCl buffer at pH 8.2 to 8.8. Saturation of the enzyme by acetyl CoA was attained at a concentration of \(10^{-4}\) M and the Michaelis constant for acetyl CoA was \(1.8 \times 10^{-5}\) M at pH 8.6 and 24°C. Saturation of the enzyme by oxaloacetate was attained at a concentration of approximately \(4 \times 10^{-4}\) M.

**Acknowledgments**

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**Literature Cited**


