DEHYDROGENASE ACTIVITY OF HYDROXYMALONATE AND RELATED ACIDS IN HIGHER PLANTS¹, ²

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Plant extracts have been found to catalyze the following reversible reaction dependent upon diposphopyridine nucleotide (DPN), in which hydroxymalonate (OHM) is oxidized to ketomalonate (KM):

\[
\begin{align*}
\text{COO} & \rightarrow \text{COO} \\
\text{HC} - \text{OH} + \text{DPN}^+ & \rightarrow \text{C}=\text{O} + \text{DPNH} + \text{H}^+ \\
\text{COO} & \rightarrow \text{COO} \\
(\text{OHM}) & \rightarrow (\text{KM})
\end{align*}
\]

The above compounds are also known as tartronic and mesoazalic acids respectively.

KM has been reported to be present in the leaves of alfalfa, Medicago sativa (11), and both KM and OHM have been identified in sugar syrups made from plants (21). OHM has also been isolated from cultures of Acetobacter acetosum grown on glucose medium, but this might be due to a non-enzymatic decomposition of 2-keto-3-gluconic acid during the heat treatment with alkali (17).

Using methylene blue as an indicator of dehydrogenase activity, Quastel and Woolridge (22) reported activity with OHM by whole cells of Escherichia coli.

OHM was found to be an inhibitor of lactic dehydrogenase in E. coli (22) and in guinea-pig brain slices (19), and also a competitive inhibitor of malic dehydrogenase of pig-heart tissue (16) and of a non-PP dependent malic oxidation in pigeon-liver extracts (25). The latter pigeon-liver extracts did not oxidize OHM either with or without added DPN. Recently, OHM has been considered as a possible coenzyme of oxalosuccinic carboxylase (31).

The present study was undertaken because of the possible interrelationships of OHM and KM with two other substrates associated with a DPN-dependent dehydrogenase activity in plants, i.e., diketosuccinate and dihydroxyfumarate (28).

MATERIALS AND METHODS

The wheat germ enzyme was prepared from wheat germ S-50 (kindly supplied by General Mills) by extracting an acetone powder in 8 times its weight of M/200 phosphate buffer (pH 7.4). Subsequent treatment with MnCl₂ and solid ammonium sulfate was similar to that reported for phosphoglucolic dehydrogenase (2), except that the fraction used was that obtained between 200 to 400 gm ammonium sulfate per liter of enzyme solution. At the last step, the ammonium sulfate precipitate was taken up in approximately 1/5 the original volume and the resulting fluid...
contained about 100 mg of protein per ml (29). Dia-
lized extracts of acetone powders (27) were used in
the distribution study.

KM was used from 3 sources, with no essential
chemical or enzymatic difference between these puri-
fied samples. Most of the experiments were per-
formed with the sodium salt of KM purchased from
Aldrich Chemical Company and recrystallized from
a saturated aqueous solution of the sodium salt by the
addition of alcohol. The 2,4-dinitrophenylhydrazone
derivative had a melting point of 202 to 204°C (24),
and an Rf value of 0.40 to 0.45 when chromatographed
in the butanol phase of a butanol–alcohol–water (50–
10–40 parts by volume) solvent, using ascending
chromatography. Only one yellow spot was obtained
which turned reddish-brown upon spraying with alco-
holic KOH. The phenylhydrazine derivative had a
melting point of 163 to 165°C (24), and a Rf value
of 0.54. A second sample was obtained as the barium
salt from alloxan, using the method of Deichsel (9).
A third sample prepared from ethylmalonate (8) was
kindly supplied by Dr. I. Zelitch. (He reported that
he had obtained the 2,4-dinitrophenylhydrazone with
the correct melting point in better than a 92% yield
by weight.)

KM was identified chromatographically as a pH
spot after spraying with brom-cresol green (3) and
as a brown-black spot after spraying with alkaline
AgNO3 (3) with Rf values of 0.2 in the mesityl oxide
phase of a mesityl oxide–formic acid–water solvent
(6). It is possible to spray first with the pH indica-
tor, followed with the AgNO3 spray to observe the
superimposed spots. The 2,4-dinitrophenylhydrazone
was identified chromatographically using the method
of Cavallini (7), and analyzed quantitatively using
the total hydrazone method (15) with an incubation
period of 1 hour (the color increases with even longer
incubation periods) with a broad peak between 455 to
400 mμ. KM was also estimated by the α-methylindole
colorimetric method of Dische (10). A yellow color
is formed in Feigl's naphthoresorcinol test (12)
modified for colorimetric analysis as for glycric acid
(28). Decarboxylation of the sodium salt with an in-
soluble yeast decarboxylase (4,5) produced glyoxylace,
which was identified chromatographically as a pH spot
and as a red color in the phenylhydrazine–potassium
ferricyanide test (5,14).

Hydroxymalonate was prepared from diketosuc-
cinate (prepared from tartaric acid) according to
Fenton (13) with a melting point of 158 to 160°C
(24), or was purchased from the Aldrich Chemical
Company. It was necessary to purify the commer-
cial samples by Norite treatment followed by recrys-
tallization from concentrated solution before crystals
were obtained with the above melting point. Chemi-
ical analysis of one of these samples gave 29.5 % C,
3.51 % H; expected analysis for anhydrous OHM;
30 % C, 3.33 % H. (These analyses were made
through the kindness of Dr. B. Vennesland.) Recrys-
tallization of the Norite treated acid as the potassium
salt by the addition of alcohol was a more convenient
method of purification. Earlier commercial samples
sold in the hydrate form were contaminated with an
enzymatically active compound similar to or identical
with tartaric acid, while recent anhydrous samples
have been free of this compound. As both Dl- and
meso-tartaric acids are also enzymatically active, it
was necessary to check the sample of OHM for con-
taminating tartaric acid.

The following tests were useful in differentiating
between these two acids. Dl- or L(+)-tartaric acid
forms an insoluble potassium acid salt without the
addition of alcohol. About a 50% alcoholic solution
is necessary to precipitate the potassium acid salt of
OHM. (Alcohol is also necessary in the case of meso-
tartaric acid.) OHM reduces ammonium molybdate
solution to blue upon heating while tartaric acid does
not react. The blue color can be observed chroma-
tographically by spraying with the molybdate spray
reagent as prepared by Bandurski and Axelrod for
phosphate compounds (1), and heating the paper for
5 minutes at 85°C. This reducing method can be
adapted for colorimetric estimation by the addition of
0.1 ml of the molybdate solution to the sample (0.1
micromole of OHM) in 1 ml of water. After heating
in a hot water bath at 85°C for 10 minutes, the blue
color is measured colorimetrically at 600 mμ. KM
can be identified as the main product after heating an acid
solution of OHM in the presence of an equimolar solu-
tion of CuSO4. A yellow color with a broad absorp-
tion peak at 430 mμ is formed in the Feigl naphtho-
resorcinol test at a concentration of 5 micromoles of
OHM per ml. This is in contrast to the greenish color
reported by Feigl (12). Tartaric acid gives a bright
green color with the highest absorption peak at about
680 mμ and a lower one at 430 mμ at a concentra-
tion of 0.5 micromoles per ml. Furthermore, the two acids
can be separated chromatographically, using a mesityl
oxide–formic acid–water solvent combination (6) with
the following Rf values: OHM 0.38, tartaric 0.2, KM
0.2, malic 0.42, glyoxylic, 0.22, diketosuccinic 0.39
(presumably decomposing to OHM). An ether-
formic acid–water solvent (100–40–20 parts by vol-
ume) likewise is useful in differentiating between these
two compounds: OHM 0.65, tartaric 0.49, KM 0.39,
malic 0.68.

Even the purest preparations (correct melting
point) show very faint pH spots near the origin (Rf
0.03) when large amounts (greater than 0.5 micro-
moles) are chromatographed. This faint spot does not
react with the molybdate or AgNO3 spray. Old
samples, frozen and thawed several times, indicate an
increase in this unidentified spot, and KM samples
showed similar slow-moving spots in old solutions.

DPN of 90 % purity was purchased from Krishell's
Chemical Company. DPNH of 90 % purity as a TRIS
(tris(hydroxymethyl)aminomethane) salt was
prepared by Dr. F. Loewus. dl- and meso-tartaric
acids were purified as K acid salts from samples pur-
chased from Aldrich Chemical Co. L (+)-tartaric acid
(Merek tartaric acid, reagent grade) was similarly
purified. dl-malic acid from Krishell Chemical Co.
was recrystallized from ethyl acetate with petroleum ether.

**Experimental Results**

**Direct Spectrophotometric Observation of DPNH Oxidation and DPN Reduction:** Typical data showing reduction of DPN⁺ spectrophotometrically in the presence of added OHM are shown in figure 1, using a wheat germ enzyme preparation. DL- and meso-tartaric acids give similar DPN reduction rates and final equilibrium values at approximately one-half the substrate concentration, assuming that the activity with DL-tartaric acid is due to the \( \nu^- \) form only. The \( \nu^+ \)-isomer alone is inactive, just as it has been reported for liver mitochondria preparations (18). With both OHM and tartaric acids, the final DPN reduction was similar aerobically and anaerobically.

Equilibrium and Michaelis constants were calculated from the above data. As these values were obtained with a crude enzyme with a high blank reaction that was subtracted from all of the data, these constants should only be considered as rough estimates. The \( K \) value for the reaction \((K)\) (DPNH)\((H^+)/[OHM])\(\) (DPN⁺) was equal to \( 1 \times 10^{-14} \) M, while \( K_m \) for OHM at pH 9 was equal to \( 3.8 \times 10^{-1} \) M.

In the presence of added KM, DPNH is rapidly oxidized. This activity could not be due to the decarboxylation of KM to glyoxylate and the subsequent oxidation of DPNH by glyoxylate reductase (32) because the wheat germ enzyme preparation which is active with KM is relatively inactive with added glyoxylate. Furthermore, the subsequent addition of KM to the glyoxylate vessel causes a rapid oxidation of the DPNH at a rate comparable to that of the cuvette with KM alone. Similar results were obtained with a pea shoot enzyme preparation, although the initial glyoxylate activity was higher. No significant difference was observed when KM was added under anaerobic conditions.

**Experiments with Coupled Systems:** The addition of OHM to wheat germ extracts can be coupled with the reduction of 2,6-dichlorophenolindophenol presumably via a diaphorase also present in the extract. Comparable amounts of \( \nu^- \)-tartaric acid added as the \( \nu^- \) form were also able to reduce the dye.

The reduction of KM to OHM could be coupled with the oxidation of ethanol to acetaldehyde in the presence of DPN. Table I shows typical data from such an experiment in which the disappearance of KM was compared with the appearance of OHM. At the end of the indicated incubation periods, the vessels were held in a hot water bath for 10 minutes in order to drive off the acetaldehyde, and the cooled extract was acidified with 0.1 ml of concentrated sulfuric acid and was centrifuged. Then, 0.05-ml aliquots were analyzed for KM by the \( \nu^- \)-methylindole test or total hydrazone method, and 0.1-ml aliquots for the molybdate reducing activity of OHM. Values were standardized against standards added to non-incubated vessels with all of the components of the experimental vessels. In a number of different experiments using wheat germ or parsley leaf enzymes, about 50% of the added KM disappeared.

**Identification of OHM Starting with KM:** Starting with KM as the initial substrate, the enzymatic production of OHM was identified chromatographically in both coupled and uncoupled systems. While OHM can be identified in the crude incubation system after chromatography by using the molybdate spray method, further purification is necessary in order to identify it as the free acid because of interference with other acids of the mixture.

![Graph](image-url)

**Table I**

<table>
<thead>
<tr>
<th>Time</th>
<th>KM disappearance</th>
<th>OHM formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>micromoles</td>
<td>micromoles</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>34.6</td>
<td>34.0</td>
</tr>
<tr>
<td>180</td>
<td>40.8</td>
<td>43.2</td>
</tr>
</tbody>
</table>

The experimental vessels contained 0.2 ml of 0.5 M TRIS buffer at pH 7.4, 0.4 mg DPN⁺, 75 micromoles of KM, 0.1 ml 95% ethanol, 51 mg of wheat germ enzyme in a total volume of 3 ml.

The reduction of glyoxylate with KM occurs in wheat germ, pea shoot, and parsley leaf extracts in the presence of DPN⁺. The reaction is uncoupled by the omission of OHM, and the rates are equal in the presence of OHM. The KM value for the reaction (KM) (DPNH)\((H^+)/[OHM])\(\) (DPN⁺) was equal to \( 1 \times 10^{-14} \) M, while the \( K_m \) for OHM at pH 9 was equal to \( 3.8 \times 10^{-1} \) M.
A typical large scale experiment was performed as follows: an incubation mixture in a 4-ml volume was prepared containing the following components: 2.0 ml of an 0.5 M phosphate buffer (pH 7.4), 300 micromoles of DPNH as the TRIS salt, 300 micromoles of the sodium salt of KM, and 13 mg of a purified protein from parsley leaves. The reaction was followed spectrophotometrically at 340 mμ until the reaction was completed. The reaction mixture was acidified by the addition of 0.3 ml of 18 N H2SO4, followed by continuous ether extraction for 60 hours from this aqueous medium. The enzymatic product co-chromatographed with OHM in 5 different solvent combinations. The pH spot and the blue molybdate spot were superimposable. Controls without DPNH and without KM showed no such spots.

Identification was likewise made in a coupled system, using both ether extraction and anion column purification in order to isolate the acid. An incubation mixture similar to that described in table I was analyzed by Dische's α-methylindole test, indicating that 50% of the KM had disappeared. The remainder of the sample was air dried on strips of filter paper (30 x 4 cm). These strips were folded into a cylinder and inserted into a Soxhlet extractor in place of the thimble and were extracted with ether for 8 hours. Further separation by anion column chromatography was necessary in order to separate the remaining KM from the OHM. The ether extract was taken up in water after evaporation to dryness and put on a Dowex-1 (or IRA-400) column (about 1 x 10 cm) in the carbonate form (made with 5% Na2CO3). After washing with water, the column was eluted with 100 ml of 2.5% (NH4)2CO3. (The KM remains on the column and can be eluted with 5% (NH4)2CO3.) The 2.5% eluate was evaporated almost to dryness on a hot plate at low temperature. After batchwise decaeration with Dowex 50 (H+ form), aliquots were chromatographed. Again the molybdate-reducing spots superimposed the pH spots, and co-chromatographed with known OHM in several solvent combinations. Control experiments were negative.

Effect of OHM on Malic Dehydrogenase Activity: Since OHM has been reported to be a competitive inhibitor of lactic and malic dehydrogenases of animal tissues (16, 19, 22, 25), the inhibition of wheat germ malic dehydrogenase was checked. At an enzyme concentration so dilute that no OHM activity can be demonstrated, the addition of OHM to malic acid showed a typical competitive inhibition as indicated in figure 2. Rates were calculated from the first 5 minutes of activity after the DPN was added. These data are graphed as a double reciprocal plot of velocity vs substrate concentration according to Line and Burk (20). Blank values of increase of absorption at 340 mμ with added substrate were negligible.

Calculations of the Michaelis-Menten constant using these data give a value for Km of about 1 x 10⁻² M for the α- form of malic acid, or presumably one-half of this value for the L- form. Other calculations for Km for the L- form are those reported for wheat seeds with a Km equal to 4 x 10⁻² M (30), and for animal tissues with a value close to 1 x 10⁻² M (16). The value of K0 for OHM (20) was equal to 1 x 10⁻² M, indicating that OHM is less active an inhibitor in the wheat germ system than in pigeon liver (25).

Distribution of OHM Activity in Plants: The dehydrogenase activity capable of converting KM to OHM is probably widely distributed in plants, because it is present in all parts of a plant during various developmental stages. The activities with oxalacetate and diketosuccinate have a similar distribution within a plant (table II), while the activities with glyoxylate or hydroxypyruvate are found predominantly in leaves (27).

Table II shows enzymatic data for parts of 5 different plants. Since oxalacetate and diketosuccinic acids are unstable, they were added as small amounts of solid. The oxidation of DPNH without added substrate was subtracted from the total change in optical density with added substrate. The enzyme was diluted so that the change in optical density was not higher than 0.020 per minute. The reaction was followed for 10 minutes, and the activity expressed as the change in optical density x minutes⁻¹ x mg protein⁻¹ x 100. All enzyme preparations were made from acetone powders. Similar preparations give values of about 30 for leaf enzyme activity with hydroxypyruvate (28). Wheat germ and pea root preparations

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**Fig. 2.** The competitive inhibition of malic dehydrogenase activity in wheat germ by OHM. The incubation mixtures contained in a 3-ml volume 0.5 ml TRIS buffer (pH 9), 0.05 ml freshly prepared 0.1 M KCN, 50 to 200 micromoles L-malic acid, 50 or 100 micromoles OHM, 10 μgm wheat germ protein, and 0.4 mg DPN added in that order. V = velocity, expressed as the change in optical density per minute at 340 mμ; S = molar substrate concentration of L-malic acid.
TABLE II
DISTRIBUTION OF DEHYDROGENASE ACTIVITIES IN HIGHER PLANTS

<table>
<thead>
<tr>
<th>Preparation</th>
<th>KETO-MALONATE</th>
<th>DIKETOSUCCINATE</th>
<th>OXALACETATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea shoot</td>
<td>9.7</td>
<td>10.2</td>
<td>310.0</td>
</tr>
<tr>
<td>Pea root</td>
<td>21.7</td>
<td>8.1</td>
<td>694.0</td>
</tr>
<tr>
<td>Pea seeds</td>
<td>2.8</td>
<td>0.8</td>
<td>120.0</td>
</tr>
<tr>
<td>Parsley shoots</td>
<td>1.8</td>
<td>1.5</td>
<td>100.0</td>
</tr>
<tr>
<td>Watercress shoots</td>
<td>6.5</td>
<td>2.4</td>
<td>337.0</td>
</tr>
<tr>
<td>Carrot root</td>
<td>0.6</td>
<td>0.2</td>
<td>37.4</td>
</tr>
<tr>
<td>Wheat root</td>
<td>3.0</td>
<td>2.6</td>
<td>105.0</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>20.9</td>
<td>10.9</td>
<td>730.0</td>
</tr>
</tbody>
</table>

The data were obtained spectrophotometrically at 340 ma in a 3-ml volume containing 0.2 ml 0.05 M phosphate buffer (pH 7.4), 200 micrograms DPNH, 10 micromoles KM or approximately 10 micromoles of diketosuccinase or oxalacetic acid as solids. Activity is expressed as the change in optical density × min⁻¹× mg protein⁻¹× 100.

were among the best sources for all 3 enzymatic reactions.

Purification of Parsley Leaf Enzyme: A partial purification of the parsley leaf preparation was undertaken to determine whether the activities for oxalacetate and KM could be separated. Although possessing a lower activity with KM than wheat germ, parsley was used because the initial purification steps are easier. Table III shows enzymatic data for some of the fractions in the purification process. The procedure was devised for the best specific activity for KM, but certain of the fractions were tested for the other activities as indicated. The first few fractions were also tested for activity with hydroxypyruvate. This activity was rapidly eliminated by the pH 5 treatment and subsequent steps.

An acetone powder made from a commercial source of parsley shoots was extracted 1 hour in an 0.001 M phosphate buffer, pH 7.4, and strained through cheesecloth and centrifuged at low speed. To this supernatant (H) were added 400 gm of solid ammonium sulfate per liter of supernatant. The centrifuged precipitate was taken up in a minimum volume of buffer and dialysed against 0.001 M phosphate buffer (P1). Then, 200 gm (NH4)2SO4 per liter were added to the solution of P1. The new precipitate, P2, was subsequently discarded. More (NH4)2SO4 was added to the above solution to make a total of 400 gm per liter of solution. The precipitate, P3, represents the ammonium sulfate precipitate obtained between 200 to 400 gm per liter. The supernatant was discarded. Fraction P3 was taken up in distilled water and the pH lowered to 5.0 with 10 % acetic acid. The residue was discarded. The supernatant, S1, contained approximately 75 % of the activity of the extract H, and was re-fractionated with ammonium sulphate. The first precipitate between 0 to 250 gm per liter was discarded. The second precipitate (P4) between 250 to 300 gm per liter contained approximately 35 % of the activity of H, and the third precipitate (P5) from 350 to 400 gm per liter about 26 % of the original activity. These two fractions represented a 30-50-fold purification. Subsequent treatment with calcium phosphate gel increased the total purification about 80-fold, but in fractions representing only about 1 % of the original activity of H. About 70 mg of Ca3(PO4)2 gel were added to 40 mg of the protein in fraction P4. The supernatant S2 had one of the highest specific activities (146) but represented only 1 % of the original total activity. The enzyme adsorbed on the gel was eluted with 3 successive 5 ml portions of 0.1 M phosphate buffer (G1, G2 and G3) containing 12 %, 7 %, and 1 % of the activity of H, respectively.

Although the three enzymatic activities with oxalacetate, diketosuccinate and KM follow the same general pattern during purification, some differences were found. The highest purification was about 50-fold for KM, 48-fold for oxalacetate, and 28-fold for diketosuccinate.

**TABLE III**
SUMMARY OF DEHYDROGENASE ACTIVITIES DURING THE PURIFICATION OF A PARSLEY LEAF PREPARATION

<table>
<thead>
<tr>
<th>Preparative stage</th>
<th>KETO-MALONATE</th>
<th>DIKETOSUCCINATE</th>
<th>OXALACETATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>1.8</td>
<td>1.5</td>
<td>100</td>
</tr>
<tr>
<td>P1</td>
<td>11.1</td>
<td>. .</td>
<td>. .</td>
</tr>
<tr>
<td>P2</td>
<td>1.9</td>
<td>. .</td>
<td>. .</td>
</tr>
<tr>
<td>P3</td>
<td>20.5</td>
<td>. .</td>
<td>. .</td>
</tr>
<tr>
<td>S1</td>
<td>38.0</td>
<td>5.0</td>
<td>940</td>
</tr>
<tr>
<td>P4</td>
<td>52.5</td>
<td>6.8</td>
<td>1669</td>
</tr>
<tr>
<td>P5</td>
<td>95.8</td>
<td>. .</td>
<td>. .</td>
</tr>
<tr>
<td>S2</td>
<td>146.0</td>
<td>42.4</td>
<td>4781</td>
</tr>
<tr>
<td>G1</td>
<td>97.0</td>
<td>19.0</td>
<td>1735</td>
</tr>
<tr>
<td>G2</td>
<td>91.0</td>
<td>28.0</td>
<td>4757</td>
</tr>
<tr>
<td>G3</td>
<td>140.0</td>
<td>25.0</td>
<td>2765</td>
</tr>
</tbody>
</table>

Activity is expressed as in table II, and the purification stages are described in the text.

**Discussion**

Is the dehydrogenase activity reported here for OHM of any physiological importance? Although OHM has been identified in biological material (11, 21, 23), the recent finding of OHM as a non-enzymatic product of a gluconic acid derivative during the extraction of the acid (17) indicates the possibility of a non-enzymatic origin of this compound in extracts of biological material. This problem is still to be re-investigated.

A second problem is whether this activity with OHM is due to a separate enzyme or to the activity of a relatively non-specific dehydrogenase. Malic dehydrogenase has a similar distribution pattern in plants, but the ratio of the activity with oxalacetate and KM does vary somewhat during purification. Furthermore, the competitive inhibition by OHM of malic dehydrogenase activity at a level of enzyme concentration too weak to give any activity with OHM alone, would argue against the activity being due to the malic dehydrogenase. Complete separation of the enzymatic activities will be necessary to prove this point.
If one assumes a physiological function for OHM and KM, their relationships with closely related compounds are of interest. The following conversions might be involved: D-tartrate $\rightarrow 2\text{H}$ dihydroxyfumarate $\rightarrow 2\text{H}$ diketosuccinate $\rightarrow \text{CO}_2$ OHM $\rightarrow 2\text{H}$ KM. There is evidence of enzymatic activity in plant extracts for all but the decarboxylation step. Although leaf extracts possess an active hydroxypyruvic reductase activity (28), no evidence has been obtained in these leaf preparations of any hydroxypyruvate arising from dihydroxyfumarate as reported by Kun and Hernandez (18).

**SUMMARY**

A dehydrogenase activity capable of a reversible conversion of hydroxymalate to ketomalonate in the presence of diphosphopyridine nucleotide has been demonstrated in a variety of plant extracts. The distribution of this activity in plants is similar to that for diketosuccinate reductase and malic dehydrogenase. An 80-fold purification of the enzyme was made. Although oxalacetate and diketosuccinate activities were still present, changes in relative activity occurred, indicating that different enzymes are involved. Furthermore, hydroxymalate is a competitive inhibitor of malic dehydrogenase activity at enzyme concentration levels too weak to give any activity with hydroxymalate alone. Although both ketomalonate (mesoxalic) and hydroxymalonate (tartronic) acids have been demonstrated by others in plant extracts, the physiological role, if any, of this activity is still to be demonstrated.

The author is indebted to Dr. B. Vennesland, Biochemistry Department, University of Chicago, under whom the earlier work was done, and to Dr. A. Magaldi who performed some of these earlier experiments.

**LITERATURE CITED**

THE REACTIONS OF THE PHOTINDUCITIVE DARK PERIOD \textsuperscript{1,2}

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Since the demonstration by Hamner and Bonner (7) of the importance of the dark period for photoperiodic induction in the short-day plant Xanthium, there have been many attempts to elucidate the reactions which take place within the plant during this period (2, 11, 13, 18). The dark period reactions appear to be concerned directly with the act of induction—the persistent change of the plant from the vegetative to the flowering condition. In Xanthium the flowering condition persists after a single completed act of induction even though the photoperiod during floral development is too long for induction to occur in vegetative plants. The condition of the plant after induction has taken place will be termed the induced state to distinguish it from the act of induction.

The induced state in Xanthium is a quantitative one. Rate of development of the floral bud is dependent upon the intensity of the original act of induction. A measure of the rate of development based upon a series of floral stages has been previously described (21). By the application of this system the quantitative nature of induction can be demonstrated by the relationship between length of the inductive dark period and subsequent rate of floral development, as measured by floral stage a number of days after induction (this is illustrated by the control points of the experiment shown in fig 4). The longer the dark period, the more rapidly the buds develop. This may be interpreted on the supposition that the rate of bud development is dependent upon the amount of flowering hormone produced, and that longer dark periods result in the production of more flowering hormone. This view is supported by the fact that buds develop at different rates when the leaves are removed from Xanthium plants at different times after the beginning of induction (9, 21, 22, 23). If leaves are removed immediately following induction the plants seldom flower. If leaves are removed after a sufficient time, however, floral buds develop at a rate almost as great as that attained by control plants with leaves. Intermediate rates of floral development characterize plants whose leaves are removed at intermediate times (this is illustrated by the points labeled “leaf removed” in fig 5). These results are in accord with the hypothesis that rate of floral development is determined by the amount of flowering hormone which reaches the growing point. The longer the leaves remain on the plant, the more hormone is translocated from them to the growing point.

The experiments below concern kinetic studies on the reactions of the dark period. Xanthium pensylvanicum Wall.\textsuperscript{4} plants were treated at various times by red light interruption of the dark period and/or the application of auxin (which inhibits the act of induction, 21). The effects of these treatments on floral induction were measured in terms of rate of subsequent floral development, which is assumed to measure the amount of flowering hormone exported from the leaf.

METHODS

Plants were grown as previously described (3, 20, 21) from seed and maintained in a vegetative condition by daylight supplemented with incandescent irradiation of approximately 100 ft lan to make up a total day length of approximately 20 hours. To facilitate auxin treatment and to insure controlled light intensity during interruption of the dark period, plants were defoliated to a single leaf after first being classified according to the size of the most rapidly expanding leaf, the one most sensitive to induction. In the experiments reported below, the plants were defoliated to the most rapidly expanding leaf and given a single dark period, unless stated otherwise in the figure headings. A weak green light (ca 2 \(\mu W/cm^2\) for 10 minutes at 2-hour intervals) was used to facilitate treatment during the dark period.

The growing points of treated plants were examined under a dissecting microscope (36 diameters magnification) approximately 9 days after induction. Rate of floral development as measured by the pres-

\textsuperscript{1}Received November 15, 1955.

\textsuperscript{2}Report of work supported in part by the Herman Frasch Foundation and in part by the National Science Foundation.

\textsuperscript{3}Arthur McCallum and Atomic Energy Commission Predoctoral Fellow during part of the work reported herein.

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\textsuperscript{4}Synonymous with X. saccharatum, the name used by various other workers in photoperiodism. Specimens of the type of plants used in these studies have been filed by K. C. Hamner at the herbarium of the University of California at Los Angeles.