13. KOCH, L. H. and MCMEEKIN, T. L. A new direct nesslerization microkjeldahl method and a modification of the nessler-folin reagent for ammonia. Jour. Amer. Chem. Soc. 46: 2066–2068. 1924.
14. KVAMME, E. and HELLMAN, L. Isolation of pyruvic and alpha-ketoglutaric acids from blood and tissues in the presence of carbon-14 acetate. Anal. Chem. 26: 1995–1997. 1954.
15. OWENS, R. G. Metabolism of fungus spores. I. Oxidation and accumulation of organic acids by conidia of *Neurospora sitophila*. Contrib. Boyce Thompson Inst. 18: 125–144. 1955.
16. PETERS, R. Significance of biochemical lesions in the pyruvate oxidase system. Brit. Med. Bull. 9: 116–121. 1953.
17. RACKER, E. Crystalline alcohol dehydrogenase from baker's yeast. Jour. Biol. Chem. 184: 313–319. 1950.
18. RUBINSTEIN, D. and DENSTEDT, O. F. The metabolism of the erythrocyte. III. The tricarboxylic acid cycle in the avian erythrocyte. Jour. Biol. Chem. 204: 623–637. 1953.
19. SHEAR, C. L. and DODGE, B. O. Life histories and heterothallism of the red bread-mold fungi of the *Monilia sitophila* group. Jour. Agr. Research 34: 1019–1042. 1927.
20. STOTZ, E. A colorimetric determination of acetaldehyde in blood. Jour. Biol. Chem. 148: 585–591. 1943.
21. STRAUSS, B. Aspects of the carbohydrate metabolism of a mutant of *Neurospora crassa* requiring acetate for growth. Arch. Biochem. Biophys. 36: 33–47. 1952.
22. STRAUSS, B. Properties of mutants of *Neurospora crassa* with low pyruvic carboxylase activity. Arch. Biochem. Biophys. 44: 200–210. 1953.
23. SUSSMAN, A. S. The effect of furfural upon the germination and respiration of ascospores of *Neurospora tetrasperma*. Amer. Jour. Bot. 40: 401–404. 1953.
24. SUSSMAN, A. S. The effect of heterocyclic and other compounds upon the germination of ascospores of *Neurospora tetrasperma*. Jour. Gen. Microbiol. 8: 211–216. 1953.
25. SUSSMAN, A. S. Changes in the permeability of ascospores of *Neurospora tetrasperma* during germination. Jour. Gen. Physiol. 38: 59–77. 1954.
26. SUSSMAN, A. S. and MARKERT, C. L. The development of tyrosinase and cytochrome oxidase activity in mutants of *Glomerella cingulata*. Arch. Biochem. Biophys. 45: 31–40. 1953.
27. WESTENBRINK, H. G. K. and STEYN-PARVÉ, ELIZABETH Methods for the determination of diphosphothiamine in animal tissues, blood and yeast. Intern. Rev. Vitamin Research 21(4): 461–479. 1950.
28. WESTERFELD, W. W. A colorimetric determination of blood acetoin. Jour. Biol. Chem. 161: 495–502. 1945.

DEHYDROGENASE ACTIVITY OF HYDROXYMALONATE AND RELATED ACIDS IN HIGHER PLANTS[1,2]

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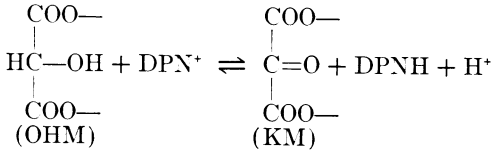
Plant extracts have been found to catalyze the following reversible reaction dependent upon diphosphopyridine nucleotide (DPN), in which hydroxymalonate (OHM) is oxidized to ketomalonate (KM):



The above compounds are also known as tartronic and mesoxalic 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-D-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-DPN 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 posible interrelationships of OHM and KM with two other substrates associated with a DPN-dependent dehydrogenase activity in plants, i.e., diketosuccinate and dihydroxyfumarate (26).

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 $MnCl_2$ and solid ammonium sulfate was similar to that reported for phosphogluconic dyhydrogenase (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

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contained about 100 mg of protein per ml (29). Dialyzed 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 purified samples. Most of the experiments were performed 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 R_f 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 alcoholic KOH. The phenylhydrazine derivative had a melting point of 163 to 165° C (24), and a R_f 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 $AgNO_3$ (3) with R_f 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 indicator, followed with the $AgNO_3$ 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 460 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 glyceric acid (28). Decarboxylation of the sodium salt with an insoluble yeast decarboxylase (4, 5) produced glyoxylate, 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 diketosuccinate (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 commercial samples by Norite treatment followed by recrystallization from concentrated solution before crystals were obtained with the above melting point. Chemical analysis of one of these samples gave 29.5 % C, 3.51 % H; expected analysis for anyhydrous OHM; 30 % C, 3.33 % H. (These analyses were made through the kindness of Dr. B. Vennesland.) Recrystallization 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 contaminating 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 chromatographically 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 660 mμ. KM can be identified as the main product after heating an acid solution of OHM in the presence of an equimolar solution of $CuSO_4$. A yellow color with a broad absorption peak at 430 mμ is formed in the Feigl naphthoresorcinol 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 concentration 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 R_f 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 volume) 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 (R_f 0.03) when large amounts (greater than 0.5 micromoles) are chromatographed. This faint spot does not react with the molybdate or $AgNO_3$ 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 purchased from Aldrich Chemical Co. L(+)-tartaric acid (Merck 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 D(−)-form only. The L(+)-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 $(KM)(DPNH)(H^+)/(OHM)(DPN^+)$ was equal to 1×10^{-14} M, while K_m for OHM at pH 9 was equal to 3.8×10^{-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 glyoxylic 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.

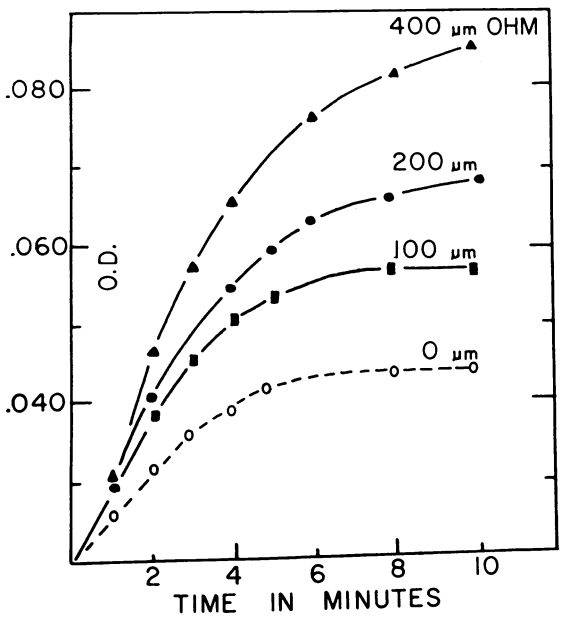

Fig. 1. Reduction of DPN^+ in the presence of wheat germ enzymes by OHM or tartaric acids. The second curve from the top (solid circles) represents a family of curves for the reduction of DPN^+ in the presence of 200 micromoles OHM, 200 micromoles DL-tartaric, or 100 micromoles of D(−)-tartaric acid. The other curves represent data for OHM only. These experiments were made in 0.08 M TRIS buffer at pH 9.0 in a 3-ml volume containing 0.4 mg DPN^+, 0 to 400 micromoles of OHM or tartaric acid, and 20 mg of wheat germ enzyme. Optical density (OD) measurements were made at 340 mμ.

Table I

KM Disappearance and OHM Formation by OHM Dehydrogenase Activity Coupled with Alcohol Dehydrogenase

Time	KM disappearance	OHM formed
min	*micromoles*	*micromoles*
0	0	0
60	34.6	34.0
180	40.8	43.2

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.

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 D(−)-tartaric acid added as the DL- 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 α-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.

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 H_2SO_4, 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 × 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 × 10 cm) in the carbonate form (made with 5 % Na_2CO_3). After washing with water, the column was eluted with 100 ml of 2.5 % $(NH_4)_2CO_3$. (The KM remains on the column and can be eluted with 5 % $(NH_4)_2CO_3$.) The 2.5 % eluate was evaporated almost to dryness on a hot plate at low temperature. After batchwise decationization 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 tisues (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 Lineweaver and Burk (20). Blank values of increase of absorption at 340 mμ without added substrate were negligible.

Calculations of the Michaelis and Menten constant using these data give a value for K_m of about 1×10^{-2} M for the DL- form of malic acid, or presumably one-half of this value for the L- form. Other calculations

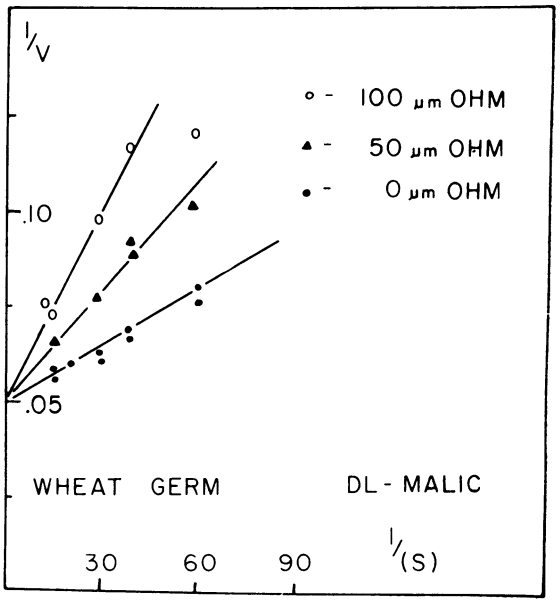

Fig. 2. The competitive inhibition of malic dehydrogenase activity in wheat germ by OHM. The incubation mixtures contained in a 3-ml volume 0.5 ml TRIS buffer (pH 9), 0.05 ml freshly prepared 0.1 M KCN, 50 to 200 micromoles DL-malic acid, 50 or 100 micromoles OHM, 10 μgm wheat germ protein, and 0.4 mg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

for K_m for the L- form are those reported for wheat seeds with a K_m equal to 4×10^{-2} M (30), and for animal tissues with a value close to 1×10^{-2} M (16). The value of K_i for OHM (20) was equal to 1×10^{-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 oxalacetic 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 × minutes^{-1} × mg protein^{-1} × 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

TABLE II

DISTRIBUTION OF DEHYDROGENASE ACTIVITIES IN HIGHER PLANTS

PREPARATION	KETO-MALONATE	DIKETO-SUCCINATE	OXAL-ACETATE
Pea shoot	8.7	10.2	310.0
Pea root	21.7	8.1	694.0
Pea seeds	2.8	0.8	120.0
Parsley shoots	1.8	1.5	100.0
Watercress shoots ..	6.5	2.4	337.0
Carrot root	0.6	0.2	37.4
Wheat root	3.0	2.6	105.0
Wheat germ	20.9	10.9	730.0

The data were obtained spectrophotometrically at 340 mμ 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 diketosuccinic or oxalacetic acids as solids. Activity is expressed as the change in optical density $\times$ min^{-1} $\times$ mg $protein^{-1}$ $\times$ 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 dialyzed against 0.001 M phosphate buffer (P_1). Then, 200 gm $(NH_4)_2SO_4$ per liter were added to the solution of P_1. The new precipitate, P_2, was subsequently discarded. More $(NH_4)_2SO_4$ was added to the above solution to make a total of 400 gm per liter of solution. The precipitate, P_3, represents the ammonium sulfate precipitate obtained between 200 to 400 gm per liter. The supernatant was discarded. Fraction P_3 was taken up in distilled water and the pH lowered to 5.0 with 10 % acetic acid. The residue was discarded. The supernatant, S_1, 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 (P_4) between 250 to 300 gm per liter contained approximately 35 % of the activity of H, and the third precipitate (P_5) 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 $Ca_3(PO_4)_2$ gel were added to 40 mg of the protein in fraction P_4. The supernatant S_2 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 (G_1, G_2 and G_3) containing 12 %, 7 %, and 1 % of the activity of H, respectively.

TABLE III

SUMMARY OF DEHYDROGENASE ACTIVITIES DURING THE PURIFICATION OF A PARSLEY LEAF PREPARATION

PREPARATIVE STAGE	KETO-MALONATE	DIKETO-SUCCINATE	OXAL-ACETATE
H	1.8	1.5	100
P_1	11.1	...	....
P_2	1.9	...	....
P_3	20.5	...	....
S_1	38.0	5.0	940
P_4	52.5	6.8	1669
P_5	95.8	...	....
S_2	146.0	42.4	4781
G_1	97.0	19.0	1735
G_2	91.0	28.0	1757
G_3	140.0	25.0	2765

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

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 80-fold for KM, 48-fold for oxalacetate, and 28-fold for diketosuccinate.

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 reinvestigated.

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 $\xrightarrow{-2H}$ dihydroxyfumarate $\xrightarrow{-2H}$ diketosuccinate $\xrightarrow{-CO_2}$ OHM $\xrightarrow{-2H}$ 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 hydroxymalonate 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 diketosuccinic 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, hydroxymalonate is a competitive inhibitor of malic dehydrogenase activity at enzyme concentration levels too weak to give any activity with hydroxymalonate alone. Although both ketomalonic (mesoxalic) and hydroxymalonic (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

1. Bandurski, R. S. and Axelrod, B. The chromatographic identification of some biologically important phosphate esters. Jour. Biol. Chem. 193: 405–410. 1951.
2. Barnett, R. C., Stafford, H. A., Conn, E. E. and Vennesland, B. Phosphogluconic dehydrogenase in higher plants. Plant Physiol. 28: 115–122. 1953.
3. Bloch, R. J. Paper Chromatography. Pp. 92–100. Academic Press, New York. 1952.
4. Brunel-Capelle, G. Sur une forme particulière de décarboxylation biologique. Compt. rend. acad. sci. 233: 1658–1660. 1951.
5. Brunel-Capelle, G. Décarboxylation biologique de l'acide mésoxalique et contribution à l'étude due métabolisme des composés thiols chez les végétaux. Thèses, Faculté des Sciences, l'Université de Paris 1955.
6. Bryant, F. and Overell, B. T. Quantitative chromatographic analysis of organic acids in plant tissue extracts. Biochim. Biophys. Acta 10: 471–476. 1953.
7. Cavallini, D. and Frontali, N. Quantitative determination of keto-acids by paper partition chromatography. Biochim. Biophys. Acta 13: 439–445. 1954.
8. Curtis, R. S. and Stracham, E. K. Condensations in the mesoxalic ester series. Jour. Amer. Chem. Soc. 33: 396–400. 1911.
9. Deichsel, T. Ueber die Mesoxalsäure. Jour. prakt. Chem. 93: 192–233. 1864.
10. Dische, Z., Weil, R. and Landsberg, E. A new color reaction for ketoacids and other carbonyl compounds. Jour. Biol. Chem. 208: 23–28. 1954.
11. Euler, H. von and Bolin, I. Zur Kenntnis biologisch wichtiger Oxydation. Zeit. Physiol. Chemie. 61: 1–11. 1909.
12. Feigl, F. Qualitative Analysis by Spot Tests, 2nd ed. P. 302. Nordeman Publ. Co., Inc, New York 1939.
13. Fenton, H. J. H. Properties and relationships of dihydroxytartaric acid. Jour. Chem. Soc. 73: 71–81. 1898.
14. Fosse, M. M. and Hieulle, A. Sur une réaction colorée, supposeé spécifique de l'aldehyde formique, produite par l'acide glyoxylique. Compt. rend. acad. sci. 179: 636–638. 1924.
15. Friedeman, T. E. and Haugen, G. E. Pyruvic acid. II. The determination of keto acids in blood and urine. Jour. Biol. Chem. 147: 415–442. 1943.
16. Green, D. The malic dehydrogenase of animal tissues. Biochem. Jour. 30: 2095–2110. 1936.
17. Hall, A. N., Kulka, D. and Walker, T. K. Formation of arabinose, ribulose, and tartronic acid from 2-keto-D-gluconic acid. Biochem. Jour. 60: 271–274. 1955.
18. Kun, E. and Hernandez, M. G. Enzymatic oxidation of tartaric acid. Federation Proc. 14: 240. 1955.
19. Jowett, M. and Quastel, J. H. Effects of hydroxymalonate on the metabolism of brain. Biochem. Jour. 31: 275–281. 1937.
20. Lardy, H. A. Respiratory Enzymes. Pp. 17–24. Burgess Publ. Co., Minneapolis, Minnesota 1949.
21. Lippmann, E. O. von. Ein Vorkommen von Tartronsäure and Mesoxalsäure. Ber. deut. chem. Ges. 46: 3862–3864. 1913.
22. Quastel, J. H. and Woolridge, R. Some properties of the dehydrogenating enzymes of bacteria. Biochem. Jour. 22: 689–702. 1928.
23. Riebin, W. B. and Hastings, A. B. Chemical and biological study of tartronic acid. Helv. Physiol. et Pharmacol. Acta 4: 52–53. 1946.
24. Rodd, E. H., Ed. Chemistry of Carbon Compounds. Pp. 1098, 1126. Elsevier Publ. Co., New York 1952.
25. Scholefield, P. G. The oxidation of malic and mesotartaric acids in pigeon-liver extracts. Biochem. Jour. 59: 177–179. 1951.
26. Stafford, H. A., Magaldi, A. and Vennesland, B. Enzymatic oxidation of DPNH by diketosuccinate and dihydroxyfumarate. Science 120: 265–266. 1954.
27. Stafford, H. A. and Magaldi, A. A developmental study of D-glyceric dehydrogenase. Plant Physiol. 29: 504–508. 1954.
28. Stafford, H. A., Magaldi, A. and Vennesland, B. The enzymatic reduction of hydroxypyruvic acid to D-glyceric acid in higher plants. Jour. Biol. Chem. 207: 621–629. 1954.

29. WARBURG, O. and CHRISTIAN, W. Isolierung und Kristallisation des Gärungsferments Enolase. Biochem. Zeits. 310: 384–421. 1942.
30. WAYGOOD, E. R. Physiological and biochemical studies in plant metabolism. Canadian Jour. Research C 28: 7–62. 1950.
31. WESSON, L. G. Tartronate as a possible coenzyme of oxalosuccinic carboxylase. Science 122: 595–597. 1955.
32. ZELITCH, I. Oxidation and reduction of glycolic and glyoxylic acids in plants. Jour. Biol. Chem. 201: 719–726. 1953.

THE REACTIONS OF THE PHOTOINDUCTIVE DARK PERIOD[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.[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 fc 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 μw/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-

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[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.