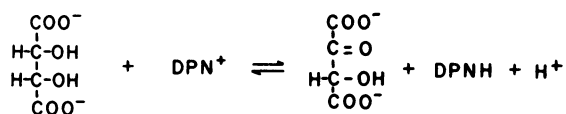


TARTARIC ACID DEHYDROGENASE ACTIVITY IN HIGHER PLANTS^{1,2}

HELEN A. STAFFORD

BIOLOGY DEPARTMENT, REED COLLEGE, PORTLAND, OREGON

An enzyme which catalyzes the oxidation of (-)- and meso-tartrate to the enol or keto form of dihydroxyfumarate (DHF) by a diphosphopyridine nucleotide (DPN) dehydrogenase can be demonstrated in higher plants. The reaction is postulated as follows:



Although the tartrates have been considered as possible metabolites in higher plants since the initial work of Pasteur in 1848, surprisingly little is known of their actual physiological significance. Vickery and Palmer (35) have briefly reviewed the history of (+)-tartrate in plants, including the problems of identification and of nomenclature. They conclude that (+)-tartrate is an uncommon plant acid produced from glucose by a few plants like grapes. In their own experimental work with tobacco leaves, these authors report that the (+)-isomer is metabolically inert, and that it has no effect on the production of the enzymatically active L-malic acid. No reference is made, however, to either the meso- or the (-)-forms as possible metabolites, the latter isomer having the configuration of the enzymatically active L-malic acid. The meso-form could have been overlooked in plants because it has solubility characteristics which differ from those of the other tartrates.

Bacteria and molds, on the other hand, can oxidize both the (-)- and (+)-isomers of tartaric acid, the (+)-isomer being the most active (34). Recently, bacterial extracts have been reported capable of converting either (-)- or (+)-tartrate to oxalacetate (17, 20), and tartrate is postulated as an intermediate in the production of oxalate from oxalacetate in *Aspergillus niger* (2).

Furthermore, the (-)- and meso-isomers of tartrate are active metabolites in extracts of animal tissues. Scholefield (28) investigated a DPN-linked dehydrogenase in pigeon liver which oxidizes meso-tartrate, while Kun et al (15, 16) reported a mitochondrial dehydrogenase in rat and beef tissue which is capable of oxidizing both the (-)- and meso-forms, but not the (+)-isomer.

The presumed oxidation product, dihydroxyfumarate (DHF) has been reported in higher plants (7, 18, 27), but the instability of this compound would make its identification very difficult. Furthermore, the presence of DHF could easily be confused with ascorbic acid because of similar chemical properties (7).

The chemistry of DHF and its non-enzymatic oxidation and decarboxylation products was first reported by Fenton in a series of articles in which the following breakdown compounds were discussed: glycolaldehyde, diketosuccinic, ketomalonic (mesoxalic), hydroxymalonic (tartronic) acids, mesoxalic-semialdehyde, glyoxal, and the 3 isomers; tartronic-semialdehyde, dihydroxyacrylic and hydroxypyruvic acids (3 to 6). Later, oxalic and glyoxylic acids were added to this list of compounds (19). These side reactions of DHF, which make this compound a difficult one to use experimentally, will be discussed later along with the possible role of DHF in plant metabolism (fig 3).

The present work on tartaric acid dehydrogenase in higher plants was started as a result of investigations concerning diketosuccinic acid reductase and hydroxymalonic acid dehydrogenase (30, 32). The activity of (-)-tartrate was briefly reported in the latter paper, and a preliminary account of the present data has already been made (33).

MATERIALS AND METHODS

Enzyme preparations from peas (*Pisum sativum*), wheat germ S-50, and beans (*Phaseolus vulgaris*) were made from acetone powders with subsequent precipitation with ammonium sulfate as previously described (32), except that treatment with MnCl_2 was omitted. The fraction used was that obtained between 200 to 400 gm ammonium sulfate per liter of enzyme solution. Typical protein content (32) of the major preparations used was as follows: wheat germ, 46 mg per ml extract; 12-day-old green shoots, 8.2 mg per ml; pea seeds soaked for 12 hours, 7.9 mg per ml.

The tartrates were recrystallized as potassium acid salts (32); (+)-tartaric acid was obtained from Merck (reagent grade), (-)-tartaric (labelled L-) and meso-tartaric acids were purchased from both Aldrich Chemical Company and Nutritional Biochemicals Corporation. Samples of DHF used were either made from (+)-tartaric acid or were purchased from Aldrich Chemical Company and were recrystallized according to Hartree (8). The purity of the samples (96 to 100%) was determined spectrophotometrically by light absorption of the enol form at 292 $m\mu$ (8) and by a $\text{K}_3\text{Fe}(\text{CN})_6$ -Prussian blue analysis for both enol and keto forms (21). The DPNH used was purchased from Sigma Chemical Company. Glycolaldehyde was made from DHF according to Fenton (4). After treatment with Dowex 1 (CO_3^- form) to remove any free acid, analysis with crystalline alcohol dehydrogenase indicated a minimum purity of 85 to 90%. Hydroxypyruvate of 95% purity by enzymatic analysis was made from methylbromopyruvate (Aldrich Chemical Co.). Other compounds used were described in previous papers (30, 31, 32).

¹ Received February 5, 1957.

² This investigation was supported by a research grant from the National Science Foundation (NSF-G 1277).

The tartrates were analyzed by the naphthoresorcinol (32) or sodium vanadate methods (34), the latter being adaptable for paper chromatography for the optically active isomers. Reduced DPN (DPNH), hydroxymalonate, and DHF were determined by the $K_3Fe(CN)_6$ —Prussian blue analysis. Hydroxymalonate, DHF, and hydroxypyruvate were analyzed by the molybdate method (32). The 2,4-dinitrophenylhydrazine colorimetric and chromatographic methods used were described previously (32).

EXPERIMENTAL RESULTS AND DISCUSSION

DEMONSTRATION OF THE AEROBIC OXIDATION OF TARTRATE TO DHF BY SPECTROPHOTOMETRIC OBSERVATION OF THE REDUCTION OF DPN: The reduction of DPN in the presence of added tartaric acid, indicated by an increase in absorption of 340 $m\mu$, is shown in figure 1. The curves illustrate the difference in activity between meso- and (-)-tartrate in wheat germ preparations. Comparable results were obtained with pea enzyme preparations. No measurable activity is observed in the presence of the (+)-isomer. The final equilibrium values with either of the active tartrates vary somewhat with different preparations, probably because of variations in side reactions which affect the products of the reaction. Under the conditions, described in figure 1, the enzyme was saturated with

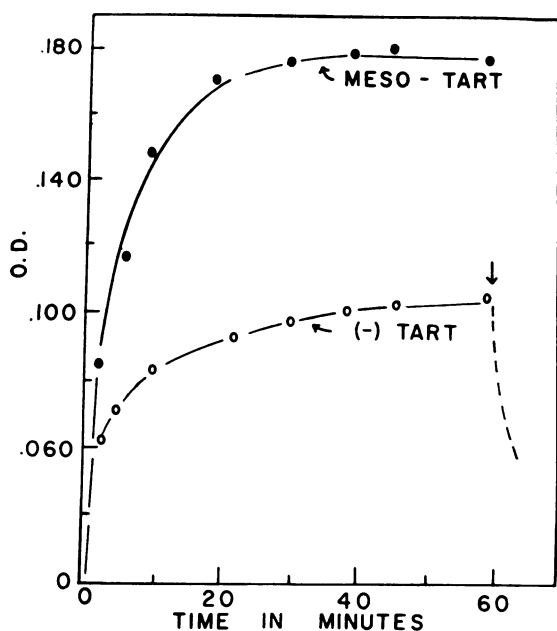


Fig. 1. Reduction of DPN by tartrate in the presence of wheat germ enzymes. The light absorption (O.D.) at 340 $m\mu$ due to DPNH is plotted against time. Cuvettes contained the following in a total volume of 3 ml: 0.5 ml of 0.5 M TRIS buffer (pH 9.0), 0.4 mg DPN⁺, 100 micromoles of meso- or (-)-tartrate, and 9.2 mg protein from wheat germ extract. Blank values of DPN reduction without added substrate have been subtracted.

TABLE I
ANAEROBIC OXIDATION OF TARTRATE IN THE PRESENCE OF $K_3Fe(CN)_6$

HRS INCUBATION	TARTRATE	MICROMOLES	
		$K_3Fe(CN)_6$ PRODUCED *	DPNH OR DHF CALCULATED **
1	Meso-	1.71	(0.43)
2	Meso-	2.92	(0.73)
4	Meso-	5.26	(1.31)
2	(-)-	0.69	(0.17)
2	(-)- + Mg ⁺⁺	0.59	(0.15)
2	Meso-	2.52	(0.63)

Thunberg tubes contained the following in a 3 ml volume: 0.5 ml 0.5 M TRIS (tris-hydroxymethyl-aminomethane), 0.2 mg DPN⁺, 23 mg wheat germ protein, 200 micromoles tartrate, 150 micromoles Mg⁺⁺, 60 micromoles $K_3Fe(CN)_6$. Blank values have been subtracted.

* Prussian blue analysis.

** Calculated from above analysis.

100 micromoles of substrate, and extra DPN did not increase the rate. When 150 micromoles of acetaldehyde were added to the cuvette with (-)-tartrate at 60 minutes, there was a rapid decrease in absorption at 340 $m\mu$ as the DPNH was oxidized by the alcohol dehydrogenase present in the enzyme mixture. Unlike the enzyme reported by Kun (15, 16), there was no noticeable effect of either added Mg⁺⁺ or ethylenediamine-tetraacetic acid in these plant preparations. Whether this is due to a difference in the activity of the enzyme itself, or to the presence of substances or side reactions that would obscure this effect has not been determined. Furthermore, the plant activity is a far more sluggish one than that reported for animal tissues. The presence of hydroxylamine to trap the DHF formed doubled the rate of the reduction of DPN. Since the sodium salts of tartrate appeared to be less active than the potassium salts, KOH was used to neutralize the tartaric acid in the work reported here. Anaerobic experiments, performed in evacuated Thunberg tubes, appeared to have no effect on the final amount of DPN reduced. TPN is inactive even when 10 × the amount of DPN used was added.

DEMONSTRATION OF THE ANAEROBIC OXIDATION OF TARTRATE IN A COUPLED SYSTEM: While neotetraazolum and 2,6-dichlorophenolindophenol were reduced anaerobically in the presence of tartrate via diaphorase, the use of $K_3Fe(CN)_6$ as the ultimate electron acceptor was the most sensitive method used. DPNH and hydroxymalonate in trichloroacetic acid filtrates give a positive test when using the Prussian blue colorimetric method to determine the $K_4Fe(CN)_6$ formed either before or after the addition of the Prussian blue reagent. (The Prussian blue color produced by hydroxymalonate is characterized by a 2- to 3-minute lag in the appearance of the color after the reagents are added.) Although both DHF and hydroxymalonate react in this test, and the values are additive when the substances are analyzed together, only a one step oxidation is observed when DHF is

the starting substrate. This indicates that the presumed oxidation product, diketosuccinate, does not decarboxylate to hydroxymalonate under the conditions of the test. Attempts to analyze the products of this $K_3Fe(CN)_6$ oxidation were not successful.

Typical results are shown in table I for a wheat germ preparation. Similar results were obtained with a pea leaf enzyme preparation. The reaction is roughly linear with time for about a 4-hour period. Assuming that both DPNH and DHF give only a one step oxidation each, these $K_4Fe(CN)_6$ values can be divided by 4 to give the amount of DPNH or of DHF produced. Again the meso- form of tartrate is more active than the (-)- form and added Mg^{++} did not alter the results appreciably.

IDENTIFICATION OF THE REACTION PRODUCTS OF THE AEROBIC OXIDATION OF TARTRATE: The immediate reaction product of tartrate oxidation is presumably DHF. If the enol rather than the keto form of DHF were the first oxidation product, an increase in absorption would be expected at 292 $m\mu$ where the enol form absorbs strongly. Under aerobic conditions, such an increase does not occur. This indicates either that the enol form is absent or that ketonization occurs too rapidly.

An indication of the formation of DHF (enol or keto form) as the major reaction product is shown in table II. In these aerobic tests with meso-tartrate as substrate, the reaction was followed spectrophotometrically at 340 $m\mu$ for 1 to 3 hours. The protein was precipitated with trichloroacetic acid (3% final concentration), and molybdate, $K_3Fe(CN)_6$ —Prussian blue and total hydrazone analyses were made on unheated and heated aliquots (held in a hot water bath at 80° C for 30 minutes). Sample data are shown

in table II for different enzyme preparations. If any of the DHF formed is converted to glycolaldehyde, this would not be accounted for in the values of this chart except in the amount of DPN reduced. Many of these preparations also contain active DPNH oxidase or DPN destroying activities which would tend to lessen the apparent amount of DPN reduced. Low $K_4Fe(CN)_6$ values in comparison with the value expected from the amount of DPNH formed according to the absorption at 340 $m\mu$ could be attributed to the side reaction to glycolaldehyde. This tends to be more evident with the longer incubation periods. Any hydroxypyruvate formed would decarboxylate to glycolaldehyde above pH 8.5. Although the $K_3Fe(CN)_6$ —Prussian blue analysis on unheated aliquots would detect DHF, DPNH and hydroxymalonate, only the latter would be detectable in the heated aliquots. These data indicate some of the variation found. For example, hydroxymalonate can be detected with long incubation periods in some preparations, but not in others. An increase in total hydrazones can be demonstrated in separate tests. Data are shown here for a pea seed supernatant fraction, but similar increases in total hydrazones were observed in particulate preparations also. No evidence is shown here for the second possible step; i.e. DHF to diketosuccinate. If this occurred, larger values for hydroxymalonate would be expected.

Analyses of the 2,4-dinitrophenylhydrazones formed by methods previously described (32) and by those of Kun (15), were not considered accurate enough for quantitative results. Even qualitative identification of these derivatives is difficult because a variety of osazones as well as hydrazones, some of which can exist as cis-trans isomers, have been reported as de-

TABLE II
AEROBIC OXIDATION OF MESO-TARTRATE BY PLANT ENZYMES

ENZYME SOURCE	MG PROTEIN USED	MIN INCUBATION	MICROMOLES PRODUCED					
			DPNH *	$K_4Fe(CN)_6$ **	DHF + OHM		OHM ‡	DHF ††
					CALCULATED †	Mo TEST †††		
Wheat germ	9.2	60	0.078	0.322	(0.081)
		75	0.043	0.230	(0.058)	0.062
Pea seed	1.6	60	0.045	0.176	(0.044)	0 ^a	...
		150	0.05	0.04
		180	0.067	0.214	(0.053)	0 ^a	...
		180	0.074	0.100	(0.025)	0.03	...
Pea shoot	3.0	90	0.051	0.258	(0.065)
		0.8	180	0.085	0.220	(0.055)

Cuvettes contained in a total volume of 3 ml: 0.5 ml TRIS buffer (pH 9.0), 0.4 mg DPN⁺, 200 micromoles of substrate, plus enzyme. Blank values were subtracted.

* $\Delta O.D.$ at 340 $m\mu$.

** Prussian blue test on unheated aliquots.

† Calculated from above value (divided by 4).

†† Molybdate test on unheated aliquots.

‡ Prussian blue test on heated aliquots.

‡‡ Total 2,4-dinitrophenylhydrazone value on unheated aliquots.

^a Not detectable with largest aliquot possible.

rivatives of DHF (6, 9, 29). Tentative evidence of the formation of 2,4-dinitrophenylhydrazone derivatives of DHF, diketosuccinate, and of glycolaldehyde in extracts of these enzyme mixtures was obtained chromatographically, but the author feels that a better understanding of DHF and its decomposition products is required before more definitive qualitative and quantitative evidence can be obtained.

DEMONSTRATION OF THE OXIDATION OF DPNH BY DHF: The spectrophotometric demonstration of the reduction of DHF to tartrate via DPNH oxidation is complicated by the presence of at least two other reactions occurring in the plant extracts used which result in products which can subsequently oxidize DPNH. These are the autooxidation of DHF to diketosuccinate, and the non-enzymatic decarboxylation of the DHF to glycolaldehyde. Other possibilities are the production of hydroxypyruvate or glyoxylate, which likewise could be involved in further oxidation of the DPNH.

The graphs shown in figure 2 and those reported previously, indicate that DPNH is oxidized both aerobically and anaerobically upon the addition of DHF. The reaction which occurs aerobically probably involves the autooxidation of diketosuccinate and the subsequent oxidation of the DPNH by the diketosuccinic acid reductase activity (30). Kun (16) reports no difficulty with this side reaction and performs his spectrophotometric tests aerobically. Activity curves are shown here for wheat germ and pea shoot enzyme preparations. Curves for pea seed preparations would be comparable to those shown for wheat germ. Unfortunately, only small amounts (about 0.2 micromole) of DHF can be added; otherwise, there would be some absorption at 340 μ due to the enol form. Anaerobic oxidation does occur in these preparations, but only at a relatively slow rate in comparison with the aerobic activity with DHF or with diketosuccinic acid reductase activity. The curve for activity with diketosuccinate is that observed when 1 micromole of substrate is added; comparable initial rates are obtained with 0.2 micromoles of substrate, but there is an indication of a decline in reaction rate with time. Starting with 0.2 micromoles of DHF in the presence of wheat germ enzyme (containing 2.3 mg protein per cuvette), about 0.02 micromoles of DPNH were oxidized in 15 minutes anaerobically, and about 0.13 micromoles after 2 hours of incubation anaerobically. This can be compared with a value of about 0.062 micromoles after 2 hours incubation with a pea shoot preparation (containing 1.7 mg protein). While these slow rates in contrast with the reductase activities of ketomalonnate and of diketosuccinate could be due to a sluggish enzyme system, the inability to add an excess of substrate and the multiplicity of reactions occurring could also account for the slowness of the rate.

The remaining curves in figure 2 indicate the possible cause(s) of this anaerobic oxidation of DPNH. In pea shoot preparations, the oxidation of DPNH appears to be due to the reversal of tartrate dehydrogenase activity, while in the presence of wheat

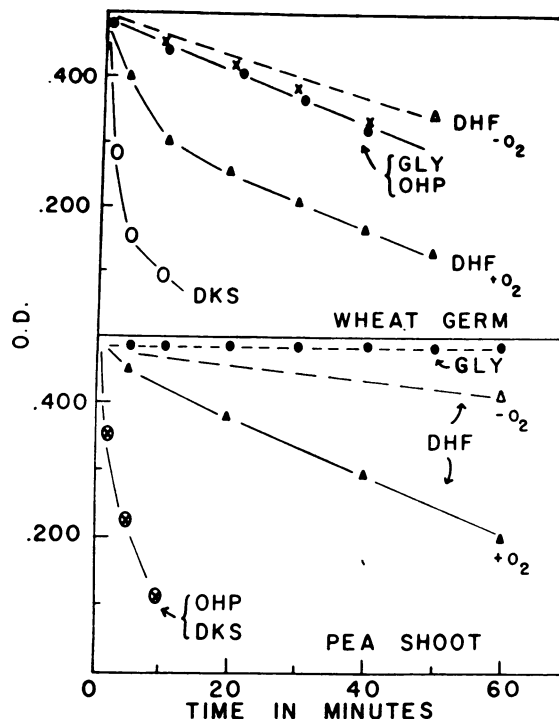


Fig. 2. Oxidation of DPNH in wheat germ and pea shoot enzyme preparations in the presence of different substrates. The light absorption (O.D.) at 340 μ due to DPNH is plotted against time. The cuvettes contained in a total volume of 3 ml: 0.2 ml of 0.5 M phosphate or TRIS buffer (pH 7.4), 0.25 micromoles DPNH, 0.2 micromoles DHF, 1 micromole diketosuccinate (DKS), 4 micromoles glycolaldehyde (GLY) and hydroxypyruvate (OHP), and 2.3 mg protein from wheat germ or 1.7 mg from pea shoot enzyme preparations. All experiments were made aerobically, except in the case of one of the curves for DHF (open triangles) which were performed anaerobically (30). Blank values of DPNH oxidation without added substrate have been subtracted.

drogenase activity, while in the presence of wheat germ preparations the oxidation could be attributed to the combined activities of this same tartrate dehydrogenase plus alcohol dehydrogenase which would reduce the decarboxylation product glycolaldehyde to ethylene glycol.

The following data suggest that neither hydroxypyruvate nor glyoxylate are formed in significant amounts. Leaves contain active glyoxylic and hydroxypyruvic acid reductases in contrast to seeds (13). If significant amounts of either glyoxylate or hydroxypyruvate were produced from DHF, one might expect to find very different rates of DPNH oxidation with the two different types of preparations. Figure 2 shows DPNH oxidation curves obtained in the presence of hydroxypyruvate in the two preparations. Similar curves would be obtained with

4 micromoles of glyoxylate, and both enzymes are quite active even with trace amounts of substrate. The anaerobic activity shown for DHF with pea shoot enzymes, therefore, would not be due to the formation of either hydroxypyruvate or glyoxylate as has been reported by Kun (15, 16). If one assumes that the wheat germ has no unique effect on the decarboxylation of DHF, the oxidation of DPNH in such preparations would likewise not be due to hydroxypyruvate, although at this high enzyme concentration there is an oxidation with hydroxypyruvate comparable to that obtained with added glycolaldehyde.

Data were obtained, however, which indicate the slow but steady production of glycolaldehyde aerobically and anaerobically in the presence and in the absence of wheat germ and pea shoot preparations. The first evidence was the production of CO₂ when either of these enzyme preparations were incubated anaerobically (N₂ gas) in Warburg vessels. Starting with 7 micromoles of DHF, and 7 mg protein from a pea shoot preparation, the evolution of CO₂ continued for a period of over two hours, with approximately 4.5 micromoles produced during the first two hours. Boiled enzyme preparations were just as active as unboiled ones, but in both there was a definite stimulus over the spontaneous rate. Such an experiment does not rule out the production of some hydroxypyruvate, although a double step decarboxylation is indicated.

The second line of evidence of the slow formation of glycolaldehyde from DHF non-enzymatically can be detected by using crystalline alcohol dehydrogenase as a reagent to determine the glycolaldehyde concentration (9). If equal amounts of DHF and glycolaldehyde (0.2 micromoles) were added to crystalline alcohol dehydrogenase at pH 7.4, the DPNH is rapidly oxidized by the glycolaldehyde while there is a slow but steady oxidation of DPNH in the presence of added DHF. This rate amounts to about 0.035 micromoles of glycolaldehyde per hour under anaerobic conditions, and slightly less under aerobic conditions.

Chemical tests for phenylhydrazones or for glycolaldehyde (diphenylamine test, 14) were difficult to interpret for reasons already discussed, and because any DHF left in the preparation could give rise to glycolaldehyde during the actual analytic test itself. After vigorous aeration of the enzymatic mixture to autoxidize any remaining DHF, about 20% of the original DHF was analyzed as glycolaldehyde.

Unsuccessful attempts were made to identify tartrate as the reaction product when DHF and DPNH were incubated with plant enzymes. When coupled with alcohol oxidation at pH 7.4 to generate the DPNH, considerable coupling occurred as indicated by the amount of acetaldehyde formed, but it was not possible to identify the product chromatographically as a tartrate (pH spray) or by the sensitive sodium vanadate test (34). One might expect that the product formed would be the meso-isomer because this is more active in reducing DPN. The vanadate test will analyze only the (-), (+), and (±)- forms of tar-

trate, and negative results indicate that little (-)-tartrate was accumulating.

Side reactions could also remove the DHF or the tartrate itself. These might include the decarboxylation of DHF to glycolaldehyde with the subsequent reduction to ethylene glycol via DPNH, or the tartrate could be converted to oxalacetate by dehydration (17, 20) followed by reduction to malate in the presence of DPNH and malic dehydrogenase.

Preliminary evidence that this latter reaction does occur in pea seed preparations has been obtained. When DPNH is incubated with (-)- or meso-tartrate, the DPNH is oxidized. The (+)- form is inactive. Further work is in progress to clarify this side reaction which may be similar to that reported in bacteria (17, 20). If this reaction converting tartrate to oxalacetate is further validated, the interest of tartaric dehydrogenase activity in plant metabolism is greatly enhanced.

DISTRIBUTION OF TARTARIC ACID DEHYDROGENASE: Tartaric acid dehydrogenase activity has been demonstrated in pea plants, wheat embryos, and bean leaves. In peas, the activity is distributed throughout the plant, being found in mature roots and shoots, as well as in seeds soaked in distilled water for 12 hours. The pattern of distribution, therefore, appears to be similar to that of malic and hydroxymalonic acid dehydrogenase activities.

The intracellular distribution of tartaric acid dehydrogenase is similar to that of malic dehydrogenase, but differs from that for hydroxymalonic acid dehydrogenase. About 20 to 30% of the original activity with meso-tartrate was associated with a particulate fraction, the remaining portion being found in the final supernatant solution. The initial separations were made after grinding the peas for 1 minute in a Waring blender in an iced mixture of 0.5 M sucrose in 0.025 M phosphate buffer (pH 7.4). The particulate fraction was a washed sediment of particles centrifuged down between 500 to 25,000 × g for 30 minutes. In order to lower blank reactions and to demonstrate a DPN-dependency in the case of the particulate fraction, it was necessary to purify all fractions by ammonium sulfate precipitation. The fraction used for assay was that precipitated between 200 to 400 grams of ammonium sulfate per liter of enzyme solution, and then resuspended in 0.025 M phosphate buffer at pH 7.4. The activities were followed spectrophotometrically at pH 9.0 in the presence of 0.4 mg DPN, 200 micromoles of substrate (plus 5 micromoles of KCN in the case of added malate). Approximately 85% of the total protein of the homogenate was recovered in the two fractions.

Although the ratio of activities with added tartrate and with hydroxymalonnate were close to 1.0 in the supernatant fractions, no activity could be detected with added hydroxymalonnate in the particulate fraction. The ratio of the activity with malate to that with tartrate varies from about 800 to 1300 in the different fractions.

In the other plant enzyme preparations used in

this study, the ratios of activity with hydroxymalonnate to that with tartrate were always close to 1 when both activities were detectable. When the ratios of the activity with malate to that with tartrate were compared, the values ranged from 200 to 2500. Although this might indicate the presence of separate enzymes, changes in the side reactions could account for such variation. The question of the number of enzymes involved, therefore, is still open.

COMPETITIVE INHIBITION OF MALIC DEHYDROGENASE ACTIVITY: In experiments performed as described for hydroxymalonnate (32), meso-tartrate was found to be a competitive inhibitor of malic dehydrogenase activity in pea seed preparations with a K_i value of approximately 2×10^{-2} M. (K_i equals the dissociation constant of the enzyme-inhibitor complex.) The values of K_i reported for inhibition by hydroxymalonnate was about 1×10^{-2} M; the difference between these values is not considered highly significant. If

present in high enough concentration in comparison with malate, therefore, both hydroxymalonnate and tartrate might function as physiological regulators of malic dehydrogenase activity. (The value of K_m for L-malate, or the dissociation constant of the enzyme-substrate complex, was reported as 0.5×10^{-2} M (32)).

SIGNIFICANCE OF DHF IN PLANT METABOLISM: Some of the numerous enzymatic and non-enzymatic reactions of DHF are summarized in figure 3. DHF is shown here as a compound capable of being oxidized, reduced, or decarboxylated, with further similar reactions involving most of the initial products. At least 7 different dehydrogenase activities in plants are associated with these compounds. Some of these breakdown products, like glycolaldehyde and hydroxypyruvate, have been postulated as possible precursors of carbohydrates (10, 12, 25), and DHF oxidase (1, 24) plus diketosuccinic acid reductase can serve as a potential oxidase system comparable to the complex

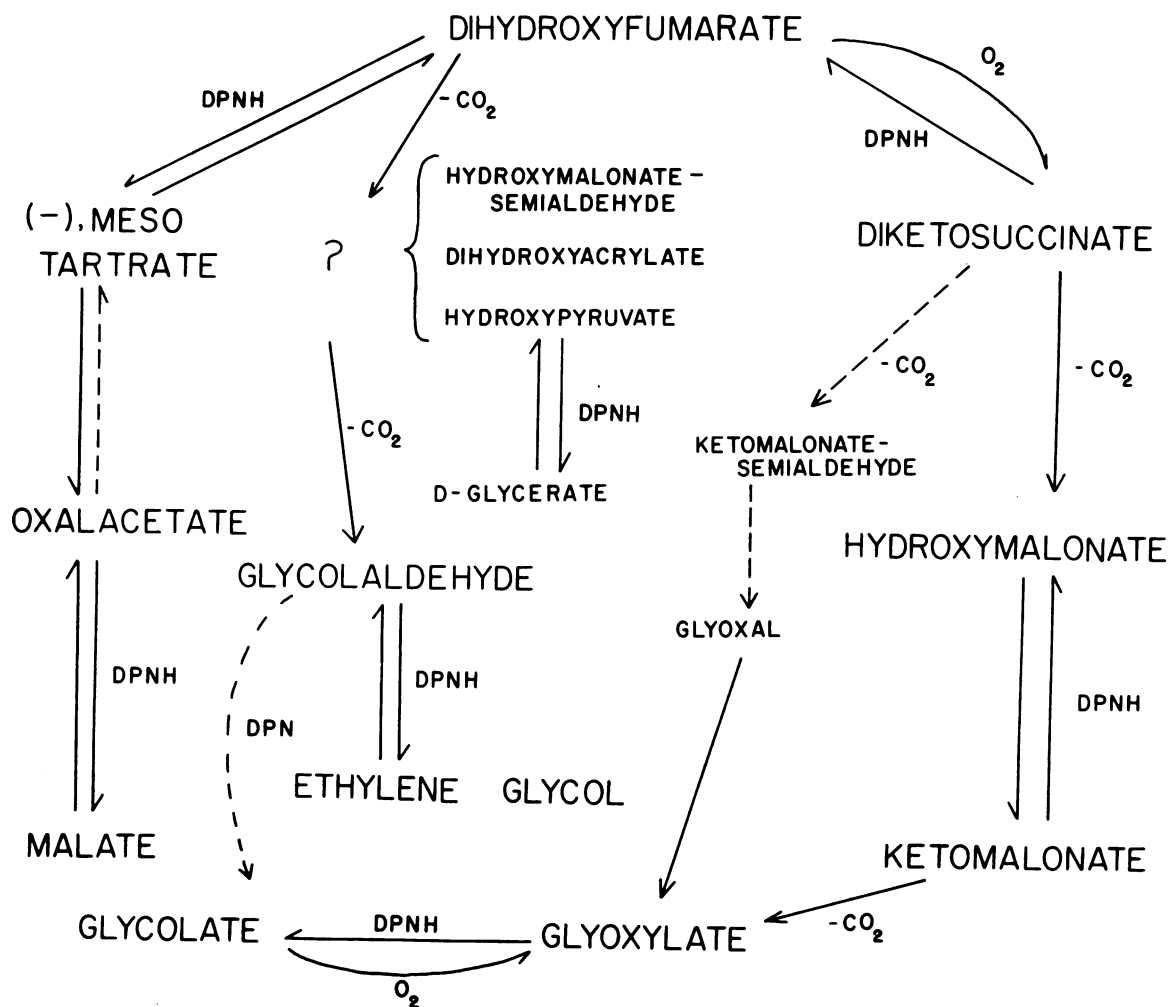


FIG. 3. Possible reactions of dihydroxyfumarate. Solid lines represent enzymatic or non-enzymatic activities reported in higher plants; dotted lines represent activities reported in animal tissues or which are postulated as possible chemical reactions.

involving glycolate and glyoxylate (36). Furthermore, transaminase reactions can connect some of these organic acids with their corresponding amino acids (26). DHF can be considered, therefore, as a possible key metabolite in plants.

The aerobic oxidation of DHF to diketosuccinate can occur non-enzymatically or enzymatically (1, 11, 18, 30). Whether the latter reaction is catalyzed by a separate oxidase or only by a peroxidase acting as an oxidase, and whether a metal ion is necessary for all such oxidations has not yet been definitely proven (24). The product of these oxidations is presumably diketosuccinate, but again this has never been unequivocally demonstrated. If any H_2O_2 is produced, the situation is further complicated by the subsequent oxidation by H_2O_2 of compounds such as hydroxymalonate to ketomalonate. The dehydrogenase reaction which reduces DHF to tartrate and the tentative evidence of the conversion of tartrate to oxalacetate in plants have already been discussed.

The direct decarboxylation products of DHF and diketosuccinate need further study, especially in relation to metal ion effects and to pH differences. The early chemical work of Fenton (4) identified glycolaldehyde and hydroxymalonate as the main decarboxylation products of the acids of DHF and diketosuccinate, respectively. Although preparations from higher plants catalyze non-enzymatically these decarboxylations at neutral pH, no enzymatically catalyzed reactions have been demonstrated, except in the more complicated Mn^{++} -phenol-peroxidase system reported by Kenten and Mann (11). Yeast preparations, on the other hand, are reported to decarboxylate DHF forming glycolaldehyde and CO_2 (23), while yeast pyruvic carboxylase can apparently act on hydroxypyruvate by converting it to glycolaldehyde (9). This latter reaction would make it difficult to determine whether hydroxypyruvate were the intermediate in the decarboxylation of DHF to glycolaldehyde in some plant preparations, although the ones used in this study had no pyruvic acid carboxylase activity without added coenzyme. While Fenton considered either hydroxypyruvic acid, dihydroxyacrylic acid or hydroxymalonate-semialdehyde as the intermediate in this double decarboxylation, he gives no evidence for their detection (6). Kun's data identifying glyoxylate and hydroxypyruvate as non-enzymatic products have already been discussed (15, 16).

The spontaneous decarboxylation of diketosuccinate to hydroxymalonate has been frequently reported (5, 6, 16), and an enzymatic decarboxylation can be demonstrated in yeast (22). Fenton cites evidence for ketomalonate-semialdehyde as an intermediate in a metal catalyzed conversion of DHF to glyoxal via diketosuccinate (5). If any such reaction occurred in plants, glyoxal could then be converted to glyoxylate (13). Another possible route of glyoxylate production is by a DPN-linked aldehyde oxidase reaction which oxidizes glycolaldehyde to glycolic

acid as reported in liver (14). No such activity could be demonstrated spectrophotometrically using the plant preparations described in this study.

The author feels that any further enzymatic work with DHF and its derivatives must be preceded by a thorough chemical study of all these compounds.

SUMMARY

A dehydrogenase activity which oxidizes (-)- or meso-tartrate to either the enol or keto form of dihydroxyfumarate has been demonstrated in pea, bean and wheat extracts. This activity has been measured spectrophotometrically with substrate amounts of DPN⁺ or colorimetrically by the Prussian blue analysis of the $K_4Fe(CN)_6$ formed in a system coupled with the oxidation of $K_3Fe(CN)_6$. Under the conditions used, neither Mg^{++} nor ethylenediamine-tetraacetic acid affects the rate of this activity. The reduction of dihydroxyfumarate to tartrate in the presence of DPNH can be demonstrated spectrophotometrically under anaerobic conditions in certain preparations. Identification of the products of the dehydrogenase activity is hampered by at least three side reactions: the decarboxylation of dihydroxyfumarate to glycolaldehyde; the autoxidation to diketosuccinate followed by decarboxylation to hydroxymalonate; the possible dehydration of tartrate to oxalacetate. No evidence of significant amounts of glyoxylate or hydroxypyruvate was obtained. The dehydrogenase activity is found in seeds, roots, and shoots of peas. Although the activity is associated mainly with the supernatant fraction, a definite activity can be demonstrated in a partially purified particulate fraction sedimented between 500 to 25,000 × g. The possibility that this activity is merely a secondary one of the similarly distributed malic dehydrogenase has not yet been eliminated. Like hydroxymalonate, tartrate is a competitive inhibitor of malic dehydrogenase activity. The possible key role of dihydroxyfumarate in plant metabolism is discussed.

LITERATURE CITED

1. BANGA, I. and PHILLIPOT, E. Über die dioxymaleinsäureoxydase. *Zeits. Physiol. Chem.* 258: 147-159. 1939.
2. CLELAND, W. W. and JOHNSON, M. Studies on the formation of oxalic acid by *Aspergillus niger*. *Jour. Biol. Chem.* 220: 595-606. 1956.
3. FENTON, H. J. H. Oxidation of tartaric acid in the presence of iron. *Jour. Chem. Soc.* 65: 899-910. 1894.
4. FENTON, H. J. H. A new synthesis in the sugar group. *Jour. Chem. Soc.* 71: 375-383. 1897.
5. FENTON, H. J. H. and RUFFEL, J. H. Mesoxalic semi-aldehyde. *Jour. Chem. Soc.* 81: 426. 1902.
6. FENTON, H. J. H. Further studies on dihydroxymaleic acid. *Jour. Chem. Soc.* 87: 804-818. 1905.
7. GATET, L. Étude du pouvoir réducteur des jus de fruits au cours de la maturation. *Enzymologia* 6: 375. 1939.
8. HARTREE, E. F. Dihydroxyfumaric acid. *Biochem. Preparations* 3: 56-58. 1953.

9. HOLZER, H., GOEDDE, H. W. and SCHNEIDER, S. Umsatz Oxybrenztraubensäure und Glycoaldehyde mit Carboxylase und Alkoholdehydrase auf Hefe. *Biochem. Zeits.* 327: 245-254. 1955.
10. HOUGH, L. and JONES, J. K. N. The synthesis of sugars from simpler substances. V. Enzymic synthesis of sedoheptulose. *Jour. Chem. Soc.* 1953: 342-345. 1953.
11. KENTEN, R. H. and MANN, P. J. G. The oxidation of certain dicarboxylic acids by peroxidase systems in the presence of manganese. *Biochem. Jour.* 53: 498-505. 1953.
12. KRAKAW, G. and BARKULIS, S. S. Conversion of glyoxylate to hydroxypyruvate by extracts of *E. coli*. *Biochim. Biophys. Acta* 21: 593-594. 1956.
13. KOLESNIKOV, P. A. Oxidation of glyoxal in extracts from green leaves. *Doklady Akad. Nauk. SSSR* 90: 221-224. 1953. (Chem. Abstr. 47: 8839b.)
14. KUN, E., DECHARY, J. M., and PIROT, H. C. The oxidation of glycolic acid by a liver enzyme. *Jour. Biol. Chem.* 210: 269-280. 1954.
15. KUN, E. and HERNANDEZ, M. G. The oxidation of tartaric acid by an enzyme system of mitochondria. *Jour. Biol. Chem.* 221: 201-211. 1956.
16. KUN, E. Enzymatic mechanism of oxidation of tartrate. *Jour. Biol. Chem.* 221: 223-230. 1956.
17. KRAMPITZ, L. O. and LYNEN, F. Formation of oxalacetate from D-tartrate. *Abstr., Federation Proc.* 15: 292. 1956.
18. KUZIN, A. S. and DOMAN, N. G. Oxidation of dihydroxymaleic acid in the green plant leaf. *Doklady Akad. Nauk. SSSR* 62: 255-258. 1948. (Chem. Abstr. 43: 726c.)
19. LACHMAN, A. Dihydroxytartaric acid. *Jour. Amer. Chem. Soc.* 43: 2091-2097. 1921.
20. LARIVIERE, J. W. M. Specificity of whole cells and cell-free extracts of *Pseudomonas putida* towards (+), (-), and meso-tartrate. *Biochim. Biophys. Acta* 22: 206-207. 1956.
21. LARNER, J., JACKSON, W. T., GRAVES, D. J. and STAMER, J. R. Inositol dehydrogenase from *Aerobacter aerogenes*. *Arch. Biochem. Biophys.* 60: 352-363. 1956.
22. NEUBERG, C. and KARZAG, L. Über Zuckerfreie Hefegärungen. *Biochem. Zeits.* 37: 171-176. 1911.
23. NEUBERG, C. and SCHWENK, E. Die Gärung der Dioxymaleinsäure. *Biochem. Zeits.* 71: 104-113. 1915.
24. MASON, H. S. Mechanisms of oxygen metabolism. *Advances in Enzymology* (In press).
25. MILHAUD, G., BENSON, A. A. and CALVIN, M. Metabolism of pyruvic acid-2-C¹⁴ and hydroxypyruvic acid-2-C¹⁴ in algae. *Jour. Biol. Chem.* 218: 599-606. 1956.
26. SALLACH, H. J. Formation of hydroxyaspartic acid from dihydroxyfumaric acid and L-glutamic acid. *Jour. Biol. Chem.* 223: 629-634. 1956.
27. SCHMALFUS, H. and KEITEL, K. Vorarbeiten für den Nachweis von Säuren in Pflanzen. *Zeits. Physiol. Chem.* 138: 156-163. 1924.
28. SCHOLEFIELD, P. G. The oxidation of malic and meso-tartaric acids in pigeon liver extracts. *Biochem. Jour.* 59: 177-179. 1955.
29. SPRINSON, D. B. and CHARGAFF, E. A study of β -hydroxy- α keto acids. *Jour. Biol. Chem.* 164: 417-432. 1946.
30. STAFFORD, H. A., MAGALDI, A. and VENNESLAND, B. Enzymatic oxidation of DPNH by diketosuccinate and dihydroxyfumarate. *Science* 120: 265-266. 1954.
31. 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.
32. STAFFORD, H. A. Dehydrogenase activity of hydroxymalonate and related acids in higher plants. *Plant Physiol.* 31: 135-141. 1956.
33. STAFFORD, H. A. Tartaric acid dehydrogenase in higher plants. *Abstr., Western Section Amer. Soc. Plant Physiol.* P. 9. Seattle, Washington, June, 1956.
34. VAUGHN, R. H., MARSH, G. L., STADTMAN, T. C. and CANTINO, B. C. Decomposition of tartrates by the coliform bacteria. *Jour. Bact.* 52: 311-325. 1946.
35. VICKERY, H. B. and PALMER, J. K. The metabolism of the organic acids of tobacco leaves. VII. The effect of culture of excised leaves in solution of (+) tartrate. *Jour. Biol. Chem.* 207: 275-285. 1954.
36. ZELITCH, I. Oxidation and reduction of glycolic and glyoxylic acids in plants. *Jour. Biol. Chem.* 201: 719-726. 1953.