Kinetics of Malic Dehydrogenase Inhibition by 2,4-Dichlorophenoxyacetic Acid [1, 2, 3]

Randolph T. Wedding & M. Kay Black

Department of Biochemistry, University of California, Riverside

In the course of an investigation of the uncoupling effect of 2,4-D on phosphorylation in plant mitochondria (13), it became evident that this compound exerted two effects on the balance between oxidation of certain of the TCA cycle substrates and associated phosphorylations. In no case was it possible to observe a stimulation of the rate of oxidation concomitant with the inhibition of phosphate esterification, but with malate and citrate a substantial inhibition of oxygen uptake by mitochondria was caused by high concentrations of 2,4-D. This inhibition was lacking when mitochondria were oxidizing succinate, α-ketoglutarate or DPNH. These facts appeared to point toward an interference by 2,4-D with the DPN-requiring dehydrogenases of the mitochondria. It was additionally apparent that this effect on oxidation was independent of uncoupling by 2,4-D, since it occurred at a higher concentration than that causing a reduction in P/O ratios, the uncoupling effect was manifested with substrates whose oxidation was not inhibited, and the inhibitory effect was apparently exerted upon the first electron transfer step in the oxidation of susceptible substrates, i.e., preceding the oxidation of reduced pyridine nucleotide, and thus before the first phosphorylation reaction.

The inhibition of respiration in intact cells by 2,4-D has been frequently reported (3), and some years previously an inhibition of the activity of malic dehydrogenase and similar enzymes by naphthalene acetic acid in cell-free preparations or partially purified enzyme preparations was noted (2). The work reported here represents a quantitative investigation of the inhibition by 2,4-D of the activity of malic dehydrogenase isolated from red beet roots and presents kinetic evidence regarding the mechanism by which this inhibition occurs.

Materials & Methods

Chemicals: The DPN, TPN and DPNH used were the most highly purified preparations available from Sigma Chemical Co. and Biochemica Boehringer. The DPN was dissolved in water, neutralized to pH 5.5 with KOH and stored at −13 C when not in use. The DPNH was dissolved in 0.02 M tris, pH 9.2 and stored at −13 C in an opaque tube. L-Malic acid and oxaloacetic acid were obtained from California Corporation for Biochemical Research. Malic acid was neutralized with KOH to pH 7.0 and stored frozen. Oxaloacetic acid was made up fresh daily and neutralized to pH 7.0 with KOH. These and other unstable solutions were stored in an ice-water bath at 0 C when in use. Glucose-6-P was obtained from Sigma Chemical Co. Sodium pyruvate and dl-isocitric acid (lactone) were obtained from the California Corporation for Biochemical Research. Solutions of these chemicals were prepared as directed for assay of the appropriate enzymes (4).

The 2,4-D and DNP used were recrystallized three times from hot water, and other phenoxy acids were recrystallized once from hot water. Phenol was purified by distillation.

Commercial Enzymes: Malic dehydrogenase from porcine heart was obtained from Biochemica Boehringer as were alcohol dehydrogenase (yeast), lactic dehydrogenase (rabbit muscle) and TPN-isocitric dehydrogenase (porcine heart). Glucose-6-P dehydrogenase Type V (yeast) was obtained from Sigma Chemical Co. These enzymes were assayed according to the standard procedures (4).

Preparation of Beet Root Mitochondrial Malic Dehydrogenase: Mitochondria were prepared from fresh roots of red beets (*Beta vulgaris*, L.) in the manner described earlier (13). Unless otherwise specified, all operations were carried out at a temperature of 0 to 1 C. An acetone powder was prepared from the once-washed mitochondria obtained from two kg of beet roots, suspended in 10 ml 0.6 M sucrose, 0.02 M tris, pH 7.0, by adding the suspension slowly with stirring to 50 volumes of acetone at −18 C. The precipitate was filtered off and dried under vacuum. The resulting acetone powder was suspended by stirring in 0.02 M K phosphate buffer, pH 7.4, made to 10 ml and centrifuged 10 min at 10,000 × *g*. The supernatant fluid was fractionated by adding 3.75 M ammonium sulfate adjusted to pH 7.4 with tris (10). The precipitate obtained between 2.3 and 3.0 M ammonium sulfate, suspended in 2.0 ml of 1.7 M ammonium sulfate, 0.02 M K phosphate buffer, pH 7.4, was rapidly raised to 60° with

---

[1] Received Oct. 18, 1962.

[2] Supported in part by Cancer Research Funds of the University of California.

[3] The abbreviations used are: 2,4-D, 2,4-dichlorophenoxyacetic acid; DCP, 2,4-dichlorophenol; DNP, 2,4-dinitrophenol; MDH, malic dehydrogenase; OAA, oxaloacetate; TCA, tricarboxylic acid.

stirring and held for two minutes at this temperature. After centrifugation the supernatant liquid was refractionated with ammonium sulfate, and the fraction precipitating between 2.3 and 2.6 M ammonium sulfate was suspended in 1 ml of 1.7 M ammonium sulfate, 0.02 M K phosphate, pH 7.4 and dialyzed overnight against the same solution. After centrifugation this concentrated suspension was diluted with the same solution to a level of activity which would permit the use of 5 to 10 μl of dilute enzyme for assay purposes.

For some of the data reported here preparations less highly purified which were obtained by dialysis immediately following heat treatment, and more highly purified by the addition of an ethanol fractionation step prior to the ammonium sulfate reprecipitation were used. These preparations, although differing in specific activity from that detailed in the following section, did not differ significantly in kinetic constants, response to the inhibitors used here, or the presence of other enzymes.

Assay Methods: In general the method used for malic dehydrogenase assay was determination of the appearance or disappearance of the 340 mμ absorption peak of DPNH. A double-beam, recording spectrophotometer with a temperature-controlled cell compartment maintained at 27 C was used, and the standard procedure involved determination of the time required for a change in absorbance of 0.05 units. For very slow reactions, or those which showed a tendency for non-linearity with time, a change of 0.02 units was used. Specific details of assay are given with the tables or figures in the results section. For other enzymes similar spectrophotometric methods were employed, using the absorbance of DPNH, TPNH or 2,6-dichlorophenolindophenol as appropriate to the particular enzyme (4,5).

One unit of malic dehydrogenase is defined as an amount of enzyme which brings about the appearance or disappearance of 1.0 μmole of DPNH per minute. Specific activity is expressed as units per mg protein. Protein was determined by the micromethod of Nielsen (9) using crystalline bovine serum albumin as a standard.

Results

Purification of Malic Dehydrogenase: The degree to which beet root mitochondrial malic dehydrogenase was purified by the procedures used is summarized in table I. For purposes of calculating relative purification the acetone powder suspension was used as a base, as no effort was made to assay MDH in the mitochondria or tissue brei. The overall purification obtained is about 100-fold. The final ratio of absorbance at 280 mμ to absorbance at 260 mμ was 1.07.

Characteristics of Beet Root Enzyme Preparation: The beet root mitochondrial malic dehydrogenase purified by the procedures described showed no detectable activity of several other enzymes which might interfere with the malic dehydrogenase reaction. Tests for the activity of malic enzyme, TPN- and DPN-isocitric dehydrogenase, lactic dehydrogenase, alcohol dehydrogenase, and glucose-6-P dehydrogenase revealed no detectable activity. Tests for oxidation of DPNH by diaphorase indicated that a very small contaminant of this enzyme, on the order of 0.5 %, was present. However, since the malic dehydrogenase assays used do not contain a suitable electron acceptor for diaphorase, it is improbable that this contamination had any effect on the MDH measurements. No detectable endogenous substrates for MDH were present.

Table I

Purification of Malic Dehydrogenase from an Acetone Powder of Beet Root Mitochondria

Procedure	Volume (ml)	Total Units	Total protein (mg)	Specific activity (units mg protein)
Acetone Powder suspension	10.1	2631	1370	1.92
Crude extract of acetone powder	10.0	2538	150	16.9
2.3-3.0 M ammonium sulfate ppt.	2.0	1506	46	32.7
Supn. from heat treatment, 2.0 min at 60 C	2.0	945	3.9	242
2.3-2.6 M ammonium sulfate ppt., dialyzed	1.0	382	1.7	225

Assays were carried out at pH 9.2, at 27 C, in a total volume of 3.0 ml, using a 1 cm light path at 340 mμ. Each cuvette contained 160 mM tris-HCl, 26.7 mM sodium malate, 2.67 mM DPN, and 2.83 mM ammonium sulfate. The DPN was added to each cuvette just prior to initiation of the reaction by the addition of 5 μl enzyme.

The enzyme was very unstable in water, phosphate buffer, 0.5 M glycyl-glycine buffer, 10 mg/ml bovine serum albumin, or 0.1 M malate. The most effective of these suspending media, malate, resulted in the loss of 30 % of the enzyme activity in 24 hours storage at 4 C. However, it was found that the enzyme, either in concentrated or dilute suspension, is stable for several weeks if stored at 0 to 4 C in 1.7 M ammonium sulfate and 0.02 M glycyl-glycine buffer, pH 7.0. Dilutions were prepared in the same solution such that aliquots of 5 to 10 μl provided sufficient activity for a single assay. Using this technique a plot of activity against enzyme concentration did not show the presence of an endogenous inhibitor or activator in the preparation and assays run with the same preparation two months apart indicated no loss in activity.

The Michaelis constants (Km) for the malic dehydrogenase from beet root mitochondria obtained by slope/intercept of fitted lines of 1/v vs 1/S for each substrate, are listed in table II, together with the constants for partially purified porcine heart

Table II

Michaelis Constants of MDH

Substrate	Km Values Beet root MDH	Porcine heart MDH	Beef heart MDH
DPN	1.9×10^{-3}	1.1×10^{-4}	9.9×10^{-5}
Malate	6.7×10^{-3}	8.9×10^{-4}	2.5×10^{-4}
DPNH	7.2×10^{-5}	9.6×10^{-5}	5.2×10^{-5}
Oxaloacetate	1.3×10^{-4}	1.4×10^{-4}	3.4×10^{-4}

Km values were determined by calculation of the intercepts of Lineweaver and Burk plots. Concentrations of companion substrates were near saturation for the beet root and porcine heart enzymes.

MDH determined in this laboratory and those for beef heart mitochondrial MDH reported by Siegal and Englard (9). It may be seen that the Km's for DPNH and OAA are of the same general order as those for enzymes from animal sources, while the Km's for DPN and malate are about one order of magnitude larger than those for pork or beef heart enzymes. This difference in Km's for the forward reaction is the only significant difference observed in these studies between the beet root MDH and that from porcine heart.

The influence of pH on the activity of beet root malic dehydrogenase in both the forward and back reaction is shown in figures 1 and 2. The optimum pH for malate oxidation is about pH 9.5 while for oxaloacetate reduction it is in the vicinity of pH 8.5. It should be pointed out that the value for the oxaloacetate reduction was obtained by adding DPNH and oxaloacetate immediately before initiating the reaction by adding enzyme. Incubation of the oxaloacetate at an unfavorable pH results in a lower apparent optimum pH. In a case of this sort a summation of the effect of pH on the activity of the enzyme and its effect on breakdown of OAA is measured. The same is true to a lesser extent of DPNH.

The second lines with filled circles in figures 1 and 2 illustrate the fact that inhibition by 2,4-D is essentially constant over a range of pH and that the point of attachment of the inhibitor does not have a different pattern of dissociation from the active site(s) of the enzyme.

Inhibition by 2,4-D: Since the inhibition by 2,4-D of malate oxidation by purified porcine heart malic dehydrogenase and by beet root mitochondria had already been demonstrated (13), the primary objectives of these studies with isolated beet root enzyme were a confirmation of the inhibitory effect on this enzyme, a quantitative evaluation of this inhibition, and a kinetic investigation of the mechanism by which 2,4-D causes the inhibition. All three of these points are covered in figures 3 to 6 which are double reciprocal or Lineweaver-Burk (6) plots of the rate of enzyme reaction against substrate concentration for each of the four substrates involved in the reversible reaction catalyzed by malic dehydrogenase. These rates were determined in the absence of 2,4-D and with two levels of 2,4-D in each case using assay conditions as specified with variation in the concentration of each substrate as appropriate.

Figure 3 shows that when the enzyme is oxidizing malate and reducing DPN, the effect of increasing DPN concentration while maintaining malate at a level near saturation is to overcome the inhibition due to 2,4-D; that is, 2,4-D is competitive with DPN in the classical sense. Figure 4 demonstrates that on the other hand, 2,4-D is a non-competitive inhibitor of malate in this reaction, or in a more precise

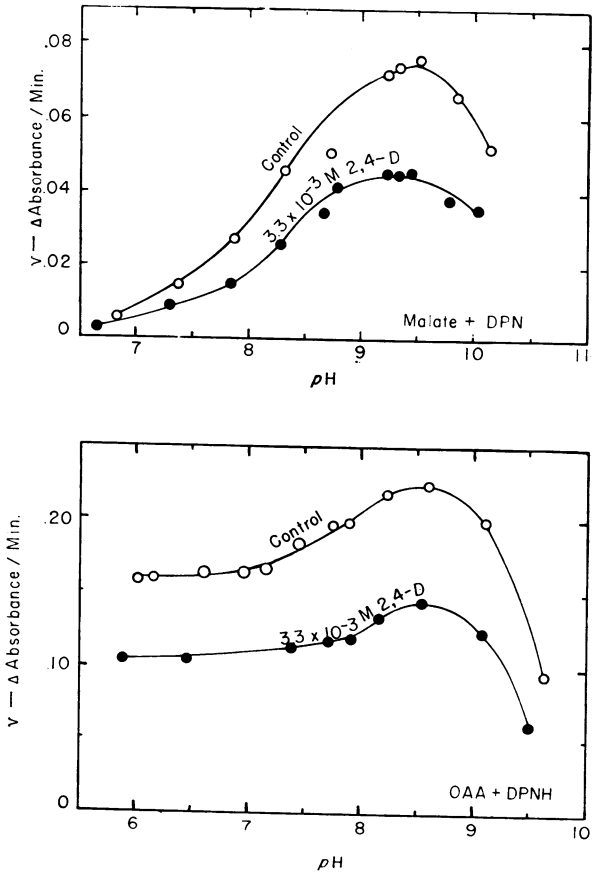

FIG. 1 (*upper*). Effect of pH on malate oxidation by beet root malic dehydrogenase. The reaction mixture was the same as for standard assay (see table I), except the DPN concentration was 1.33 mM. Additional KOH up to a concentration of 17.8 mM was added to obtain pH's higher than 9.2. The final pH was determined immediately after each assay was completed, using a Beckman Model G pH meter.

FIG. 2 (*lower*). Effect of pH on oxaloacetate reduction by beet root malic dehydrogenase. The reaction mixture contained the same concentration of tris and ammonium sulfate as the standard reaction mixture. Immediately before initiating the reaction by enzyme addition, oxaloacetate to give a concentration of 0.4 mM, and DPNH to give a concentration of 0.25 mM, were added. pH's below 7 were obtained by adding HCl to a concentration of not more than 16 mM.

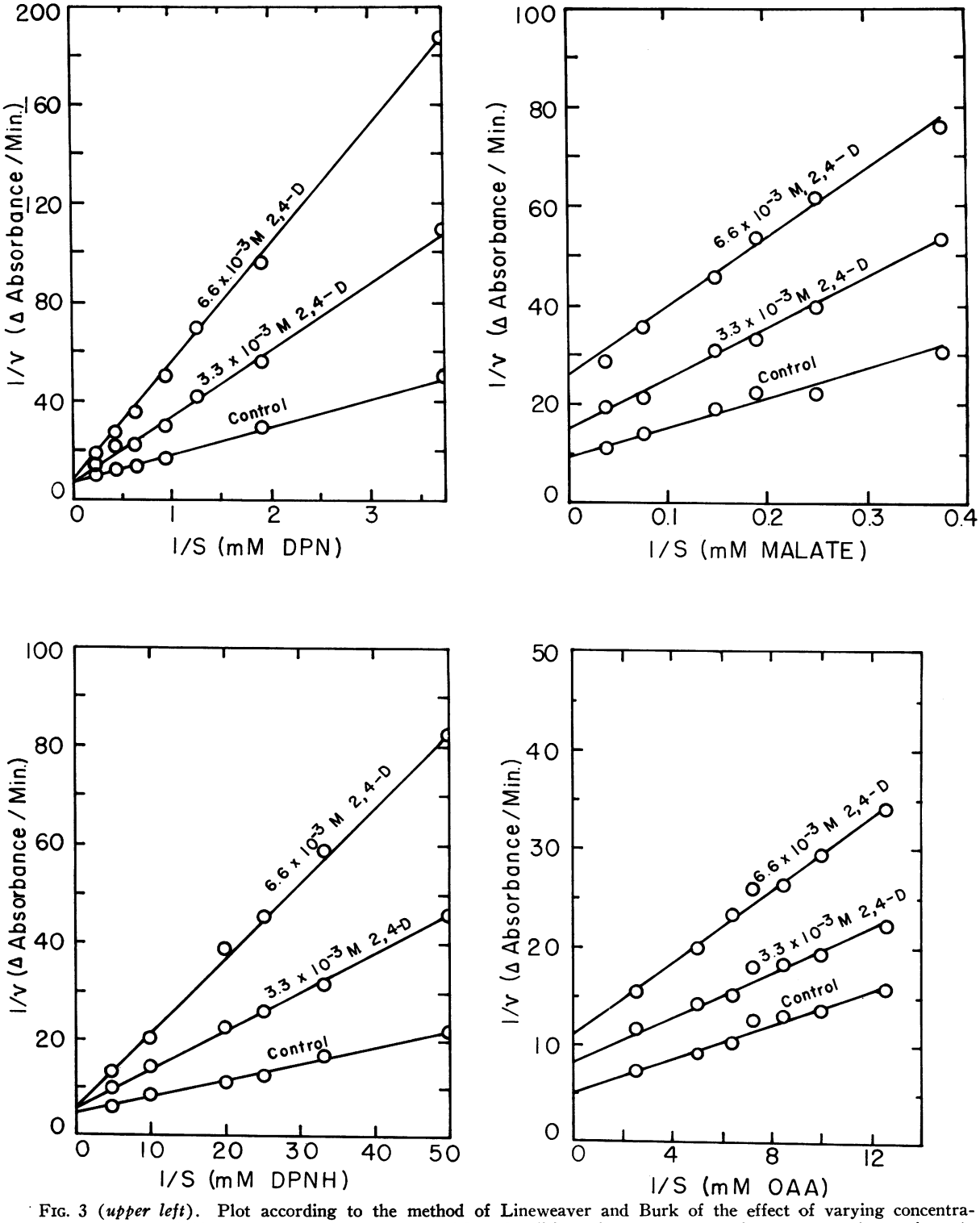

FIG. 3 (*upper left*). Plot according to the method of Lineweaver and Burk of the effect of varying concentrations of DPN on inhibition of beet root MDH by 2,4-D. Conditions for assay were the same as those given in table I.

FIG. 4 (*upper right*). Double reciprocal (Lineweaver and Burk) plot of the effect of varying concentrations of malate on inhibition of beet root MDH by 2,4-D. Conditions for assay same as in figure 3 except DPN concentration was 1.33 mM.

terminology (10), 2,4-D inhibition is antagonized by DPN and is indifferent to malate. When the reaction is run in the reverse direction so that OAA is reduced and DPNH is oxidized, a similar pattern is found. Figure 5 shows that with respect to reduced pyridine nucleotide the 2,4-D inhibition is substrate-antagonized or competitive, while figure 6 again demonstrates that the relation between the organic acid substrate, OAA, and 2,4-D is a non-competitive or substrate-indifferent one.

Testing for the nature of the inhibition using the method of Hunter and Downs (6) also indicates that 2,4-D is competitive with DPN and DPNH and non-competitive with malate and oxaloacetate. Plots by this technique also reveal that the inhibitor constants, Ki, for both DPN and DPNH are approximately the same, 2.2×10^{-3} M.

Interaction of 2,4-D with Pyridine Nucleotide: In a general way the competitive relationship found in these experiments for the reciprocal effects of 2,4-D and pyridine nucleotides can occur in three ways: The inhibitor (2,4-D) can complex with the free enzyme at the site which is normally reserved for DPN or DPNH. Such a complex, if dissociable, would be reduced by an increase in the substrate concentration and thus cause the reduction in inhibition found in these experiments. This category can be further broken down into those cases in which the inhibitor reacts with free enzyme to form a completely inhibited enzyme complex, which is incapable of further activity, and those cases in which the complex of inhibitor with enzyme can be replaced by the normal complex of enzyme with substrate, i.e., the inhibitor and substrate are capable of mutually displacing one another on the enzyme. This last would result in a situation of partial inhibition. These two types may be called complete E-type and partial E-type inhibition (10) respectively. The third mechanism which can produce Lineweaver-Burk and Hunter-Downs plots of the type found here is one in which the inhibitor complexes with the substrate independently of the enzyme and subsequently inhibits the reaction either by reducing the effective concentration of substrate, or because the inhibitor-substrate complex itself is an inhibitor for the enzyme (probably, although not necessarily, occupying the normal substrate site on the enzyme), or by a combination of the two mechanisms.

Any of these three possibilities could produce the type of data which have thus far been presented, but Reiner (10) has derived a technique which makes it possible to distinguish between the several types of inhibition by kinetic methods. This consists in plotting inhibitor concentration (I) against the inhibited fraction of enzyme activity divided by the fraction of activity which is not inhibited (i/1-i). A plot of this type yields a line which is curvilinear in I for low values of i/1-i, with a final linear portion at high levels of i/1-i, i.e., when the amount of inhibition is high.

This plot has characteristics such that if the inhibition is of the complete E-type, extrapolation of the final linear portion to the abscissa will intercept at a positive value of I which is proportional to enzyme concentration. If the inhibition is of the type designated partial E-type, extrapolation of the final linear portion of the line will intercept the abscissa at a negative value. In the third case, that of inhibition via substrate, extrapolation gives a final positive intercept which is proportional to substrate concentration.

Data obtained with beet root malic dehydrogenase plotted in the manner described by Reiner are shown in figure 7. It may be seen that extrapolation of the linear portion to the Y axis gives a positive intercept, and that this intercept is proportional to substrate concentration—a value of 2.5 for 0.67 mM DPN as against 5.0 for 1.3 mM DPN. It may also be seen that doubling the amount of enzyme used (filled circles) does not significantly change the curve when DPN concentration is held constant. This shows that the mechanism is not complete E-type since the intercept is not affected by enzyme concentration, nor partial E-type since the intercept is positive, and because the intercept is proportional to substrate (in this case, DPN) concentration, an inhibition through complexing with the substrate directly is indicated.

There remains the question of whether the inhibition is due simply to starvation of the enzyme for DPN as a result of the complexing activity of 2,4-D, to the fact that the 2,4-D-DPN complex is itself an inhibitor for the enzyme, or to a combination of these two possibilities. Reiner's derivation shows that in the case of starvation a curve of the type found here would be expected, as would also be produced by a combination of both mechanisms. Reiner's model provides a means of distinguishing the starvation case from the combination of both mechanisms. This depends on the slope of the final linear portion of the I vs i/1-i plot with substrate concentration. In the combination of both mechanisms he shows that a plot of this slope against substrate concentration will be curvilinear and that

FIG. 5 (*lower left*). Lineweaver-Burk plot of the effect of varying concentrations of DPNH on 2,4-D inhibition of beet root MDH. Assay conditions as described in table I, except pH was 7.4, malate and DPN were omitted, and oxaloacetate concentration was 0.8 mM with variable concentrations of DPNH.

FIG. 6 (*lower right*). Lineweaver-Burk plot of the effect of varying concentrations of oxaloacetate on inhibition of beet root MDH by 2,4-D. Assay conditions as in figure 5 except DPNH concentration was 0.25 mM.

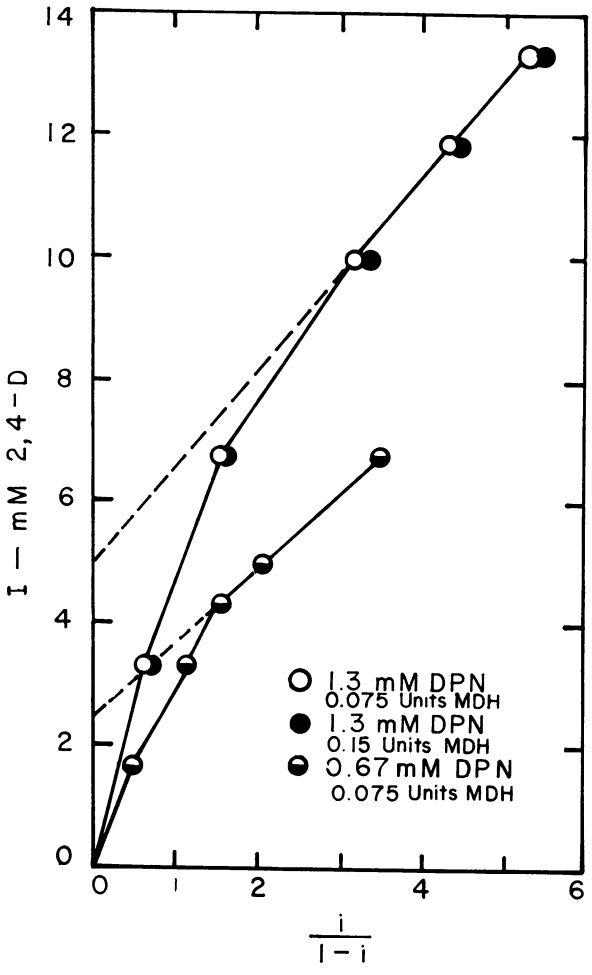

FIG. 7. Effect of DPN concentration and enzyme concentration on the relative inhibition of beet root MDH by 2,4-D plotted according to the method of Reiner. Assay conditions as in table I.

such a plot will be linear if only starvation is involved. In our case the plot of final slope against DPN concentration yields a curved line. On this basis we believe that the 2,4-D-DPN complex does not attach as readily to the enzyme as DPN alone, and that the complex, once attached, serves as an inhibitor for MDH.

All of the experiments described above concerning the inhibition of beet root MDH by 2,4-D, the relation of this inhibition to nucleotide and organic acid substrates, and the mechanism of this inhibition, have also been performed with purified porcine heart malic dehydrogenase with results which are qualitatively and quantitatively the same as those reported for the beet root enzyme. There is, therefore, no evidence that this inhibitory action of 2,4-D is in any way unique to MDH from plant sources.

Effect on Other Pyridine Nucleotide-Requiring Enzymes: As an indication of how widespread this effect of 2,4-D might be with other enzymes using pyridine nucleotide as one substrate, the inhibitory effect of this material was also measured with several commercial enzyme preparations. These included alcohol dehydrogenase (yeast), TPN-isocitric dehydrogenase (porcine heart), lactic dehydrogenase (rabbit muscle) and glucose-6-phosphate dehydrogenase (yeast). These enzymes were assayed by recommended procedures (4). It was found that alcohol dehydrogenase was substantially inhibited by 2,4-D in a competitive fashion. Lactic dehydrogenase showed a similar inhibition, but to a lesser degree. The TPN-isocitric dehydrogenase was slightly inhibited and glucose-6-phosphate dehydrogenase was not inhibited by 2,4-D concentrations up to 10^{-2} M. These determinations do not provide sufficient data to permit conclusions as to the ubiquity of this inhibitory effect on PN-requiring enzymes, but they do indicate that the effect is not specific for malic dehydrogenase.

Inhibitory Effectiveness of Related Compounds: Since it had already been shown that with mitochondria, 2,4-dinitrophenol (13) and 2,4-dichlorophenol were even more effective than 2,4-D in inhibiting the oxidation of malate, it seemed improbable that this inhibition was related to the growth-promoting properties of 2,4-D. However, to confirm these previous observations and to establish the relative effectiveness of similar compounds, the inhibition constants of several substituted phenoxyacids and phenols for the oxidation of malate were determined. These constants were calculated by the method of Hunter and Downs (6), fitting lines to experimental values of $I\ \alpha/(1 - \alpha)$, where α is defined as rate in the presence of inhibitor/uninhibited rate, and calculating the intercept of the line on the vertical axis, which is equal to Ki. All of the compounds listed in table III were inhibitory to malic dehydrogenase

Table III

Inhibitor Constants for Substituted Phenols & Phenoxy Compounds

Compound	Ki	Effectiveness as an inhibitor relative to 2,4-D
Phenol	2.3×10^{-2}	0.10
2,4-Dichlorophenol	1.2×10^{-3}	1.83
2,4,5-Trichlorophenol	1.9×10^{-4}	11.58
2,4-Dinitrophenol	2.5×10^{-4}	8.80
Phenoxyacetic Acid	3.8×10^{-3}	0.58
4-Chlorophenoxyacetic Acid	3.1×10^{-3}	0.71
2,4-Dichlorophenoxyacetic Acid	2.2×10^{-3}	1.00
2,4,6-Trichlorophenoxy-acetic Acid	1.7×10^{-3}	1.29
2,4,5-Trichlorophenoxy-acetic Acid	1.2×10^{-3}	1.83
2,3,4,6-Tetrachlorophen-oxyacetic Acid	3.8×10^{-4}	5.79

Conditions for the assays as specified in table I, except that six DPN concentrations were used, from 0.27 to 2.67 mM. Inhibited reactions contained 3.3 mM inhibitor, except in the case of DNP, where a concentration of 0.17 mM was used.

in some degree, and the positive slope of their Hunter-Downs lines indicated that they were competitive with DPN.

In table III the Ki's and the corresponding inhibitory effectiveness relative to 2,4-D are listed for ten phenols and phenoxyacid compounds. In general the picture is that unsubstituted phenoxyacetic acid is more effective than unsubstituted phenol (6 ×), but chloro-substitution causes a greater increase in toxicity with phenol than with phenoxyacetic acid, since 2,4,5-trichlorophenol is almost 7 × more inhibitory than 2,4,5-trichlorophenoxyacetic acid. Thus chloro-substitution increases inhibitory effectiveness with both types of compounds, but relatively more with phenol than with phenoxyacetic acid. On the basis of one comparison, that between 2,4-dichlorophenol and 2,4-dinitrophenol, it appears that nitro-substitution is more effective (in this case about 5 ×) than chloro-substitution.

It may be noted that 2,4-dichlorophenol is approximately twice as effective as 2,4-D, and this fact is an indication that the results reported earlier in this paper did not result from an impurity of DCP in the 2,4-D, since the 2,4-D was recrystallized three times from hot water and no detectable phenol could be found in the 2,4-D. If it were assumed that DCP was the effective agent, an impurity of 50 % would be required to produce the inhibition found here.

These data provide little evidence for the existence of an ortho effect in the inhibition of MDH as might be expected if this response were related to the growth-stimulating effects of 2,4-D. Phenoxyacetic acid with a 2,4,6-trichloro substitution is intermediate in its effectiveness between 2,4-D and 2,4,5-trichlorophenoxyacetic acid, but 2,3,4,6-tetrachlorophenoxyacetic acid, which also has both ortho positions masked, is more effective than either. It is apparent that if a free ortho position does favor the complexing of these materials, its effect is much less than additional chloro substitution.

Any attempt to relate the inhibition of malic dehydrogenase by these compounds to their electrochemical or other properties must await the acquisition of similar data with a wider range of related compounds.

It should be emphasized in consideration of the data presented in table III, that with a mechanism of inhibition such as that postulated here, in which the inhibitor complexes first with a substrate for the reaction, after which the inhibited substrate further complexes with the enzyme, the Ki cannot be interpreted as a dissociation constant for either complex, but rather represents a mean dissociation constant for the two complexes. Since this is the case, the Ki's reported here, while they do give some measure of the overall effectiveness of the inhibitors, do not necessarily represent a realistic estimate of the relative affinity of these compounds either for DPN or for the enzyme.

Discussion

The inhibition of beet root mitochondrial malic dehydrogenase by 2,4-D and structurally related compounds is of some interest in relation to the phytotoxic properties of 2,4-D and substituted phenols, and together with the known uncoupling abilities of these compounds, may provide an explanation of the primary cause of the death of plant cells exposed to high concentrations of these materials. The level of inhibition found is inversely related to the amount of DPN present, so that at low levels 2,4-D is a fairly effective inhibitor. For example, at 10^{-4} M DPN, a concentration of 2,4-D in the range usually used for weed killing purposes (3.3×10^{-3} M or 729 ppm) inhibits the oxidation of malate 80 %. At a DPN concentration of 10^{-3} M, this level of 2,4-D still causes a 20 % inhibition of MDH activity. When the cellular level of DPN is low, due perhaps to piling up of reduced nucleotide as a result of rapid metabolism, 2,4-D would cause severe reductions in the oxidation of malate and in the overall operation of the TCA cycle. This effect, added to the reduction in available energy supply due to the concomitant uncoupling of phosphorylation, would drastically reduce the level of available metabolic energy, and could well result in the death of the cell.

These observations may also provide an explanation for the previously observed shift from TCA metabolism to the pentose phosphate pathway under the influence of 2,4-D (8). The inhibition of malic dehydrogenase, and of isocitric dehydrogenase as well (13), would partially block the flow of substrate through the TCA cycle and thus induce an increase in the utilization of glucose for energy production via the pentose phosphate pathway.

Of perhaps more general interest are the indications obtained by kinetic methods that the mechanism of 2,4-D inhibition of malic dehydrogenase involves the non-enzymatic complexing of 2,4-D with DPN rather than a complexing of 2,4-D directly with the enzyme. It should be emphasized that kinetic evidence is at best indirect and the confirmation of the inhibitory mechanism postulated here must await more direct evidence such as demonstration of a spectral shift or isolation of a complex by chemical methods. The Ki's determined for this inhibition, since they are the mean of at least two dissociation steps, give no real clue as to the possible stability of a DPN- 2,4-D complex. Attempts to demonstrate the existence of a complex by the induction of a shift in the spectrum of either DPN or 2,4-D over the range from 220 mμ to 400 mμ occasioned by mixing the two compounds have been unsuccessful, but efforts to obtain direct evidence for the formation of a complex are continuing.

From tests with a small number of other dehydrogenases it is clear that the inhibitory effect of 2,4-D is not limited to malic dehydrogenase, since alcohol dehydrogenase and lactic dehydrogenase were both competitively inhibited by 2,4-D. Whether the

inhibitory complex is formed with TPN is not clear, since TPN-isocitric dehydrogenase appeared to be slightly inhibited by 2,4-D, while glucose-6-P dehydrogenase was not inhibited at all. It is probable that inhibitory effectiveness depends not only on the ability of 2,4-D to complex with the pyridine nucleotide, but also on the affinity of the complex for a specific enzyme and the ability of the enzyme to process the complex to the usual products. The data available appear to indicate that 2,4-D complexes more readily with DPN than TPN, and that enzymes differ in the degree to which the complex, once formed, inhibits further activity.

The question of whether the observed inhibition of malic dehydrogenase by 2,4-D and the putative inhibition mechanism consisting of a non-enzymatic complex formation with DPN has any significance in the growth process initiated by 2,4-D is one which at this point is open only to speculation. It seems improbable that the inhibition of MDH by 2,4-D has any bearing on growth promotion by this compound: First, the inhibition would be found in the cell only at concentrations of 2,4-D higher than those usually causing growth responses or at extremely low endogenous levels of DPN. Second, compounds which have little or no growth promoting effect, such as DNP, DCP, and phenoxy acids with both ortho-positions blocked do have a very potent inhibitory effect.

On the other hand, this indication of the formation of a complex of 2,4-D with DPN, together with the earlier direct demonstration of complexing between DPN and indole compounds (1), does open the possibility that a complex of auxin with pyridine nucleotide may be the substance which uniquely fits an enzyme controlling a key reaction in the growth. Although it is possible that control of growth by a complex of this type might be a negative one, that is, the complex would specifically inhibit a reaction to cause a shunting of metabolic energy into reactions leading to growth, it is more attractive to postulate that auxin would exert a positive metabolic control in that only the auxin-DPN complex would serve as substrate or cofactor for a reaction which would either directly or indirectly open the gate to a flow of energy into the growth process.

Summary

A malic dehydrogenase isolated from beet root mitochondria has been purified 100-fold, some of its characteristics determined, and the purified preparation used in a study of the kinetics of the inhibition by 2,4-D of malate oxidation and oxaloacetate reduction and the associated oxidation-reduction of diphosphopyridine nucleotide.

It was found that 2,4-D inhibits the reaction catalyzed by malic dehydrogenase proceeding in either direction. This inhibition was shown to be competitive for diphosphopyridine nucleotide or reduced diphosphopyridine nucleotide and non-competitive for malate or oxaloacetate.

A kinetic investigation of the mechanism of this inhibition indicates that the probable mechanism is through a non-enzymatic complexing of 2,4-D with pyridine nucleotide, and that the complex so formed is an inhibitor for the enzyme.

Several structurally similar compounds of the phenoxy acid and phenol types were also found to cause a competitive inhibition of malic dehydrogenase. The number and type of substituents appear to be major factors in increasing the inhibitory effectiveness of these compounds.

Literature Cited

1. Alivisatos, S. G. A., G. A. Mourkides, & A. Jibril. 1960. Non-enzymatic reactions of indoles with coenzyme I. Nature 186: 718–719.
2. Berger, J. & G. S. Avery, Jr. 1944. Glutamic & isocitric dehydrogenases in the Avena coleoptile & the effect of auxins on these enzymes. Am. J. Botany 31: 11–19.
3. Bonner, J. & R. S. Bandurski. 1952. Studies of the physiology, pharmacology & biochemistry of the auxins. Ann. Rev. Plant Physiol. 3: 59–86.
4. Colowick, S. P. & N. O. Kaplan. 1955. Methods in Enzymology. Vol. I. Acad. Press, Inc., New York, N. Y.
5. Colowick, S. P. & N. O. Kaplan. 1955. Methods in Enzymology. Vol. II. Acad. Press, Inc., New York, N. Y.
6. Dixon, M. & E. C. Webb. 1958. Enzymes. Acad. Press, Inc., New York, N. Y.
7. Hiatt, A. J. & H. J. Evans. 1960. Influence of salts on activity of malic dehydrogenase from spinach leaves. Plant Physiol. 35: 662–672.
8. Humphreys, T. E. & W. M. Dugger, Jr. 1957. The effect of 2,4-dichlorophenoxyacetic acid on the respiration of etiolated pea seedlings. Plant Physiol. 32: 530–536.
9. Nielsen, H. 1958. Quantitative micro-determination of proteins & peptides. Acta Chem. Scand. 12: 38–43.
10. Reiner, J. 1959. Behavior of Enzyme Systems. Burgess Publ. Co., Minneapolis, Minn.
11. Siegel, L. & S. England. 1961. Beef-heart malic dehydrogenases. I. Properties of the enzyme purified from extracts of acetone-dried powders. Biochim. Biophys. Acta. 54: 67–76.
12. Umbreit, W. W., R. H. Burris, & J. F. Stauffer. 1957. Manometric Techniques. 3rd Ed. Burgess Publ. Co., Minneapolis, Minn.
13. Wedding, R. T. & M. Kay Black. 1962. Response of oxidation & coupled phosphorylation in plant mitochondria to 2,4-dichlorophenoxyacetic acid. Plant Physiol. 37: 364–370.