FORMATE OXIDATION BY PARTICULATE PREPARATIONS FROM HIGHER PLANTS [1]

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Enzyme systems capable of oxidizing formate to CO_2 are of wide occurrence in higher plants (3). Seed extracts have been used primarily as the source of enzyme. Extracts of rat liver and kidney were also able to carry out this oxidation (9). The enzyme from leguminous seeds, such as pea and bean, has been studied in considerable detail (1, 3, 4, 9). The formic dehydrogenase activity in various organs of the pea has been followed throughout the life cycle (3). The highest activity was found in the mature seed. This activity diminished upon germination and growth. Seeds of 93 species were tested and 54 were shown to be capable of oxidizing formate (3). The intracellular localization of the enzyme has not been reported for either plant or animal tissues.

The present study reports the presence of a formate-oxidizing system in cabbage leaf localized in the cytoplasmic particles sedimenting between $1000 \times G$ and $23,000 \times G$ during centrifugation. Characteristics of this enzyme have been examined in some detail and compared with the classical pea seed dehydrogenase. The latter has also been found in the present study to be mainly in the particulate fraction. The enzyme is even more widely distributed than previously believed, since active preparations have been obtained from plants which had given negative results with seed extracts. Among these are cabbage and several other members of the Cruciferae. In every case the activity is localized in the particulate fraction obtained by centrifugation.

EXPERIMENTAL METHODS

PREPARATION OF ENZYME: All plant sources were purchased at local markets. Washed cytoplasmic particle suspensions were prepared as described previously (11), with slight modifications depending upon the material. Soluble cytoplasmic proteins of pea and cabbage were precipitated from the supernatant fluid obtained after centrifugation at $23,000 \times G$. This solution was made 75 % saturated with solid $(NH_4)_2SO_4$ at 4° C. After 2 hours in the cold, the precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed against several changes of 0.01 M Na_2HPO_4 overnight (4). Insoluble material was centrifuged down and discarded, and the solution was lyophilized. An acetone powder of cabbage particles was made from a preparation which had been suspended in and then dialyzed against 0.01 M phosphate at pH 7.0 for 60 to 90 minutes to remove any remaining sucrose. Four volumes of −20° C. acetone were added slowly with stirring; the mixture was kept at ice temperature for 30 minutes, filtered, washed, and dried in vacuo in the cold. A 1 % suspension of the acetone powder was made in 0.01 M phosphate pH 7.0. After 20 minutes in the cold, it was centrifuged and the clear solution used as the enzyme source. A digitonin extract of cabbage particles was prepared by adding 2 volumes of a 1 % digitonin suspension and shaking occasionally for 10 minutes at 4° C. The insoluble material was removed by centrifugation. The solution was made 75 % saturated with solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in a small volume of 0.01 M phosphate pH 7.0, and dialyzed several hours against the same buffer. Some material precipitated within the dialysis sack and was discarded. The clear dialysate was used as the enzyme.

STANDARD ASSAY PROCEDURES: The reaction was carried out in conventional Warburg vessels with continuous shaking at 30° C. The activity was determined by the conversion of $HC^{14}OONa$ to $C^{14}O_2$, which was trapped by absorption in 0.2 ml 20 % KOH in the center well. The reaction was terminated by tipping in 0.3 ml 5 *N* H_2SO_4 from the sidearm (10 % trichloracetic acid was used on occasion). After shaking at least 10 minutes more, the contents of the center well were collected, the well washed with distilled water several times, and the washings combined with the KOH. The absorbed $C^{14}O_2$ was precipitated as $BaCO_3$, plated, and counted as described by Stumpf (13). Protein was determined by the Biuret method as described by Weichselbaum (17). After heating at 37° C for 30 minutes, the samples were cooled to room temperature and read by means of an Evelyn colorimeter at 540 mμ. Armour crystalline bovine plasma albumin was used as the standard. The Ag-Ba double salt of phosphoenolpyruvate (PEP), and the barium salt of phosphoglyceric acid (PGA), were converted to the Na salt before use.

RESULTS

LOCALIZATION OF ENZYME IN CABBAGE LEAF: The formate-oxidizing system was almost completely localized in the particulate fraction isolated from cab-

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TABLE I

INTRACELLULAR DISTRIBUTION OF CABBAGE LEAF FORMATE-OXIDIZING SYSTEM

REACTION SYSTEM	TOTAL COUNTS/MG PROTEIN	
	PARTICLES	CYTOPLASM
Complete	7,450	126
Minus DPN	6,680	28
Minus DPN + ATP	4,050	31
+ ATP	4,350	556
Heated complete	8	...

The complete reaction mixture contained 100 μmoles phosphate pH 7.0; 1 μmole Na formate-C^{14} (120,000 total counts); 0.2 μmole DPN; 20 μmoles GSH; enzyme and distilled water to a final volume of 2 ml. 10 μmoles neutralized Na ATP were added where indicated. An aliquot of the washed particle suspension was inactivated by heating in a boiling water bath for 5 minutes for use in the heat-treated system. The incubation time was 60 minutes. 9.7 and 7.7 mg protein were present in the particulate and cytoplasmic experiments, respectively.

bage leaf homogenates (table I). Adding DPN to the washed particles resulted in a slight stimulation. The activity of the lyophilized cytoplasmic proteins increased 4.5 times on addition of DPN, but this was still only 2 % as active as the particles. Cabbage cytoplasm has a very active nucleotide pyrophosphatase (11). On adding ATP, which would tend to protect the DPN, the cytoplasmic activity was raised another four times, but this was still only 8 % of the activity exhibited by the particles. Interestingly enough, adding ATP to the particulate system resulted in a marked inhibition. The lack of any significant stimulation of the particulate activity by DPN could mean either an enzyme different from the classical DPN-requiring formic dehydrogenase is involved, or that the particle contained sufficient bound DPN so that exogenously added coenzyme had little effect. Several experiments with freshly prepared cabbage particles showed that a 10 to 20 % stimulation was usually obtained by adding DPN. The particles, however, are very active in its absence. Solubilization studies to be described later will demonstrate the DPN requirement much more clearly. The particulate system also showed a consistent slight stimulation on adding GSH; therefore, it was customarily added. The reaction time was 60 minutes in the experiment described in table I. Subsequent experiments were usually of 30 minutes duration since this was the period during which formate oxidation proceeded linearly, however, there was no increased effect of DPN with the shorter incubation time.

OPTIMUM PH: A sharp optimum in activity was exhibited at pH 7.0 in phosphate buffer (fig 1). This differed from the pea seed dehydrogenase which had an optimum activity at pH 6.0, decreasing gradually at more alkaline pH (3). Since the cabbage enzyme was particulate-bound, this optimum may reflect the optimum for formate permeability and not oxidation. Phosphate was much better than tris(hydroxymethyl)aminomethane or collidine buffers at the same pH. This was not the result of a phosphate requirement since tris(hydroxymethyl)aminomethane + 10 μmoles of phosphate gave no stimulation over the buffer alone.

OXIDATION AS FUNCTION OF TIME: The conversion of formate to CO_2 was approximately linear with time for the first 30 minutes. There was a leveling off of oxidation after about 50 % of the formate had been oxidized. In several duplicate runs the leveling off always came at the point where approximately half the added formate had been oxidized. Tipping in additional formate-C^{14} at this point did not increase the amount of $C^{14}O_2$ formed. This could result from a gradual inactivation of the enzyme or the DPNH-oxidizing system.

K_s OF PARTICULATE SYSTEM: A Lineweaver-Burk plot (8) is shown in figure 2. The K_s was found to be 5×10^{-3} M. Adler and Sreenivasaya (1) obtained a value of 7×10^{-3} M for the pea seed dehydrogenase.

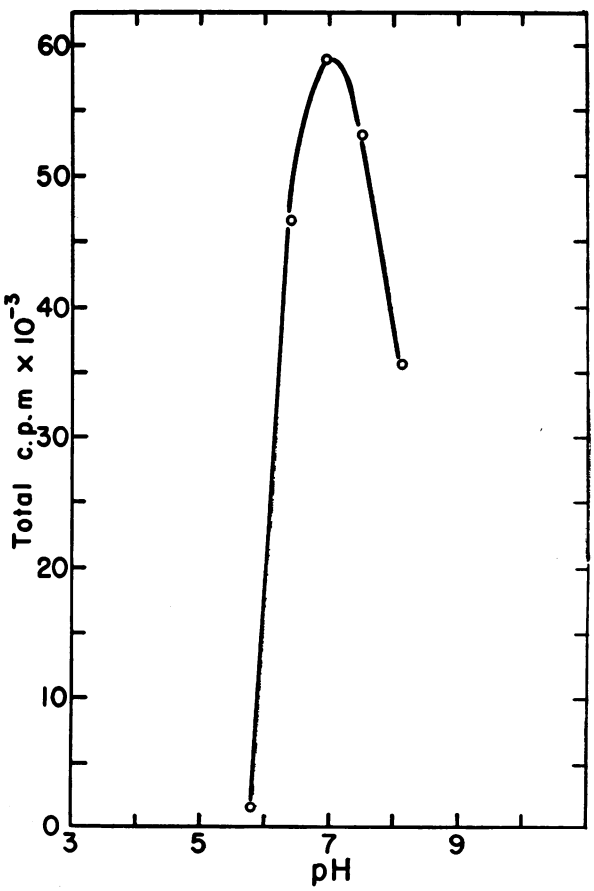

FIG. 1. Formate oxidation as a function of pH. Vessels contained the complete reaction mixture described in table I. Phosphate buffer used for whole range of pH studied. A washed particle suspension containing 10.5 mg protein was used as the enzyme source in each vessel. Incubated for 60 minutes at 30° C.

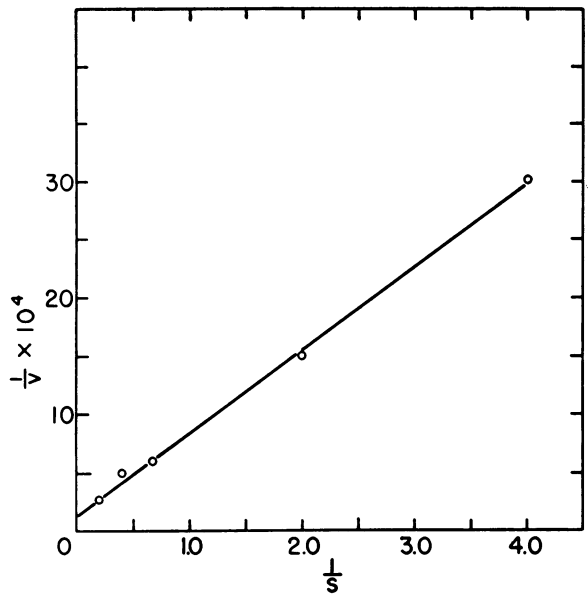

FIG. 2. Effect of substrate concentration on velocity. Na formate-C^{14} used as substrate, given in μmoles per ml; velocity is in total cpm of $BaC^{14}O_3$ formed in the first 10 minutes; DPN, phosphate buffer pH 7.0, GSH are as in table I; and 7.4 mg protein as washed particle suspension. The final volume was 2 ml.

INHIBITOR STUDIES: Pea formic dehydrogenase has been found to be very susceptible to CN^- and azide (4). These inhibitors are rather unusual for a dehydrogenase. Cabbage particles were pre-incubated with various inhibitors at pH 7.0 for 10 minutes and then added to the formate reaction mixture. Table II shows that KCN and Na azide were extremely effective inhibitors at 10^{-3} M final concentration. The oxidation of formate was almost completely inhibited. Semicarbazide and hydroxylamine produced significant inhibitions at 5×10^{-3} M. To a certain extent 8-hydroxy quinoline stimulated the activity. If the particles were not pre-incubated with the inhibitor, but added directly to the reaction mixture plus inhibitor, substantially the same results were obtained, except that inhibition by hydroxylamine was only 29 % and the 8-hydroxy quinoline stimulation declined to 19 %.

TABLE II

EFFECT OF VARIOUS INHIBITORS ON FORMATE OXIDATION

INHIBITOR AND FINAL CONCENTRATION	RELATIVE ACTIVITY
No inhibitor	100
1×10^{-3} M KCN	1
1×10^{-3} M Na azide	0.1
5×10^{-3} M semicarbazide	41
5×10^{-3} M hydroxylamine	58
1×10^{-3} M 8-hydroxy quinoline	125

The reaction mixture contained 100 μmoles phosphate pH 7.0; 1 μmole Na formate-C^{14} (62,000 total counts); 0.2 μmole DPN; 20 μmoles GSH; particle suspension containing 6.8 mg. protein; distilled water to final volume of 2 ml. The particles were incubated with the phosphate buffer and the indicated inhibitor for 10 minutes prior to addition to the reaction mixture. The particles used for the control reaction without any added inhibitor were incubated with buffer and distilled water to the same final volume as where inhibitor was used. The center well contained a KCN : KOH mixture described by Krebs (6) when KCN was added to the reaction mixture. Incubation time was 30 minutes.

OXIDATION UNDER AEROBIC VS. ANAEROBIC CONDITIONS: The necessity for oxygen as a requirement for the conversion of formate to CO_2 was examined. The reaction was carried out in double sidearm Warburg vessels. The enzyme suspension was placed in one sidearm and tipped in to start the reaction. One pair of vessels was gassed with nitrogen for 5 minutes. One of the pair contained methylene blue to act as an electron acceptor. Another pair of vessels of similar composition was not gassed with nitrogen, but kept under the same conditions of manipulation and equilibration. A third pair of vessels was used as a control in that the enzyme suspension was tipped in at once.

TABLE III

FORMATE OXIDATION UNDER ANAEROBIC CONDITIONS

REACTION SYSTEM	FLASK ATMOSPHERE	TOTAL COUNTS/ MG PROTEIN
Complete, enzyme tipped in immediately	Air	706
Same, plus methylene blue	"	665
Complete, enzyme tipped in after gassing 5 min with N_2 and 10 min equilibration	Nitrogen	216
Same, plus methylene blue	"	789
Complete, enzyme tipped in after 15 min in air in sidearm	Air	582
Same, plus methylene blue	"	610

Complete system contained 100 μmoles phosphate, pH 7.0; 1 μmole Na formate (62,000 total counts); 20 μmoles GSH; 0.2 μmole DPN; 0.5 ml washed particle suspension containing 9.6 mg protein; distilled water to a final volume of 2 ml. Where indicated, 0.2 ml of a 0.5 % methylene blue solution was added. After the enzyme was tipped in, the reaction, was carried on for 30 minutes at 30° C before stopping the incubation by tipping in the H_2SO_4 from the other sidearm.

This was to determine whether there was a large amount of inactivation of the enzyme during the time of the gassing and equilibration period. The results are summarized in table III. If the gas phase was air, then there was no effect (or only a slight one) on adding methylene blue. The oxidation of formate was reduced 60 to 70 % after gassing with nitrogen. Adding methylene blue raised the total oxidation back to the amount found under aerobic conditions.

TABLE IV

NUCLEOTIDE INHIBITION OF FORMATE OXIDATION

NUCLEOTIDE ADDED	RELATIVE ACTIVITY
None	100
ATP	77
ADP	62
AMP	47
Adenosine	84
Adenine	86
Yeast AMP	101
UTP	109

The reaction mixture was the same as that given in table III. 10 μmoles of the respective nucleotides were added as indicated. Incubation time, 30 minutes. 6.0 mg protein per vessel as a washed cytoplasmic particle suspension.

ADENINE NUCLEOTIDE INHIBITION: The inhibition of the particulate formate oxidation by ATP (table I) was examined in more detail. The effect of various nucleotides on the production of $C^{14}O_2$ is shown in table IV. The inhibition appears to be specific for both the presence of the adenine moiety and a phosphate on the 5′ position of the ribose. Uridine triphosphate (UTP) and yeast adenylic acid had no inhibitory effect. There was an increasing inhibition on going from ATP to adenosine-5′-phosphate (AMP). This may be a reflection of the ease of permeation by AMP compared to adenosine diphosphate (ADP) or ATP.

PEP carboxylase and PEP carboxykinase have been shown to be widely distributed in plant tissue (10). These enzymes catalyze reactions A and B, respectively.

A. PEP + CO_2 → oxalacetate + inorganic phosphate

B. PEP + ADP + CO_2 ⇄ oxalacetate + ATP

Plant PEP carboxykinase has a specific requirement for adenine nucleotides (10), whereas the avian and animal enzymes show a guanosine or inosine nucleotide specificity (2, 7). If reaction B above is the cause of the nucleotide inhibition, then added PEP should also give an inhibition. Table V clearly demonstrates a large inhibition on addition of PEP.

TABLE V

EFFECT OF PEP AND PGA ON PRODUCTION OF LABELED CO_2

COMPONENT ADDED	RELATIVE ACTIVITY
None	100
+ 10 μmoles ADP	62
+ 2 μmoles PEP	21
+ 2 μmoles PEP + 10 μmoles ADP	18
+ 2 μmoles PGA	36
+ 2 μmoles pyruvate	101

The reaction mixture was the same as described in table III. Incubation time was 30 minutes.

The production of $C^{14}O_2$ was inhibited 80 % in the presence of 10^{-3} M PEP. PGA was also inhibitory at the same concentration but to a lesser extent. The presence of enolase and phosphoglyceric acid mutase is indicated by this result. When pyruvate was added in the same concentration, there was no effect. If the inhibition is due to the fixation of the CO_2 produced, the action of PEP is not due to hydrolysis to pyruvate and fixation by means of malic enzyme. An attempt to demonstrate incorporation of label into the organic acid fraction in the presence of AMP or PEP was not successful. The fact that no activity could be demonstrated in the organic acid fraction might be a reflection of the rapid removal of the oxalacetate formed, such as by a rapid transamination to aspartate. Aspartate is labeled very rapidly by $C^{14}O_2$ in certain cases (15, 16).

SOLUBILIZATION OF FORMIC DEHYDROGENASE: The enzyme was solubilized from an acetone powder of the particles and by a digitonin extraction as described previously. Table VI compares the activity of the washed particles, the digitonin and acetone powder extracts of the particles, and the cytoplasm. The DPN requirement is readily apparent after solubilization. Triphosphopyridine nucleotide (TPN) cannot substitute for DPN. This specificity is the same as that found for pea formic dehydrogenase (9). The acetone powder extract showed a different effect on adding ATP than the digitonin extract. The stimulation of activity of the former was similar to the stimulation exhibited by the cytoplasmic system upon addition of ATP. The oxidation of formate by the digitonin extract was inhibited when ATP was present.

DISTRIBUTION OF PARTICULATE SYSTEM: A brief survey of several plant families was made to determine the presence and intracellular localization of formate oxidation. Pea seed was studied because it is the customary source of formic dehydrogenase. Special emphasis was placed on testing members of the Cruciferae (cabbage, turnip, radish, cauliflower) because of the negative results reported with seed extracts (3). Squash, representing the Cucurbitaceae, had also given negative results with seed extracts. Spinach leaf was included because it is commonly used as a source of chloroplasts and chloroplast fragments for enzymatic studies. In every case formate was oxidized and the activity almost exclusively localized in the particulate fraction (table VII). No attempt was made to determine and use the optimum conditions for oxidation for each preparation, so the specific activities are probably lower than could be obtained.

The pea seed dehydrogenase was the least concentrated in the particles, but this fraction still had three times the activity of the cytoplasm on a per milligram protein basis. The pea and cabbage cytoplasm fractions were prepared by a procedure which had been shown to stabilize the formic dehydrogenase of pea seed (4).

TABLE VI

COMPARISON OF SOLUBILIZED FORMIC DEHYDROGENASE WITH PARTICULATE AND CYTOPLASMIC SYSTEM

REACTION SYSTEM	TOTAL COUNTS/MG PROTEIN			
	WASHED PARTICLES	ACETONE POWDER EXTRACT	DIGITONIN EXTRACT	CYTOPLASM
Control	1,510	1,252	216	28
+ DPN	1,885	2,066	1,653	126
+ ATP	1,065	...	205	31
+ DPN + ATP	1,148	3,391	1,247	556
+ TPN	...	...	218	...
+ TPN + ATP	...	...	...	102

Control reaction mixture consisted of phosphate buffer, Na formate-C^{14}, GSH, and enzyme, as in table III. 0.2 μmole DPN or TPN and 10 μmoles ATP added where indicated. Incubation time was 60 minutes, except for the digitonin extract which was only 30 minutes.

DISCUSSION

The present work has shown that the widespread ability of plants to oxidize formic acid is not confined to the seeds. Extremely active preparations were made from other organs of plants which had no activity on the basis of seed extracts. The localization of formate oxidation in the particulates isolated by differential centrifugation from plant or animal[3] homogenates completes a unified biochemical pattern for localization of enzymes oxidizing this compound. Stickland (14) found that bacterial preparations also possessed formate-oxidizing ability which was localized in an insoluble cell fraction. Previous work on pea formic dehydrogenase was done under conditions which would not distinguish a particulate system from a soluble one. The procedure which had customarily been used was that of extracting a homogenate with an aqueous salt solution. Such procedures have frequently been used to solubilize enzymes from particulate structures (12). The results in table VII also indicate that pea seed particles are more fragile than others and the enzyme would tend to be solubilized more readily during homogenization.

Certain conclusions may be drawn concerning the electron transport system after formic dehydrogenase. Under anaerobic conditions methylene blue is as effective as oxygen as an electron acceptor. This indicates that a diaphorase must be present in the particles. Another implication of the results in table III is that endogenous electron acceptors must be constituents of the particles since under a nitrogen atmosphere the oxidation of formate was not lowered to a zero value.

The solubilized system showed a quite different ATP response depending upon the method of extraction. Acetone powder extracts behaved similarly to the cytoplasm in exhibiting a strong stimulation of formate oxidation when ATP was added. Digitonin extracts had the same response to added ATP as the washed particles. Cabbage particles have been shown to contain active pyrophosphatases (11). In the case of the acetone powder extracts the added ATP is probably protecting the DPN from destruction. The lack of effect of added ATP with digitonin extracts would imply that the latter treatment either inactivates or does not extract these enzymes.

[3] Mazelis, M. Unpublished experiments with mouse liver and kidney homogenates.

TABLE VII

INTRACELLULAR LOCALIZATION OF FORMATE OXIDATION IN ORGANS FROM SEVERAL HIGHER PLANTS

ENZYME SOURCE	TOTAL COUNTS/MG PROTEIN					
	WASHED CYTOPLASMIC PARTICLES			CYTOPLASM		
	No DPN	+DPN	+DPN +ATP	No DPN	+DPN	+DPN +ATP
Cabbage leaf	1,575	1,655	1,135	20	40	300
Pea seed	606	1,140	...	26	384	185
Spinach leaf	194	301	171	36	64	...
Radish root	673	1,229	921	26	47	...
Cauliflower bud	1,270	1,608	1,360	2	...	29
Turnip root	214	703	202	26	...	50
Squash fruit	1,284	2,715	1,605	180	206	18

Phosphate buffer, formate C^{14}, and GSH as in table III. 0.2 μmoles DPN and 10 μmoles ATP added where indicated. Cytoplasm was the lyophilised preparation described in "Methods" in the case of cabbage and pea seed. In all other cases an aliquot of the supernatant fluid obtained after removal of the particles from the homogenate by centrifugation at 23,000 × G was used as the source of enzyme. Incubated for 60 minutes at 30° C.

Many metabolic reactions lead to the formation of C_1 units which can be converted to formate (5). The widespread occurrence of PEP carboxylase and PEP carboxykinase in higher plants (10), and the particulate nature of enzymes fixing CO_2 in the presence of PEP (10, 18) has been well established. The presence of these enzyme complexes could be a means whereby the carbon in the formate or C_1 pool would be conserved.

Summary

Formate is oxidized by enzymes localized in the particles sedimenting at 23,000 × G from sucrose homogenates of organs of higher plants.

The enzyme has been found in various roots, buds, leaves, seeds, and fruits, and is DPN-specific.

The production of CO_2 from formate by cabbage particles is inhibited strongly in the presence of 10^{-3} M PEP or PGA, or 5×10^{-3} M adenine nucleotides, presumably by refixation of the CO_2 formed.

The enzyme is readily solubilized by a digitonin treatment of the particles.

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