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Studies on Plant Formic Dehydrogenase

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Thunberg (1921, 1936) first discovered a formic dehydrogenase in the seeds of *Phaseolus vulgaris* (French bean). The same enzyme, obtained from peas by Fodor & Frankenthal (1930), was found to differ from the formic dehydrogenase of bacteria in that it required a coenzyme present in boiled pea juice. Andersson (1934) and Lichtenstein (1936) were able to identify this factor as coenzyme I (Co I). In 1937, Adler & Sreenivasaya, again using peas, studied the formic system in some detail, drawing attention to the strong inhibition obtained with low concentrations of cyanide. The widespread occurrence of the dehydrogenase in plant seeds and variation in the amount of enzyme during the life cycle of the pea has been reported (Davison, 1949*a*). The enzyme is most active in the mature dried seed and diminishes in activity with germination and growth. It increases again in the pod and developing seed.

The work described in this paper has been directed to finding a function for formic dehydrogenase in the metabolism of the plant. The enzyme has been purified 21-fold in 42% yield. Some observations on the properties of the dehydrogenase, including the Co I specificity, the effect of some inhibitors, especially some metal-binding reagents, and experiments on the coupling of the formic system with other dehydrogenases, are reported. Attempts to reverse the oxidation of formate by the dehydrogenase are described. The reduction of certain dyes by the system has been investigated, and attempts have been made to reverse the reaction by poising against one of these dyes (benzyl viologen). Other experiments designed to show such a reversal are those on the effect of carbon dioxide on Co I reduction, and the coupling of the system with the oxidation of triosephosphate. The possible significance of the system in the metabolism of the plant is discussed.

METHODS AND MATERIALS

Pea seeds. Pisum sativum (var. Meteor) were used.

Bean seeds. Phaseolus multiflorus (var. Giant White Czar) were used throughout this work.

Coenzyme I. This was prepared by the method of Williamson & Green (1940), modified by Ochoa (1948). It was 41% pure when estimated by reduction either with lactic dehydrogenase and lactate in the presence of cyanide, or with formic dehydrogenase and formate.

Coenzyme II. This was prepared by the method of Warburg, Christian & Griese (1935), modified by Haas (1947), and found to be 50% pure when estimated by the heart *iso*citric dehydrogenase-aconitase preparation of Adler, Euler, Günther & Plass (1939). The method of estimation, as with Co I, depends on the measurement of reduced coenzyme in an open tube in the Beckman quartz

spectrophotometer at 340 mμ. The value for purity for coenzyme II (Co II) is subject to slight error, since the enzyme preparation contained a fairly active Co II reductase which is slightly autoxidizable.

Heart diaphorase. This was prepared by the method of Straub (1939).

I am grateful to Mr E. J. Morgan of this department for the preparation of the coenzymes and the heart diaphorase.

Estimation of formic dehydrogenase

In place of methylene blue used by previous investigators, thionine was chosen as the reversibly oxidizable dye in most of the estimations, since it was found to be more efficient for plant dehydrogenases. The reduction of the dye was followed in evacuated Thunberg tubes at 38°. The test system was that given in Table 1. Under these experimental conditions the time for sensibly complete reduction of the dye is inversely proportional to the amount of enzyme added. One enzyme unit is defined as the quantity of enzyme reducing 1 μmol. of dye in 1 min.; thus if x μmol. are reduced in t min. the number of enzyme units present is x/t. The activity of the enzyme is expressed as units/mg. N in the preparation, i.e. μmol. thionine/mg. N/min.

Excess heart diaphorase was used because plant Co I diaphorase varied in the different fractions, and there was never enough present to activate the system completely. These observations differ from those of Adler & Sreenivasaya (1937), who found that yellow enzyme caused increased O_2 uptake when the system was tested aerobically, but did not affect the reduction of methylene blue. In a few later experiments a partially purified Co I diaphorase from pea seedlings (Davison, 1950) was used in place of the heart diaphorase.

In many experiments the reaction

$$HCOOH + \tfrac{1}{2}O_2 \rightarrow CO_2 + H_2O$$

has been followed by O_2 uptake in Warburg manometers fitted with flasks carrying two side arms. The incubation temperature was 38° and the test system was that given in Fig. 1. All formate added is completely oxidized, and a theoretical O_2 uptake obtained. Since the purified enzyme will not oxidize other related compounds such as acetic, oxalic, lactic and pyruvic acids, formaldehyde and acetaldehyde, it may possibly prove an effective tool for the estimation of formate.

To absorb CO_2 produced in the reaction, NaOH was placed in the centre well, and, in experiments using cyanide, this was replaced by a cyanide-alkali mixture (Krebs, 1935).

In the earlier stages of purification the cloudiness of the preparations precluded the use of the Beckman spectrophotometer in following the reduction of Co I, although this method was used with later clear preparations.

Purification of formic dehydrogenase. Preliminary experiments using pea and white bean seeds showed that extraction of a dry powder obtained by grinding the seeds finely in a coffee grinder and sieving through bolting silk (50–60 meshes/in.) was more effective than the use of the Waring blender in homogenizing soaked seeds. Drying the seeds for 3 hr. at 40° did not affect the activity of the enzyme and facilitated grinding.

Alkaline phosphate was used by Adler & Sreenivasaya (1937) in preparing formic dehydrogenase. Although this was not the most efficient extractant when compared with water and phosphate buffers of various pH values, it was used in the large-scale preparations because extracts made with 0·1 M-Na_2HPO_4 were more stable to dialysis and storage and it also provided the necessary buffering action when $(NH_4)_2SO_4$ was used for subsequent precipitation. With water extracts it was found that treatment with 80% $(NH_4)_2SO_4$ followed by dialysis against distilled water destroyed formic dehydrogenase activity, completely in pea preparations and partially in bean preparations. This inactivation could be partly prevented by dialysis against running tap water, which had some buffering action, and completely prevented by dialysis against 0·01 M-Na_2HPO_4. The same pH inactivation effect was encountered in experiments aiming to clarify the cloudy water extract by dialysis against water in which various amounts of CO_2 were dissolved. Dialysis for 1 or 2 hr. against distilled water 1/10 saturated with CO_2 did not affect the enzyme. Dialysis for 4 hr. decreased the activity by 50%, and after 8 hr. no activity remained.

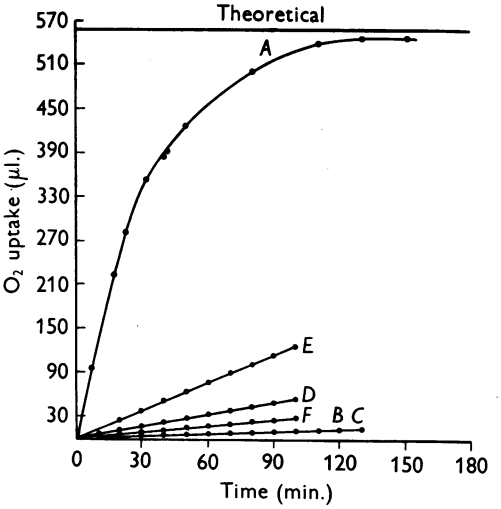

Fig. 1. Aerobic estimation of formic dehydrogenase. The complete test system: 1·8 ml. enzyme preparation (a freeze-dried preparation from fraction (8), 15 mg./ml.); 0·6 ml. 0·3 M-phosphate buffer, pH 7·0; 0·3 ml. Co I, 5 mg./ml.; 0·1 ml. heart diaphorase, containing 1 μg. bound riboflavin; 0·1 ml. 0·005 M-methylene blue; 0·1 ml. 0·5 M-sodium formate (≡560 μl. O_2) in the side bulb; distilled water to a final volume of 3·0 ml. In the centre well, 0·2 ml. 20 % (w/v) NaOH. Formate tipped in from side bulb to start reaction. Curve *A*, complete system; curve *B*, no formate; curve *C*, no coenzyme; curve *D*, no heart diaphorase; curve *E*. no methylene blue; curve *F*, no diaphorase or methylene blue.

For large-scale purification beans were chosen because here the initial blank was smaller, the formic enzyme was more stable to acid conditions, and there was less loss on storing extracts. The enzyme was purified as follows.

Sifted bean meal (150 g.) was extracted with 450 ml. 0·1 M-Na_2HPO_4 for 1 hr. at room temperature with occasional stirring. The homogenate was squeezed through muslin, centrifuged for 15 min. at 2800 rev./min., and the supernatant (1) treated with solid $(NH_4)_2SO_4$ to 0·8 saturation. The precipitate was filtered off on kieselguhr, and the cake rubbed up with 75 ml. 0·03 M-Na_2HPO_4. This suspension

was dialysed against running tap water overnight. The use of alkaline phosphate in taking up the precipitate, and running water for dialysis was adopted instead of distilled water since use of the latter decreased the yield, due to acid conditions. The dialysate was spun for 30 min. at 3600 rev./min. and a large amount of inert protein removed. The supernatant (2), about 200 ml., was used for fractionation with calcium phosphate gel (30 mg. dry wt./ml.). Calcium phosphate gel (20 ml./100 ml. supernatant) was added to fraction (2), the suspension mixed and allowed to stand at room temperature for 30 min. The gel was spun off and the supernatant (3) treated again with 20 ml. calcium phosphate/100 ml. supernatant. The supernatant (4) from this adsorption was then treated with 30 ml. calcium phosphate/100 ml. The supernatant (5), after spinning, was treated a second time with 30 ml. calcium phosphate/100 ml. supernatant. Supernatant (6) from this adsorption was then treated with 40 ml. calcium phosphate/100 ml., the enzyme being brought down on the gel at this stage. The supernatant was discarded, and the gel eluted with three successive lots of 25 ml. 0·1 M-Na_2HPO_4 for 15 min. each time. This preparation (7) was neutralized to pH 7·0 with 0·3 M-KH_2PO_4 and dialysed against distilled water at 4° overnight. The dialysed preparation (8) may be stored at −10° for several weeks without loss of activity, or, alternatively, freeze-dried and the dry powder stored. Preparation (7), after neutralizing, may also be stored as a dry powder, but the enzyme does not keep well stored in solution at −10° in the presence of phosphate. The activity and yield at the various stages of purification are given in Table 1. In the final preparation (8) the enzyme was purified 21-fold and a 42% yield obtained.

Table 1. *Purification of formic dehydrogenase*

(Test system: in the Thunberg tube: 1·0 ml. preparation; 0·3 ml. 0·3 M-phosphate buffer, pH 6·0; 0·3 ml. 0·5 M-sodium formate; 0·1 ml. heart-diaphorase preparation, containing 1·2 μg. riboflavin. In the hollow stopper: 0·3 ml. Co I, 5 mg./ml.; 0·4 ml. 0·005 M-thionine. The tubes were incubated for 5 min. at 38° before the Co I and dye were tipped in. Reduction times were between 1 and 4 min. for the series. Fraction (1) is corrected for the blank obtained with this preparation (decolorization times 1·5 and 30 min. in the presence and absence of formate). After this fraction there was no appreciable blank. Overall purification 21-fold; overall yield 42%.)

Fraction no.	Activity (μmol./min./mg. N)	Total enzyme units
1	0·0642	275
2	0·1451	192
3	0·188	186
4	0·266	184
5	0·3605	173
6	0·676	165
7	1·065	151
8	1·347	115

RESULTS

Coenzyme specificity

In experiments on the coenzyme specificity of formic dehydrogenase, Adler & Sreenivasaya (1937) used a preparation of Co II contaminated with Co I, but since the activity of this preparation could be accounted for entirely by its Co I content, they concluded that the enzyme was specific for Co I. This has been confirmed using fraction (8), and a Co II preparation free from Co I. Co II, in four times the concentration of Co I used in Table 1, was completely ineffective in the reduction of thionine, and no appreciable reduction of Co II could be observed in the Beckman spectrophotometer at 300 mμ. in the presence of the enzyme and formate. Mathews & Vennesland (1950) have also confirmed this specificity and report that it is possible to detect as little as 0·01 mg. Co I in the presence of 1·0 mg. Co II, making the enzyme a useful tool for the detection of Co I.

Effect of metal ions

In view of the cyanide sensitivity of formic dehydrogenase, the effect of various metal ions at a concentration of 1×10^{-3} M was tried on fraction (8) in Thunberg experiments. Fe^{++}, Mg^{++} and Mn^{++} had no effect; Fe^{+++} and Cu^{++} were strongly inhibitory, reduction times of >120 min. being obtained, in comparison with 2 min. in the absence of metal additions.

Other inhibitors

Although Fodor, Frankenthal & Kuk (1930) did not obtain cyanide inhibition of formic dehydrogenase using methylene blue as hydrogen acceptor, Adler & Sreenivasaya (1937) found that both methylene blue reduction and oxygen uptake by the formic system are inhibited by 10^{-3} M-cyanide. The results of Adler & Sreenivasaya have been confirmed and $1·3 \times 10^{-3}$ M-cyanide was found to inhibit formic dehydrogenase completely with both methylene blue and oxygen as final hydrogen acceptors. Mathews & Vennesland (1950) also report that the reduction of Co I in their spectrophotometric test was inhibited more than 95% by 10^{-3} M-cyanide.

Besides cyanide, other metal-combining compounds have been tried on the purified formic dehydrogenase (Table 2). Azide, in a concentration of $1·3 \times 10^{-3}$ M, completely inhibits the oxidation of formate, both aerobically and anaerobically. The cyanide and azide inhibition was found to be completely removed by dialysis. Other heavy-metal combining substances, such as hydroxylamine, 8-hydroxyquinoline and *o*-phenanthroline had no effect. The effect of carbon monoxide was tried and a mixture of 4 parts carbon monoxide to 1 part oxygen showed a 15% inhibition of the oxygen uptake by the enzyme system, but this inhibition was diminished only very slightly by light. Diethyldithiocarbamate ($0·5 \times 10^{-2}$ M) was found to activate the enzyme slightly both aerobically (18% activation) and anaerobically (12%). 2:3-Dimercaptopropanol (BAL) had no effect aerobically at a concentration

of 1×10^{-3}M. Anaerobically, difficulty was experienced with the non-enzymic reduction of dyes which are rapidly decolorized by the strongly reducing BAL. However, on a percentage blank basis, the substance did not have any effect on the enzyme.

Table 2. *Effect of inhibitors of formic dehydrogenase*

(Contents of manometer flasks: 1·0 ml. enzyme fraction (2); 0·6 ml. 0·3M-phosphate buffer, pH 7·0; 0·3 ml. 0·5M-sodium formate; 0·1 ml. heart-diaphorase preparation containing 1·2 μg. bound riboflavin; 0·3 ml. CoI, 5 mg./ml.; 0·1 ml. 0·005M-methylene blue; 0·3 ml. water or inhibitor. In the centre well, 0·2 ml. 20 % alkali or alkali-cyanide mixture (Krebs, 1935). The inhibitor was incubated with the enzyme preparation at 38° for 10 min. before tipping in the formate from the side bulb.)

Final concentration of the inhibitor	O_2 uptake in 60 min. (μl.)
No inhibitor	134
$0·5 \times 10^{-2}$M-Diethyldithiocarbamate	159
Carbon monoxide (80 %) in the gas phase (dark)	109
Carbon monoxide (80 %) in the gas phase (light)	114
1×10^{-3}M-BAL	139
$1·3 \times 10^{-3}$M-Azide	0
$1·3 \times 10^{-3}$M-Cyanide	13

Malonate in concentrations of $1·3 \times 10^{-3}$M did not inhibit the aerobic system; and 4×10^{-2}M-semicarbazide had no effect. Iodoacetate, even up to a concentration of 0·1M, had no effect, but two other —SH reagents were inhibitory. *p*-Chloromercuribenzoate was found to inhibit the anaerobic system completely at 1×10^{-3}M, and iodosobenzoate inhibited about 60 % at this concentration.

Attempted reversal of the oxidation of formate

In Fig. 2 the positions of the half-reduced oxidation-reduction systems, carbon dioxide-formate, CoI-reduced CoI, and benzyl viologen-reduced benzyl viologen, have been plotted on an *r*H scale. Calculated from the distance between the coenzyme and formate curves the equilibrium constant of the reaction between these two systems, at pH 7·0 and 30°, is approximately $1·1 \times 10^4$. Attempts to reverse this reaction were made in three types of experiment.

(1) *Poising the system against a dye of low E'_0, and the effect of carbon dioxide on the percentage reduction of the dye*

Several dyes were tried to see if the formic system could be poised against one of them (Table 3). Neutral red ($E'_0 = -0·325$ V.) was not reduced at all. Janus green ($E'_0 = -0·255$ V.) was reduced slowly to the red amino compound, but not further to the leuco form. Although the reduction was rather slow with benzyl viologen ($E'_0 = -0·359$ V.) and methyl viologen ($E'_0 = -0·446$ V.), both these dyes were found to be satisfactory in that the final colour was less than the full reduction colour.

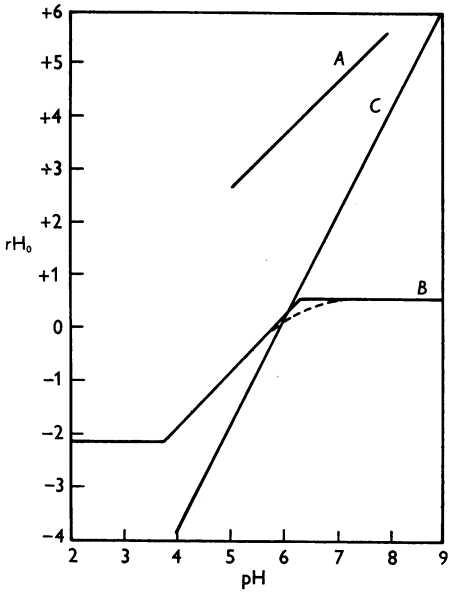

Fig. 2. *r*H curves for half-reduced oxidation-reduction systems. Curve *A*, Co I; data from Borsook (1940). Curve *B*, formate-bicarbonate-carbonate; from the thermochemical data on free energies (Woods, 1936). Curve *C*, benzyl viologen; data from Michaelis & Hill (1933).

Table 3. *Reduction of various dyes by the formic system*

(Thunberg contents: 1·0 ml. freeze-dried preparation from fraction (8), 10 mg./ml.; 0·6 ml. 0·2M-phosphate buffer, pH 7·0; 0·3 ml. 0·5M-sodium formate; 0·1 ml. heart diaphorase (1·2 μg. bound riboflavin), or 0·1 ml. water. In the hollow stopper: 0·3 ml. CoI, 5 mg./ml.; 0·4 ml. 0·0025M-dye. Incubation at 37°.)

Dye	Reduction time (min.) With flavoprotein	Reduction time (min.) Without flavoprotein
Thionine	0·58	1
Methylene blue	3	4·5
Benzyl viologen	15	45
Methyl viologen	25	50
Benzyl viologen + thionine	0·58	1
Neutral red	>180	>180
Janus green	10	90

That benzyl viologen is not inhibitory is seen in the experiment where it is used in addition to thionine, when the thionine was decolorized before the benzyl viologen started to show its blue reduction colour. The possibility that the coenzyme was being destroyed during the longer times with benzyl viologen was tested by reopening the tubes to

re-oxidize the dye. On re-evacuating, the same colour again developed, and at approximately the same rate.

Fig. 3 gives the results of experiments on the percentage reduction of benzyl viologen, with increasing pH, in the presence of equimolecular amounts of formate and dissolved carbon dioxide. A curve representing the theoretical reduction, calculated from curves *B* and *C* of Fig. 2, is also given.

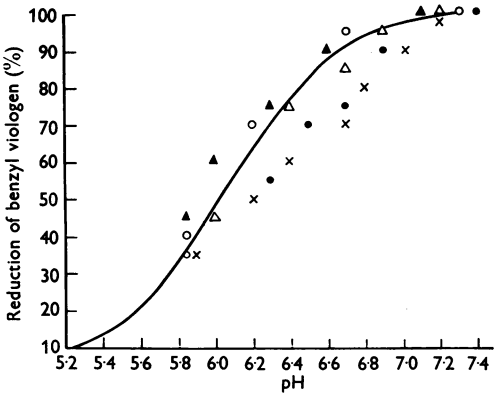

Fig. 3. Percentage reduction of benzyl viologen at different pH values. The whole line represents the theoretical percentage reduction of the dye as calculated from the curves of Fig. 2. The points marked are those obtained in five experimental runs. Thunberg contents: 1·0 ml. fraction (8); 2·0 ml. $NaHCO_3$ (molarity depending on the pH required and calculated from the Henderson-Hasselbach equation); 0·3 ml. 0·3 M-sodium formate; 0·1 ml. heart diaphorase (1·2 μg. bound riboflavin); 0·2 ml. Co I, 5 mg./ml.; 0·4 ml. 0·0025 M-benzyl viologen. Temperature of incubation, 30°. The tubes were evacuated and gassed with CO_2, giving a concentration of dissolved CO_2 of 0·02 M. The gas was freed from O_2 by passing over heated copper, then through a flask containing freshly cut yellow phosphorus. A small piece of yellow phosphorus was placed in the hollow stopper of each tube, and the dye added to the other components just before evacuation. Standards for comparison of the colour were made by reducing with $Na_2S_2O_4$ known concentrations of benzyl viologen corresponding to a range of percentage reduction colours.

When these experiments were repeated in the absence of carbon dioxide, the percentage reduction of the dye was increased by about 20%. These results, however, were within the limits of experimental error, as seen from the wide scatter of points in Fig. 3. The presence of adenosinetriphosphate (ATP) had no effect on the percentage reduction of the dye, either in the presence or absence of carbon dioxide.

(2) *Effect of carbon dioxide on the percentage reduction of Co I*

A Thunberg tube fitted with an optical cell at the base was used for these experiments, in which the reduced Co I was measured by its absorption at 340 mμ. in the Beckman spectrophotometer. Adler & Sreenivasaya (1937) found that 85% of added Co I was reduced by the formic system, but this was assuming 100% purity for the Co I. Using fraction (8), either in the above apparatus or in an open tube, Co I added was completely reduced in the presence of formate. When bicarbonate buffer was used and the Thunberg tube gassed with carbon dioxide, no decrease in the reduction of Co I could be observed. In the absence of formate there was no re-oxidation of added reduced Co I by the formic dehydrogenase and carbon dioxide.

(3) *Attempted reversal by coupling with the oxidation of triosephosphate*

Attempts were made to reverse the formate oxidation by coupling carbon dioxide reduction with a reaction producing reduced Co I, and high-energy phosphate in the form of ATP. Such a reaction is the oxidation of triosephosphate to phosphoglyceric acid. Since inorganic phosphate is taken up in this oxidation and passed on to adenosinediphosphate (ADP) (which in turn, in the presence of added yeast hexokinase and glucose, will hand it on to form glucose-6-phosphate), if there is any coupling with a system which will re-oxidize the reduced Co I, this will be shown by an uptake of inorganic phosphate. Hexosediphosphate was used as substrate in trying to show this, in the presence of bean extract, Mg^{++} ions, ATP, Co I, yeast hexokinase, glucose and bicarbonate buffer, the manometers being gassed with carbon dioxide. There was no significant decrease in inorganic phosphate after 1 hr. incubation, i.e. the carbon dioxide-formate system was not re-oxidizing the reduced Co I. That all the enzymes necessary for the production of triosephosphate from hexosediphosphate and for the subsequent oxidation of triosephosphate were present in the crude extract was seen when acetaldehyde and alcohol dehydrogenase (also present) were used as the re-oxidizing system. Here there was a 20% reduction of the inorganic phosphate in 30 min.

Carrier-linked dismutations

It has been shown that the oxidation of formate can be linked in crude pea extract with the reduction of acetaldehyde and of fumarate, through the mediation of Co I, or Co I plus some other carrier (Davison, 1949*b*). Another dismutation which can easily be demonstrated is that between formate and formic dehydrogenase as the negative system, and α-ketoglutarate, ammonia and glutamic dehydrogenase as the positive system. This reaction goes particularly well because the formic system is strongly reducing, and the equilibrium of the glutamic system is far towards the side of glutamate formation (Euler, Adler, Günther & Das, 1938).

Sifted bean meal (10 g.) was stirred up with 20 ml. 0·2M-phosphate buffer, pH 7·4, and left to stand 2 hr. The homogenate was centrifuged and the supernatant used in the experiment described in Table 4.

Table 4. *Coenzyme-linked dismutation between formic and glutamic dehydrogenases*

(Contents of manometer flasks: 1·0 ml. bean supernatant; 0·3 ml. 0·09M-$NaHCO_3$ (to give a pH of 7·2 when gassed with 5% CO_2 in N_2 at 38°); 0·4 ml. CoI, 5 mg./ml.; 0·2 ml. α-ketoglutarate; 0·8 ml. 0·3M-NH_4Cl; 0·3 ml. 0·4M-sodium formate (in the side bulb); water to 3·0 ml.)

Omissions from flask	CO_2 evolved in 60 min. (μl.)
No omissions	272
Ammonia	38
α-Ketoglutarate	16
Coenzyme I	22
Ammonia and α-ketoglutarate	12

The increase in carbon dioxide output with the complete system is due to increased oxidation of formate in the presence of a system capable of re-oxidizing reduced CoI.

DISCUSSION

Three distinct enzyme systems capable of oxidizing formic acid have now been described. The plant formic dehydrogenase is characterized by its dependence on CoI, and is the only coenzyme-linked dehydrogenase found to be inhibited by cyanide. The formic dehydrogenase of *Escherichia coli* (Gale, 1939) does not need CoI, and has been shown to be cytochrome-linked. Here, cyanide does not inhibit the reduction of methylene blue, but strongly inhibits the aerobic oxidation of formate because of its effect on the cytochrome system (Cook, Haldane & Mapson, 1931).

In animals, Wishart (1923) showed that extracts of rabbit liver will catalyse the reduction of methylene blue by formate. Recently, Mathews & Vennesland (1950) have obtained soluble preparations from rat liver and kidney which would oxidize formate. The oxidation was inhibited about 85% by 10^{-3}M-cyanide. It is not clear what the immediate hydrogen acceptor is here; the system was not activated by CoI, but differed from the bacterial enzyme in that either ATP or adenylic acid were necessary for oxygen uptake.

Mathews & Vennesland report that ATP has no effect on the activity of the plant formic dehydrogenase, and this has been confirmed by anaerobic experiments using benzyl viologen. To investigate the possibility of some other mechanism for the formation of formyl phosphate in plants, extracts of bean have been incubated with formate, ATP, Mg^{++} ions, CoI and hydroxylamine. If any formyl phosphate were formed this would be trapped by the hydroxylamine. After 30 min. incubation the solutions were examined for hydroxamic acid by the method of Lipmann & Tuttle (1945). None was present.

In considering the possible significance of formic dehydrogenase in higher plants, if the enzyme is looked upon as a carboxylase, the suggestion that it may be involved in carbon dioxide fixation at once arises. Two pathways involving carboxylases are known to occur in higher plants for the heterotrophic fixation of carbon dioxide. Vennesland & Felsher (1946), Vennesland (1949) and Vennesland, Gollub & Speck (1949), showed that the reversible breakdown of oxaloacetate to pyruvate and carbon dioxide occurs in a wide variety of seeds, seedlings, leaves and roots. The carbon dioxide fixation reaction required the presence of ATP. The oxaloacetic carboxylase is closely associated with a malic dehydrogenase, and, in the presence of reduced CoII, the reduction of oxaloacetate to malate takes place. In parsley roots, carbon dioxide fixation has been shown to occur by a reversal of the oxidative decarboxylation of *iso*citrate (Ceithaml & Vennesland, 1949). With a preparation from this source oxalosuccinate is formed from α-ketoglutarate and carbon dioxide, in the presence of cobalt or manganese ions. If reduced CoII is added to this system, *iso*citrate is formed from the oxalosuccinic acid.

The possibility of a third mechanism for carbon dioxide fixation in plants was suggested by the work of Woods (1936) with *Escherichia coli*, in which formic acid is produced from carbon dioxide and hydrogen. However, it was found that the participation of CoI in the oxidation of formate by plants changed the equilibrium constant from 3·81 in the case of the bacterial reaction to $1·1 \times 10^4$ when the plant system was used. It has not been possible to obtain the reduction of carbon dioxide to formate by means of the plant formic dehydrogenase. No evidence was obtained that the formate carbon dioxide system can be reversed by the effect of carbon dioxide on the percentage reduction of benzyl viologen, against which the system may be poised. The presence of ATP had no effect on the reduction of the dye either in the presence or absence of carbon dioxide. Nor did coupling with the oxidation of triosephosphate push the reaction in the direction of carbon dioxide fixation. Possibly some physiological reversal does occur in the intact seed, but, if so, it has not been possible to demonstrate this *in vitro*. Since the completion of this work, Mathews & Vennesland (1950) have reported independently that they have not been able to obtain the reduction of carbon dioxide in the presence of a partly purified formic dehydrogenase from pea seeds, nor have isotope experiments of the same authors indicated any significant exchange of labelled carbon between $^{14}CO_2$ and sodium formate in the presence of the enzyme.

Data indicating a much more likely role for formic dehydrogenase are those presented on the use of the enzyme in forming reduced Co I, which can then react with the corresponding oxidized compounds to form ethanol, succinate and glutamate in the presence of their respective dehydrogenases, and, in the case of succinate, some other carrier. Such anaerobic dismutations have an obvious significance in the respiration of large seeds. The ease with which the glutamic reaction goes, especially, and the importance of glutamic acid in the seed, points to a possible role for the strongly reducing formic system in the metabolism of the plant.

Tied up with this is the question of the origin of formate in plants. Formate is said to arise, together with ethanol and acetic acid, during the anaerobic respiration of potatoes and seeds (Stoklasa, Ernest & Chocenský, 1907). Stoklasa & Ernest (1908) found that, when the oxygen supply was limited, formic and acetic acids were excreted from the roots of buckwheat and barley, and formic acid from the roots of oats and maize. Formate is known to arise by the oxidation of glycollic acid in leaves (Clagett, Tolbert & Burris, 1949). However, in the present work, glycollic oxidase has not been found in pea seeds, and in tobacco leaves containing a very active glycollic oxidase there was no formic dehydrogenase. Of some possible precursors of formate, pyruvate is completely decarboxylated by pea and bean extracts; acetate, formaldehyde and glycine do not support oxygen uptake in the presence of Co I, and therefore it is not likely that formate is being produced from these compounds.

In animal tissues formate has been shown to give rise to labile methyl groups (Sakami & Welch, 1950). Also formate, or a one-carbon intermediate of its metabolism, was shown by Sakami (1950) to be formed from acetone by *in vivo* isotope experiments with rats. Formic acid, together with glutamic acid and ammonia, are claimed to be the products of the breakdown of histidine by liver histidase (Walker & Schmidt, 1944). However, none of these reactions has yet been investigated in plants, so that the origin of formate and the role of its dehydrogenase in seeds remain open questions.

SUMMARY

1. A method of purification for formic dehydrogenase from bean seeds is described.

2. The coenzyme I specificity of the enzyme has been confirmed.

3. The effects of various inhibitors on the enzyme have been examined.

4. No reduction of carbon dioxide to formate by means of this enzyme has been obtained.

5. The coenzyme-linked reaction between formate oxidation and the reductive amination of α-ketoglutarate has been shown to occur rapidly in extracts of bean seed.

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