The Inhibition of Plant Aconitate Hydratase (Aconitase) by Fluorocitrate

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Fluoroacetate is a potent poison of animals. It is metabolized to fluorocitrate, which is a powerful inhibitor of mammalian aconitate hydratase [aconitase; citrate (isocitrate) hydrolyase] (Peters, 1952). Fluoroacetate and fluoroacetamide, as systemic insecticides for plants, are taken up by the roots or absorbed by the leaves and are then translocated to other parts of the plant. The plants show signs of injury only at doses 50–100 times that needed to kill *Aphis fabae* when feeding on the plants (David & Gardiner, 1951). Two distinct species of plants synthesize fluoroacetate, namely *Dichapetalum cymosum* (Marais, 1944) and *Acacia georgina* (Oelrichs & McEwan, 1961; Murray, McConnell & Whitem, 1961). *Dichapetalum toxicarium* can synthesize ω -fluoro-oleic acid (Peters, Hall, Ward & Sheppard, 1960). It is apparent that plants are much less sensitive to fluoroacetate than are animals. To ascertain a reason for the lower sensitivity of plants we have examined the inhibition of aconitate hydratase from plant sources; we find it to be much less strongly inhibited by fluorocitrate than is pig-heart aconitate hydratase (Peters, 1961).

EXPERIMENTAL

Chemicals. Monopotassium $L_3(+)$ -isocitrate from Crassulaceae (three- D_3 -isocitrate according to Vickery, 1962) was a gift from Dr H. B. Vickery. Trisodium fluorocitrate (about 40% pure) was the synthetic preparation used by Ward & Peters (1961) and was activated immediately before use according to their procedure.

Enzyme preparation. Sycamore (*Acer pseudoplatanus* L.) cells isolated from cambial tissue were grown in tissue culture for 10–14 days as described by D. T. A. Lamport (in preparation). The cells were separated from the growth medium by filtration and suspended in 2 vol. of 0.1M-tris-hydrochloric acid buffer, pH 8.0, containing 5 mM-ethylenediaminetetra-acetate. The soluble proteins were extracted by shaking with glass beads (2 mm. diam.) in a cell mill (Zillig & Holzel, 1958) for 2 min. at 0° at 50 cyc./sec. The clear supernatant obtained by centrifuging in a Spinco no. 40 head at 35000 rev./min. (80 730 g_{av}) for 30 min. was saturated with solid ammonium sulphate at 0°. The precipitate was spun down and resuspended in 0.05M-tris-hydrochloric acid buffer, pH 8.0. Some material did not redissolve and was removed by centrifuging. The supernatant was used immediately as such and was kept at 0°. The precipitation by ammonium sulphate is necessary

to remove low-molecular-weight compounds which adsorb strongly at the wavelength (240 m μ) used for activity measurements. All preparations were unstable; particularly when prepared from older cells, the preparation turned brown on exposure to air and lost activity. The more stable preparations used in the kinetic work did not lose activity measurably in the 12 hr. experiment.

Pea aconitate hydratase. The testa were removed from peas (*Pisum sativum*) germinated at room temperature in the dark for 10 days, and the entire seedling was ground in a cold mortar with sand and a solution containing 0.5M-sucrose, 0.2M-tris-hydrochloric acid and 5 mM-ethylenediaminetetra-acetate (Bacon, Palmer & DeKock, 1961). After being squeezed through muslin the extract was centrifuged at 11 000g for 30 min. at 0° and the supernatant used as such.

Enzyme assay. The activity was determined by the method of Racker (1950) by measuring the change in extinction at 240 m μ in a Beckman DK 2 recording spectrophotometer at 25°. Preincubation with inhibitor was carried out in silica spectrophotometer cells in a water bath at 25°.

RESULTS

Fig. 1 indicates a Michaelis constant for sycamore aconitate hydratase of 85 μ M- $L_3(+)$ -isocitrate. Bacon *et al.* (1961) reported that the Michaelis constant for three plant aconitate hydratases lay between 5 and 10 mM-($\pm$)-isocitrate. The lack of linearity of the V^{-1}/S^{-1} plot of the inhibited activity in Fig. 1 indicates that equilibrium conditions are not set up immediately on addition of enzyme to both substrate and inhibitor. This lack of equilibrium is due to the slow setting-up of inhibition. Fig. 2 shows that inhibition increases with time of preincubation of enzyme with inhibitor before the reaction is started by adding substrate. Other time-dependent inhibitions of enzyme activity have been reported, e.g. the inhibition of cholinesterase by di-isopropyl phosphorofluoridate (Mackworth & Webb, 1948) and the inhibition of yeast alcohol dehydrogenase by 1,10-*o*-phenanthroline (Hock & Vallee, 1956; Williams, Hock & Vallee, 1958). However, the resultant inhibition in those cases is of the non-competitive type, whereas in this instance the inhibition after preincubation with inhibitor is largely reversible by increased substrate concentrations (Fig. 3) and is certainly not of the non-competitive type.

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Pea aconitate-hydratase preparation is less convenient than the sycamore preparation because of a very high extinction at 240 μ . Comparison of the inhibition of both preparations under the same conditions showed that they were inhibited to nearly the same extent; so that the behaviour of the sycamore aconitate hydratase towards fluorocitrate is not unique. The enzymes (0.05 ml.) were incubated in 3 ml. of 25 mM-tris-hydrochloric acid buffer, pH 8.0, at 25° for 15 min. with or without 13.6 μ g. of fluorocitrate and the activity was measured on addition of neutralized $L_s(+)$ -isocitrate to a final concentration of 0.725 mM. Under these conditions pea aconitate hydratase was inhibited 67 % and sycamore aconitase 62 % (each the average of three determinations).

Experiments were carried out to ascertain whether sycamore was sensitive to fluorocitrate. Fluoroacetate (10 mM) was without effect on the respiration of sycamore cells, suspended

in 0.05M-potassium dihydrogen phosphate adjusted to pH 7.0 with sodium hydroxide, during a 3 hr. incubation in air at 25° in Warburg respirometers. This may be due to poor penetration by ionized fluoroacetate, as shown by Aldous & Rozee (1956) for yeast. However, the sycamore cells were killed by exposure to pH 2.1, which is the pH necessary to ensure the presence of an appreciable concentration of un-ionized fluoroacetate. Accordingly fluoroacetate (10 mM) was added to the growth medium. At this concentration no growth occurred during 10 days.

DISCUSSION

The results show that the inhibition of sycamore aconitate hydratase is qualitatively similar to the inhibition of pig-heart aconitate hydratase, in that the inhibition is slow to develop but, once developed, can still be largely reversed by high substrate concentrations (Morrison & Peters, 1954; Peters, 1961). However, only a 60 % inhibition was achieved by preincubating sycamore aconitate hydratase with 20 μ g. of fluorocitrate, whereas Peters (1961) obtained a 64 % inhibition of pig-heart aconitate hydratase with only 0.01 μ g. of the

Fig. 1. Inhibition of sycamore aconitate hydratase by fluorocitrate. For the complete system, monopotassium $L_s(+)$ -isocitrate, neutralized with potassium hydroxide to pH 8.0 (10 mg./ml.), 0.01–0.1 ml./cuvette, tris-hydrochloric acid buffer (0.05M, pH 8.0) to a final volume of 3 ml. and, as inhibitor, trisodium fluorocitrate (25 or 50 μ g./0.01 ml.), were added. Enzyme preparation (0.1 ml.) was added to start the reaction. ○, ●, No inhibitor; Δ, 25 μ g. of fluorocitrate; ▲, 50 μ g. of fluorocitrate. Two enzyme preparations were used as indicated by the use of open and closed symbols. ○ represents the average of seven determinations. ●, Δ and ▲ represent the average of three determinations. Temperature, 25°.

Fig. 2. Effect of time of preincubation at 25° of sycamore aconitate hydratase with inhibitor on activity. Complete system: enzyme preparation 0.1 ml., inhibitor 0.01 ml., tris-hydrochloric acid buffer (0.05M, pH 8.0) 2.8 ml., monopotassium $L_s(+)$ -isocitrate 0.71 mg. in 0.1 ml. (neutralized to pH 8.0 with potassium hydroxide). ▲, No inhibitor; Δ, 10 μ g. of fluorocitrate; ○, 20 μ g. of fluorocitrate; ●, 50 μ g. of fluorocitrate/cuvette. Temperature, 25°. In the experiments with no preincubation started by the addition of enzyme, points represent the average of four determinations. In the experiments with preincubation started by addition of substrate, points represent one determination.

same preparation of fluorocitrate at pH 7.4. Thus plant aconitase is some 2000-fold less sensitive to fluorocitrate than is aconitate hydratase from animal sources. This may explain, in part, the lower sensitivity of plants to fluoroacetate; but the mystery remains why *D. cymosum* and *Acacia georgina*, which make fluoroacetate, are not poisoned by their own product.

SUMMARY

1. The growth of sycamore-cell suspensions is completely inhibited by 10 mM-sodium fluoroacetate.

2. Sycamore aconitate hydratase, K_m 85 μ M- $L_+(+)$ -isocitrate, is inhibited by fluorocitrate at much higher concentrations (about 2000-fold) than those needed to inhibit pig-heart aconitate hydratase.

3. The inhibition is slowly set up, requiring 10–20 min. at 25° to reach a maximum, but is largely reversed by high concentrations of $L_+(+)$ -isocitrate.

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Fig. 3. Effect of varying concentrations of substrate on sycamore aconitase preincubated with fluorocitrate for 10 min. Complete system: monopotassium $L_+(+)$ -isocitrate adjusted to pH 8.0 with potassium hydroxide (10 mg./ml.) 0.01–0.071 ml./cuvette, trisodium fluoroacetate 10 or 20 μ g./0.01 ml./cuvette, enzyme preparation 0.1 ml., tris-hydrochloric acid buffer (0.05M, pH 8.0) to make a final volume of 3 ml. The reaction was started by the addition of substrate. ○, No inhibitor; ▲, 10 μ g. of fluorocitrate; △, 20 μ g. of fluorocitrate/cuvette. Temperature, 25°. Points represent the average of at least three determinations.