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Competitive Inhibition of Succinoxidase by Acetylene Dicarboxylate.* (25027)

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Among many compounds tested as inhibitors of the succinoxidase system there are a number of dicarboxylic acids closely related to succinate which inhibit competitively, *e.g.*, malonate, oxalacetate, glutarate, etc. One compound that has been largely overlooked except for a brief report by Dietrich *et al.*(1) is the acetylene derivative, acetylene dicarboxylic acid (ADC). Since this compound has been used to prepare tetradeuterosuccinic acid and could conceivably be present in small quantity in the product, it was of interest to reexamine its inhibition of the dehydrogenation of succinate.

Methods. Dipotassium ADC was prepared by neutralizing the commercial monopotassium salt with KOH, and precipitating K_2ADC from a 25% solution by addition of 5 volumes of ethanol. After 3 such precipitations the salt was dried *in vacuo*. Succinoxidase was assayed by the method of Schneider and Potter(2). Homogenized rat kidney was used as the source of the enzyme system. The mechanism of inhibition was studied both by varying the substrate concentration in the presence of constant inhibitor concentration, and *vice versa*. The methods of Lineweaver and Burk(3) and Goldstein(4) were used to analyze the nature of the inhibition.†

Results. The order of addition of substrate and inhibitor is of considerable importance (Fig. 1). When ADC was added 20 minutes after substrate and enzyme were mixed, rate of oxygen consumption decreased slowly; and only after 5 hours did it fall to the level observed when the substrate was added after the inhibitor and enzyme were mixed.

Fig. 2 is a Lineweaver-Burk plot of the

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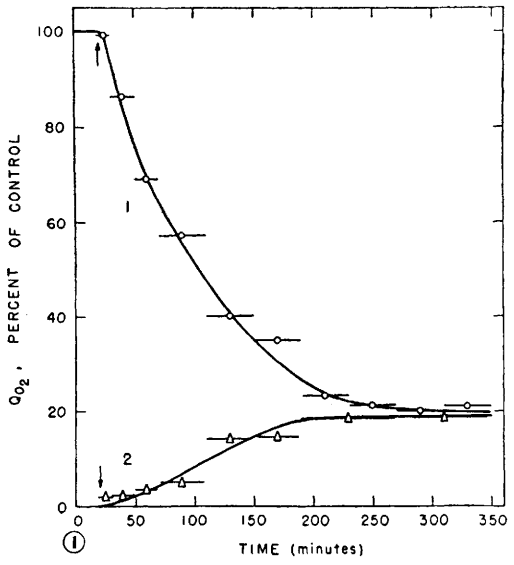
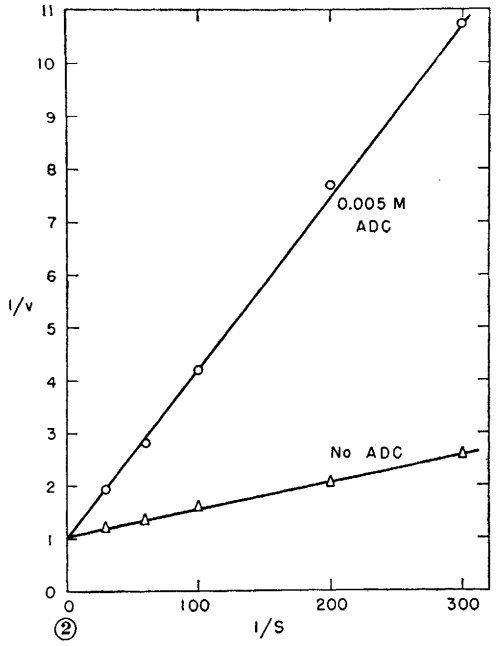
† The following symbols are used: K_S, dissociation constant of enzyme-inhibitor complex (Michaelis constant); K_I, dissociation constant of enzyme-ADC complex; S, molar concentration of succinate; I, concentration of ADC; v, rate of oxygen consumption (extrapolated to zero time from 10, 20, and 30 minutes readings); V_m, theoretical maximum rate of oxygen consumption (at "infinite" substrate concentration).





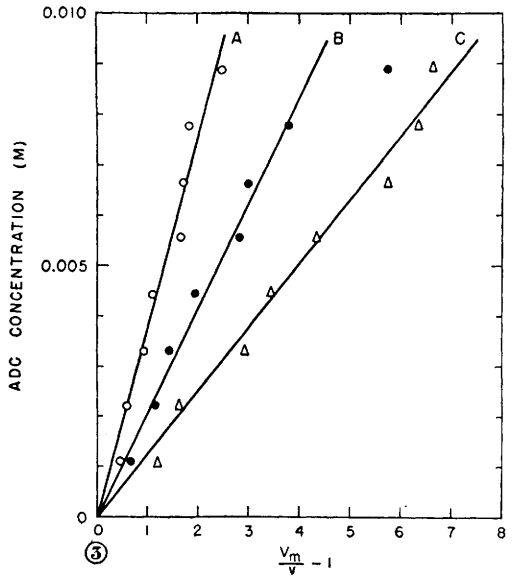


FIG. 1. Effect of order of addition of succinate and ADC. Curve 1, succinate added at zero time, ADC after 20 min. (arrow); Curve 2, ADC added at zero time, succinate after 20 min. Ordinate is rate of oxygen consumption, as % of control, over a time interval the length of which is indicated by horizontal lines through each point. Concentrations of succinate and ADC were 0.083 and 0.0033 M, respectively.

FIG. 2. Inhibition of succinoxidase by ADC (Lineweaver-Burk plot). Both succinate and ADC were added to the enzyme simultaneously. The slope with no ADC $(K_S/V_m) = 0.00465$; the slope with 0.005 M ADC $(K_S/V_m)(1 + I/K_I) = 0.03263$.

FIG. 3. Inhibition of succinoxidase by ADC (Goldstein plot). Succinate, ADC, and enzyme incubated 18 hr at 1°C before assay. Curve A: Succinate conc. 0.0833 M; slope $(S(K_I/K_S)) = 0.00377$. Curve B: Succinate conc. 0.05 M; slope = 0.00209. Curve C: Succinate conc. 0.0167 M; slope = 0.00063. In curve C, the values of $(V_m/v - 1)$ have been divided by 2 to conserve space.

inhibition produced by simultaneous addition of inhibitor and substrate. From these data K_S was calculated to be 0.00412, and K_I 0.00081, about a 1:5 ratio. In this experiment substrate concentration was varied, while inhibitor concentration remained constant. A very similar value, $K_I = 0.00079$, was obtained in an experiment with constant substrate and variable inhibitor concentration; equation $7A_IA_S$ of Goldstein was used in this experiment. There is no doubt that the inhibition is competitive.

Considerably lower values for K_I (1/90 to 1/30 the value for K_S) were obtained when the substrate was added after inhibitor and enzyme were mixed, although a 3-fold variation in K_I values was observed in different experiments. Therefore, the substrate, in-

hibitor, and enzyme were mixed and stored at 1°C for 18 hours before assay. Fig. 3 illustrates the results of such an experiment in which 3 concentrations of succinate and 8 concentrations of ADC were employed. From these data, K_I was estimated to be 0.000171 ± 0.000063, about 1/25 that of K_S.

Discussion. Dietrich *et al.*(1) reported that ADC, like malonate, was a competitive inhibitor of pigeon breast succinoxidase. However, their data leave something to be desired, since the best straight line which can be drawn in their Lineweaver-Burk plot of reaction velocity in the absence of inhibitor gives a *negative* value for $1/V_m$. From another experiment in their paper, a value of $K_S = 0.03$ was estimated; this value is some 20 times higher than that observed by other investigators(see(5)). Using this value of K_S, one can calculate that K_I for both ADC and malonate is about 0.005-0.007, so that the ratio of K_S to K_I is about 5. Other workers have reported values of 30 to 50 for the K_S/K_I ratio for inhibition of succinoxidase by malonate(5,6). Finally, Dietrich *et al.*(1) did not specify the order of addition of succinate and ADC; on the basis of their K_S/K_I ratio, it would seem that the inhibitor was added to the enzyme either at the same time or after the substrate was added, since this ratio of 5 was what we observed in the experiment reported in Fig. 2.

Thus under the conditions presumably employed by Dietrich *et al.* or by us in the experiment shown in Fig. 2, ADC is a competitive inhibitor of succinoxidase with an affinity about 5 times that of succinate. However, when the components of the system are incubated overnight prior to assay, the affinity of ADC appears to be about 25 times that of succinate. ADC is thus about as effective as malonate and considerably less effective than oxalacetate as an inhibitor of succinic dehydrogenase.

On the basis of experiments similar to that reported in Fig. 1, we have estimated using Goldstein's equation 16A(4), the rate constants for dissociation of the enzyme-substrate complex, ES → E + S, and of the enzyme-inhibitor complex, EI → E + I. The absolute values are of doubtful value because of the presence of other reactions; however, on a relative basis, the former rate was 44 times the latter. This difference thus explains why a long incubation time is necessary to obtain more nearly correct values for the ratio of dissociation constants, K_S/K_I.

Summary. Acetylene dicarboxylate (ADC) is a competitive inhibitor of succinoxidase. The apparent dissociation constant of the ADC-enzyme complex is about 1/5 that of the succinate-enzyme complex when the inhibitor is added to the enzyme after or along with the substrate. However, when the enzyme, inhibitor, and substrate are incubated 18 hours before assay, the value of K_I is about 1/25 that of K_S.

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