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STUDIES ON SUCCINATE DEHYDROGENASE

VI. INHIBITION BY MONOCARBOXYLIC ACIDS

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SUMMARY

1. Bicarbonate is a competitive inhibitor of succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1).

2. Bicarbonate is bound to the enzyme at two sites, with virtually equal affinities.

3. A method for the determination of the inhibitor constant for this type of inhibition is proposed. The K_i (intrinsic dissociation constant at each binding site) was found to be 12 mM.

4. Formate (K_i = 120 mM), glycolate (K_i = 120 mM) and glyoxylate (K_i = 21 mM) are all competitive inhibitors binding at two sites. Acetate and propionate do not inhibit.

5. It is suggested that an interaction between the enzyme and a hydroxyl group in the α-position leads to a binding energy of 0.9–1.4 kcal/mole, in addition to the binding between carboxylate groups and basic groupings in the enzyme.

INTRODUCTION

There are a number of observations suggesting that succinate oxidation in plants is inhibited by bicarbonate**. Succinate is accumulated in plants kept in an atmosphere of CO_2 (refs. 1, 2) and inhibition of succinate oxidation by bicarbonate has been shown with mitochondria isolated from Ricinus seeds[3–5] and cauliflower[6]. BENDALL *et al.*[5] showed that the inhibition is competitive with respect to succinate. The inhibition has also been reported with submitochondrial particles from beef heart[7] and rat-liver mitochondria[8].

KASBEKAR[8] suggested that the inhibition might be due to formation of oxaloacetate from CO_2 and pyruvate.

In this paper, it is shown that bicarbonate and structurally related compounds are inhibitors of soluble, purified succinate dehydrogenase.

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** In this paper, the equilibrium mixture of CO_2, H_2CO_3 and HCO_3^- will be referred to as 'bicarbonate'.

RESULTS

Inhibition by bicarbonate

From the double-reciprocal plots in Fig. 1 it is clear that bicarbonate is a competitive inhibitor of succinate dehydrogenase. From this experiment, K_i values were calculated using the formula: $K_m{}^i = K_m \{1 + ([I]/K_i)\}$, where K_m and $K_m{}^i$ are the Michaelis constants in absence and presence of inhibitor, respectively. These values

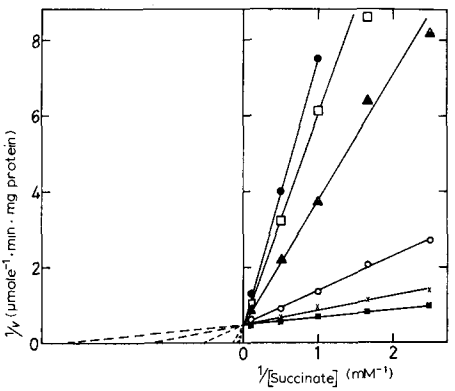


Fig. 1. Inhibition of succinate dehydrogenase by bicarbonate. The bicarbonate concentrations were: ■, none; ×, 10 mM; ○, 20 mM; ▲, 40 mM; □, 50 mM; ●, 60 mM.

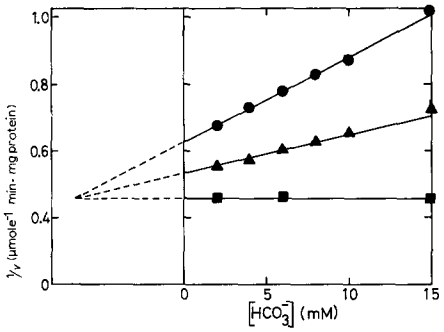
are given in Table I. The value of the inhibitor constant calculated in this way decreases with increasing bicarbonate concentration. In Fig. 2 it is seen that in a Dixon plot ($1/v$ *versus* $[HCO_3^-]$) a straight line is obtained with bicarbonate concentrations up to 15 mM. With higher bicarbonate concentrations, however, the Dixon plot shows an upward curvature (see Fig. 3A). When, as in Fig. 3B, $1/v$ is plotted against $[HCO_3^-]^2$, a straight line is obtained with a downward deviation at low bicarbonate concentrations. These findings can be explained by assuming that bicarbonate can be bound to the enzyme at two different sites. This leads to the following general mechanism:

$$E + I \rightleftharpoons EI$$
$$EI + I \rightleftharpoons EI_2$$
$$E + S \rightleftharpoons ES$$
$$ES \rightleftharpoons E + P$$

TABLE I

K_i VALUES FOR BICARBONATE, AS CALCULATED FROM THE EXPERIMENT SHOWN IN FIG. 1

$[HCO_3^-]$ (mM)	K_i (mM)	
	Calculated from: $K_m{}^i = K_m \left[1 + \frac{[I]}{K_i}\right]$	Calculated from: $K_m{}^i = K_m \left[1 + \frac{[I]}{K_i}\right]^2$
10	6.2	16
20	5.0	16
40	2.4	12
50	1.8	12
60	1.5	12



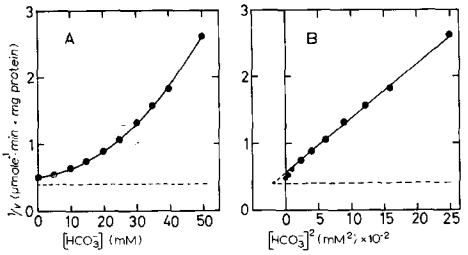


Fig. 2. Dixon plots of the inhibition by bicarbonate. ■, 100 mM succinate; ▲, 3 mM succinate; ●, 1 mM succinate.

Fig. 3. Dixon plots of the inhibition by high concentrations bicarbonate. — — —, 100 mM succinate (here the reaction velocity was independent of the bicarbonate concentration up to 60 mM); ●—●, 3 mM succinate. A, $1/v$ plotted against $[HCO_3^-]$. B, $1/v$ plotted against $[HCO_3^-]^2$.

It is important to note that the first equilibrium is 2-fold degenerate. If both inhibitor molecules are bound with equal affinities, this mechanism leads to the following rate equation (for initial rates):

$$v = \frac{V}{1 + \frac{K_m}{s}\left(1 + \frac{2[I]}{K_i} + \frac{[I]^2}{K_i^2}\right)} = \frac{V}{1 + \frac{K_m}{s}\left(1 + \frac{[I]}{K_i}\right)^2} \tag{1}$$

where V is the maximal velocity at infinite substrate concentration (s). According to this equation, a straight-line Dixon plot ($1/v$ *versus* $[HCO_3^-]$) would be expected at low bicarbonate concentrations where the square term $[I]^2/K_i^2$ can be neglected. The abscissa of the intersection point of the Dixon plots is equal to $[I] = -\frac{1}{2} K_i$. In this way it can be calculated from Fig. 2 that $K_i(HCO_3^-)$ is 13 mM.

The inhibition constant may also be calculated from the Lineweaver–Burk plot shown in Fig. 1, by use of the equation

$$K_m{}^i = K_m\left(1 + \frac{[I]}{K_i}\right)^2$$

Table I shows that, calculated in this way, K_i is independent of the inhibitor concentration and is equal to 12–16 mM.

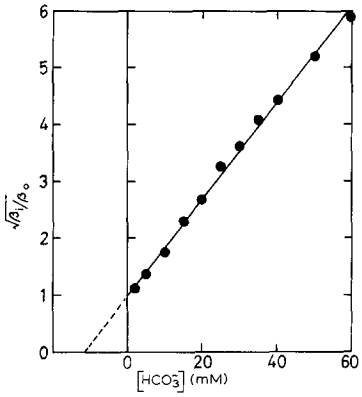
A more general procedure is the following. We introduce the function $\beta = \{(V/v) - 1\}$. From Eqn. 1 it follows that, in the presence of inhibitor:

$$\beta_i = \left(\frac{V}{v_i} - 1\right) = \frac{K_m}{s}\left(1 + \frac{[I]}{K_i}\right)^2 \tag{2}$$

Now K_m/s is equal to $\beta_0 = \{(V/v) - 1\}$ for the uninhibited reaction. Thus we can write:

$$\beta_i/\beta_0 = \left(1 + \frac{[I]}{K_i}\right)^2, \text{ or } \sqrt{(\beta_i/\beta_0)} = 1 + \frac{[I]}{K_i} \tag{3}$$

A plot of $\sqrt{(\beta_i/\beta_0)}$ against $[I]$ gives a straight line, with a slope of $1/K_i$, intersecting the ordinate at the point $\sqrt{(\beta_i/\beta_0)} = 1$, and the abscissa at the point $[I] = -K_i$. This



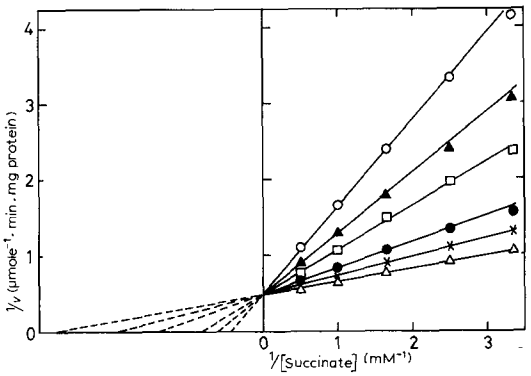


Fig. 4. Plot of $\sqrt{(\beta_i/\beta_0)}$ against $[HCO_3^-]$ (for details see the text). The data of Figs. 1, 2 and 3 were used.

Fig. 5. Inhibition of succinate dehydrogenase by formate. The formate concentrations were: △, none; ×, 25 mM; ●, 50 mM; □, 100 mM; ▲, 150 mM; ○, 200 mM.

way of plotting the results has the advantage that the measurements at different inhibitor and substrate concentrations can be brought together into one plot.

In Fig. 4 a plot of $\sqrt{(\beta_i/\beta_0)}$ against $[HCO_3^-]$ is shown. From this we can calculate that $K_i(HCO_3^-)$ is 11 mM.

In contrast to many other competitive inhibitors of succinate dehydrogenase[9,10] bicarbonate has no effect on the absorption spectrum of the enzyme. Thus, it is not possible to determine the dissociation constant of the enzyme–bicarbonate complex by spectrophotometric titration.

Competitive inhibition at two sites, with a K_i of 12 mM, was also found with the Keilin and Hartree heart-muscle preparation.

The method described by DALZIEL AND LONDESBOROUGH[11] was used to determine whether the inhibition is due to the anion HCO_3^- or to CO_2. When bicarbonate was added to a complete reaction mixture, the reaction was immediately inhibited and the amount of inhibition did not change in time. When, however, gaseous CO_2 was led through the solution, the inhibition set in slowly, and was maximal after about 1.5 min, presumably due to the slow conversion of CO_2 into HCO_3^-. This result strongly suggests that HCO_3^- is the inhibitor. This conclusion is supported by the fact that structural analogues of HCO_3^-, *viz.* formate, glyoxylate and glycolate, are competitive inhibitors of succinate dehydrogenase acting at two sites.

Effect of monocarboxylic acids

Fig. 5 shows that formate is a competitive inhibitor of succinate dehydrogenase. Using the formula $K_m{}^i = K_m\{1 + ([I]/K_i)\}^2$, an average K_i value of 125 mM may be calculated. From the plot of $\sqrt{(\beta_i/\beta_0)}$ against formate, shown in Fig. 6, it is clear that formate is also bound at two equivalent sites. From this plot an inhibition constant of 125 mM may be calculated. Formate is thus a much weaker competitive inhibitor than bicarbonate.

No inhibition was found with acetate and propionate. Glyoxylate (Fig. 7) and glycolate are two-site competitive inhibitors, with K_i values of 21 and 120 mM, respectively.

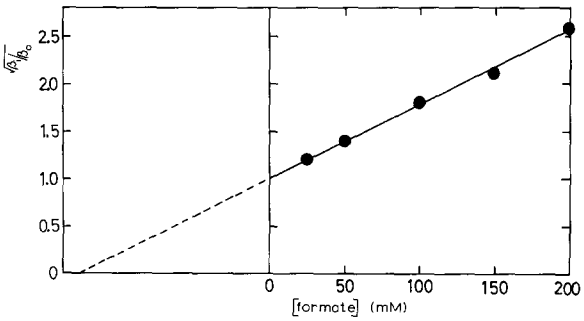


Fig. 6. Plot of $\sqrt{(\beta_i/\beta_o)}$ against [formate], calculated from Fig. 5.

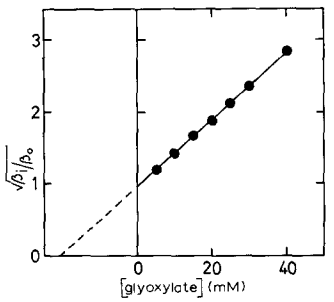


Fig. 7. Plot of $\sqrt{(\beta_i/\beta_o)}$ against [glyoxylate], calculated from the same type of experiment as in Fig. 5.

DISCUSSION

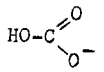
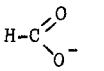
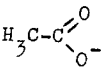
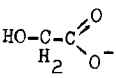
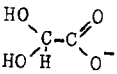
It may be concluded that bicarbonate and related compounds are bound at two different sites on succinate dehydrogenase, and that the affinity is equal for both sites. This follows from the straight lines obtained in the plots of $\sqrt{(\beta_i/\beta_o)}$ against $[I]$.

The substrates known for succinate dehydrogenase, *viz.* succinate, *S*-chlorosuccinate and *S*- and *R*-malate (*cf.* ref. 12) and most competitive inhibitors, *e.g.* malonate, fumarate and oxaloacetate, are dicarboxylic acid anions (exceptions are the competitive inhibitors pyrophosphate[13,14], phosphate[15] and fluoride[15]). It is probable that substrates and competitive inhibitors react with the enzyme by means of their carboxylic groups. This has led to the proposal[16,17] that the enzyme contains two cationic groups capable of interacting with these carboxylic groups. From our results it seems likely that monocarboxylic acids compete with succinate for these two sites of attachment, virtually without having any interaction with the flavin and iron moieties in the enzyme; the latter can be concluded since these compounds, unlike many other competitive inhibitors[9,10] have no effect on the absorption spectrum of the enzyme. It is interesting that fluoride, a competitive inhibitor of succinate dehydrogenase[15] can also react with the enzyme at two equivalent binding sites (W. P. ZEYLEMAKER AND A. D. M. KLAASSE, unpublished experiments), suggesting also an interaction with the same two cationic groups.

From Table II it is seen that the K_i of formate is lower than that of bicarbonate by a factor of 10, which corresponds to a difference in binding energy of about 1.4 kcal/mole. This difference may be due to an interaction between the hydroxyl group of bicarbonate and a group in the enzyme molecule. The K_i values of glycolate and glyoxylate differ by a factor of 5.7, corresponding to a difference in binding energy of about 0.9 kcal/mole. At the pH used in our experiments (7.8) glyoxylate is completely in the hydrate form[18], which has two hydroxyl groups, whereas glycolate has only one. This may be the reason for the stronger binding of glyoxylate. By the same reasoning it can be argued that the K_i for acetate would be lower than that of glycolate by at least a factor of 5.7, which would mean that K_i for acetate would be about 0.7 M. Such a weak inhibition would not be detected in our experiments. It is interesting to note that an interaction between a hydroxyl group in the α-position to a

TABLE II

INHIBITOR CONSTANTS FOR DIFFERENT MONOCARBOXYLIC ACIDS

Compound		K_i *(mM)*
Bicarbonate		12
Formate		125
Acetate		700
Glycolate		120
Glyoxylate (hydrate)		21

carboxyl group and the enzyme was also found with oxaloacetate[19]. Here two enzyme–oxaloacetate complexes were found, the binding energies of which differ by a factor of 10. A possible explanation is that in the first complex the interaction between the enzyme and oxaloacetate occurs *via* the carboxylate groups, and in the second complex the enolic α-hydroxyl group interacts with the enzyme. It is interesting that the difference in binding energy of these two complexes is about the same as that between the glycolate and glyoxylate, and between the formate and bicarbonate complexes. Because one molecule of succinate dehydrogenase can bind two molecules of these inhibitors, it may be concluded that there are at least two entities in the enzyme molecule capable of reacting with α-hydroxyl groups of the inhibitors. It seems likely that these entities are also involved in the reaction of succinate dehydrogenase with *S*- and *R*-malate. These compounds are substrates of the enzyme, both being oxidized to oxaloacetate[10].

An interesting feature of the two-site competitive inhibition is an apparent co-operative effect: the inhibition constant, when calculated as for a one-site inhibition, decreases with increasing inhibitor concentration (*cf.* Table I), leading to a curved Dixon plot (Fig. 3A).

The inhibition of succinate dehydrogenase by bicarbonate might possibly have a physiological function. KREBS AND VEECH[20] estimated the concentration of CO_2 in the liver cell to be 1.16 mM at pH 7.0 and 25°, corresponding to a concentration of bicarbonate of about 8 mM. This concentration of bicarbonate would cause a considerable inhibition of succinate dehydrogenase, if the *in vivo* concentration of succinate is not much higher than K_m for succinate.

A manometric assay for succinate dehydrogenase activity, using ferricyanide and bicarbonate, has been used by several authors (*cf.* refs. 16, 21, 22). Because of the high concentrations of bicarbonate in this system (*viz.* between 20 and 40 mM), the true activity of succinate dehydrogenase is not always measured by this method.

METHODS

Succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) was isolated from pig heart by the method of WANG *et al.*[23] as previously described[9,24]. The enzyme was kept in small amounts in liquid nitrogen, and always used within 15 min after thawing.

Reaction velocities were measured in a medium containing 2.5 mM potassium ferricyanide, 0.1 M phosphate buffer (pH 7.8), 1 mM EDTA, 1 mg/ml bovine serum albumin, and different concentrations of succinate. The reactions were started by the addition of enzyme (0.17–0.2 mg/ml). In all experiments the temperature was 25°.

Bicarbonate solutions were prepared as follows: $KHCO_3$ (Analar) was dissolved in 0.1 M phosphate buffer (pH 7.8), and the pH was then brought to 7.8 by the addition of 0.1 M KH_2PO_4. The solutions were freshly prepared before every experiment, and kept in closed tubes with a small gas volume above the liquid. According to the Henderson–Hasselbach equation, at this pH, 96% of the bicarbonate is present as HCO_3^-, 4% as CO_2, and less than 0.3% as CO_3^{2-} (*cf.* refs. 25, 26). The diffusion of CO_2 from the solution can under these conditions be neglected (*cf.* refs. 11, 25). Thus, it was assumed that the HCO_3^- concentration was 96% of that calculated from the amount of $KHCO_3$ added.

Protein was determined by the biuret method, after precipitation with 5% trichloroacetic acid.

Succinate was from Boehringer, bovine serum albumin from Sigma, potassium ferricyanide from Merck. All other chemicals were from the British Drug Houses.

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