Inhibition by Malonate of Succinic Dehydrogenase in Heart-muscle Preparations

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Studies of enzyme inhibition and of the action of enzymes on a variety of substrates can lead to information which may be valuable in the elucidation of the mechanism of enzyme action, since the results indicate a series of molecular structures with which the enzyme combines and thus lead to some conception of the nature of the enzyme active centre.

If a compound is recognized as being a reversible competitive inhibitor, it becomes necessary to express its action in terms of some constant, in order that a series of such inhibitors may be arranged according to potency on some rational and quantitative basis. The most satisfactory method of expressing the potency of a reversible competitive inhibitor is in terms of the dissociation constant of the enzyme-inhibitor complex (K_i), rather than as a degree of inhibition at arbitrarily chosen concentrations of substrate and inhibitor.

As a preliminary to an investigation of this nature to be carried out on succinic dehydrogenase it was felt to be desirable to study the inhibitory action of malonate. The inhibitory action of malonate on succinic dehydrogenase was recognized as being competitive by Quastel & Wooldridge (1928), and it has since become a classical example of a structural analogue acting as a competitive inhibitor. The literature, however, reveals surprising disagreement on the inhibitory power of this substance. The relative affinity of succinic dehydrogenase for malonate has been given values as different as 50 (Potter & DuBois, 1943), 13 (Keilin & Hartree, 1949), 10 (Krebs & Johnson, 1948) and 60 (Krebs, Gurin & Eggleston, 1952). The enzyme preparations used were rat-liver homogenate, heart-muscle preparation, minced pigeon-breast muscle and a yeast preparation, respectively. In some preliminary experiments reported here, a relative affinity as low as 4·7 was obtained. The reason for these discrepancies probably lies in the fact that different types of enzyme preparation were used, and different methods employed for measuring their activity. Slater & Bonner (1952) have shown that in the case of succinic dehydrogenase K_m (the Michaelis constant) cannot simply be regarded as the dissociation constant of the enzyme-substrate complex, but that it corresponds to Briggs & Haldane's (1925) expression $(k_2+k_3')/k_1$, where k_1, k_2 and k_3' are the velocity constants indicated:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3'} E+\text{products}.$$

Since k_3' is larger than k_2, and is dependent on the characteristics of individual enzyme preparations, and on the method of measuring their activity (Slater & Bonner, 1952), K_m determined by conventional methods (Lineweaver & Burk, 1934) is not a constant, and consequently affinity ratios (K_m/K_i) are also variable.

This paper deals with preliminary attempts to determine the K_m/K_i ratio by a direct method in the case of succinate and malonate, with determinations of K_m and K_i separately in a number of preparations, and with the use of malonate in further experiments to determine the velocity constants k_1, k_2 and k_3' by the method described by Slater & Bonner (1952). In the course of this work, some of the discrepancies in the K_m/K_i ratio pointed out above became intelligible in the light of the theoretical explanation outlined (as was predicted by Slater & Bonner (1952)). Furthermore, it is apparent that the limit of K_m, as k_3' approaches zero, is k_2/k_1, the dissociation constant of the enzyme-substrate complex. Comparison of this quantity with K_i should give a true ratio of affinities of succinic dehydrogenase for malonate and succinate.

A preliminary account of this work has already appeared (Thorn, 1953).

METHODS

Enzyme preparation. The preparation was made from pig-heart muscle by the methods of Keilin & Hartree (1947) and Slater (1949*a*). Fat-free dry weights were determined according to Slater (1949*a*), and the phosphate content of the preparations was determined by estimating inorganic phosphate colorimetrically in the trichloroacetic acid supernatant liquids from the dry-weight determinations.

Measurement of succinic dehydrogenase activity. Two main methods were employed, namely, manometric and absorptiometric methods.

(i) Manometric methods. (*a*) Warburg manometers were used, the temperature being 37° and the gas phase, air. Phosphate buffer, pH 7·3, succinate, malonate and KCN, all adjusted to pH 7·3, and methylene blue were contained in

the main compartments of the flasks, and the enzyme was added from the side arms after temperature equilibration. Total fluid volume was 2·5 ml. Final concentrations were: phosphate, 0·15 M; KCN, 0·001 M; methylene blue, 0·001 M; succinate and malonate as shown. The rate of O_2 consumption for the first 20 min. after addition of enzyme was sensibly constant, and was used as the measure of reaction velocity.

(*b*) A similar manometric method was used, but in order to eliminate the effect of competitive inhibition by phosphate (Slater & Bonner, 1952) a borate buffer, pH 7·3, was used instead. The concentration of inorganic phosphate in the reaction mixture, calculated from the amount of inorganic phosphate introduced with the enzyme preparation, was always of the order of $1·7 \times 10^{-3}$ M, and was sufficiently small compared with the K_i of phosphate to have a negligible effect on the determined K_m of the enzyme. Histidine (Bonner, 1951) was also added in order to produce a standard activation of the enzyme in the presence of variable small quantities of phosphate introduced with the enzyme preparation. Total fluid volume was 3·5 ml. Final concentrations were: borate, 0·1 M; histidine, pH 7·3, $8·1 \times 10^{-3}$ M; KCN, 0·01 M; succinate, malonate and methylene blue as shown.

(ii) Absorptiometric method. The rate of reduction of potassium ferricyanide was measured essentially as described by Slater & Bonner (1952), except that the Spekker absorptiometer with the Ilford 601 violet filter was used in place of a photoelectric spectrophotometer set at 400 mμ. All the constituents of the reaction mixture, except the enzyme, were pipetted into glass-stoppered tubes. Enzyme was added to a tube at zero time, and the contents well mixed and tipped into a 1 cm. cell provided with a glass plate as a lid. The temperature of the cell contents was taken after about 1 min. and the cell was placed in the absorptiometer. After readings had been taken until about 5 min. after addition of enzyme, the cell was removed from the instrument and the temperature of its contents again taken. The average rate of change of optical density between 1 and 5 min. after addition of enzyme was used for the calculation of enzyme activities. If precautions were taken to prevent a rise in temperature in the vicinity of the instrument, as a result of the heat of the lamp, the temperature change in the cell during the 4 min. reaction period was not usually more than 0·3°, and the individual runs of an experiment did not differ by more than 0·5° from the average temperature for the whole experiment. Temperature corrections were not applied. The temperatures in this series of experiments lay between 23 and 27·5°, and are stated in the text. The blank cell contained all reagents (including enzyme) except succinate, and with it the instrument was set to a suitable drum reading, usually 0·15, in order to make use of the most convenient part of the scale. Total fluid volume was 7 ml. Final concentrations were: $K_3Fe(CN)_6$, 0·001 M; borate, KCN and histidine as in manometric method (*b*); succinate and malonate as shown.

Measurement of succinic oxidase activity. A manometric method was employed. The details of the method were exactly as described in the manometric method (*b*) for succinic dehydrogenase, except that water was added in place of KCN and methylene blue.

Reagents. Glass-redistilled water was used in making up all solutions.

Succinate. Solutions of succinate were prepared from succinic acid (A.R. grade) and neutralized to pH 7·3 with NaOH.

Malonate. Solutions of malonate were prepared from malonic acid (m.p. 133·5–135° decomp.) and neutralized to pH 7·3 with NaOH.

Phosphate buffers. These were mixtures of KH_2PO_4 and Na_2HPO_4 (both A.R. grade).

Histidine. Solutions of histidine were prepared from L-histidine monohydrochloride M.B.T. (British Drug Houses Ltd.) and were neutralized to pH 7·3 with NaOH.

$K_3Fe(CN)_6$. Solutions of $K_3Fe(CN)_6$ were prepared from the A.R. grade salt, which was recrystallized before use.

The pH of solutions was determined using a glass electrode.

THEORETICAL

The competitive inhibition of succinic dehydrogenase by malonate may be represented by the following scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3'} E + \text{products}, \qquad K_m = \frac{k_2 + k_3'}{k_1},$$

$$E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI, \qquad K_i = \frac{k_5}{k_4},$$

where E is succinic dehydrogenase, ES is the enzyme-succinate complex, and EI the inactive enzyme-malonate complex. All the methods used are based on the standard Michaelis-Menten expression for the velocity of an enzyme reaction in the presence of a competitive inhibitor, which can be derived from this scheme, namely

$$v = \frac{Vx}{x + K_m(1 + i/K_i)}, \tag{1}$$

where x and i are the concentrations of succinate and malonate respectively, V is the velocity of the reaction at infinite substrate concentration, and v the observed velocity.

Method 1. *Determination of* K_m *and* K_i

K_m and K_i were determined by the methods of Lineweaver & Burk (1934), the basis of which may be simply derived by inverting equation (1). From these values can be obtained the ratio K_m/K_i. This will be described as the 'calculated' value for the ratio, to distinguish it from the directly determined value of the ratio which was obtained by one of the two methods described below.

Method 2. *Determination of the ratio* K_m/K_i *directly*

Equation (1) may be rearranged to the form

$$\frac{V}{v} = 1 + \frac{K_m}{x}\left(1 + \frac{i}{K_i}\right). \tag{2}$$

This equation corresponds to equation (11) of Slater & Bonner (1952), except that it refers to the case of a single competitive inhibitor instead of to the more complex system studied by these workers.

The right-hand side of the equation can be multiplied out to give

$$\frac{V}{v} = 1 + \frac{K_m}{x} + \frac{K_m}{K_i}\frac{i}{x}, \qquad (3)$$

from which it can be seen that if x is maintained constant and i is varied from 0 to any suitable value, a plot of V/v against i will give a straight line with intercept $1 + K_m/x$, and slope $K_m/K_i x$. From the slope of the line, and the given value of x, the desired ratio can be determined.

Method 3. *Approximate determination of the ratio* K_m/K_i

If equation (3) is divided by the expression $1 + K_m/x$, the following equation is obtained

$$\frac{V}{v}\left(\frac{x}{K_m + x}\right) = 1 + \frac{K_m}{K_i}\frac{i}{x}\left(\frac{x}{K_m + x}\right).$$

On the left-hand side of the equation, the product of V and $\left(\frac{x}{K_m + x}\right)$ is the rate of the enzyme reaction at substrate concentration x in the absence of inhibitor. This rate will be designated v_0. The equation may then be rearranged to give:

$$\left(\frac{v_0}{v} - 1\right)\frac{x}{i} = \frac{K_m}{K_i}\left(\frac{x}{K_m + x}\right). \qquad (4)$$

This means that, provided the substrate concentration is sufficiently large to make the factor $\left(\frac{x}{K_m + x}\right)$ approximately unity, the left-hand side of the equation equals the desired ratio. An estimate of the ratio can thus be obtained from the degree of inhibition of the reaction at one concentration of substrate and inhibitor. It can be seen from the equation that if the reaction rate is reduced to one-half by the presence of the inhibitor, the left-hand side of the equation becomes x/i, demonstrating the well known fact that the ratio of affinities is equal to the ratio of the concentrations of substrate to inhibitor at 50 % inhibition. Equation (4) is equivalent to the formula given by Krebs *et al.* (1952) with their proviso that virtually all the enzyme must be present either in combination with substrate or inhibitor.

RESULTS

Preliminary experiments

In this set of experiments the manometric method (*a*) was used for estimating succinic dehydrogenase activity. Since separate determinations of K_m and K_i involved measurement of rates of oxygen consumption at low substrate concentrations, at which the rate of reaction fell after about 5 min., the main work was directed to determining the ratio K_m/K_i directly, which can be done under conditions where the rate of reaction remains constant for a convenient length of time. One determination of K_m by the method of Lineweaver & Burk (1934) was carried out, however, and gave a value of 5×10^{-4} M. (Since the reaction mixture contained 0·15 M-phosphate, this value must be corrected for the effect of the competitive inhibition of phosphate (Slater & Bonner, 1952). The corrected value of K_m thus becomes 2×10^{-4} M.) No significance is attached to this value, but it provided an approximate figure for calculating V in the following experiments on the direct determination of K_m/K_i.

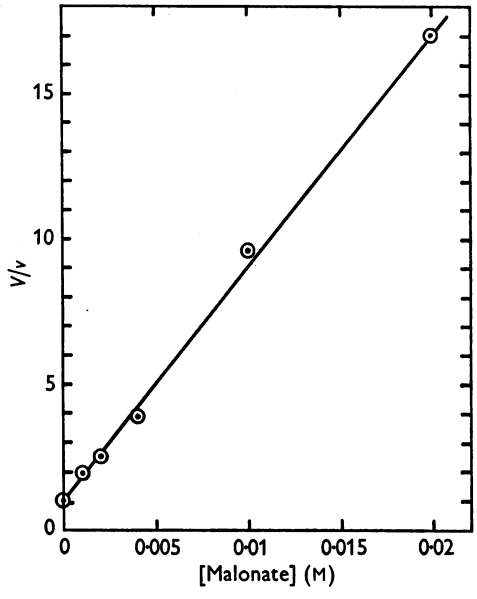

Fig. 1. Effect of malonate concentration on inhibition of succinic dehydrogenase in the presence of a constant amount of succinate. V = maximum velocity; v = observed velocity in the presence of the concentration of malonate shown. Succinic dehydrogenase activity measured manometrically at 37° in the presence of phosphate, pH 7·3, 0·15 M; succinate, 0·01 M; malonate as shown; methylene blue, 0·001 M; KCN, 0·001 M, heart-muscle preparation, approx. 0·6 mg./ml.

A number of experiments was performed to determine the ratio K_m/K_i directly. The kinetic basis of these experiments is given in the Theoretical section, Method 2. One such experiment is illustrated in Fig. 1, where the concentrations of succinate, malonate and methylene blue are given. From the slope of the line and the succinate concentration, the ratio K_m/K_i works out at about 8. (This method gives results which do not require correction for the effect of competitive inhibition by phosphate, since the magnitude of the intercept, not that of the slope, is affected.) The average value obtained for the ratio K_m/K_i, using 0·001 M-

methylene blue, was 6. However, the most significant observation was that the value of the ratio varied with methylene-blue concentration. An experiment illustrating this point is shown in Table 1. The kinetic basis of the calculations is given in the Theoretical section, Method 3. The effect of methylene-blue concentration was shown more fully at a later stage in the investigation.

Table 1. *Effect of methylene-blue concentration on the relative affinity of succinic dehydrogenase for malonate and succinate* (K_m/K_i)

(Activity of succinic dehydrogenase measured manometrically at 37°. All flasks contained phosphate buffer, pH 7·3, 0·15 M; succinate, 0·025 M; KCN, 0·001 M; heart-muscle preparation, 0·67 mg./ml.; malonate (0·01 M) and methylene blue where shown. Total volume 2·5 ml. $\frac{K_m}{K_i} = \left(\frac{v_0}{v} - 1\right)\frac{[\text{succinate}]}{[\text{malonate}]}$, see Theoretical section, Method 3.)

[Methylene blue] ($M \times 10^4$)	Succinic dehydrogenase activity (μl. O_2/hr.) Without malonate (v_0)	With malonate (v)	% Inhibition	K_m/K_i
4	272	94	65	4·7
8	338	102	70	5·8
10	370	105	72	7·5

Determination of K_m and K_i

In these experiments the absorptiometric method of estimating succinic dehydrogenase activity, using potassium ferricyanide as electron acceptor, was employed. This method gives better estimates of initial velocity at low substrate concentrations than does the manometric method. One experiment, demonstrating the effect of substrate concentration on the rate of the reaction in the presence and absence of malonate, is shown in Fig. 2. The results of a number of determinations are given in Table 2, together with the temperatures at which the experiments were carried out, and the calculated K_m/K_i ratios.

The range of values for K_m is from $2·5 \times 10^{-4}$ M to $5·3 \times 10^{-4}$ M, and is to be compared with a figure of $2·3 \times 10^{-4}$ M reported by Slater & Bonner (1952) under similar experimental conditions. The range of values of K_i is from $5·4 \times 10^{-6}$ M to $9·8 \times 10^{-6}$ M. The

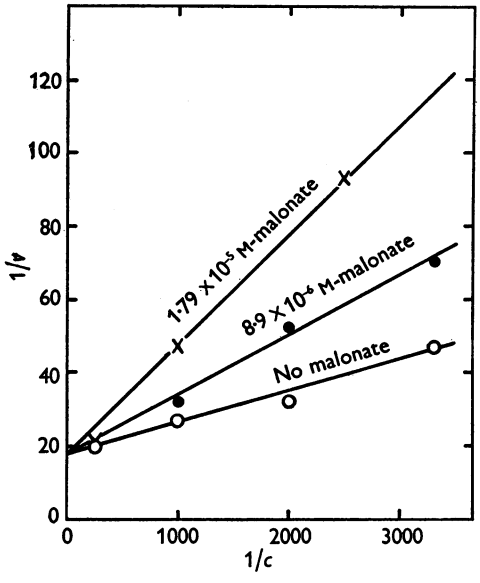

Fig. 2. Competitive inhibition of succinic dehydrogenase by malonate, plotted according to Lineweaver & Burk (1934). c = Molar concentration of succinate; v = change in Spekker reading/min. (Ilford 601 filter). Succinic dehydrogenase activity measured absorptiometrically at 25·3–25·7° in the presence of borate, pH 7·3, 0·1 M; histidine, $8·1 \times 10^{-3}$ M; $K_3Fe(CN)_6$, 0·001 M; KCN, 0·01 M; heart-muscle preparation, 0·42 mg./ml.

spread of the two sets of results is similar and must be attributed at present to experimental error. It might be anticipated that K_m values of different preparations would be different, owing to the complex nature of the reaction and to the participation of a possibly variable quantity, k'_3, in K_m (Slater & Bonner, 1952), but K_i would not be expected to vary,

Table 2. *Values of K_m of succinic dehydrogenase with succinate, and K_i of malonate*

(Succinic dehydrogenase activity measured absorptiometrically with $K_3Fe(CN)_6$ in the presence of cyanide.)

Enzyme preparation	Experiment	Temperature (°)	K_m ($\times 10^4$ M)	K_i ($\times 10^6$ M)	K_m/K_i (calc.)
9	13F	24·8	3·3	9·4	35
	14F	25·5	2·5	—	—
10	16F	24·7	2·8	6·0	47
	17F	23·1	2·7	5·4	50
11	18F	24·5	2·9	6·2	47
	20F	26·9	3·2	9·8	33
13	24F	24·4	5·3	—	—
14	27F	25·6	5·1	9·0	57
15	29F	24·1	5·1	—	—

unless malonate behaves in some anomalous manner. Experiments described below, however, suggest that malonate does in fact behave in a truly competitive manner. The K_m and K_i values shown in Table 2 do not vary consistently with temperature; variation in temperature cannot, therefore, account for the spread of the results. In view of the spread of the results, and the uncertainty in the exact mechanism of participation of potassium ferricyanide in the reaction (Slater, 1949*b*; Tsou, 1951), too much reliance should not be placed on these figures.

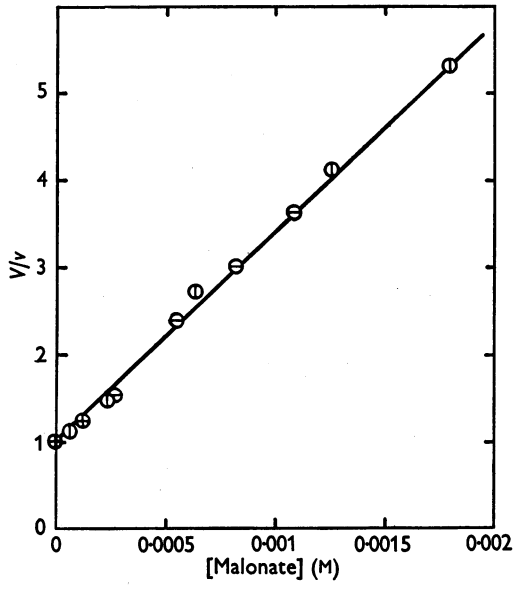

Fig. 3. Effect of malonate concentration on inhibition of succinic dehydrogenase in the presence of a constant amount of succinate. V = maximum velocity; v = observed velocity in the presence of the concentration of malonate shown. Succinic dehydrogenase activity measured absorptiometrically at 27·1–27·4° (ⓘ), and at 26·5–26·7° (⊖), in the presence of borate, pH 7·3, 0·1 M; histidine, $8{\cdot}1 \times 10^{-3}$ M; succinate, 0·025 M; $K_3Fe(CN)_6$, 0·001 M; KCN, 0·01 M; heart-muscle preparation, 0·32 mg./ml.

Further experiments on competitive inhibition by malonate

The absorptiometric method of measuring succinic dehydrogenase activity was used to determine the K_m/K_i ratio directly. The kinetic basis is that given in the Theoretical section, Method 2. The experimental points in Fig. 3 are from two experiments in which malonate concentration was varied at constant succinate concentration. The slope of the line and the succinate concentration give a K_m/K_i ratio of 60, which is to be compared with a calculated ratio of 57 from Exp. 27F in Table 2 since these experiments were carried out with the same preparation. Furthermore, the fact that a straight line is obtained shows that the inhibition involves one molecule of malonate, as might be expected.

The effect of enzyme concentration on inhibition by a given concentration of malonate is shown in Fig. 4. This was a manometric experiment carried out on the complete succinic oxidase system. The points deviate somewhat from linearity, but not to an extent which would suggest that malonate behaved in a 'pseudo-irreversible' manner (Ackermann & Potter, 1949).

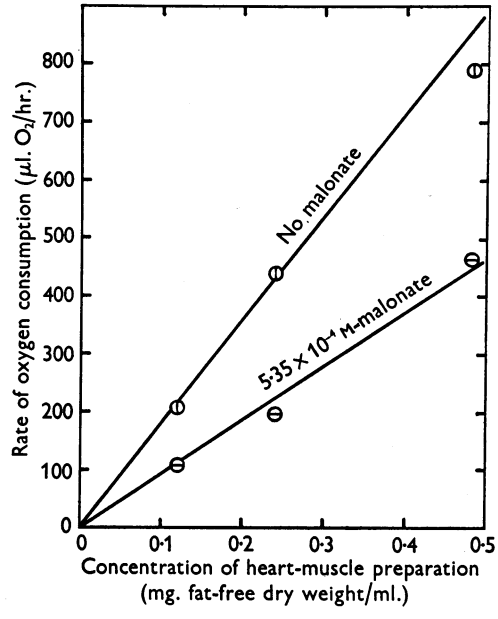

Fig. 4. Effect of enzyme concentration on inhibition of succinic oxidase system by malonate. Succinic oxidase activity measured manometrically at 37° in the presence of borate, pH 7·3, 0·1 M; histidine, $8{\cdot}1 \times 10^{-3}$ M; succinate 0·025 M; malonate as shown.

Determination of the constants k_1, k_2 and k_3'

The constants k_1, k_2 and k_3' were determined exactly as described by Slater & Bonner (1952), except that malonate was used as competitive inhibitor in place of phosphate and fluoride. The reaction medium contained borate buffer and histidine for the reasons given in the Methods section. One experiment is set out in Table 3, which corresponds to Table 6 in the paper by Slater & Bonner (1952).

If the data of Table 3, and similar data from an experiment with preparation 14, are treated exactly as described by Slater & Bonner (1952), values of k_2e and $k_3'e$ (where e is the enzyme concentration) are obtained. From these values, and the relevant figures for K_m shown in Table 2, k_1e can also be calculated. Making the same assumptions as to

Table 3. *Inhibition of succinic dehydrogenase and of the complete succinic oxidase system by malonate*

(Both activities measured manometrically at 37°. Borate, pH 7·3, 0·1 M; succinate, 0·025 M; histidine, $8·1 \times 10^{-3}$ M; KCN, where succinic dehydrogenase activity was measured, 0·01 M; malonate ($5·36 \times 10^{-4}$ M) and methylene blue where shown; heart-muscle preparation, 0·24 mg./ml. Total volume 3·5 ml. $V = v_0 \left(\frac{K_m + 0·025}{0·025}\right)$, $K_m = 5·1 \times 10^{-4}$ with succinic oxidase system, $5·1 \times 10^{-4} \times \frac{v_0}{2 \times 404}$ with succinic dehydrogenase.)

Enzyme activity measured	[Methylene blue] ($M \times 10^4$)	Activity (μl. O_2/hr.) Without malonate (v_0)	With malonate (v)	% inhibition	V	V/v
Succinic dehydrogenase	1·5	107	90	15·9	107	1·19
Succinic dehydrogenase	3·0	131	107	18·3	132	1·23
Succinic dehydrogenase	10	222	167	24·8	223	1·28
Succinic dehydrogenase	30	257	183	28·8	258	1·41
Succinic oxidase system	—	404	192	52·6	412	2·15

Table 4. *Values of k_1, k_2 and k_3' for succinic oxidase*

Preparation	Q_{O_2}	Temperature (°)	k_1 (l.mole^{-1} sec.$^{-1}$)	k_2 (sec.$^{-1}$)	k_3' (sec.$^{-1}$)
14	510	37	$3·5 \times 10^4$	1·1	17
15	490	37	$3·2 \times 10^4$	0·88	15
Slater & Bonner (1952)	950	38	7×10^4	2	32

enzyme concentration as these workers, the approximate values of k_1, k_2 and k_3' given in Table 4 were found. It can be seen at once that there is virtually no difference between the results with preparations 14 and 15, and that these figures, together with the Q_{O_2} values, are about half those of Slater & Bonner (1952).

It should be noted that in these calculations the figure for K_m of succinic oxidase at 37° was taken as $5·1 \times 10^{-4}$ M. This is in fact the value for the K_m of succinic dehydrogenase at about 25°, with potassium ferricyanide as hydrogen acceptor (see Table 2, preparations 14 and 15). This value was used because it was not possible to determine K_m for succinic oxidase accurately, since only the manometric method was available, and this method was not reliable for determining rates of oxygen consumption at low substrate concentrations. (The published figure for K_m of the succinic oxidase system (Slater & Bonner, 1952), obtained by ultraviolet spectrophotometry, is $4·8 \times 10^{-4}$ M at 25°, which is similar to the figure which has been used above.) In any event, since k_2 and k_3' are derived directly from the experimental data, only k_1 would be greatly affected by errors in the value of K_m, and the striking difference between k_2 and k_3' is the most interesting result.

In view of the indirect methods used in determining k_1, k_2 and k_3', and the assumptions involved (as to enzyme concentration and the value of K_m for succinic oxidase at 37°), the agreement between the results reported here and those of Slater & Bonner (1952) is considered good. In fact, the differences between the absolute values for k_1, k_2 and k_3' obtained in this paper and by Slater & Bonner (1952) can be attributed to the questionable assumption that the concentration of succinic dehydrogenase (per mg. fat-free dry weight) was the same in all preparations, although the Q_{O_2} values differed by a factor of 2. If, on the other hand, it is assumed that the concentration of succinic dehydrogenase is proportional to the Q_{O_2}, the differences between the two sets of results disappear, and the values should then be regarded, not as absolute, but, more correctly, as indications of the order of magnitude of the constants. The most significant part of the results is that the relative magnitudes of k_1, k_2 and k_3' are in both cases virtually identical; in particular, that the rate of breakdown of the enzyme-substrate complex to give products is sixteen times faster than its rate of breakdown to give the unchanged substrate.

An estimate of the ratio of the dissociation constants of the enzyme-succinate and enzyme-malonate complexes

It has been pointed out in the Introduction that the limit of K_m as k_3' approaches zero is k_2/k_1. It follows that K_m/K_i ratios determined under conditions of successively decreasing maximum velocities ($V = k_3'e$) should tend towards the ratio of the dissociation constants of the enzyme-succinate and enzyme-malonate complexes, or conversely, towards what might be described as the true ratio of affinities of succinic dehydrogenase for malonate and succinate. A method of varying V (and hence k_3') is to use different methylene-blue concentrations. Data such as those in Table 3 may therefore be used

in this extrapolation to zero k_3'. Since the succinate concentration is much greater than K_m, it is possible to work out the ratio K_m/K_i for succinic dehydrogenase at each methylene-blue concentration, and for succinic oxidase (see Theoretical section, Method 3). In Fig. 5 the values of the ratio are plotted against the corresponding values of Q_{O_2}, where the Q_{O_2} has been calculated from the maximum velocity V in Table 3, and is hence directly proportional to k_3'. The points originate from experiments with preparations 14 and 15, and it can be seen that they fall close to a straight line whose

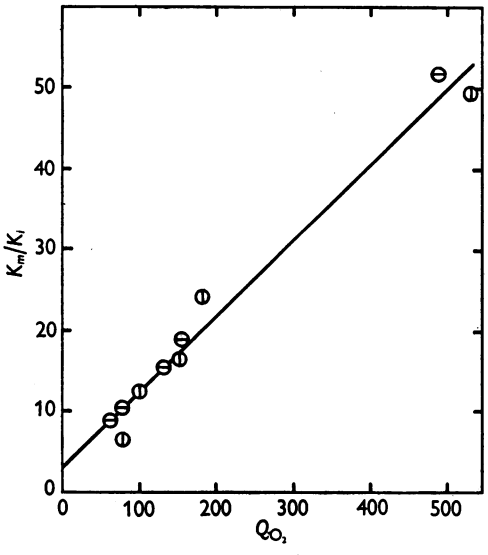

Fig. 5. Variation of relative affinity of succinic dehydrogenase for malonate and succinate (K_m/K_i) with Q_{O_2} (calculated from maximum velocity, V). From data of Table 3 (⊖), and similar data of another experiment (⊕).

ordinate intercept is about 3. This implies that in the absence of further reaction (zero Q_{O_2}, hence zero k_3', and $K_m = k_2/k_1$ = dissociation constant of enzyme-substrate complex) the ratio of affinities of succinic dehydrogenase for malonate and succinate is 3.

DISCUSSION

The inhibition of succinic dehydrogenase by malonate has long been known to be of the reversible competitive type, and the present work was originally intended to determine the relative affinity of succinic dehydrogenase for malonate and succinate. This object implies the comparison of the dissociation constants of the enzyme-succinate and enzyme-malonate complexes. It has been pointed out that there are considerable discrepancies in the various estimates of the ratio of these quantities. The discovery of Slater & Bonner (1952) that K_m for succinic dehydrogenase is not the dissociation constant of the enzyme-succinate complex (k_2/k_1) but the fraction $(k_2+k_3')/k_1$, and that normally k_3' is considerably greater than k_2, but can vary according to the experimental conditions chosen, suggested a better understanding of the problem.

It is clear from the experiments reported here that there is proportionality between k_3' and the ratio K_m/K_i. The extrapolated value of K_m/K_i when k_3' is zero appears to be the true ratio of affinities of the enzyme for malonate and succinate. The extrapolated value of K_m/K_i is about 3, implying that the affinity of the enzyme for malonate is about three times its affinity for succinate. The magnitude of this ratio of affinities is in marked contrast to the figures of 50 (Potter & DuBois, 1943), 60 (Krebs *et al.* 1952) and similar high values recorded under some conditions in this paper. These findings, of course, do not in any way alter the fact that the various relative affinities previously reported in the literature are the experimentally important quantities relevant to the types of preparation in which they were determined. The comparatively small relative affinity of 3 given here is of theoretical interest, and the work reported is a demonstration of the different view which has to be taken of the kinetics of succinic dehydrogenase, namely that k_3' may play an important rather than an insignificant part in determining the value of K_m. This view was first put forward by Briggs & Haldane (1925) on theoretical grounds, and has since been shown by Slater & Bonner (1952) to apply in the case of this enzyme.

The high relative affinities reported by Potter & DuBois (1943), using rat-liver homogenates as a source of enzyme, and by Krebs *et al.* (1952), using a yeast preparation, are what might be expected for highly active succinic oxidase systems, in the light of the results given here. However, K_m of the enzyme in homogenates is given by Ackermann & Potter (1949) as about 10^{-2} M, and the K_i of malonate about 10^{-4} M. Similarly, data given by Krebs *et al.* (1952) on the effect of succinate concentration on the oxygen consumption of the yeast system yield a value for K_m of about 10^{-2} M. Even if these K_m values were corrected for the effect of competitive inhibition by phosphate (Slater & Bonner, 1952) they would still be of a different order of magnitude from those found in heart-muscle preparations. The explanation of these facts, and of the affinity ratio of 10 found by Krebs & Johnson (1948), using pigeon breast-muscle suspensions, is not apparent from the work reported here, and must await further investigation.

SUMMARY

1. Using potassium ferricyanide as hydrogen acceptor, the Michaelis constant (K_m) of succinic dehydrogenase in six different heart-muscle pre-

parations had values ranging from $2{\cdot}5 \times 10^{-4}$M to $5{\cdot}3 \times 10^{-4}$M.

2. In the same series of experiments, the inhibitory constant (K_i) of malonate had values ranging from $5{\cdot}4 \times 10^{-6}$M to $9{\cdot}8 \times 10^{-6}$M.

3. The ratio of affinities of succinic dehydrogenase for malonate and succinate (K_m/K_i), determined directly, varied from 4·7 to 60, depending on the nature and concentration of the hydrogen acceptor. This variation can be explained by differences in the value of the rate constant (k_3') for the decomposition of the enzyme-substrate complex to give the products of the reaction.

4. The true ratio of affinities of succinic dehydrogenase for malonate and succinate, in the absence of further reaction of succinate, is about 3.

5. Values of the rate constants of the uninhibited reaction have been calculated, and were found to agree substantially with those previously reported.

6. The results have been briefly discussed in relation to estimates of the ratio of affinities (K_m/K_i) by other workers using different types of preparation.

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REFERENCES

Ackermann, W. W. & Potter, V. R. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 1.
Bonner, W. D., jun. (1951). *Biochem. J.* **49**, viii.
Briggs, G. E. & Haldane, J. B. S. (1925). *Biochem. J.* **19**, 338.
Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 500.
Keilin, D. & Hartree, E. F. (1949). *Biochem. J.* **44**, 205.
Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 614.
Krebs, H. A. & Johnson, W. A. (1948). *Tabul. biol., Hague*, **19**, part 3, 100.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Potter, V. R. & DuBois, K. P. (1943). *J. gen. Physiol.* **26**, 391.
Quastel, J. H. & Wooldridge, W. R. (1928). *Biochem. J.* **22**, 689.
Slater, E. C. (1949*a*). *Biochem. J.* **45**, 1.
Slater, E. C. (1949*b*). *Biochem. J.* **45**, 14.
Slater, E. C. & Bonner, W. D., jun. (1952). *Biochem. J.* **52**, 185.
Thorn, M. B. (1953). *Biochem. J.* **53**, i.
Tsou, C. L. (1951). *Biochem. J.* **49**, 512.

The Metabolism of Ethers in the Rabbit

1. ANISOLE AND DIPHENYL ETHER

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Investigations in this laboratory of the metabolic fate of aromatic compounds containing 'potential centres for conjugation' (see Bray, Ryman & Thorpe, 1948; Thorpe, 1950) have been concerned mainly with compounds containing groups which may be converted to carboxyl in the animal body, e.g. $-CH_3$; $-CONH_2$; $-CN$. Another common conjugable group, the hydroxyl group, might theoretically be formed *in vivo* from ethers. The evidence in the literature suggests that the ether link in unsubstituted alkyl phenyl ethers is not hydrolysed to any appreciable extent *in vivo*, since Kossel (1880, 1883) and Lehmann (1889) found that a glucuronide of ethyl *p*-hydroxyphenyl ether was a major metabolite of phenetole (ethyl phenyl ether) in the dog. With substituted alkyl phenyl ethers, however, there is evidence that considerable dealkylation may occur *in vivo*, as in the following examples: the formation of *p*-iodophenol from *p*-iodoxyphenyl methyl ether (Röhmann, 1905); of *p*-aminophenol from phenetidine (*p*-aminophenyl ethyl ether) and phenacetin, its *N*-acetyl derivative (Müller, 1888; Mörner, 1889; Smith & Williams, 1949*a*, *b*); of *p*-hydroxydiphenyl from *p*-methoxydiphenyl and of *p*-hydroxyphenyl *p*-methoxyphenyl ether from di-(*p*-methoxyphenyl) ether (Stroud, 1940*a*). Stroud did not observe fission of a phenyl group and there appears to be no example in the literature of such fission of diphenyl ethers. It was thus of interest to investigate the metabolism of aromatic ethers in detail. Anisole (methyl phenyl ether) and diphenyl ether have been chosen