The Effect of Fluoride on the Succinic Oxidase System

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Although it has been known since the work of Battelli & Stern (1910) that the intracellular oxidation of succinate is inhibited by fluoride, the mechanism of this inhibition is little understood. The intracellular oxidation of succinate by oxygen is catalysed by a complex of enzymes, the succinic oxidase system. Our present knowledge of this system is summarized by the reaction scheme succinate → succinic dehydrogenase → cytochrome *b* → intermediary factor → cytochrome *c* → cytochrome *a* → cytochrome a_3 → O_2, where the arrows describe the pathway taken by two hydrogen atoms of succinate (or the electrons derived therefrom).

Potter & Schneider (1942) refer to unpublished work of O'Kane, who found that fluoride inhibits succinic dehydrogenase competitively with respect to succinate. This suggests that the locus of the fluoride action is the reaction between succinate and its dehydrogenase. Dufait & Massart (1939) and Borei (1945), on the other hand, found that the complete succinic oxidase system was more susceptible to fluoride than the dehydrogenase component. This seemed to indicate that some other component of the system, as well as the dehydrogenase, was attacked by fluoride. From other experiments, Borei concluded that cytochrome *c* was this component, but Slater (1949*a*, *b*) considered this unlikely and suggested that the intermediary factor, acting between cytochrome *b* and cytochrome *c*, might be the sensitive component. Subsequent experiments (Slater, 1950*a*) showed, however, that the dihydrocozymase oxidase system, which includes both cytochrome *c* and the same intermediary factor, was not affected by fluoride (see also Potter, 1939). It follows, therefore, that neither cytochrome *c* nor the intermediary factor can be the point of attack of fluoride on the succinic oxidase system.

Further studies are clearly necessary to resolve these discrepancies. Bonner's (1951) recent finding that phosphate is required for the inhibitory action of fluoride made this particularly desirable, since it indicates a reaction between phosphate and some component of the enzyme system. It is now established that phosphate is esterified during the aerobic oxidation of succinate by mitochondrial preparations (e.g. Cross, Taggart, Covo & Green, 1949), although phosphate is not necessary for the oxidation itself (Keilin & Hartree, 1949; Bonner, 1951, 1952). It seems possible that the reaction between the enzyme system and phosphate which is necessary for the inhibition by fluoride might be related to some reaction between phosphate and a component of the enzyme system, which must occur if the oxidation of succinate is accompanied by esterification of phosphate. The fact that high concentrations of fluoride are customarily employed in studies of oxidative phosphorylation was an additional reason for studying its mode of action.

A preliminary account of this work has already appeared (Slater & Bonner, 1951).

METHODS

Keilin-Hartree heart-muscle preparation was the preparation containing very little inorganic phosphate, made as described by Bonner (1952). Concentrations of heart-muscle preparation are expressed as mg. fat-free dry wt./ml.

Enzyme activities were all measured at pH 7·2–7·4.

Succinic oxidase system. Two methods were employed.

(i) Manometric method. After temperature equilibration, the reaction was commenced by addition of succinate from a dangling tube and the O_2 uptake was followed. Except where otherwise stated, cytochrome *c* was not added. The total fluid volume was 3·3 ml.

(ii) Spectrophotometric method. This depends upon measurement of the rate of formation of fumarate by determining the rate of increase of optical density at 230 mμ. This procedure has been used by Chance (1952) for the succinic oxidase system and by Racker (1950) for measuring fumarase activity. The method is only valid for the succinic oxidase system if the preparation is essentially free of fumarase. This was found to be the case with the Keilin & Hartree preparation, but not with the phosphorylating preparation used by Slater (1950*b*). A 1 cm. silica cell contained succinate and any other additions in a total volume of 2·8 ml. The reference cell contained no succinate. At zero time, 0·2 ml. of heart-muscle preparation, suitably diluted (usually 50-fold), was added to each cell and the optical density followed (in a Hilger 'Uvispek' spectrophotometer) as a function of time. The temperature of the solution was measured. The activity was expressed as increase of optical density at 230 mμ./min., corrected to 20°, applying the temperature coefficient found by Slater (1950*a*).

Succinic dehydrogenase. Two methods were employed.

(i) Manometric method. This was as previously described (Slater, 1949*f*), with the exception that the dangling tube contained the diluted heart-muscle preparation, not the succinate. This was to prevent inactivation, during temperature equilibration, of the succinic dehydrogenase by the

cyanide (0·01 M), which Tsou (1951) has found occurs only in the absence of succinate. This method measures the rate of reduction of methylene blue by succinate, which may be a function not only of the succinic dehydrogenase concentration, but also of the cytochrome *b* (Slater, 1949*f*; Tsou, 1951).

(ii) Spectrophotometric method. The rate of reduction of $K_3Fe(CN)_6$ (0·001 M) was measured in the presence of sufficient KCN (0·01 M) to inhibit cytochrome oxidase, by following the rate of decrease of optical density at 400 mμ. A 1 cm. cell contained the succinate, cyanide and ferricyanide in a total volume of 2·8 ml. The reference cell contained water only. At zero time, 0·2 ml. of heart-muscle preparation, suitably diluted (usually tenfold), was added to both cells and the optical density at 400 mμ. followed as a function of time. The rate of change of optical density decreased somewhat during the measurement. The average rate between 1 and 5 min. after addition of the enzyme was used for calculation of the activities. The temperature of the solution was measured and the rate corrected to 20°, assuming the same temperature coefficient as found for the complete succinic oxidase system by Slater (1950*a*). It may not be valid to use the same temperature correction, but the range of temperature in the one experiment was small, so that any error caused by uncertainty in the correction will be negligible. It is not known what components of the succinic oxidase system are involved in the reduction of $K_3Fe(CN)_6$ (cf. Slater, 1949*b*), but it is probable that succinic dehydrogenase is the most important.

Reagents

Sodium succinate. This was standardized manometrically.

Phosphate buffer, pH 7·24. In order to ensure that this contained no pyrophosphate, which is a strong competitive inhibitor of succinic dehydrogenase (Leloir & Dixon, 1937; Tsou, 1951), the buffer was prepared in the following manner. Orthophosphoric acid (49 g.) was dissolved in approximately 500 ml. water. The solution was boiled for several hours, then diluted to about 900 ml., brought to pH 7·24 (glass electrode) with concentrated NaOH and diluted to 1000 ml. The phosphate concentration, measured by the molybdenum blue method, was 0·447 M.

EXPERIMENTS

Mechanism of the action of fluoride

The action of fluoride, in the presence of phosphate, on that portion of the succinic oxidase system responsible for the reduction of potassium ferricyanide, is shown in Fig. 1. It is clear that the inhibition by fluoride is a rapid reaction. The same degree of inhibition was found irrespective of whether fluoride was added to the enzyme 10 min. after or before the succinate. This is in disagreement with Borei (1945), who found that prior treatment of the enzyme preparation with succinate gave protection against fluoride.

Also in contrast with Borei (1945), it was found that the inhibition was completely reversed by dialysis against phosphate buffer. The reversibility of the inhibition is shown also by the experiment described in Fig. 2. Concentrated heart-muscle preparation (1 ml.) was treated with 1·0 M-NaF (0·1 ml.) and 0·15 M-phosphate, pH 7·3 (0·9 ml.), for 1 hr. at 22°. The concentration of fluoride during the treatment was 0·05 M and that of phosphate, 0·067 M. The activity of the complete succinic oxidase system was measured after dilution of this mixture 187-fold; the final fluoride concentration was then $2·7 \times 10^{-4}$ M. A control was treated in the same way, without fluoride, except for the addition of $2·7 \times 10^{-4}$ M in the test for enzyme activity. The

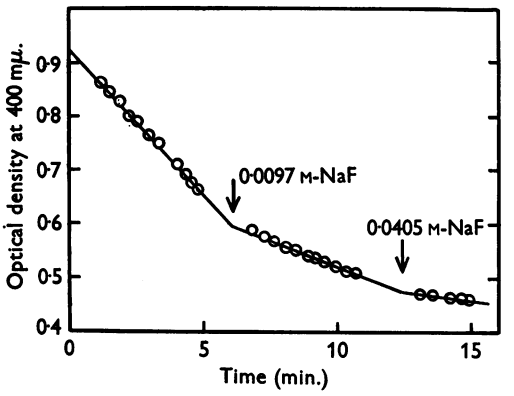

Fig. 1. Inhibition by fluoride of succinic dehydrogenase, measured spectrophotometrically at 20°. Phosphate, 0·14 M; succinate, 0·026 M; $K_3Fe(CN)_6$, 0·001 M; KCN, 0·01 M; heart-muscle preparation, 0·25 mg./ml.

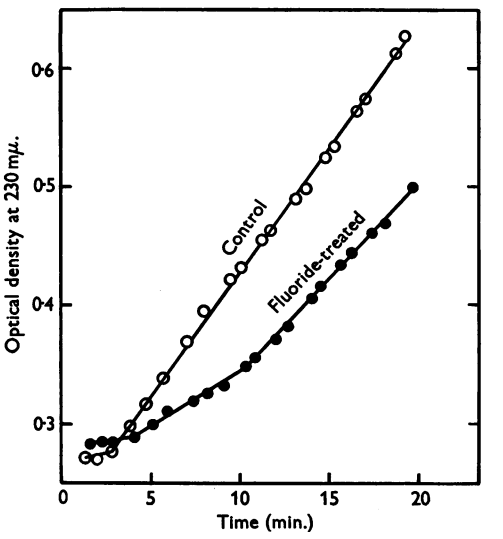

Fig. 2. Reversibility of fluoride inhibition of succinic oxidase system. See text for treatment of 'control' and 'fluoride-treated'. Activity of succinic oxidase system measured spectrophotometrically at 21·3°; phosphate, 0·12 M; succinate, 0·027 M; heart-muscle preparation, 0·062 mg./ml.

succinic oxidase activities of the two preparations are shown in Fig. 2. It can be seen that, after an initial lag, the fluoride-treated preparation has nearly the same activity as the control. Thus, even prolonged treatment with 0·05 M-fluoride, which is sufficient to inhibit completely the enzyme (see below), has practically no irreversible effect.

The effect of phosphate on the degree of inhibition by fluoride is shown in Table 1. As already found by Bonner (1951), phosphate is necessary for any appreciable inhibition by fluoride; with increasing phosphate, the inhibition by fluoride is increased.

Arsenate can replace phosphate, but is less effective (Table 2).

creasing succinate concentration, becoming zero at infinite succinate concentration.

Table 1 shows that, in the absence of fluoride, phosphate is itself somewhat inhibitory (cf. Keilin & Hartree, 1949; Slater 1949*c*). This inhibition has been further studied in Fig. 4. Unlike the experiments summarized in Tables 1 and 3, the complete succinic oxidase system was not studied in

Table 1. *Effect of phosphate on the degree of inhibition of the succinic oxidase system by fluoride*

(Activity of succinic oxidase system measured manometrically at 25°. All flasks contained succinate, 0·021 M; borax-boric acid buffer, pH 7·3, 0·1 M; histidine, pH 7·3, $8·1 \times 10^{-3}$ M; heart-muscle preparation, 0·36 mg./ml.; NaF (0·01 M) and phosphate where shown.)

$$V = v_p\{1 + 0·023(1 + p/0·1)\}$$ (see text, p. 191).

[Phosphate] (M)	Succinic oxidase system (μl. O_2/hr.) No NaF (v_p)	+NaF (v)	Inhibition by NaF (%)	V	V/v
0	349	315	9·6	357	1·13
0·03	319	198	38·0	327	1·65
0·06	274	108	60·6	284	2·62
0·10	252	92·5	63·2	264	2·86
0·15	235	57	76	248	4·35
0·20	209	46	78	224	4·89

Table 2. *Comparison of effect of phosphate and arsenate on the inhibition of the succinic oxidase system by fluoride*

(Activity of succinic oxidase system measured spectrophotometrically at 20–22°. Succinate, 0·027 M; heart-muscle preparation, 0·031 mg./ml. Activity expressed as increase of optical density at 230 mμ./min.)

[Phosphate] (M)	[Arsenate] (M)	[NaF] (M)	Succinic oxidase system	Inhibition by NaF (%)
0·12	0	0	0·0178	72
0·12	0	0·0097	0·0050	
0	0·12	0	0·0190	29
0	0·12	0·0097	0·0132	

Table 3. *Effect of different concentrations of fluoride on the degree of inhibition of the succinic oxidase system*

(Activity of succinic oxidase system measured manometrically at 25°. All flasks contained succinate, 0·021 M; borax-boric acid buffer, pH 7·3, 0·1 M; histidine, pH 7·3, $8·1 \times 10^{-3}$ M; heart-muscle preparation, 0·36 mg./ml. Phosphate (0·1 M) and fluoride where shown.)

$$V = v_f\{1 + 0·023(1 + f/0·02)\}$$ (see p. 192),
$$v' = 331\ v/226$$ (see p. 192).

[Fluoride] (M)	Succinic oxidase system (μl. O_2/hr.) No phosphate (v_f)	+phosphate (v)	Inhibition (%)	V	v'	V/v'
0	331	226	31·9	340	331	1·02
0·002	296	185	37·5	302	271	1·11
0·005	293	114	61·1	300	167	1·80
0·008	276	84	69·6	294	123	2·30
0·012	326	72	77·9	338	105	3·22
0·015	277	49	82·3	288	72	4·01

The effect of various concentrations of fluoride, at a fixed concentration of phosphate (0·1 M), is shown in Table 3.

In Fig. 3 the effect of fluoride on the enzyme system in a phosphate medium at different substrate concentrations is shown, plotted according to the method of Lineweaver & Burk (1934). The results clearly show that the inhibition is competitive, i.e. the degree of inhibition decreases with in-

this experiment, but only that portion of it responsible for the reduction of potassium ferricyanide, which is probably mainly succinic dehydrogenase. The reason for this change was that it seemed likely from previous work (see Slater, 1949*c*, Fig. 2) that phosphate had a much stronger action on the complete system than on the dehydrogenase, whereas it was clear from the fact that fluoride was a competitive

inhibitor that it acted exclusively on succinic dehydrogenase (see below). Fig. 4 shows that phosphate is a competitive inhibitor of succinic dehydrogenase.

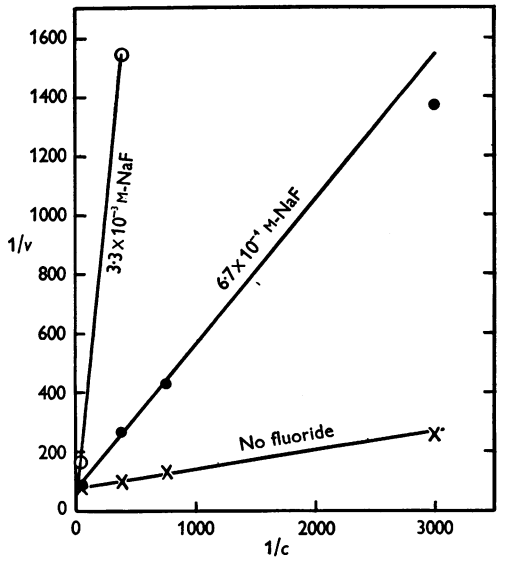

Fig. 3. Competitive nature of fluoride inhibition of succinic oxidase system. Activity measured spectrophotometrically at 19·1–21·2° and calculated to 20°; phosphate, 0·12 M; heart-muscle preparation, 0·06 mg./ml. c = molar concentration of succinate, v = increase/min. of optical density at 230 mμ.

In the absence of phosphate, fluoride is a weak competitive inhibitor of succinic dehydrogenase.

Phosphate and fluoride are not the only inorganic anions which inhibit succinic dehydrogenase. Chloride and nitrate are considerably more inhibitory than phosphate, but this inhibition is largely non-competitive; the degree of inhibition is less at high than at low substrate concentrations, but is still high at infinite substrate concentrations.

The addition of magnesium had no effect on the inhibition by fluoride and phosphate (Table 4). In this respect, the inhibition of succinic dehydrogenase by fluoride differs from that of enolase, which inhibition also requires phosphate (Warburg & Christian, 1942). Manganese also had no effect.

Component of succinic oxidase system affected by fluoride

Added cytochrome c did not affect the degree of inhibition by fluoride (Table 5), showing that it is not the susceptible component of the system. This experiment does not agree with a similar one by Borei (1945).

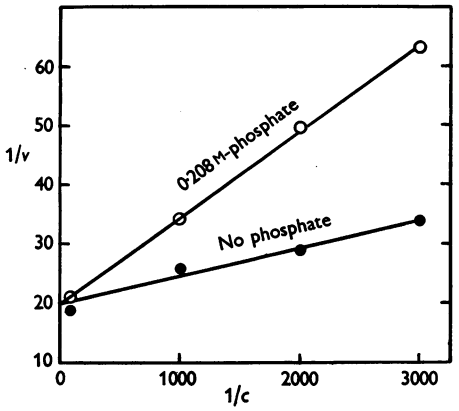

Fig. 4. Competitive inhibition of succinic dehydrogenase by phosphate. Activity measured spectrophotometrically at 24·2–25·2° and corrected to 20°; histidine, pH 7·3, $8·1 \times 10^{-3}$ M; heart-muscle preparation, 0·16 mg./ml. pH of all reagents adjusted to pH 7·3 (glass electrode). c = molar concentration of succinate, v = increase of optical density at 400 mμ./min.

Borei's finding that the succinic dehydrogenase activity, as customarily measured, is considerably less sensitive to fluoride than the succinic oxidase system is confirmed by the experiment summarized in Table 6, in which the effect of a wide range of methylene blue concentrations (resulting in a 2·5 range of rate of O_2 uptake) is shown. It is seen that with increasing activity of the system in the absence of fluoride, the percentage inhibition by fluoride increases, but even at the highest concentration of methylene blue used, when the

Table 4. *Effect of magnesium and manganese on the inhibition by fluoride of the succinic oxidase system*

(Activity of succinic oxidase system measured manometrically at 38° (Exp. 1) or 25° (Exp. 2). Phosphate, 0·13 M; succinate, 0·024 M; heart-muscle preparation, 0·26 mg./ml. (Exp. 1) or 0·36 mg./ml. (Exp. 2).)

[Mg] (M)	[Mn] (M)	[NaF] (M)	Succinic oxidase system (μl. O_2/hr.)	Inhibition by NaF (%)
		Exp. 1		
0	0	0	474	57·6
0	0	0·01	200	
0·001	0	0	480	58·4
0·001	0	0·01	200	
		Exp. 2		
0	0	0	224	74·1
0	0	0·01	58	
0	0·001	0	235	76
0	0·001	0·01	56	

Table 5. *Effect of cytochrome c on the degree of inhibition of the succinic oxidase system by fluoride*

(Activity of succinic oxidase system measured manometrically at 38°. Phosphate, 0·13 M; succinate, 0·024 M; heart-muscle preparation, 0·28 mg./ml.)

[Cyt. *c*] (M × 10^5)	[NaF] (M)	Succinic oxidase system (μl. O_2/hr.)	Inhibition by NaF (%)
0	0	371	70·4
0	0·01	110	
6	0	430	71·2
6	0·01	124	

O_2 uptake of the control approached that found in the measurement of the activity of the complete succinic oxidase system, the percentage inhibition was far below that found with the complete system.

The direct comparison of O_2 uptakes in the measurements of the succinic dehydrogenase and the complete succinic oxidase systems is, however, not valid; the former should be halved. This is because the final reaction with oxygen in the succinic dehydrogenase measurement is with leuco-methylene blue and the product of this oxidation is H_2O_2, thus

$$\text{succinate} + \text{meth. blue} \rightarrow \text{fumarate} + \text{leuco-meth. blue}$$
$$\text{leuco-meth. blue} + O_2 \rightarrow \text{meth. blue} + H_2O_2$$
$$\text{i.e. succinate} + O_2 \rightarrow \text{fumarate} + H_2O_2$$

The formation of H_2O_2 in this reaction was demonstrated by the addition of a large amount of pure catalase, which halved the O_2 uptake (Fig. 5). Heart-muscle preparation contains a little catalase, but this would be only very slightly active in the presence of 0·009 M-cyanide, which is used in the estimation of succinic dehydrogenase. The amount of catalase added in the experiment shown in Fig. 5 was such that considerable activity remained even in the presence of this concentration of cyanide. In the measurement of the succinic oxidase system, O_2 reacts with cytochrome oxidase and is reduced to water, not H_2O_2. The overall reaction is, therefore,

$$\text{succinate} + \tfrac{1}{2}O_2 \rightarrow \text{fumarate} + H_2O.$$

Thus the succinate equivalent of the O_2 consumed in the dehydrogenase test is only half that of the same amount of O_2 in the measurement of the succinic oxidase system.

Even at the highest level of methylene blue used in Table 6, the succinic dehydrogenase activity in the absence of fluoride is, then, much less than the activity of the succinic oxidase system. Thus, the succinic dehydrogenase is working at far less than its full capacity in the dehydrogenase test, which seemed a possible explanation of why it was less susceptible to fluoride. This possibility is confirmed mathematically in the next section. The experimental data are found, in fact, to fit very closely the equation derived on the assumption that the reaction between succinic dehydrogenase and its substrate is the only point of attack of fluoride.

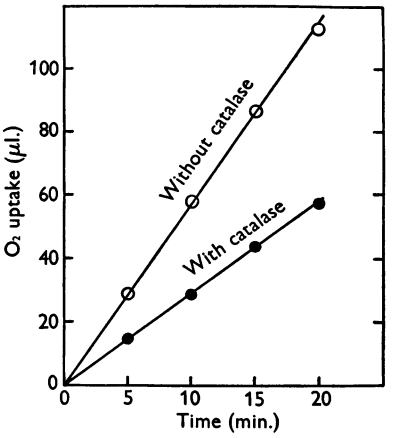

Fig. 5. Effect of catalase on O_2 uptake in manometric estimation of succinic dehydrogenase. Phosphate, 0·13 M; succinate, 0·026 M; methylene blue, $9{\cdot}2 \times 10^{-4}$ M; KCN, $9{\cdot}2 \times 10^{-3}$ M; heart-muscle preparation, 0·21 mg./ml.; catalase, 0·6 mg./ml.

Thus the relative insensitivity of the dehydrogenase compared with the succinic oxidase system is only apparent and not real, and is due to the unsatisfactory nature of the measurement of dehydrogenase activity.

Malonate, the well known competitive inhibitor of succinic dehydrogenase, had previously been found by Slater (1949 *e*) to inhibit the O_2 uptake in the measurement of the succinic oxidase system more than in the dehydrogenase measurement. Undoubtedly, the explanation is the same as that given for fluoride.

The fact that succinic dehydrogenase is working far below its full capacity when it reacts with methylene blue causes difficulty only when a reversible competitive inhibitor of the

Table 6. *Inhibition of succinic dehydrogenase and of the complete succinic oxidase system by fluoride*

(Both activities measured manometrically at 38°. Phosphate, 0·13 M; succinate, 0·024 M; fluoride, 0·0091 M; heart-muscle preparation, 0·21 mg./ml.)

$V = v_p(1 + 95\ K_m)$ (see p. 192),

$K_m = 4{\cdot}8 \times 10^{-4}$ with succinic oxidase system, $3{\cdot}6 \times 10^{-7}\ v_p$ with succinic dehydrogenase (see p. 192).

Activity	[Methylene blue] (M × 10^4)	Activity (μl. O_2/hr.) No NaF (v_p)	Activity (μl. O_2/hr.) +NaF (v)	Inhibition by NaF (%)	V	V/v
Succinic dehydrogenase	1·5	208	159	23·6	209	1·31
	3·0	267	193	27·7	269	1·39
	9·1	361	241	33·2	365	1·51
	30	453	291	36·0	460	1·58
Succinic oxidase system	.	663	268	59·6	690	2·58

dehydrogenase is being studied. The degree of inhibition by compounds which act by irreversibly removing a fraction of the enzyme, e.g. sulphydryl-combining reagents (Slater, 1949*d*) will be independent of the activity.

Effect of fluoride on the succinic oxidase system in a phosphorylating preparation

The experiments described above were all carried out with the Keilin & Hartree heart-muscle preparation, which, although it oxidizes succinate very rapidly, is unable to couple this oxidation with the synthesis of adenosinetriphosphate (ATP). It was of interest to see if fluoride had the same effect on a phosphorylating as on a non-phosphorylating preparation, since the former must contain enzyme systems linked with the phosphorylating reaction absent from the latter preparation, and it appeared possible that these additional systems might provide an oxidative pathway not susceptible to fluoride. The experiment summarized in Table 7 shows, however, that the succinic oxidase system in a phosphorylating mitochondrial preparation was inhibited to the same extent as that in the non-phosphorylating Keilin & Hartree preparation. Similarly, inhibition of the phosphorylation by the addition of dinitrophenol to or the omission of the adenylic system from, the mitochondrial preparation did not appreciably affect the degree of inhibition by fluoride.

Table 7. *Inhibition of succinic oxidase system by fluoride in both phosphorylating and non-phosphorylating systems*

(1·82 mg. pig-heart mitochondria (*M*); 1·91 mg. horse-heart Keilin-Hartree (*K-H*) preparation. Phosphate, 0·0348 *M*; succinate, 0·0267 M; cytochrome *c*, $5{\cdot}8 \times 10^{-5}$ M in all flasks. Those provided with 'adenylic system etc.' contained, in addition, adenylic acid, $1{\cdot}6 \times 10^{-3}$ M; ADP, $0{\cdot}63 \times 10^{-3}$ M; ATP, $0{\cdot}29 \times 10^{-3}$ M; glucose, 0·083 M; $MgCl_2$, 0·047 M and hexokinase. Dinitrophenol (DNP), 5×10^{-4} M, and NaF, 0·04 M where stated. Total volume, 3 ml. Time of exp. 30 min. Temperature, 25° with *M*; 38° with *K-H* preparation. ΔHMP (hexosemonophosphate) measured as described by Slater (1950*b*, 1951).)

Preparation	Adenylic system, etc.	DNP	NaF	O_2 uptake (μg. atoms)	ΔHMP (μmoles)	P/O	Inhibition of O_2 uptake by NaF (%)
M	–	–	–	9·51	.	.	62
M	–	–	+	3·58	.	.	
M	+	–	–	9·91	10·5	1·03	59
M	+	–	+	4·07	4·54	1·11	
M	+	+	–	9·56	0·04	0	54
M	+	+	+	4·44	0	0	
K-H	–	–	–	24·0	.	.	58
K-H	–	–	+	10·0	.	.	

CALCULATIONS

Inhibition constants

Fluoride (F), phosphate (P), but especially the two together, compete with succinate (S) for succinic dehydogenase (E). Thus we have

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES, \quad (1)$$

$$ES \xrightarrow{k_3'} \text{products} + E, \quad (2)$$

$$E + P \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP, \quad (3)$$

$$E + F \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EF, \quad (4)$$

$$EP + F \underset{k_9}{\overset{k_8}{\rightleftharpoons}} EPF \underset{k_{10}}{\overset{k_{11}}{\rightleftharpoons}} EF + P. \quad (5)$$

$k_1, k_2, \ldots, k_{11}$ are true rate constants, with the exception of k_3'; k_3' is not a true rate constant, but includes concentrations of reactants of the individual hydrogen-transferring reactions, whose sum is reaction 2. The back reaction of Eqn. (2) has been ignored, since initial rates of reaction when the fumarate concentration is low have been used throughout this study. Fig. 5, for example, shows no decrease of the rate of reaction due to fumarate production in the first 15 min.

Accepting this mechanism, it is possible to calculate from the experimental data the inhibition constants $K_P = k_5/k_4$; $K_F = k_7/k_6$; $K_{PF} = k_9/k_8$ and $K_{FP} = k_{11}/k_{10}$. The velocity (v) of the oxidation of succinate to fumarate is given by the expression (see Appendix, p. 195)

$$v = V/\{1 + (K_m/s)(fp/K_P K_{PF} + p/K_P + f/K_F + 1)\}, \quad (10)$$

where s, f and p are concentrations of succinate, fluoride and phosphate, $K_m = (k_2 + k_3')/k_1$ is the Michaelis constant in the absence of inhibitors and V is the velocity at infinite succinate concentration.

The Michaelis constant in the presence of inhibitor (K_m') equals $K_m(fp/K_P K_{PF} + p/K_P + f/K_F + 1)$.

K_P can be calculated from the data of Fig. 4. The straight line intercepts the x-axis at a distance equal to $-1/K_m'$ from the origin. In this way, the following values were found ($f = 0$)

p	$K_m' \times 10^4$
0	2·3
0·208	7·25

i.e. $2{\cdot}3 \times 10^{-4} = K_m$,

$7{\cdot}25 \times 10^{-4} = K_m(1 + 0{\cdot}208/K_P)$.

By difference,

$$4{\cdot}95 \times 10^{-4} = 0{\cdot}208\, K_m/K_P = 0{\cdot}208 \times 2{\cdot}3 \times 10^{-4}/K_P,$$

i.e. $K_P = 0{\cdot}1$.

In a similar way, K_F was found to be 0·02.

In the experiments used for the following calculations, the complete succinic oxidase system was studied, not just that portion responsible for the reduction of ferricyanide. The K_m for the complete system, which is considerably different from the value given above (see p. 194), can be calculated from the data of Fig. 3. In the absence of fluoride,

$$K'_m = K_m + pK_m/K_P.$$

Substituting the value of K'_m found ($1{\cdot}11 \times 10^{-3}$), of p (0·123) and K_P found above, $K_m = 4{\cdot}8 \times 10^{-4}$.

K_{PF} can be calculated if $K_m/K_P K_{PF}$ is known. This expression can be determined from the effect of different concentration of fluoride on the Michaelis constant (Fig. 3). This experiment was carried out with the same preparation as was used to determine K_m.

Fig. 3 yields the following values of K'_m

p	f	$K'_m \times 10^3$
0·123	0	1·11
0·123	$0{\cdot}67 \times 10^{-3}$	7·40

i.e. $$1{\cdot}11 \times 10^{-3} = K_m(1 + 0{\cdot}123/K_P)$$

$$7{\cdot}40 \times 10^{-3} = K_m(1 + 0{\cdot}123/K_P + 0{\cdot}67 \times 10^{-3}/K_F + 8{\cdot}25 \times 10^{-5}/K_P K_{PF}).$$

By difference,

$$6{\cdot}29 \times 10^{-3} = K_m(0{\cdot}67 \times 10^{-3}/K_F + 8{\cdot}25 \times 10^{-5}/K_P K_{PF}),$$

i.e.

$$6{\cdot}29 \times 10^{-3} = 0{\cdot}67 \times 10^{-3}\, K_m/K_F + 8{\cdot}25 \times 10^{-5}\, K_m/K_P K_{PF}$$
$$= 0{\cdot}02 \times 10^{-3} + 8{\cdot}25 \times 10^{-5}\, K_m/K_P K_{PF},$$

and $$K_m/K_P K_{PF} = 76.$$

Substituting the values of K_m and K_P found above,

$$K_{PF} = 6{\cdot}7 \times 10^{-5}.$$

Since $K_P K_{PF} = K_F K_{FP}$ (Eqn. (14), Appendix),

$$K_{FP} = K_P K_{PF}/K_F = 3{\cdot}3 \times 10^{-4}.$$

The value of the expression $K_m/K_P K_{PF}$ can also be calculated from the data of Tables 1 and 3. The values obtained have not been used in the above calculations for two reasons. In the first place, the experiments summarized in Tables 1 and 3 were carried out with a preparation different from that used above. Secondly, there are side reactions which complicate the calculations and require certain assumptions, the validity of which is uncertain. Nevertheless, these assumptions do not invalidate the comparison of the data of Table 1 with that of Table 3, both of which were obtained with the same enzyme preparation, and the calculations provide certain information not available from the other experiments.

Equation (11) (see Appendix) gives the relation between V, the velocity at infinite succinate concentration and v, the observed velocity.

$$V/v = 1 + (K_m/s)(1 + p/K_P + f/K_F + fp/K_P K_{PF}). \quad (11)$$

In the absence of fluoride, the velocity, v_p, is given by the expression

$$V/v_p = 1 + (K_m/s)(1 + p/K_P). \quad (11a)$$

Substituting the values for K_m and K_P, found above, and the concentration of succinate in Table 1,

$$V/v_p = 1 + 0{\cdot}023(1 + p/0{\cdot}1). \quad (11b)$$

When $p = 0$ and 0·2 respectively, $V/v_p = 1{\cdot}023$ and 1·069, i.e. the ratio of the activities of succinic dehydrogenase when the phosphate concentration is zero and 0·2 M respectively is $1{\cdot}023/1{\cdot}069 = 0{\cdot}96$. Actually, the second column of Table 1 shows that this ratio is $209/349 = 0{\cdot}60$. It is clear, then, that phosphate has an effect on the complete succinic oxidase system additional to that on succinic dehydrogenase and this must be taken into account when using the figures of Table 1 to calculate the effect of phosphate on the degree of inhibition by fluoride. It should be noted that yet another

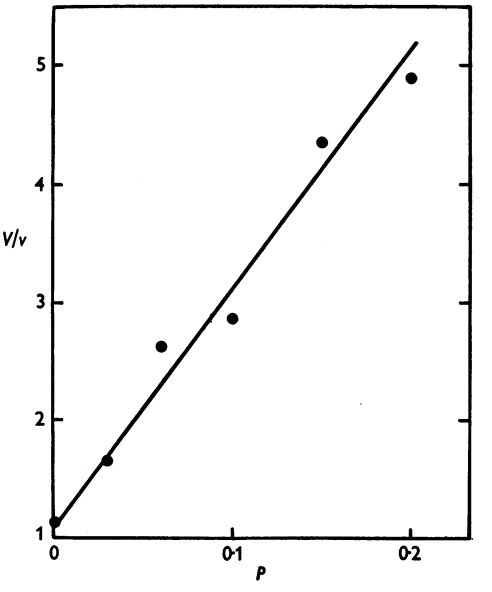

Fig. 6. Data of Table 1 graphed. p = phosphate concentration (M).

effect of phosphate on the succinic oxidase system, namely a stimulation of the succinic oxidase system (Keilin & Hartree, 1949), is eliminated in these experiments by the addition of histidine. Bonner (1951) has found that histidine replaces phosphate in this respect, so that in its presence phosphate has no stimulatory effect.

Equation (11) may be rewritten

$$V/v = 1 + (K_m/s)(1 + f/K_F) + p(K_m/s)(1/K_P + f/K_P K_{PF}).$$

Thus, if V/v is plotted against p, a straight line should be obtained with intercept on the y-axis equal to

$$1 + (K_m/s)(1 + f/K_F)$$

and slope $(K_m/s)(1/K_P + f/K_P K_{PF})$ (cf. Burton, 1951). The data of Table 1 have been treated in this way in Fig. 6. V, the velocity at infinite concentration of succinate, has been calculated separately for each concentration of phosphate from the values of v_p in the second column of Table 1 by Eqn. (11b).

Fig. 6 shows no trend away from a straight line, although the individual points deviate somewhat from this line. The slope equals 20, i.e. $(K_m/s)(1/K_P + f/K_P K_{PF}) = 20$. Substituting for K_m, K_P, s and f, we find $K_m/K_P K_{PF} = 42$.

The experiment summarized in Table 3 can be treated similarly. In the absence of phosphate, but the presence of fluoride, the velocity of the reaction, v_f, is given by the expression

$$V/v_f = 1 + (K_m/s)(1 + f/K_F).$$

Substituting for K_m, K_F and s,

$$V/v_f = 1 + 0{\cdot}023\,(1 + f/0{\cdot}02). \qquad (11c)$$

Equation (11) may be re-written

$$V/v = 1 + (K_m/s)\,(1 + p/K_P) + f(K_m/s)\,(1/K_F + p/K_P K_{PF}),$$

and if V/v is plotted against f, a straight line with intercept $1 + (K_m/s)\,(1 + p/K_P)$ and slope $(K_m/s)\,(1/K_F + p/K_P K_{PF})$ should be obtained. In Table 3, V is calculated from v_f by Eqn. (11c). A correction must be applied to the values of v in the third column of this table to allow for the inhibition by phosphate in the absence of fluoride. It is apparent from the above that this inhibition is not due to the slight inhibition of succinic dehydrogenase, but must be due to the second effect on the complete succinic oxidase system. It is assumed that this is superimposed on the effect being studied. It cannot be influenced by the addition of fluoride, which acts only on succinic dehydrogenase. The value of the correction is given by the ratio of v_f and v in the absence of fluoride, namely 331/226.

In Fig. 7, the plot of V/v' against f fits a straight line closely, although this line does not meet the y-axis at the predicted point. The slope of the straight line is 200. Thus $(K_m/s)\,(1/K_F + p/K_P K_{PF})$ equals 200 and, by substitution, $K_m/K_P K_{PF} = 42$.

Thus the value of $K_m/K_P K_{PF}$ calculated from the experiment in which fluoride was varied at fixed phosphate concentration agrees closely with that calculated from the experiment in which phosphate was varied at fixed fluoride concentration.

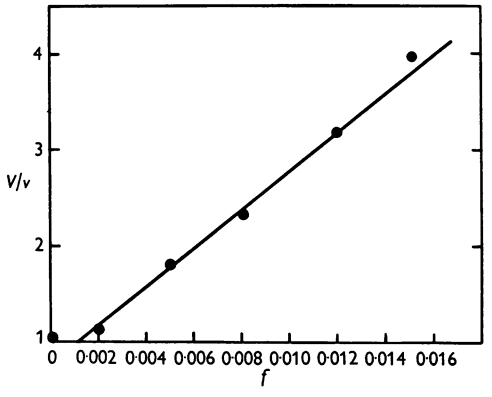

Fig. 7. Data of Table 3 graphed.
f = fluoride concentration (M).

Relative inhibition of succinic oxidase system and of succinic dehydrogenase

Equation (12) (see Appendix) shows that V/v is a linear function of V, i.e. the degree of inhibition will increase with increasing activity of the control. The data of Table 6 have been plotted in this way in Fig. 8, taking account of the fact that the values for V given in Table 6 should be divided by two when the dehydrogenase activity is measured. V has been calculated from Eqn. (11a) (see p. 191). Putting $s = 0{\cdot}024$, $p = 0{\cdot}13$ and $K_P = 0{\cdot}1$, we find, $V/v_p = 1 + 95K_m$. The value of K_m was taken as $4{\cdot}8 \times 10^{-4}$ with the succinic oxidase system (see p. 191) and

$$4{\cdot}8 \times 10^{-4} \times v_p/2 \times 663 = 3{\cdot}6 \times 10^{-7}\, v_p$$

in the dehydrogenase experiments, since it was assumed in the light of the findings below, that K_m is approximately proportional to k'_3, which is proportional to V. The factor 2 is introduced for the same reason as above.

The data fit a straight line very closely.

Calculation of the rate constants of the uninhibited reaction

It is possible to calculate from Fig. 8 and some of the previous calculations, the rate constants of the uninhibited reaction.

It is shown in the Appendix that when $V/v = 1$, V equals $-k_2 e$. Thus from the extrapolation shown in Fig. 8, $-k_2 e = -48$, i.e. $k_2 e = 48\,\mu$l. O_2/hr./3·3 ml. $= 3{\cdot}6 \times 10^{-7}$ moles succinate/l./sec.

$V = k'_3 e = 690\,\mu$l. O_2/hr./3·3 ml. $= 5{\cdot}15 \times 10^{-6}$ moles/l./sec.

The Michaelis constant,

$$K_m = (k_2 + k'_3)/k_1 = 4{\cdot}8 \times 10^{-4},$$

i.e.

$$(k_2 e + k'_3 e)/k_1 e = 4{\cdot}8 \times 10^{-4},$$

$$k_1 e = (k_2 e + k'_3 e)/4{\cdot}8 \times 10^{-4}$$
$$= 5{\cdot}51 \times 10^{-6}/4{\cdot}8 \times 10^{-4}$$
$$= 1{\cdot}15 \times 10^{-2}\ \text{sec.}^{-1}.$$

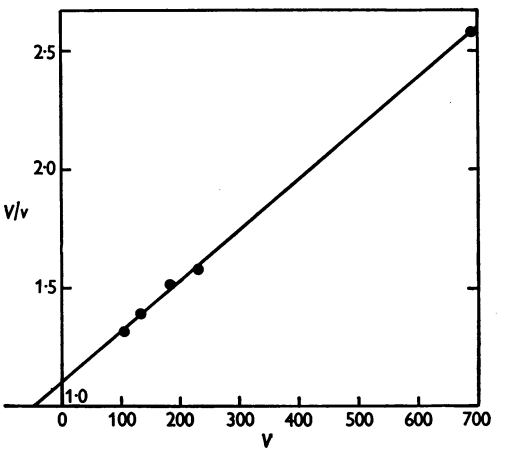

Fig. 8. Data of Table 6 graphed.

For the calculation of the actual rate constants, the enzyme concentration is required. There is no way of determining this at present. However, the above values are useful for determining the ratios of the constants, which are discussed below (see p. 194). It should be noted that the constants refer to 38°, although in the derivation of k_1, the value of K_m at 20° has been used. If the K_m is different at 38°, an error will be introduced into the calculation of k_1, but this will not be very great.

Some idea of the order of magnitude of the actual rate constants can be obtained if it is assumed that the concentration of succinic dehydrogenase in the heart-muscle preparation is of the same order of magnitude as that of cytochrome c. The fact that all the cytochrome components are present in the heart-muscle preparation in approximately the same concentration (Slater, 1949*f*; Chance, 1952) gives some justification for this assumption. The concentration of cytochrome c, calculated from the fat-free

dry weight and the data of Slater (1949*f*), was $0{\cdot}16 \times 10^{-6}$ M in the experiment summarized in Table 6. This gives the following values: k_1, 7×10^4 l. mole^{-1} sec.$^{-1}$; k_2, 2 sec.$^{-1}$; k'_3, 32 sec.$^{-1}$. These values are probably within a factor of 10 of the true values, but should not be taken as more accurate than this.

DISCUSSION

Mechanism of action of fluoride

It has been shown that fluoride and phosphate separately have weak inhibitory effects on succinic dehydrogenase, competing with succinate for the enzyme. Fluoride and phosphate together, however, inhibit much more strongly. The fact that the inhibition constant calculated from an experiment in which phosphate was varied at fixed fluoride concentration agrees closely with that obtained when fluoride was varied at fixed phosphate concentration supports the mechanism proposed on p. 190. Experiments have not been carried out at different pH's, so it is not known which phosphate ion is inhibitory or whether it is the fluoride ion or the free acid which combines with the enzyme. Since, however, fluoride is almost exclusively in the form of fluoride ion at pH 7·3 (less than 0·01 % in the form of hydrogen fluoride, Borei, 1945), it is very probable that it is the ion which is involved. The straight-line relationship shown in Figs. 6 and 7 indicate that one phosphate ion and one fluoride are involved in the inhibition. This is of interest, since inhibitions by fluoride often involve two fluoride ions, e.g. that of enolase (Warburg & Christian, 1942) and of the oxidation of quinol by cytochrome *c* and cytochrome oxidase (Borei, 1945).

The data of Table 6 and Fig. 8 clearly show that fluoride acts on the succinic oxidase system solely by virtue of its attack on succinic dehydrogenase. These experiments, therefore, give no support to Borei's (1945) view that cytochrome *c* is the fluoride-sensitive component of respiratory systems. Table 5 demonstrates this directly. Similarly, it follows that neither the cytochrome oxidase reaction nor the reaction between the endogenous cytochrome *c* in the heart-muscle preparation and cytochrome *c* oxidase are affected by fluoride. In this respect, endogenous cytochrome *c* differs from added soluble cytochrome *c*, since Borei found that the oxidation of quinol by soluble cytochrome *c* and cytochrome *c* oxidase was inhibited by fluoride and this inhibition was competitive with respect to cytochrome *c*. Borei's experiment shows that fluoride affects the reaction between soluble cytochrome *c* and the oxidase. Borei considered that this inhibition was due to a reaction between fluoride and cytochrome *c*, but since there is no other evidence for this, Slater (1949*a*) suggested that the action of fluoride was on the oxidase, similar to an effect of phosphate. It is interesting that Borei found that phosphate increased the inhibition by fluoride of this oxidation.

Succinic dehydrogenase resembles enolase in that phosphate is required for the inhibition by fluoride. This raises the question whether the dehydrogenase contains, like enolase, magnesium or possibly another metal. Such suggestions have been made previously. Massart (1939), on the basis of the inhibition by fluoride, suggested that the enzyme was a manganese compound. Because the addition of calcium increased the succinic oxidase activity of homogenates, Potter & Schneider (1942) believed that calcium was a component of the system and suggested that fluoride combined with the calcium. Later, however, it was shown (Swingle, Axelrod & Elvehjem, 1942; Keilin & Hartree, 1949) that the activation by calcium had another explanation, and there is no reason to believe that calcium is a component of the system. The lack of effect of magnesium or manganese on the degree of inhibition of succinic dehydrogenase by fluoride is in contrast with the behaviour of enolase. It cannot be excluded that the dehydrogenase is a magnesium or manganese compound but, if this is the case, the metal must be more firmly bound than in any known magnesium or manganese enzyme, since the exhaustive washing of the heart muscle would be expected to remove a considerable proportion of the metal from such enzymes.

Effect of fluoride on intracellular respiration

Fluoride in comparison with other inhibitors is not a very powerful inhibitor of succinic dehydrogenase, even in the presence of phosphate. However, if the succinate concentration is not that commonly employed when it is used as substrate in enzyme experiments but is equal to that found in cells, it becomes a powerful inhibitor. Since the oxidation of succinate is an important step in respiratory metabolism, the effect of fluoride on the respiration of intact cells (literature reviewed by Borei, 1945) might be due to its effect on succinic dehydrogenase. This would be consistent with the fact that the inhibition is reduced by any influence that increases the cells' turnover of substrate.

Enzyme kinetics

Michaelis & Menten (1913), in proposing the enzyme-substrate theory of enzyme action, which may be formulated

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES},$$

$$\mathrm{ES} \xrightarrow{k_3} \text{products},$$

assumed that k_1 and k_2 were large compared with k_3, so that the concentration of ES was kept at a value dictated by the equilibrium of the first reaction.

Using this assumption, they derived the well known formula relating enzyme activity to substrate concentration. This relationship could, in fact, be expressed by the value of a constant, now known as the Michaelis constant (K_m), which equalled the substrate concentration at which the activity was half that obtained with infinite substrate concentration. According to Michaelis & Menten's equation, $K_m = k_2/k_1$ = the dissociation constant of the enzyme-substrate compound.

Briggs & Haldane (1925) showed that the relationship between enzyme activity and substrate concentration had the same form, if Michaelis & Menten's simplifying assumption was not made. They derived, from steady-state kinetics, the same formula as Michaelis & Menten, but

$$K_m = (k_2 + k_3)/k_1,$$

which becomes k_2/k_1 only if k_2 is large compared with k_3. Thus, in general, although it may be considered a kinetic dissociation constant when ES is reacting, K_m need not be the same as the dissociation constant of the compound in the absence of further reaction. The difference between the Michaelis and dissociation constants has also been commented upon by Hearon (1949).

Chance's (1943, 1949) measurements of the actual rate constants of horse-radish peroxidase provided the first quantitative evidence in support of the essential features of the Michaelis-Menten theory. At the same time, they showed that for this enzyme the simplifying assumption of the theory did not apply, since k_2 is in fact very small compared with k_3.

The present study, by less direct methods, has shown that, in the case of succinic dehydrogenase also, k_2 is much smaller than k_3. Thus, for both these enzymes, K_m bears no relation to the dissociation constant of the enzyme-substrate compound. The actual magnitude of the difference in the case of succinic dehydrogenase is shown by the following figures; k_2/k_1 = dissociation constant of the compound $ES \rightleftharpoons E + S$, in the absence of further reaction of ES (i.e. in the absence of hydrogen acceptor) $= 3 \times 10^{-5}$; Michaelis constant $= 4{\cdot}8 \times 10^{-4}$.

Since K_m depends on the value of k_3', which is affected by the concentration of the enzyme systems responsible for the transfer of hydrogen atoms from the ES compound to the hydrogen acceptor, there is no reason to believe that K_m will be the same for different enzyme preparations, even under otherwise identical conditions. It would be expected, however, that the dissociation constant (k_2/k_1) of succinic dehydrogenase would be a constant property of this single enzyme molecule.

The rate constants which have been calculated are based on the assumption that the Michaelis theory applies to this enzyme. The existence of competitive inhibitors, such as malonate, oxalacetate, pyrophosphate, fluoride and phosphate, makes this extremely likely, but the rate constants must eventually be determined by direct measurement, when methods are available.

Oxidative phosphorylation

In order to explain the phosphorylation of adenosinemonophosphate (AMP) or adenosinediphosphate (ADP) which accompanies the oxidation of succinate, one must assume some reaction between phosphate and the succinic oxidase system. Also to be taken into account is the fact that phosphate is not necessary for the oxidation (Keilin & Hartree, 1949; Bonner, 1951). The inhibition by phosphate alone indicates some reaction between phosphate and succinic dehydrogenase, but the reaction is non-specific and the affinity very low. In the presence of fluoride, however, phosphate has a much greater affinity for the enzyme. The possibility exists that in a phosphorylating system some other component might act like fluoride and help to bind the phosphate firmly, followed by other reactions leading to the phosphorylation of AMP or ADP. There is, as yet, no evidence to support this suggestion.

SUMMARY

1. Spectrophotometric methods for measuring the activities of the succinic oxidase system and succinic dehydrogenase are described.

2. In the manometric method of determining succinic dehydrogenase, hydrogen peroxide is an end product. Thus oxygen uptakes obtained by this method should be halved to make them comparable with activities of the complete succinic oxidase system, which yields water as the end product.

3. Fluoride and phosphate separately have only very slight effects on succinic dehydrogenase, revealed only at low substrate concentrations.

4. The inhibition by fluoride and phosphate, acting together, is much greater. It is a rapid reaction and is largely reversed by dilution.

5. All three inhibitions are completely competitive with respect to succinate. One molecule of phosphate and of fluoride react with each enzyme molecule.

6. It is concluded that succinic dehydrogenase is the only component of the succinic oxidase system susceptible to fluoride, despite the fact that succinic dehydrogenase, as customarily measured, is inhibited to a smaller degree than the complete system.

7. The reasons for the smaller inhibition in the latter case are (*a*) succinic dehydrogenase is not working at its full activity in the test, because the concentration of the hydrogen acceptor limits the rate of the reaction; (*b*) the simplifying assumptions

of the Michaelis-Menten theory do not apply to succinic dehydrogenase.

8. Arsenate behaves like phosphate but to a lesser degree.

9. Manganese and magnesium did not affect the inhibition by fluoride and phosphate.

10. Fluoride had the same inhibitory action on a phosphorylating mitochondrial preparation as on a non-phosphorylating preparation.

11. Inhibitory constants, Michaelis constants and rate constants of the uninhibited reaction have been calculated.

12. The relation between the Michaelis constant and dissociation constant of the enzyme-substrate compound is discussed.

We wish to thank Prof. D. Keilin, F.R.S., for his interest and advice in the investigation. One of us (E.C.S.) acknowledges with thanks receipt of an Australian National University Research Fellowship during part of this investigation and a personal grant from the Agricultural Research Council during the remaining part. One of us (W.D.B.) is indebted to the U.S. Public Health Service for a Fellowship during the course of the investigation.

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APPENDIX

Derivation of kinetic equations

The proposed mechanism (see p. 190) is

$$\underset{(e-a-b-c-d)}{E} + \underset{(s)}{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(a)}{ES}, \quad (1)$$

$$\underset{(a)}{ES} \xrightarrow{k_3'} \text{products} + E, \quad (2)$$

$$\underset{(e-a-b-c-d)}{E} + \underset{(p)}{P} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} \underset{(b)}{EP}, \quad (3)$$

$$\underset{(e-a-b-c-d)}{E} + \underset{(f)}{F} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} \underset{(c)}{EF}, \quad (4)$$

$$\underset{(b)}{EP} + \underset{(f)}{F} \underset{k_9}{\overset{k_8}{\rightleftharpoons}} \underset{(d)}{EPF} \underset{k_{10}}{\overset{k_{11}}{\rightleftharpoons}} \underset{(c)}{EF} + \underset{(p)}{P}. \quad (5)$$

The concentrations of the reactants are shown in brackets; e is the concentration of total enzyme $(E+ES+EP+EF+EPF)$.

Put $(k_2+k_3')/k_1=K_m$; $k_5/k_4=K_P$; $k_7/k_6=K_F$; $k_9/k_8=K_{PF}$; $k_{11}/k_{10}=K_{FP}$.

In the steady state,

$$da/dt = k_1 s(e-a-b-c-d) - (k_2+k_3')\,a = 0,$$

therefore $$(e-a-b-c-d) = (k_2+k_3')\,a/k_1 s = aK_m/s. \quad (6)$$

The concentrations of EP, EF and EPF will be governed by the equilibrium of reactions (3), (4) and (5). Thus

$$b/p(e-a-b-c-d) = 1/K_P,$$

i.e. $$b = (p/K_P)\,aK_m/s, \quad (7)$$

$$c/f(e-a-b-c-d) = 1/K_F,$$

i.e. $$c = (f/K_F)\,aK_m/s, \quad (8)$$

$$d/fb = 1/K_{PF},$$

i.e. $$d = fb/K_{FF} = (fp/K_P K_{FF})\, aK_m/s. \quad (9)$$

Substituting in (6) the values for b, c and d obtained in Eqns. (7)–(9), we find

$$a = e/\{1 + (K_m/s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF})\}.$$

The rate of the reaction,

$$v = ds/dt = k_3' a,$$

i.e.

$$v = k_3' e/\{1 + (K_m/s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF})\}.$$

When $s = \infty$, $v = V = k_3' e$,

i.e.

$$v = V/\{1 + (K_m/s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF})\}, \quad (10)$$

or $$V/v = 1 + (K_m/s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}). \quad (11)$$

Expressing K_m as rate constants,

$$V/v = 1 + (k_2 + k_3') \times (1 + p/K_P + f/K_F + fp/K_P K_{FF})/k_1 s = 1 + (k_2/k_1 s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}) + (V/k_1 se)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}), \quad (12)$$

i.e. at fixed p, f and s, V/v will be a linear function of V.

When $V/v = 1$,

$$(k_2/k_1 s)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}) = -(V/k_1 se)\,(1 + p/K_P + f/K_F + fp/K_P K_{FF}),$$

i.e. $$-V = k_2 e, \quad (13)$$

i.e. the extrapolated value of V obtained by continuing the straight line obtained by plotting V/v against V to the value $V/v = 1$ will be equal to $-k_2 e$.

Returning to Eqn. (5),

$$d/cp = 1/K_{FP},$$

i.e. $$d = cp/K_{FP} = (pf/K_F K_{FP})\, aK_m/s,$$

substituting the value for c, given by Eqn. (8). But, by Eqn. (9),

$$d = (pf/K_P K_{FF})\, aK_m/s.$$

Therefore $$K_F K_{FP} = K_P K_{FF}. \quad (14)$$

The Action of Thrombin on Fibrinogen

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A number of workers in the field of blood coagulation (Schmidt, 1895; Mellanby, 1908; Hammarsten, 1914; Nolf, 1938) have sought to explain the conversion of fibrinogen to fibrin in terms of a proteolytic change. Though this concept has been contradicted recently (Jaques, 1938; Astrup, 1950), the fact that certain proteases, e.g. papain (Eagle & Harris, 1937) and some snake venoms (Eagle, 1937) can bring about clotting served to uphold the idea. However useful the information gained by analogy from other enzymes, direct proof was necessary to show that thrombin acts in a similar manner. It was therefore desirable to undertake a systematic comparison of the free amino groups of both fibrinogen and fibrin. Sanger's (1945) method was used because of its high sensitivity and usefulness in the identification of the amino-acids involved.

A preliminary account of part of this work has already been published in conjunction with Dr K. Bailey and Mr F. R. Bettelheim (Bailey, Bettelheim, Lorand & Middlebrook, 1951), who worked independently.

EXPERIMENTAL

Materials

Fibrinogen. Since there is no real criterion of purity for fibrinogen, several methods of preparation were tried which were known to produce material of clottability exceeding 90%. In most cases fresh bovine plasma collected in 0·2% (w/v) sodium oxalate was used as the source of fibrinogen.

(I) Bovine plasma was precipitated with 0·25 saturated $(NH_4)_2SO_4$, the precipitate was centrifuged and redissolved in one-third of the original plasma volume of 0·9% (w/v) NaCl containing 0·2% (w/v) sodium oxalate, and the solution was then filtered. The precipitation with 0·25 saturated $(NH_4)_2SO_4$ was repeated three times, and the final precipitate was dissolved in 0·9% (w/v) NaCl and dialysed at 2° against a similar solution for 48 hr. It was then filtered through paper pulp which had been washed with 0·9% (w/v) NaCl.

(II) The first precipitation with $(NH_4)_2SO_4$ was the same as in the previous case; the separated precipitate was dissolved in one-third of the original plasma volume of 0·9% (w/v) NaCl and was filtered through paper pulp. The solution was then cooled to 0° and diluted to 10 vol. with cold distilled water. The fibrinogen was precipitated by the addition