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On the Cyanide Inactivation of Succinic Dehydrogenase and the Relation of Succinic Dehydrogenase to Cytochrome *b*

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Unlike most oxidizing enzymes, the dehydrogenases were considered to be insensitive to cyanide, until Dixon & Keilin (1936) discovered that xanthine dehydrogenase is slowly but irreversibly inactivated by cyanide. In fact, the cyanide inactivation of xanthine dehydrogenase is in a class of its own. The usual type of cyanide inhibition of oxidizing enzymes is instantaneous, readily reversible and only partial, a definite percentage inhibition to each cyanide concentration. In the case of xanthine dehydrogenase, the inhibition is irreversible and it proceeds to completion if, prior to the addition of substrate, the enzyme is allowed to remain with cyanide for a sufficiently long time.

The treatment which Dixon & Keilin (1936) used with xanthine dehydrogenase was applied to a number of enzymes, including succinic dehydrogenase, by Leloir & Dixon (1937) and no inactivation by cyanide was obtained. This was later confirmed by Potter (1941), who found that the enzyme system responsible for the reduction of cytochrome *c* by succinate was not affected by incubation with cyanide. On the other hand, using a higher cyanide concentration, Banga & Porges (1938) and recently Slater (1949*b*) reported some inhibition of succinic dehydrogenase by cyanide.

In the present investigation it is shown that succinic dehydrogenase is slowly and irreversibly inactivated by cyanide. It is also shown that the enzyme can be effectively protected from the action of cyanide by succinate and sodium dithionite; dicarboxylic acids other than succinic are, however, incapable of giving any protection. A preliminary account of some of these findings has appeared elsewhere (Tsou, 1951*a*).

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MATERIALS AND METHODS

Enzyme preparations. The heart-muscle and kidney preparations (Keilin & Hartree, 1947; Slater, 1949*b*) containing the succinic dehydrogenase-cytochrome system were prepared as previously described (Tsou, 1951*b*).

Reagents. Solutions of cyanide, cysteine and pyrophosphate were prepared and neutralized immediately before use. Cystine solution was prepared according to Hopkins & Morgan (1938).

Succinic oxidase and cytochrome oxidase activity was estimated essentially as described by Slater (1949*a*, *b*).

Succinic dehydrogenase activity was estimated by the Thunberg technique at 38°. Unless otherwise specified, final concentrations were: phosphate buffer, pH 7·3, 0·1 M; succinate, 0·04 M; enzyme, 1–2 mg. fat-free dry weight; methylene blue (MB), 0·125 mM, or dichlorophenol indophenol, 0·5 mM. 90% reduction of the dye was taken as the end point, and activity was expressed in terms of Q_{MB} as defined by Corran, Dewan, Gordon & Green (1939) on the hydrogen basis.

Inactivation by cyanide. The correct amount of neutralized 0·1 M-cyanide to give the required concentration was added to heart-muscle or kidney preparations, previously brought to the desired temperature, in 0·1 M-phosphate buffer, pH 7·3. After thorough mixing, the sample was incubated in a bath of constant temperature. Portions were withdrawn at suitable intervals and the activity was estimated as described above. Controls were carried out where the enzyme was incubated with the same concentration of KCl instead of KCN.

Freeing the inactivated enzyme from cyanide. The enzyme preparation which had been incubated with cyanide for a specified period was dialysed first against running tap water for 4 hr. followed by two changes of 0·1 M-phosphate buffer of pH 7·3. It was allowed to remain in the second change of buffer overnight. As a control, a sample of the same enzyme preparation was incubated with KCl instead of KCN and then dialysed simultaneously. When a protective agent was also present during the incubation, the cyanide-treated enzyme preparation was first dialysed against a solution of the protective agent and then dialysed against running tap water and buffer as described above.

RESULTS

Course of inactivation. The inactivation of heart-muscle succinic dehydrogenase by cyanide is shown in Fig. 1. The succinic dehydrogenase in the kidney preparation is also affected by cyanide, although at a slower rate than the heart-muscle enzyme. Fig. 2 compares the cyanide inactivation of succinic dehydrogenase in these two preparations. Unlike the Ogston & Green (1935) preparation used by Leloir & Dixon (1937), both the heart-muscle and kidney preparations are perfectly stable at the temperature used (38°) in the absence of cyanide.

Table 1 shows that, as in the case of xanthine dehydrogenase, the effect of cyanide on succinic dehydrogenase was only produced after it had acted upon the enzyme for a definite time in the absence of substrate (tube 3). No inactivation was obtained when cyanide was introduced together with substrate and acceptor (tube 4), nor when the enzyme was incubated with cyanide in presence of the substrate (tube 2).

In addition to cyanide, the action of other respiratory inhibitors such as carbon monoxide,

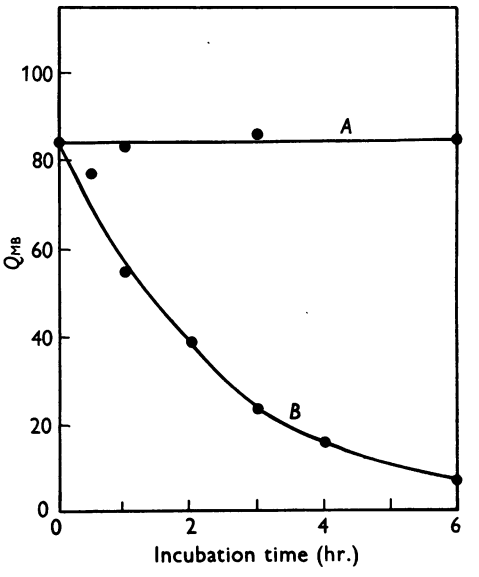

Fig. 1. Inactivation of succinic dehydrogenase by cyanide. Heart-muscle preparation in 0·1 M-phosphate buffer, pH 7·3, was incubated at 38°, alone (curve *A*) and with 0·005 M-cyanide (curve *B*). Portions were taken at suitable intervals for activity estimation.

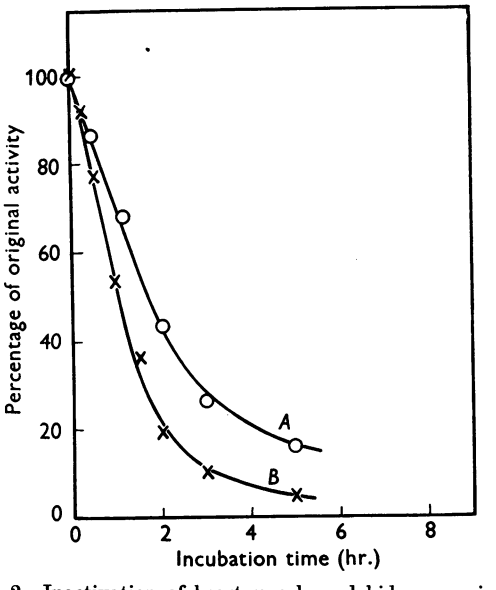

Fig. 2. Inactivation of heart-muscle and kidney succinic dehydrogenase by cyanide. Heart-muscle preparation (curve *B*) and kidney preparation (curve *A*), both in 0·1 M-phosphate buffer, pH 7·3, were incubated with 0·01 M-cyanide at 38°. Portions were taken at suitable intervals for activity estimation. In order to compare the two curves, activity of both preparations is expressed in percentage of original activity. Both preparations were perfectly stable in absence of cyanide.

Table 1. *Inactivation of succinic dehydrogenase by cyanide*

(Experiments were carried out in Thunberg tubes, one of them (no. 4) with two hollow stoppers. The tubes contained 0·1 ml. dehydrogenase (enz.); 0·04 M-succinate (suc.); 0·3 mM-methylene blue (MB); 0·02 M-KCN, neutralized; and 0·1 M-phosphate buffer, pH 7·3 (buf.). When KCN in solid form was added (5 mg.) in one of the hollow stoppers, an equivalent amount of H_2SO_4 (0·7 ml., 0·1 N) was added to the other. Volumes were made up to 4 ml. with water. After being allowed to stand at room temperature for 4 hr. the tubes were evacuated and warmed to 38°. The contents of the main tubes and the hollow stoppers were mixed and the reduction time measured.)

Tube no.	Main tube	Hollow stoppers A	Hollow stoppers B	Reduction time min.	Reduction time sec.
1	Enz. + buf.	MB + suc.	—	2	25
2	Enz. + buf. + HCN + suc.	MB	—	2	30
3	Enz. + buf. + HCN	MB + suc.	—	28	—
4	Enz. + buf.	MB + suc. + H_2SO_4	KCN	2	20

sulphide and azide on the enzyme was also studied. None of them, however, shows any action on the enzyme even after prolonged incubation either in presence or in absence of the substrate.

Irreversibility. When the cyanide-treated muscle preparation was freed from cyanide by dialysis the cytochrome-oxidase activity was completely restored, whereas the succinic dehydrogenase remained inactive. This is shown in Table 2. In order to ensure the complete removal of cyanide, the cyanide-treated enzyme preparation was dialysed as described before and then incubated with a large excess of methaemoglobin at room temperature for 24 hr. Methaemoglobin is known to have a high affinity for cyanide and should have reacted with any remaining cyanide. Estimation by the Thunberg method revealed that 24 hr. incubation with excess methaemoglobin had no effect whatsoever on the inactivated succinic dehydrogenase of the cyanide-treated and dialysed preparation as is shown in Table 3.

Table 2. *The activity of cyanide-treated heart-muscle preparation*

(Heart-muscle preparation was incubated with 0·01 M-cyanide at pH 7·3 for 18 hr. at room temperature. Cyanide was removed by dialysis at the end of incubation. As a control the same preparation was incubated with 0·01 M-KCl and dialysed simultaneously. Activity of succinic dehydrogenase (Suc.D.) is expressed as Q_{MB}; activities of succinic oxidase (Suc.O.) and cytochrome oxidase (Cyt.O.) are expressed as Q_{O_2} in μl./mg. fat-free dry wt./hr.)

System	Substrate	Added cytochrome c (mM)	Activity: Control	Activity: Cyanide treated
Suc.D.	Succinate	—	79	1
Suc.O.	Succinate	—	402	Nil
Suc.O.	Succinate	0·06	575	Nil
Cyt.O.	Ascorbate	—	25	27
Cyt.O.	Ascorbate	0·06	1850	1720
Cyt.O.	*p*-Phenylenediamine	—	460	480
Cyt.O.	*p*-Phenylenediamine	0·06	1490	1400

Table 3. *Effect of methaemoglobin on the cyanide-inactivated succinic dehydrogenase*

(Conditions of cyanide treatment and dialysis as in Table 2. Control with KCl as usual.)

Treatment	Succinic dehydrogenase (Q_{MB}): Control	Succinic dehydrogenase (Q_{MB}): Cyanide treated
Before treatment	79	79
After incubation with KCl and KCN respectively	79	1·5
After dialysis	77	1·2
After incubation with methaemoglobin at room temperature for 24 hr.	78	1·4

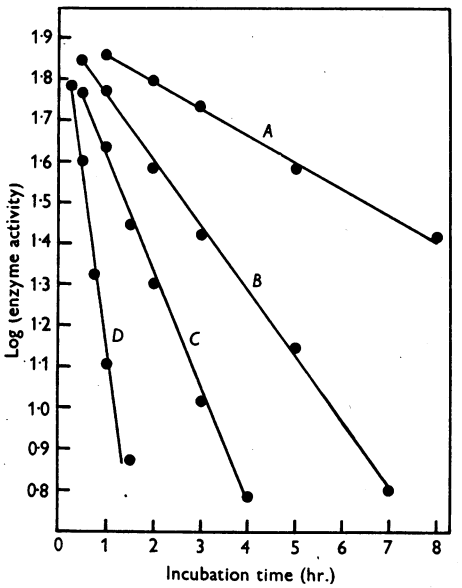

Fig. 3. Relation between the concentration of cyanide and the rate of inactivation of succinic dehydrogenase. Heart-muscle preparation was incubated at 22° with 0·005 M (*A*), 0·01 M (*B*), 0·02 M (*C*), 0·05 M (*D*) cyanide. Enzyme activity was expressed in Q_{MB}.

Inactivation kinetics. It was found that the reaction between succinic dehydrogenase and

cyanide followed the first-order equation 1 when cyanide was in excess over the enzyme

$$-\frac{d \ln E}{dt} = k[\mathrm{KCN}], \qquad (1)$$

where E is the concentration of the enzyme. A plot of log E against time gave a series of straight lines at different cyanide concentrations (Fig. 3). It follows from (1) that the time for 50 % inactivation ($t_{0.5}$) is given by

$$t_{0.5} = \frac{\ln 2}{k\,[\mathrm{KCN}]}. \qquad (2)$$

Hence a plot of $t_{0.5}$ against the reciprocal of cyanide concentration gives a straight line with a slope of $\ln 2/k$ (Fig. 4). The values of the reaction velocity constant k at 22° and 38°, calculated from the slopes of the straight lines obtained in Fig. 4 were,

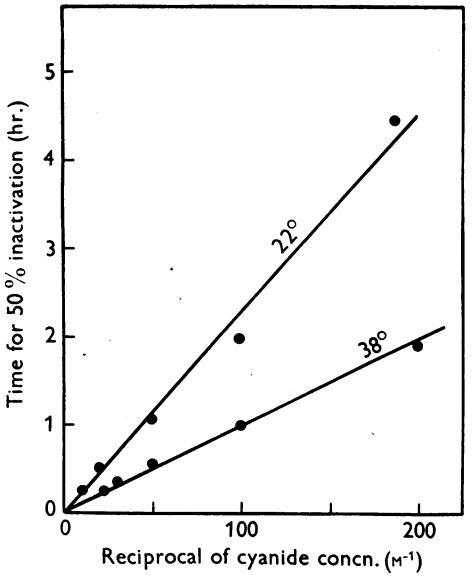

Fig. 4. Relation between the concentration of cyanide and the rate of inactivation of succinic dehydrogenase. Time for 50 % inactivation at 22° and 38° is plotted against reciprocal of cyanide concentration.

respectively, 0·51 and 1·15 l./mol./min. The average value obtained from Fig. 3 for 22° was 0·52. The energy of activation calculated from the Arrhenius equation was 9500 cal.

Effect of pH *of incubation.* Table 4 shows that change of pH of incubation from 6·5 to 8 had no effect on the rate of inactivation. As a difference of one pH unit in this region reflects approximately a tenfold difference in free cyanide ion concentration, the fact that a change in pH from 6·5 to 8 did not affect the rate of inactivation of the enzyme could best be explained by assuming the reaction as one between hydrocyanic acid and the enzyme.

Table 4. *Inactivation of succinic dehydrogenase at different* pH *of incubation*

(A heart-muscle preparation was prepared in the usual way. The final pH 5·5 precipitate was suspended in an equal volume of water, divided into three portions and each was then mixed with one-quarter its volume of 0·25 M-phosphate buffer of pH 6·5, 7·3 and 8·2, respectively. Potentiometric measurements with a glass electrode gave pH 6·43, 7·17 and 8·04 respectively for the three final preparations. The incubation was carried out in 2 ml. syringes. Each syringe received 1·8 ml. of the enzyme mixed with 0·2 ml. of 0·1 M-cyanide. The needles of the syringes were inserted into rubber bungs to prevent the escape of hydrocyanic acid vapour. KCl controls were carried out as usual. Activity estimations were carried out at pH 7·3. The Q_{MB} values after 2 and 4 hr. incubation at room temperature are listed as follows.)

	pH of incubation					
	6·43		7·17		8·04	
Incubation time (hr.)	KCl	KCN	KCl	KCN	KCl	KCN
0	78	79	82	81	80	80
2	80	41	78	40	80	38
4	79	17	81	18	79	14

Protection of the enzyme from inactivation. The protection of succinic dehydrogenase by succinate has already been shown in Table 1. Table 5 shows that 0·01 M-succinate completely protects the enzyme from the action of cyanide. The degree of protection by succinate depends upon its concentration as is shown in Fig. 5. Although a concentration of 2 mM-succinate was enough to give complete protection, a measurable degree of protection was given by as little as 0·02 mM.

In marked contrast to xanthine dehydrogenase, which can be protected from cyanide inactivation by several chemically related purine compounds, no other dicarboxylic acids, except succinic acid, were found to be effective in the case of the succinic enzyme. Malonate and fumarate, known to have a high affinity for the enzyme, were both ineffective; so were pyrophosphate, cystine and cysteine. This is shown in Tables 6–8.

Apart from succinate, only sodium dithionite was able to protect succinic dehydrogenase from the action of cyanide, although dithionite oxidized by aeration was ineffective. This is shown in Table 9.

Relation of succinic dehydrogenase to cytochrome b

A freshly prepared heart-muscle preparation in 0·1 M-phosphate buffer, pH 7·3, was incubated with 0·01 M-cyanide for 24 hr. at room temperature. At the end of the incubation, this preparation had a Q_{MB} of 1·4 which was less than 2 % of the original activity ($Q_{MB} = 86$). On the addition of a few drops of dihydrocozymase, there appeared a strong

reduced cytochrome *c* band together with a somewhat weaker reduced *b* band. Moreover, the cytochrome *b* present in the cyanide-treated enzyme preparation can also be reduced by the addition of a few drops of an untreated enzyme followed by succinate, although this reduction is rather slow.

Table 5. *Protection of succinic dehydrogenase from the action of cyanide by succinate*

(To 8 ml. of heart-muscle preparation were added 1 ml. of 0·1 M-succinate and 1 ml. of 0·1 M-cyanide. A second 8 ml. portion of the enzyme received 1 ml. of water and 1 ml. of 0·1 M-cyanide; and a third 8 ml. portion, 1 ml. water and 1 ml. of 0·1 M-KCl. The three mixtures were allowed to stand at room temperature for 18 hr., and then dialysed as previously described.)

Treatment		Activity (Q_{MB})
Before treatment		75
After incubation with:	KCN + succinate	75
	KCN alone	1
	KCl alone	74

If succinic dehydrogenase were identical with cytochrome *b*, it would be possible, on incubation with cyanide, to observe a parallel relation between the decrease in the succinic dehydrogenase activity and the intensity of the α-band of reduced cytochrome *b*. However, careful matching of the intensity of the α-band of reduced *b* in heart-muscle preparation treated with cyanide for different times failed to reveal any parallel relation. The matching was carried out against a standard of normal heart-muscle preparation with a low dispersion microspectroscope provided with a double-wedged trough. Even the inactivated preparation, with an activity of less than 2 % that of the original, could still be reduced by succinate, although it took some 45 min. for the reduced *b* band to appear, with an intensity about 15 % that of the original. This was not surprising as with this slow rate of reduction the autoxidation of cytochrome *b* in the presence of cyanide must also be taken in consideration. In the complete absence of air, the reduction of cytochrome *b* by succinate

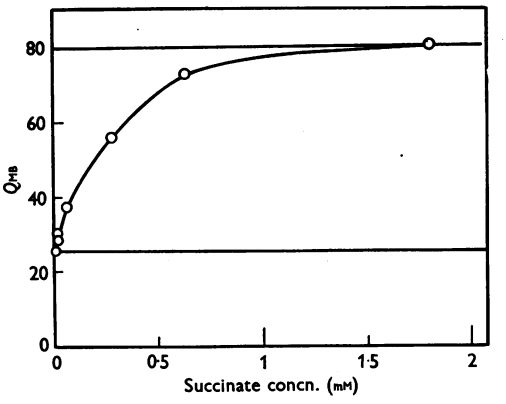

Fig. 5. Protective effect of different concentrations of succinate on the rate of cyanide inactivation of succinic dehydrogenase. The activity is plotted against succinate concentration. The upper horizontal line represents the Q_{MB} obtained without cyanide, thus corresponding to complete protection, while the lower line shows the Q_{MB} obtained after 3 hr. incubation with 0·01 M-cyanide without succinate and thus corresponds to zero protection. The intermediate points are the Q_{MB} obtained after similar incubation with cyanide in presence of different succinate concentrations.

Table 6. *Effect of malonate on the inactivation of succinic dehydrogenase by cyanide*

(Portions of enzyme preparation were incubated with 0·01 M-cyanide (1*b*, 2*b* and 3*b*) or with 0·01 M-KCl (1*a*, 2*a* and 3*a*) at room temperature. Portions 2*a* and 2*b* also contained 0·017 M-malonate and portions 3*a* and 3*b*, 0·05 M-malonate. At suitable intervals, 0·2 ml. samples were taken from each portion for activity estimation.)

Time of incubation (hr.)	Activity (Q_{MB}) 1*a* KCl	1*b* KCN	2*a* KCl	2*b* KCN	3*a* KCl	3*b* KCN	Percentage inactivation in presence of malonate (M) None	0·017	0·05
0	77	76	46	47	29	27	—	—	—
1	76	54	46	34	26	20	29	28	29
2	76	36	50	23	26	13	53	52	54
4	78	20	46	12	28	8	74	75	72

Table 7. *Effect of fumarate on the inactivation of succinic dehydrogenase by cyanide*

(Conditions as in Table 6, except that instead of malonate, portions 2*a* and 2*b* contained 0·017 M-fumarate and portions 3*a* and 3*b*, 0·05 M-fumarate.)

Time of incubation (hr.)	Activity (Q_{MB}) 1*a* KCl	1*b* KCN	2*a* KCl	2*b* KCN	3*a* KCl	3*b* KCN	Percentage inactivation in presence of fumarate (M) None	0·017	0·05
0	76	77	74	75	76	74	—	—	—
1	79	54	76	50	76	52	31	34	30
2	78	37	77	36	72	35	52	52	53
4	78	20	75	20	73	21	74	73	72

Table 8. *Effect of pyrophosphate, cysteine and cystine on the inactivation of succinic dehydrogenase by cyanide*

(The enzyme was incubated with 0·01 M-cyanide plus 0·01 M-pyrophosphate, 0·004 M-cysteine or 0·002 M-cystine for 6 hr. at room temperature. 0·2 ml. portions were taken for activity estimation at the end of incubation. Controls with KCl were carried out.)

	Activity (Q_{MB})	
	KCl	KCN
No addition	81	4·5
Pyrophosphate	74	4·0
Cysteine	72	3·2
Cystine	60	3·0

Table 9. *Effect of sodium dithionite on the inactivation of succinic dehydrogenase by cyanide*

(A heart-muscle preparation (4 ml.) was placed in the main tube of each of three Thunberg tubes each provided with two hollow stoppers. Solid KCN (0·5 mg.) was placed in one limb of each stopper and an equivalent amount of H_2SO_4 in the other. To the first tube was added 1 ml. of water, to the second, 1 ml. of a freshly prepared solution containing 10 mg. dithionite and to the third, 1 ml. of the same dithionite solution thoroughly aerated until it no longer had any reducing power. The three tubes were then evacuated, washed once with O_2-free nitrogen and again evacuated. The contents of the main tubes and hollow stoppers of the three Thunberg tubes were then mixed and allowed to stand for 18 hr. at room temperature. At the end of incubation, spectroscopic observations showed the presence in tube no. 2 of the cytochrome a_3-cyanide complex which indicated that dithionite had not combined with cyanide, the former being in excess. The three tubes were opened and dialysed until free from both cyanide and sodium dithionite.

Tube no.	Incubated with	Activity (Q_{MB}) after dialysis
Control	KCl	81
1	KCN alone	1
2	KCN + $Na_2S_2O_4$	82
3	KCN + aerated $Na_2S_2O_4$	2

in the cyanide-treated heart-muscle preparation, although extremely slow, eventually reached completion. Cytochrome *b* thus reduced was readily oxidized by methylene blue.

The pyrophosphate inhibition of succinic dehydrogenase

Leloir & Dixon (1937) expressed the view that pyrophosphate acts as an inhibitor of succinic dehydrogenase in the same way as malonate, i.e. competing with succinate for the enzyme by virtue of its two adjacent acid groups. In the present study, inhibition of succinic dehydrogenase by pyrophosphate in the presence of different pyrophosphate and succinate concentrations was measured by the Thunberg method. The results of measurements are plotted by Lineweaver and Burk's method in Fig. 6. They show conclusively that the pyrophosphate inhibition of succinic dehydrogenase is competitive in nature.

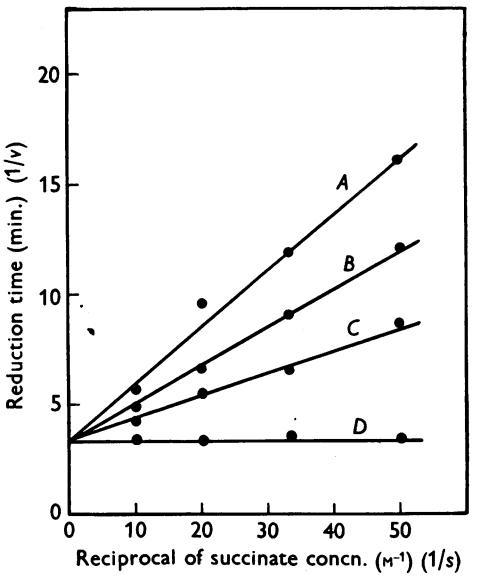

Fig. 6. The pyrophosphate inhibition of succinic dehydrogenase. The reciprocal of activity, in reduction time, is plotted against the reciprocal of substrate concentration. The fact that the four lines, representing different pyrophosphate concentrations (curves *A*, *B*, *C* and *D* represent 0·02, 0·0125, 0·0075 M and no pyrophosphate respectively), meet the ordinate at the same point shows that the inhibition is competitive.

DISCUSSION

Strictly speaking, the term 'succinic dehydrogenase' applies to the enzyme which is specifically concerned in the activation of succinate and probably acts also as the immediate hydrogen or electron acceptor. However, in fact, this term is generally used to denote that part of the succinic oxidizing enzyme system which is responsible for the catalysis of the anaerobic oxidation of succinate by dyes, such as methylene blue or dichlorophenol indophenol. Some workers even use the term succinic dehydrogenase when they are actually referring to the system that catalyses the aerobic oxidation of succinate. It seems most desirable, therefore, to emphasize here that in the present work the term 'succinic dehydrogenase' is used to denote the enzyme which is responsible for the specific activation of succinate and which, as evidence to be summarized later will show, also acts as the immediate hydrogen or electron acceptor. The term 'succinic dehydrogenase system', on the other hand, is used whenever confusion with the former term is likely to arise to denote the system which catalyses the anaerobic oxidation of

succinate by dyes. This system may include, besides succinic dehydrogenase, cytochrome *b* (Slater, 1949*b*). The differentiation between these two terms is necessary in view of the evidence presented in this paper which shows that succinic dehydrogenase is not identical with cytochrome *b*.

The protection of an enzyme from destruction by its substrate is a well known phenomenon. However, in most cases, protection is also given by compounds which are closely related in chemical structure to the substrate and are competitive inhibitors. In these cases the simple explanation that both the substrate and the closely related substances can combine with the enzyme thus blocking the active centre of the enzyme from attack by the destructive agents is adequate. In the present case the failure of dicarboxylic acids other than succinic acid and of pyrophosphate to protect succinic dehydrogenase from destruction by cyanide indicates that an altogether different explanation is necessary.

An alternative explanation is that cyanide reacts only with the oxidized form of the enzyme. Once the enzyme is reduced it no longer reacts with cyanide. This explanation also makes it necessary to place succinic dehydrogenase actually in the electron-transferring chain of the oxidation of succinate, that is to say, the enzyme, apart from being responsible for the activation of succinate, is also actually its immediate electron or hydrogen acceptor. Evidence in support of this view is as follows:

(1) Although 2×10^{-3} M-succinate is sufficient to protect the enzyme from the action of cyanide, and a measurable degree of protection can be obtained with a succinate concentration as low as 2×10^{-5} M, malonate and fumarate (0·05M) and pyrophosphate (0·01M) are quite incapable of giving any protection. All these acids are known to have a high affinity for the enzyme.

(2) In addition to succinate, the enzyme is protected only by sodium dithionite; an aerated solution of sodium dithionite, however, is incapable of giving any protection.

(3) If the enzyme is incubated in the complete absence of air with cyanide and succinate at a concentration sufficient only to give a partial protection, not only the rate of inactivation is reduced, but the inactivation remains incomplete.

Therefore it seems evident that succinate and sodium dithionite protect by keeping the enzyme in the reduced state which is not attacked by cyanide. If succinate is present at a concentration sufficient only to give a partial protection, the enzyme is kept in a partly oxidized and partly reduced state, and only the oxidized form is affected by cyanide.

The failure of Leloir & Dixon (1937) to obtain any inactivation by cyanide was probably due to the spontaneous inactivation of the enzyme, and the residual substrate present in the preparation then available to these workers (Ogston & Green, 1935). The findings of the present investigation are not incompatible with those of Potter (1941) that cyanide does not affect the enzyme responsible for the reduction of cytochrome *c* by succinate. Potter is mainly concerned with the amount of cytochrome *c* reduced by the enzyme system, whereas a partial inactivation of succinic dehydrogenase would affect the rate of cytochrome *c* reduction only.

It is practically certain that the reaction between cyanide and succinic dehydrogenase does not play any part in the mechanism of the cyanide inhibition of respiration since the enzyme is inactivated to an appreciable extent only after being allowed to remain in contact with cyanide in the complete absence of substrate for a long time.

Several suggestions have been made on the question of the chemical nature of succinic dehydrogenase and some of these suggestions will be briefly examined in the light of the present study.

Metal enzyme. Banga & Porges (1938) studied the inhibitory effect of some forty compounds and claimed to have obtained a correlation between the inhibitory and copper-combining powers of these compounds. Contrary to the view held by Leloir & Dixon (1937), Massart, Dufait & van Gremberger (1940) believed that the pyrophosphate inhibition of succinic dehydrogenase was non-competitive. They argued, therefore, that pyrophosphate acted by complex formation with a metal, probably manganese. However, Massart *et al.* (1940) did not produce any convincing evidence to show the pyrophosphate inhibition to be non-competitive. As the pyrophosphate inhibition has now been shown to be competitive, the only evidence in support of the claim of Massart *et al.* is removed. It is difficult to reconcile the view that copper is an integral part of this enzyme with the known strong inhibitory effect of this metal (Hopkins, Morgan & Lutwak-Mann, 1938; Horecker, 1939; Slater, 1949*c*) and the fact that diethyldithiocarbamate does not inhibit in the absence of air (Keilin & Hartree, 1940).

Cytochrome b. It has been pointed out that cytochrome *b* is involved in the anaerobic oxidation of succinate by methylene blue (Slater, 1949*b*). Bach, Dixon & Zerfas (1946) and Ball, Anfinson & Cooper (1947) have made the suggestion that succinic dehydrogenase may be identical with cytochrome *b*. Recently, Pappenheimer & Hendee (1949) examined the succinic dehydrogenase system in *Corynebacterium diphtheriae* in some detail, and expressed the opinion that succinic dehydrogenase is identical with cytochrome *b*. However, these workers produced very little evidence to show the quantitative correlation between succinic dehydrogenase activity and the intensity of the α-band of

ferrocytochrome *b*. As a result of the present work, the following evidence may be summarized in favour of the view that succinic dehydrogenase is not identical with cytochrome *b*.

Spectroscopic observations failed to reveal any parallel relation between the intensity of the α-band of ferrocytochrome *b*, when reduced by succinate, and the succinic dehydrogenase activity. When the succinic dehydrogenase was partially inactivated the rate of reduction of cytochrome *b* was delayed. The intensity of the α-band of ferrocytochrome *b*, when it eventually appeared, was as great as in the untreated enzyme.

When the enzyme was almost completely inactivated (activity less than 2% of the original) an appreciable amount of cytochrome *b* could still be reduced either by dihydrocozymase (Slater, 1950) or by succinate together with a few drops of untreated enzyme.

All cases of cyanide inhibition of haem proteins are reversible, no irreversible inactivation of a haem protein by cyanide being known.

If this view is accepted, the findings of Stoppani (1947, 1949) can also be easily understood. Stoppani found that narcotics inhibited the reduction of methylene blue but not the reduction of ferricyanide by succinate, and a liver preparation could only utilize ferricyanide as the acceptor, although the latter has not been confirmed by Keilin & Hartree (1949). Stoppani's findings can be explained by assuming that ferricyanide is capable of reacting directly with the dehydrogenase, whereas methylene blue can only react with cytochrome *b* (analogous to the coenzyme-linked dehydrogenases and diaphorase, Dixon & Zerfas, 1940) and that narcotics affect the interaction of the dehydrogenase and cytochrome *b*.

It may also be pointed out here that all the experimental facts now available do not exclude the possibilities of (1) a single enzyme molecule with two active centres concerned with succinic dehydrogenase and cytochrome *b* activity respectively, or (2) a separate factor operating between the true dehydrogenase and cytochrome *b*, this factor being affected by cyanide. The first possibility does not appear to be very likely, and unless some evidence is provided it seems unnecessary to postulate a separate factor.

Flavin. Keilin & Hartree (1940) observed two flavin bands at 455 and 495mμ. in their heart-muscle preparation and showed that these bands disappear on reduction with succinate. Axelrod, Potter & Elvehjem (1942) reported that livers of rats fed on a riboflavin-deficient diet were low in succinic oxidase activity. Since all the other known components of the succinic oxidase system are not flavin compounds, it is not improbable that the observed effect should be on the dehydrogenase itself. One may also recall that the 'cytochrome *b*' preparation of Yakushiji & Mori (1937) was supposed to contain a flavin. The only other dehydrogenase which is inactivated by cyanide in this peculiar way is xanthine dehydrogenase with flavin-adenine dinucleotide as its prosthetic group. It must be pointed out, however, that there are significant differences in the cyanide inactivation of xanthine and succinic dehydrogenases and the point of attack of cyanide on either enzyme is not yet clear. Recently, preparations of succinic dehydrogenase in soluble form have been briefly reported (Scott, 1950; Morton, 1950); however, final judgement on this matter must await further information.

SUMMARY

1. Succinic dehydrogenase was slowly but irreversibly inactivated by cyanide. The rate of inactivation depended upon the temperature of incubation and the cyanide concentration. The time required for 50% inactivation was inversely proportional to the cyanide concentration.

2. The rate of inactivation was independent of the pH of incubation from pH 6·5 to 8, indicating that the reaction involved hydrocyanic acid and not cyanide ion.

3. The enzyme could be protected from inactivation by succinate and sodium dithionite but not by malonate (50 mM), fumarate (50 mM), pyrophosphate (10 mM), cystine (2 mM) and cysteine (4 mM). The minimum concentration of succinate required to give complete protection was 2 mM and the minimum concentration to give a measurable protection, 0·02 mM.

4. Evidence has been summarized to show that succinic dehydrogenase, apart from being concerned with the specific activation of succinate, is actually its immediate electron acceptor, and only the oxidized form of the enzyme is susceptible to the attack by cyanide.

5. Additional evidence was obtained showing that the pyrophosphate inhibition of succinic dehydrogenase is competitive in nature.

6. Matching of the intensity of the α-band of reduced cytochrome *b* of cyanide-treated enzyme preparations showed no parallel relation with the succinic dehydrogenase activity of these enzyme preparations. After nearly complete inactivation of the dehydrogenase, cytochrome *b* can still be oxidized and reduced by various means. This evidence, and the fact that all cases of cyanide inhibition of haem proteins are reversible, led to the conclusion that succinic dehydrogenase is not identical with cytochrome *b*.

7. The possible chemical nature of succinic dehydrogenase has been discussed.

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Studies on Plant Formic Dehydrogenase

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Thunberg (1921, 1936) first discovered a formic dehydrogenase in the seeds of *Phaseolus vulgaris* (French bean). The same enzyme, obtained from peas by Fodor & Frankenthal (1930), was found to differ from the formic dehydrogenase of bacteria in that it required a coenzyme present in boiled pea juice. Andersson (1934) and Lichtenstein (1936) were able to identify this factor as coenzyme I (Co I). In 1937, Adler & Sreenivasaya, again using peas, studied the formic system in some detail, drawing attention to the strong inhibition obtained with low concentrations of cyanide. The widespread occurrence of the dehydrogenase in plant seeds and variation in the amount of enzyme during the life cycle of the pea has been reported (Davison, 1949*a*). The enzyme is most active in the mature dried seed and diminishes in activity with germination and growth. It increases again in the pod and developing seed.

The work described in this paper has been directed to finding a function for formic dehydrogenase in the metabolism of the plant. The enzyme has been purified 21-fold in 42% yield. Some observations on the properties of the dehydrogenase, including the Co I specificity, the effect of some inhibitors, especially some metal-binding reagents, and experiments on the coupling of the formic system with other dehydrogenases, are reported. Attempts to reverse the oxidation of formate by the dehydrogenase are described. The reduction of certain dyes by the system has been investigated, and attempts have been made to reverse the reaction by poising against one of these dyes (benzyl viologen). Other experiments designed to show such a reversal are those on the effect of carbon dioxide on Co I reduction, and the coupling of the system with the oxidation of triosephosphate. The possible significance of the system in the metabolism of the plant is discussed.

METHODS AND MATERIALS

Pea seeds. Pisum sativum (var. Meteor) were used.

Bean seeds. Phaseolus multiflorus (var. Giant White Czar) were used throughout this work.

Coenzyme I. This was prepared by the method of Williamson & Green (1940), modified by Ochoa (1948). It was 41% pure when estimated by reduction either with lactic dehydrogenase and lactate in the presence of cyanide, or with formic dehydrogenase and formate.

Coenzyme II. This was prepared by the method of Warburg, Christian & Griese (1935), modified by Haas (1947), and found to be 50% pure when estimated by the heart *iso*citric dehydrogenase-aconitase preparation of Adler, Euler, Günther & Plass (1939). The method of estimation, as with Co I, depends on the measurement of reduced coenzyme in an open tube in the Beckman quartz