STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

VI. Inhibitor Studies with Succinic Dehydrogenase*

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In 1938, Hopkins and coworkers (1, 2) showed that succinic dehydrogenase could be inactivated by oxidized glutathione (GSSG) and could be reactivated by reduced glutathione (GSH). They interpreted these results to mean that the active enzyme requires intact —SH groups and that when these are converted to the —S—S— form of the enzyme, the dehydrogenase is inactivated. Any assumption that the functioning of the enzyme involved an oscillation between the SH and the —S—S— form of the enzyme seemed to be definitely eliminated, however, by the fact that the —S—S— form could not be reduced by succinate. Thus the function of the SH group in succinic dehydrogenase has remained an unsolved problem.

Although many proteins contain SH groups, very little is known about the structural relationship of the SH group to the rest of the molecule. Even in the case of egg albumin, in which the SH groups have received the most careful study, the mechanism by which the SH groups of native egg albumin are shielded from some sulfhydryl reagents and not from others remains obscure (3). In the case of succinic dehydrogenase, the presumptive SH group (1, 2) is associated with function, and the reaction of the protein with sulfhydryl reagents should be demonstrable on the basis of determinations of the amount of active enzyme remaining. Furthermore, since it is an oxidative enzyme, the measurement of oxygen uptake makes possible a continuous appraisal of the amount of active enzyme at any given moment. We have previously established the test conditions for the measurement of the activity of this enzyme (4, 5). The rate of oxygen uptake is a valid measure of succinic dehydrogenase activity in this system since cytochrome *c* and cytochrome oxidase, which are needed to complete the reaction with oxygen, are present in excess. The activity of the enzyme is so great under the proper conditions that the extraneous matter present in the enzyme preparation does not interfere with the study of the reaction.

At present, inhibitor studies appear to constitute the only available means of establishing the presence of SH groups in succinic dehydrogenase and of determining their rôle in the function of the enzyme.

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In the work to follow, the nature of the SH groups in succinic dehydrogenase has been studied by testing the inhibitory action of some 38 different chemical compounds over a wide range of concentrations and in the presence and absence of the substrate. In addition, malonate, which is itself an inhibitor, was shown to decrease the action of other inhibitors. On the basis of the inhibitor experiments, it has been possible to approximate the structure of the succinate-activating center of the dehydrogenase and to provide an explanation for the earlier results of Hopkins, thus reopening the possibility that the SH group may be involved in hydrogen transport in this enzyme.

EXPERIMENTAL

Enzyme System.—Studies in this laboratory (4, 5) have led to the development of an enzyme preparation in which the components of the succinoxidase system are believed to approximate their native functional activity more closely than in systems hitherto studied. Rat liver homogenates were freshly prepared and kept in ice water until pipetted. The reaction mixtures contained 0.8 ml. of 0.1 M sodium phosphate pH 7.4, 0.4 ml. of 10^{-4} M cytochrome *c*, 0.3 ml. of 4×10^{-3} M $CaCl_2$, 0.3 ml. of 4×10^{-3} M $AlCl_3$, 0.3 ml. of 0.5 M recrystallized sodium succinate pH 7.4, 0.1 ml. of 10 per cent liver homogenate in 0.033 M sodium phosphate pH 7.4, plus water and inhibitor solution to give a final volume of 3.0 ml. About once a week the calcium, aluminum, and cytochrome were made up into one solution of which 1 ml. was pipetted into each flask. All solutions were stored in the cold and kept in ice water during the pipetting. The rate of oxygen uptake was measured in a conventional Warburg apparatus at 38°C. The need for the various components has been previously demonstrated (4, 5). The enzyme system as set up will not oxidize succinate beyond the fumarate stage.

Inhibitor Experiments.—During the course of this work, a wide variety of compounds were tested for toxicity to the succinoxidase system. The results are summarized as briefly as possible in Table I. Although the concentration of inhibitor was varied over a wide range, only one concentration is reported. In most cases, the presence of succinate decreased the action of the inhibitors; therefore, the enzyme was incubated in the absence of its substrate, and the activity was measured by delaying the succinate addition. In these cases, less inhibitor was required for a given amount of inhibition than in the case of experiments in which succinate was present at the beginning. In the delayed succinate experiments, the action of the inhibitor was usually complete by the time the substrate was added, while in those cases where succinate was present from the beginning, the per cent inhibition usually increased with time. In the latter experiments, it is thus necessary to specify the time at which the per cent inhibition was computed, and in Table I the percent inhibition is stated for the second and fourth 10 minute intervals. Although most of the inhibitors would give 100 per cent inhibition if added in sufficient quantity, an attempt was made to report those concentrations which gave 50 to 80 per cent

TABLE I

Inhibition of Succinoxidase

Test system described in text. Inhibition calculated from Q_{O_2} values, with controls in each run.

No.	Compound	Substrate added at 20 min.		Substrate added at the beginning		
		Inhibitor concentration	Inhibition	Inhibitor concentration	Inhibition 10–20 min.	Inhibition 30–40 min.
	Quinones and related compounds					
			per cent		*per cent*	*per cent*
1	Quinone	M/100,000	65	M/30,000	50	68
2	1,4 Naphthoquinone	M/100,000	43	M/30,000	21	30
3	2-Methyl naphthoquinone	M/10,000	53	M/1,000	44	45
4	9:10 Anthraquinone	Saturated	0	Saturated	0	0
5	Hydroquinone	M/100,000	65	M/30,000	11	35
6	Catechol	M/100,000	35	M/30,000	3	5
7	Resorcinol	M/10,000	0	M/1,000	0	0
	Azo compounds and diamines					
8	*p*-Phenylenediamine	M/100,000	65	M/30,000	26	54
9	*p*-Aminophenol	M/30,000	43	M/100,000	17	46
10	*o*-Phenylenediamine	M/30,000	37	M/1,000	7	7
11	*N*-Methyl-*p*-phenylenediamine	M/100,000	65	M/30,000	43	72
12	*p*-Aminodimethylaniline	M/100,000	71	M/30,000	45	77
13	2-Methyl N^4, N^4 dimethyl 1,4 phenylene-diamine (meta)*	M/100,000	72	M/30,000	15	26
14	2-Methyl N^1,N^1-dimethyl 1,4 phenylenediamine (ortho)*	M/100,000	68	M/30,000	0	0
15	*p*-Aminoacetanilid	M/1,000	4	M/100	50	53
16	Sulfanilamide	M/100	0	M/100	0	0
17	*p*-Nitrosodimethylaniline	M/2,000	4	M/2,000	22	18
18	*p*-Aminoazobenzene	M/2,000	43	M/2,000	36	35
19	*p*-Dimethylaminoazobenzene‡	M/200	55	M/200	25	24
20	Methyl orange	M/1,000	33	M/200	42	48
21	Methylene blue	M/3,000	63	M/3,000	20	20
	Sulfhydryl reagents					
22	Iodine	M/3,000	52	M/3,000	4	4
23	Iodoacetate	M/1,000	52	M/100	17	31
24	Iodoacetamide	M/1,000	90	M/100	68	83
25	Maleic acid	M/25	17			
26	*p*-Chloro-mercuro-benzoic acid	M/100,000	30	M/30,000	63	70
27	Ferricyanide	M/1,000	89	M/1,000	32	58
	Metal cations					
28	$Copper^{++}$	M/100,000	64	M/30,000	67	83
29	$Zinc^{++}$	M/100,000	30	M/30,000	56	63
30	$Iron^{+++}$	M/3,000	29	M/1,000	32	58
	Toxic anions					
31	$Selenite^{--}$	M/100,000	46	M/30,000	11	20
32	$Arsenite^{---}$	M/10,000	25	M/1,000	16	42

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‡ Dissolved in corn oil and homogenized with the tissue. Corn oil or dye non-toxic alone.

inhibition for the majority of the compounds and to report the inhibition obtained at this same concentration with compounds which were less toxic.

Although it is not possible to discuss each inhibitor at length and to give all the reasons which led to their selection, it may be stated that each compound serves as a control for one or more other compounds in the series, and all of the compounds studied are reported for the sake of completeness. Of the 38 compounds listed, certain key compounds enable one to deduce the nature of the active center in succinic dehydrogenase.

It is desirable at the outset to emphasize the oxidative capacity of the enzyme system. Many of the compounds used are in the reduced form (*p*-phenylenediamine, *p*-aminophenol, hydroquinone, etc.), but in the presence of cytochrome *c* they are rapidly oxidized. It seems certain that the toxicity of these compounds arises from their properties in the oxidized state, as Potter (6) demonstrated in studies with the urease system. This conclusion is supported by the results with the succinoxidase system.

The key compound of the series is *quinone* (*p*-benzoquinone) (No. 1 in Table I), and 21 of the 38 compounds studied are related to this substance. Of the 21 compounds, those which are toxic either contain the quinonoid structure or are converted to a quinonoid structure in this system. It seems likely that any quinonoid compound will inhibit succinic dehydrogenase but that additional factors such as solubility, molecular size, and configuration lower the toxicity as the compound deviates from the properties of quinone. Thus it will be seen that quinone is the most toxic compound in the quinonoid series, although many of the diamines are equally toxic (the difference between 65 per cent and 72 per cent inhibition is not considered significant). Naphthoquinone (No. 2) is nearly as toxic as quinone, but the introduction of a methyl group to give *2-methyl naphthoquinone* (artificial vitamin K) (No. 3) decreases the toxicity almost tenfold.[1] It has been shown (7) that quinone will combine with the SH group, and Fieser (8) has carried out a number of chemical studies demonstrating the same reaction with 2-methyl naphthoquinone. Although the 2-methyl naphthoquinone was also shown to react with certain alcohols and amino acids, it is interesting to note that Fieser concluded, "it is likely that methylnaphthoquinone can combine with proteins most readily by utilization of the sulfhydryl groups rather than the ϵ-amino groups." In the case of the next compound, *9:10 anthraquinone* (No. 4), no toxicity whatsoever could be observed (Table I), and it is obvious that the reaction postulated for quinone could not occur with this compound.

[1] The toxicity observed clinically with high doses of this compound (8) may be due to interaction with succinic dehydrogenase or a similar enzyme. The tenfold decrease in toxicity resulting from the introduction of one methyl group provides a explanation for the absence of clinical toxicity with *natural* vitamin K since it contains a long side-chain at the 3 position in addition to the 2-methyl group, and reaction with a sulfhydryl group is manifestly impossible.

Turning now to the various quinols, it is seen that *hydroquinone* (quinol) (No. 5) is just as toxic as quinone under strongly oxidizing conditions. When oxidation is less because of succinate, the compound is less toxic than quinone. This result, plus the data on quinone, proves that the action of succinate is twofold: it protects the enzyme against the inhibitors directly, and it slows the conversion of reduced compound to the toxic oxidized form. Catechol (No. 6), which is oxidized to the orthoquinone, further demonstrates this point. Resorcinol (No. 7), which is not autoxidizable and does not reduce cytochrome *c*, is not oxidized in this system. Thus it cannot be converted to the quinonoid structure and is completely non-toxic.

A number of compounds in which the OH groups of the quinols are replaced by amino groups have as the key compound *p*-phenylenediamine (No. 8); this is analogous to hydroquinone. A mixed compound is *p*-aminophenol (No. 9) in which only one of the OH groups is replaced by $-NH_2$. The ortho structure is represented by *o*-phenylenediamine (No. 10). Methyl groups can be substituted for hydrogen on the ring or in the amino groups (compounds 11 to 14) without loss in toxicity. Compound 14 is not autoxidizable and gives no inhibition when its oxidation by cytochrome is hindered by succinate. All of the *toxic* amino compounds are oxidized to the *quinonoid* structure. Various deviations which decrease the ease of conversion to the quinonoid structure result in great decreases in toxicity. This is most striking in the case of *p*-aminoacetanilide (No. 15) which is essentially non-toxic and is not oxidized to the quinonoid structure in this system. It may be contrasted with *N*-methyl-*p*-phenylenediamine (No. 11) which has a very similar structure but is very toxic and is readily oxidized to the quinonoid diimine. Further illustration of this point is sulfanilamide (No. 16) and *p*-nitrosodimethylaniline (No. 17) which are not oxidized and the azotized compounds *p*-aminoazobenzene (No. 18) and *p*-dimethylaminoazobenzene (No. 19). The latter compound is quite insoluble in water and, when sulfonated, forms methyl orange (No. 20) which is water-soluble and much more toxic than its parent compound though it has less than 1 per cent of the toxicity of quinone. It forms the quinonoid structure but is probably less toxic because of the size of the molecule. The same is probably true for methylene blue (No. 21) which is toxic and possesses the quinonoid structure. Cedrangolo and Adler (9) have shown that methylene blue will oxidize the SH of cysteine and glutathione and that it will inhibit triosephosphate dehydrogenase. They suggested that the mechanism of the inhibition was interaction with the SH of the enzyme. A number of other oxidation-reduction dyes have been shown (10) to be toxic for the succinic system, with toxicity being a function of the potential of the dye. Phenothiazone (11) and pyocyanine (12) also inhibit succinic dehydrogenase. All of the toxic compounds mentioned thus far possess the quinonoid structure. Although they might conceivably react with some group other than the SH group of the enzyme, the evidence seems to point to the SH group.

However, it seems desirable to consider the effect of other compounds which are toxic but which do not possess the quinonoid structure. A number of reagents have been used to titrate the SH groups of proteins such as egg albumin or to inhibit enzymes on the basis of interaction with SH. The literature on most of these compounds has been reviewed by Hellerman (13, 14). The compounds include iodine (No. 22), iodoacetic acid (No. 23), iodoacetamide (No. 24), maleic acid (No. 25), *p*-chloro-mercuro-benzoic acid (No. 26), and ferricyanide (No. 27). Several of these compounds have been shown to react *stoichiometrically* with the SH groups of denatured egg albumin (3) but with varying degrees of completeness in the case of the native egg albumin. It seems reasonable to conclude that they react with the SH groups of succinic dehydrogenase in a similar manner and that the latter are simply less accessible to these compounds than to quinone.

Many metallic ions are known to react with sulfhydryl groups (2, 13, 14). Experiments with copper (No. 28), zinc (No. 29), and iron (No. 30) are included in Table I. The ferric ion is much less toxic than zinc and copper. The latter is stoichiometrically equivalent to quinone, and the rate of inactivation is about the same. The ability of copper to react with thiols has been amply demonstrated by Pirie (15) who prepared a number of crystalline copper derivatives of SH compounds. In addition to the heavy metal cations which react with thiols, certain anions will also combine with the SH grouping. These are the selenite ion (No. 31) and the arsenite ion (No. 32). Bersin (16) reported that thiol compounds will combine with selenite, while Johnson and Voegtlin (17) demonstrated their combination with arsenite. The latter workers prepared a number of crystalline derivatives and postulated that the toxic action of arsenite is due to chemical combination with cellular SH compounds essential to life.

The above 32 compounds represent quinonoid structures, SH reagents, metal cations, and anions. The most toxic compounds are quite similar in their action on the basis of rate of reaction, final inhibition, and effective molarity. There can be little doubt that the common denominator of all these inhibitors is their reaction with the sulfhydryl group of succinic dehydrogenase. The situation is similar to that with egg albumin, concerning which Anson (3) stated, "the SH group, however, is the only protein group known to react with both oxidizing agents and heavy metal compounds."

In the case of the studies with urease (6), it was concluded that the mechanism of reaction between the SH groups and the inhibitors was more likely to be a combination rather than an oxidation of EnSH to En—S—S—En. The same conclusion is reached in the present instance and, since the line of reasoning is the same, it need not be repeated here. The inactivation by combination with SH probably occurs with all the quinonoid compounds, SH reagents, metal cations, and toxic anions and is represented diagrammatically in

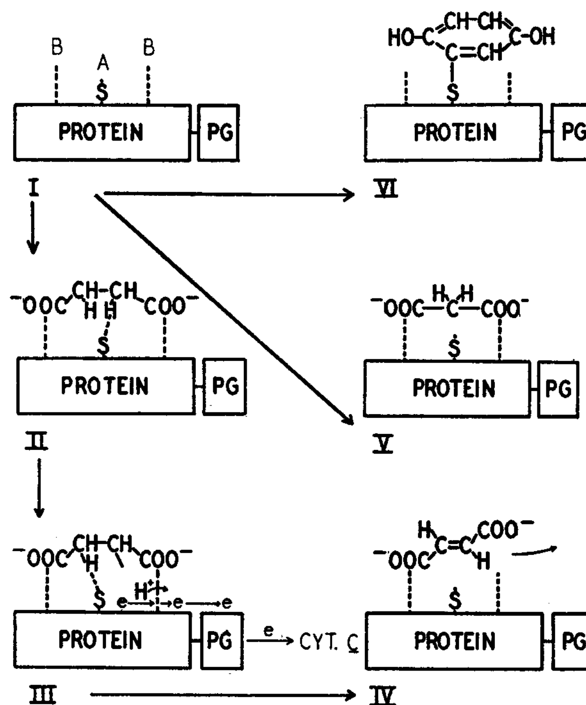

FIG. 1. Schematic representation of the structure of succinic dehydrogenase on the basis of *mutually exclusive* inhibitor reactions. The protein is succinic dehydrogenase, and PG represents its hypothetical prosthetic group.

I = enzyme showing the succinic acid activating center. A is a sulfhydryl amino acid, *e.g.*, cysteine, in a peptide chain; B and B′ are the carbonyl affinity points, *e.g.*, —NH— groups of adjacent peptide linkages, capable of forming hydrogen bonds with the carbonyl oxygens.

II = enzyme-succinic acid complex in first stage of succinic acid oxidation.

III = enzyme-succinic acid complex in a possible intermediate stage of succinic acid oxidation.

IV = enzyme-fumaric acid complex resulting from the oxidation of succinic acid.

V = enzyme-malonic acid complex. Only the carbonyl affinity points are involved. Inhibition reversible. Enzyme-sulfhydryl *shielded* by malonate and thereby protected against sulfhydryl reagents.

VI = enzyme-quinol complex, as an example of inhibition by a sulfhydryl reagent (quinone). Analogous complexes formed with other quinonoid compounds, thiol reagents, thiols, heavy metals, arsenite, and selenite.

Fig. 1, stage VI, using quinone as the inhibitor. In the case of urease, it was possible to *prevent* but not to reverse the inactivation due to quinones by adding cysteine to the reaction mixture. This fact gives further support to the

postulated mechanism. However, such experiments are not possible with the present succinic system since, in contrast to the urease system, cysteine is oxidized to a form which inactivates the succinic dehydrogenase as is shown in the next section.

Inhibition by Sulfhydryl Compounds.—In the original experiments by Hopkins *et al.* (1, 2), it was found that R—S—S—R would inactivate succinic dehydrogenase and that RSH would restore the activity. We have tested our succinoxidase system and have found that both cysteine and cystine inactivate the enzyme. Reduced glutathione will cause some inactivation (see Table II). The apparent disagreement with Hopkins *et al.* is only superficial, however, and is easily understood when the respective experimental techniques are considered. Hopkins and his coworkers added large amounts of the sulfhydryl compounds to washed muscle pulp, incubated the mixture, and then washed out the excess of soluble sulfhydryl compound on a Buchner funnel. Thus, the SH compound was never present when the activity of the enzyme was being tested. In our work, we used considerably less enzyme in much greater dispersion so that in fact it could not be washed on a funnel and recovered. Thus, we add much less sulfhydryl and leave it in the final reaction mixture, where it is converted into the oxidized form. Separate experiments in the absence of the enzyme show that RSH is oxidized by cytochrome *c*, and it seems highly probable that the free sulfhydryl radical RS· must be formed as an intermediate in such a reaction even though it dimerizes rapidly to R—S—S—R (14). It seems more likely that the enzyme is inactivated by the formation of En—S—S—R than by oxidation to En—S—S—En (in Hopkins' experiments as well as our own). In either case, the reactivation is undoubtedly due to the splitting of the disulfide linkage by RSH to reconstitute En—SH. Table II also shows

TABLE II

Inhibition of Succinoxidase by Sulfhydryl Compounds

Substrate addition delayed for 30 minutes. Egg albumin "molarity" is the cysteine molarity of the albumin used. Test system as described in text. Activity based on the 10 to 40 minute period after addition of substrate.

Inhibitor molarity	Inhibition				
	Cystine	Cysteine	Reduced glutathione	Egg albumin	
				Native	Denatured
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
M/500		93	18	0	0
M/1,000		69	0	0	0
M/3,000	51*	55*	0	0	0

* Inhibition was zero when succinate was added originally.

that native egg albumin, which will not reduce cytochrome *c*, is non-toxic at concentrations where cysteine is effective. Denatured egg albumin has free SH groups which rapidly reduce cytochrome *c*, but due to steric hindrance these oxidized SH groups cannot react with the SH of native succinic dehydrogenase as effectively as cysteine. However, when the concentration of egg albumin, native or denatured, is raised to higher levels, succinic dehydrogenase can be inhibited. This fact is perhaps analogous to the fact that large quinonoid molecules are much less toxic than quinone but are nevertheless toxic (Table I). The rate of inactivation of the dehydrogenase by the smaller sulfhydryl compounds in the presence of cytochrome system is about the same as the inactivation by copper, quinone, and all of the other inhibitors which are believed to combine with the SH group of the enzyme. It is generally agreed that inactivation of an enzyme by R—S—S—R is due to interaction with the sulfhydryl of the enzyme (1, 2, 13, 14), and such a conclusion is indicated here. Thus, inhibition of the enzyme by the oxidized sulfhydryl compounds appears to proceed by the same mechanism as in the case of the quinonoid compounds, heavy metals, sulfhydryl reagents, arsenite, and selenite.

Inhibition by Malonate.—In contrast to all of the previously mentioned inhibitors which are believed to inactivate the succinic dehydrogenase by combining with the SH group of the enzyme, malonic acid appears to inhibit succinic dehydrogenase by an entirely different mechanism. Malonate is a strong inhibitor of the succinic dehydrogenase because it possesses two —COOH groups and a configuration very similar to that of succinate (18, 19). Previous experiments with minced tissue or slices appear to be complicated by diffusion effects and require up to 0.025 M malonate to block the enzyme completely (20). According to Krebs and Eggleston (20), the inhibition is competitive; *i.e.*, it does not depend upon the absolute concentration of malonate but on the ratio, succinate/malonate. These workers found an inhibition of 50 per cent when the ratio was 9.5/1. The data in Table III confirm their observation that inhibition by malonate is competitive. However, when these data are used to calculate the succinate/fumarate ratio at the points of 50 per cent inhibition, one obtains values of 57.2, 51.8, and 50.6 for the three succinate concentrations employed. These ratios represent a close approximation of the ratios between the dissociation constants for the enzyme-succinate and enzyme-malonate complexes and are about 10 times as high as those reported by Krebs and Eggleston. The difference is probably due to the fact that in our preparation the enzyme is operating at maximum activity. The data support the view that malonate and succinate form similar dissociable complexes with succinic dehydrogenase and that the same affinity points are involved. The nature of these complexes is illustrated diagrammatically in Fig. 1, stages II (succinate) and V (malonate). That the affinity points involve the two carboxylic acid groups is indicated by previous work (2, 18, 19). There is no evidence contrary

to the statement by Hopkins *et al.* (2) that "malonic acid . . . apparently establishes no special relations with thiol groups."

Shielding Action of Malonate.—Hopkins *et al.* reported that malonate and succinate would prevent the inactivation of succinic dehydrogenase by GSSG. We have confirmed the protection by succinate against the SH inhibitors (Table I) and against cysteine and cystine (Table II) and now present evidence demonstrating protection of the enzyme by malonate against the action of quinone. (See Fig. 2.) Similar results were obtained with *p*-phenylenediamine. The data in Fig. 2 also show the difference between the malonate and the quinone inhibition. The results are expressed in terms of the Q_{O_2}, and the observed rate for each 10 minute period is plotted against time. The Q_{O_2} value

TABLE III

Inhibition of Succinoxidase by Malonate. Effect of Substrate Concentration

Test system described in text. Succinate and malonate both added prior to enzyme. Q_{O_2} calculated on basis of 1st 40 minutes.

Substrate concentration	Malonate concentration			
	0	M/300	M/1,000	M/3,000
	Q_{O_2}	Q_{O_2}	Q_{O_2}	Q_{O_2}
M/15	101	30	61	79
M/20	102	24	49	74
M/30	101	13	36	63

thus gives the amount of active enzyme present at any given moment. A detailed examination of Fig. 2 shows the following:

(*a*) Quinone inhibits progressively as shown in curve 2, with almost no inhibition originally and approaching complete inhibition after 60 minutes. When the enzyme is not protected by succinate, the rate of inactivation is at least twice as rapid, as shown by curve 4. These results show that succinate protects the enzyme against quinone and that, when the quinone combines with enzyme, the combination remains inactive. Apparently quinone can react only with enzyme molecules which are not combined with succinate.

(*b*) Malonate exerts its action at once, and the per cent inhibition remains constant (curve 3); the inhibition is no greater when the malonate is incubated with the enzyme in the absence of succinate (curve 5). These results, together with the data in Table II, show that malonate forms a dissociable complex with the enzyme which is comparable to the true enzyme-substrate complex and, furthermore, that the same affinity points are involved for succinate and malonate; that is, malonate does not damage the enzyme but merely competes with succinate for the succinate affinity points.

(*c*) Malonate can prevent quinone from acting on succinic dehydrogenase

whether succinate is added at once (curve 6) or after an incubation period (curve 7). Since the dissociation constant for the enzyme-malonate complex is only about one-fiftieth that of the enzyme-succinate complex (see above), the failure of quinone to inhibit in the presence of malonate is probably due to the fact that there are virtually no uncombined enzyme molecules available

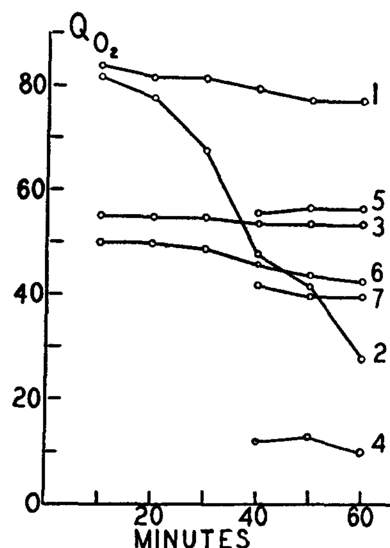

FIG. 2. Shielding of succinic dehydrogenase by malonate against quinone. Rate of inactivation of succinoxidase system (*i.e.*, rate of change in Q_{O_2}) as effected by malonate and quinone, separately and together, with original and delayed succinate additions. See Fig. 1, stages I, II, V, and VI for graphic interpretation of results. Test system as described in text. Final inhibitor concentrations: malonate, M/3,000; quinone, M/50,000. 1, control—neither inhibitor added; succinate originally present. (Loss in enzyme activity is negligible when succinate addition is delayed 35 minutes.) 2, quinone and succinate originally present. 3, malonate and succinate originally present. 4, quinone originally present; succinate added 35 minutes later (rate during next 10 minutes not plotted). 5, malonate originally present; succinate added 35 minutes later (rate during next 10 minutes not plotted). 6, malonate, quinone, and succinate originally present. 7, malonate and quinone originally present; succinate added 35 minutes later (rate during next 10 minutes not plotted).

for the quinone to attack; that is, the malonate shields the enzyme against the quinone. Curves 6 and 7 could also be explained by assuming that malonate reacts with quinone directly and thereby prevents it from exerting its effect. That such is not the case, however, was proved by the following experiment which was suggested by previous experiments with urease (6) showing that this enzyme is very sensitive to quinone: urease was tested with malonate and

quinone since, if the action of malonate in the succinic dehydrogenase system were due to reaction with quinone rather than combination with the dehydrogenase, it should also protect urease against quinone. The results showed that malonate does not inhibit urease and therefore has no affinity for this enzyme, and furthermore, it has no ability to protect urease against the action of quinone.

DISCUSSION

The data presented above deal with the inhibition of succinic dehydrogenase by a group of diverse substances which are believed to act by combining with the SH group of the enzyme, and in contrast to these, a compound, malonic acid, which is believed to act by competing for the carboxylic acid affinity points which form the basis of the succinate-enzyme complex. These fundamentally different mechanisms of combination may be shown schematically as in Fig. 1, in which quinone as an example of the SH inhibitors is shown in combination with the enzyme SH group (stage VI), while succinate and malonate combine with the enzyme on the basis of their —COOH groups (stages II and V).

Experimentation with inhibitors *whose action is mutually exclusive* seems to afford one of the most promising approaches now available for the study of the stereochemistry of enzymes. Though other possibilities most certainly may exist, it is difficult to explain the ability of malonate to protect succinic dehydrogenase against quinone in any terms other than those depicted in Fig. 1, in which the SH group is located *between* the carboxyl affinity points in such a way that, when malonate or succinate is combined with the enzyme, the SH group is *shielded* against attack by the SH reagents.

Hopkins *et al.* demonstrated three facts of paramount interest to this problem: (*a*) malonate will protect active succinic dehydrogenase against inactivation by GSSG; (*b*) enzyme which has been inactivated by treatment with GSSG may be reactivated by treatment with GSH; and (*c*) malonate will *not* prevent GSH from reactivating enzyme which has been inactivated by GSSG. These authors were unable to explain these seemingly paradoxical results and stated only, "That malonic acid protects from GSSG so completely and from GSH not at all suggests structural relations which may prove instructive." We believe that their observations constitute a contribution of fundamental importance which our own work confirms[2] and extends. Furthermore, on the basis of the concept illustrated in Fig. 1, it is easy to see why the results of Hopkins were obtained: if the SH group is located between the carboxyl affinity points, it is obvious that neither succinate nor malonate could ap-

[2] We do not present data on reactivation of GSSG-inactivated enzyme by GSH and the effect of malonate upon this reaction because of technical differences between our preparation and that of Hopkins.

proach the carboxyl affinity points (B and B′) when the enzyme is combined with an SH reagent while, on the other hand, if the enzyme is combined with succinate or malonate, the SH reagents cannot approach and react with the SH group.

While it is impossible to provide an answer as to the possible nature of the enzyme groupings which constitute the carboxyl affinity points, it is interesting to attempt a reconstruction of a peptide chain containing cysteine as the SH bearer. When this was done with accurate (Hirschfelder) atomic models, it was found that the —NH— groups adjacent to cysteine were opposite the carbonyl oxygens of the carboxyl groups, while the SH group fell between the —CH_2—groups of succinate. In the case of malonate, the carbonyl oxygens again approximated the —NH— groups, but the —CH_2— group was directed away from the SH, and both hydrogen atoms were on the side away from the SH.

The remainder of Fig. 1 is pure speculation but, since the structural relations shown in stages I, II, V, and VI seem to fit so well with the facts, it seems plausible to suggest that the location of the SH of the enzyme is of functional significance; *i.e.*, that it is involved in the actual mechanism of oxidation of succinate as is indicated in stages II, III, and IV. According to this concept, the enzyme would function by oscillating between the EnSH and EnS· forms, rather than between the thiol and disulfide forms. Succinate would be oxidized to the free radical monodehydrosuccinate in the first step of the oxidation, which would proceed in accordance with the principle of compulsory univalent oxidation described by Michaelis and Smythe (21). The electron might pass to the prosthetic group of the dehydrogenase intramolecularly without the intermediate dissociation of the semioxidized succinate. The idea of intramolecular electron transfer through a protein is not new but has been discussed by Muller (22) and by Szent-Gyorgyi (23) in general terms. The second electron would be disposed of in the same manner as the first, and the two hydrogen atoms would enter the medium as hydrogen ions. Thus the mediation between the two-electron dehydrogenation of the substrates and the one-electron transfer of the cytochrome system could be accomplished.

Whether the above concept is correct or not will remain for future research to ascertain. At any rate, the fact that the enzyme in the disulfide form (whether this be En—S—S—En or En—S—S—R) cannot be reduced by succinate seems, in view of the evidence presented in this paper, no longer to be a sufficient basis for the elimination of the SH groups from the oxidative mechanism of succinic dehydrogenase. The concept that the function of this enzyme involves the alternate oxidation and reduction of EnSH and En—S· may provide the explanation for the rôle of thiol groups in the mechanism of hydrogen transfer, which has been the goal of Hopkins since his discovery of glutathione and for which his papers on succinic dehydrogenase appear to have opened the way.

SUMMARY

1. The mechanism of succinic dehydrogenase action was studied by means of inhibitors.

2. The enzyme is inhibited by a large number of diverse compounds whose only common denominator appears to be their ability to react with SH groups. These compounds include quinonoid structures, sulfhydryl reagents, sulfhydryl compounds, copper, zinc, selenite, and arsenite.

3. In contrast to the above inhibitors, the action of malonate does not appear to involve sulfhydryl groups and is explained on the basis of its affinity for the enzyme groups which react with the carboxyl groups of succinate.

4. The action of malonate and the sulfhydryl reactants is *mutually exclusive*, and this fact suggests the conclusion that the sulfhydryl group of the enzyme is located between the carboxyl affinity points.

5. On the basis of the deduced structure of the succinate-activating center of the enzyme, it is suggested that the enzyme may function by oscillating between the EnSH and EnS· forms, rather than by a thiol-disulfide equilibrium.

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