SUMMARY

1. The presence of an active mono-amine oxidase in uterus has been confirmed. Evidence is presented indicating that in the human uterus, and possibly also in the rat uterus, it is mainly localized in the endometrium.

2. Human, rat, rabbit and guinea-pig placentas have also been found to be potent sources of the enzyme.

3. The level of enzymic activity in rat placenta (expressed per g. of tissue) appears to increase as the placenta matures *in utero*.

4. The activity of the placental enzyme, like that of the enzyme in liver, is proportional to the oxygen tension of the medium in which it acts, a fall of oxygen tension causing a marked fall in rate of enzyme action.

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Effect of Sulphydryl-combining Compounds on the Activity of the Succinic Oxidase System

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Thunberg (1916) was the first to suggest that the tissue catalyst responsible for the oxidation of succinate contained a sulphydryl group, but his evidence, viz. that colloidal sulphur inhibited the enzyme, was not very convincing. There was, in fact, better evidence available to Thunberg, since Battelli & Stern (1910) had already shown that arsenite inhibited this enzyme, and Ehrlich (1909) had suggested that the toxic action of arsenicals on tissues might be due to their combination with sulphydryl groups. This suggestion of Ehrlich has recently received convincing experimental support by the work of Peters and his colleagues (Peters, Stocken & Thompson, 1945).

Hopkins and his co-workers (Hopkins & Morgan, 1938; Hopkins, Morgan & Lutwak-Mann, 1938) were

the first to demonstrate the existence and the importance of SH groups in succinic dehydrogenase. These workers showed that succinic dehydrogenase was completely inhibited by incubation with a high concentration of oxidized glutathione (GSSG), and that the activity was completely restored by incubating the inhibited enzyme with reduced glutathione (GSH) under anaerobic conditions. They suggested that GSSG acted by oxidizing the SH groups of succinic dehydrogenase to disulphide (S.S) groups and that this reaction was reversible.

Since the publication of the papers of Hopkins and his co-workers, many studies of the action of other SH-combining compounds on succinic oxidase

have appeared (e.g. Morgan & Friedmann, 1938; Potter & Dubois, 1943; Barron & Singer, 1945; Barron & Kalnitsky, 1947; Mackworth, 1948). Five different types of compounds have been used, viz. alkylating agents, oxidizing agents, arsenicals, mercaptide-forming agents and maleic acid.

Despite these many studies of the action of SH agents, an examination of the literature revealed that certain points required further investigation. The most important of these was the reversibility of the inhibition, concerning which the literature presents a confusing picture. Hopkins *et al.* (1938) showed that treatment with GSH completely reactivated the succinic oxidase system in washed minced muscle after it had been inactivated by GSSG. Potter & Dubois (1943), on the other hand, using a tissue homogenate as the source of the enzyme, were unable, by means of GSH, to reverse the inhibition caused by various oxidizing agents. They explained the disagreement between their findings and those of Hopkins *et al.* by pointing out an important difference in the experimental procedures. Hopkins *et al.* treated the inactivated enzyme with GSH under anaerobic conditions, and, after this treatment, removed the excess GSH by filtering and washing the muscle pulp. Since this procedure is not possible when dispersions are used as the source of the enzyme, Potter & Dubois did not remove the GSH before measuring the succinic oxidase activity. GSH is fairly rapidly oxidized when added to such tissue preparations in the presence of air (especially if excess cytochrome *c* is added), and so cannot be an effective reactivating agent. In fact, Potter & Dubois (1943) and Ames & Elvehjem (1944) found that GSH and cysteine actually inhibited the succinic oxidase activities of tissue preparations. The presence of the thiol in the reaction mixture during the measurement of the activity of the enzyme introduces a further difficulty, viz. that the absorption of oxygen causes an error in the determination of the succinic oxidase activity. In opposition to the findings of Potter & Dubois, Barron & Singer (1945) claim that the succinic oxidase activity of a muscle dispersion, previously inhibited to the extent of 80% by treatment with benzoquinone or *p*-carboxyphenylarsine oxide, was completely restored by treatment with GSH (50 times the concentration of the inhibitor). These authors state that the 'reactivation produced by glutathione was instantaneous and in no way connected with its oxidation by metal catalysts in the enzyme, since the enzyme suspension showed no appreciable oxygen uptake on addition of glutathione without succinate during the course of the experiment (20 min.)'. Barron, Miller, Bartlett, Meyer & Singer (1947) later showed that the dithiol 2:3-dimercaptopropanol (BAL) was more effective than GSH in reversing the inhibition by arsenicals.

A further point requiring study is the component of the succinic oxidase system which is attacked by the inhibitor. Some authors, although using the rate of oxygen uptake in the presence of succinate as the measure of the enzyme activity, speak of the enzyme concerned in this oxidation as succinic dehydrogenase. Actually, an enzyme complex, of which succinic dehydrogenase is only one component, is necessary to catalyse the aerobic oxidation of succinate.

An additional reason for these experiments was to study the action of SH-combining compounds on the succinic oxidase system in Keilin & Hartree's heart-muscle preparation, which, although it is the most active preparation of this enzyme system, has not been used by previous investigators studying these inhibitors.

Three types of inhibitors were used, viz. (1) oxidizing agents: *o*-iodosobenzoate and GSSG; (2) *p*-aminophenylarsenoxide; (3) *p*-chloromercuribenzoate. Alkylating agents were not studied, since this inhibition is not easily reversible. A preliminary account of some of these findings has already appeared (Slater, 1948).

METHODS

Enzyme preparation. Keilin & Hartree's (1947) heart-muscle preparation was used. The methods of preparing the enzymes and determining the activities have already been described (Slater, 1949*a, b*).

Glutathione was prepared by the methods of Hopkins (1929) and Pirie (1930). Aqueous solutions were neutralized immediately before use. GSSG was prepared by shaking a neutralized solution of GSH in a manometric flask until no more O₂ was absorbed.

BAL was kindly provided by Prof. R. H. S. Thompson. Aqueous solutions were prepared immediately before use.

o-Iodosobenzoic acid was prepared by the oxidation of *o*-iodobenzoic acid with fuming HNO₃, according to the method of Meyer & Wachter (1892). It was 90% pure according to the amount of I₂ liberated from acidified KI.

p-Chloromercuribenzoate was kindly supplied by Dr S. V. Perry.

Procedure. The general procedure adopted was to treat a concentrated enzyme preparation with the inhibitor for a definite length of time and then rapidly to dilute about 50-fold before measuring the enzyme activity. Except in the case of *p*-chloromercuribenzoate, this rapid dilution practically stopped the action of the inhibitor on the enzyme. Unless otherwise stated, 1 ml. of Keilin & Hartree's (1947) heart-muscle preparation was treated with the inhibitor solution; after standing for the required time, the solution was diluted to 5 ml. with 0.18M-phosphate buffer, pH 7.3, and 0.2 ml. samples were pipetted into the manometer flasks and immediately diluted to 3.1 ml. with phosphate buffer and the other solutions necessary for the measurement of the enzyme activity, which was carried out as soon as possible.

This method is valid only if dilution of the enzyme does not cause any reversal of the inhibitions. This was found to be the case.

EXPERIMENTAL

A. Effect of sulphhydryl-combining agents on the succinic oxidase system

(1) *Rate of inhibition.* The effect of the time of contact of the inhibitor with the enzyme at 37° on the degree of inhibition is shown for four inhibitors in Fig. 1. The figures at zero time were obtained by adding the inhibitor and immediately diluting. The small inhibitions found with *o*-iodosobenzoate and *p*-aminophenylarsenoxide at zero time probably occurred during this dilution or after the dilution.

Fig. 1. Rate of inactivation, at 37°, of succinic oxidase system by 0.2 μ equiv. *o*-iodosobenzoate per ml. heart-muscle preparation (curve A); 0.5 μ equiv. *p*-chloromercuribenzoate per ml. heart-muscle preparation (curve B); 0.2 μ equiv. *p*-aminophenylarsenoxide per ml. heart-muscle preparation (curve C); 20 μ equiv. GSSG per ml. heart-muscle preparation (curve D).

Fig. 2. Rate of inactivation, at 16°, of the succinic oxidase system by 2 μ equiv. *o*-iodosobenzoate per ml. heart-muscle preparation.

Inhibition by *p*-aminophenylarsenoxide and by *o*-iodosobenzoate, and particularly by GSSG (even in large excess) was a slow process; maximum inhibition was found with the arsenical and *o*-iodosobenzoate after 1 hr. The reaction between the enzyme and *p*-chloromercuribenzoate was much more rapid. It cannot be stated from Fig. 1 whether this in-

hibition occurred practically instantaneously before or after dilution. It can, however, be definitely concluded that, under comparable conditions, the rate of inhibition of the enzyme by *p*-chloromercuribenzoate is much greater than that by the other inhibitors tried. The succinic dehydrogenase, which, as is shown below, is less susceptible to the mercurial than is the complete succinic oxidase system, was just as rapidly inactivated by the mercurial.

In Fig. 2, the rate of inhibition of the enzyme by *o*-iodosobenzoate at 16° is shown. It appears from the shape of the curve that the inhibition at this temperature was due to two reactions; an initial fairly rapid reaction which was complete after 10 min., followed by a slower reaction, which was not complete after 2 hr.

(2) *Effect of amount of inhibitor.* The effect of different amounts of *o*-iodosobenzoate, *p*-aminophenylarsenoxide and *p*-chloromercuribenzoate on the succinic oxidase activity of the same heart-muscle preparation is shown in Fig. 3. Sufficient time was allowed for complete reaction between the

Fig. 3. Effect of different amounts of *p*-aminophenylarsenoxide (curve A), *o*-iodosobenzoate (curve B), and *p*-chloromercuribenzoate (curve C) on the succinic oxidase system. *p*-Amino-phenylarsenoxide and *o*-iodosobenzoate: reaction carried out at 37° for 60 min.; *p*-chloromercuribenzoate: 37° for 15 min.

inhibitor and the enzyme. A considerable amount of the arsenical or *o*-iodosobenzoate was required before appreciable inhibition occurred; the inhibition increased markedly with further small increases in the amount of inhibitor until nearly complete inactivation was obtained. The unusual shape of the curves is probably due to the presence, in the heart-muscle preparation, of substances (possibly denatured proteins, which are known to contain many reactive SH groups) which combine preferentially with these two inhibitors and thus protect the enzyme from inactivation by small concentrations of the inhibitor. The heart-muscle preparation often became less susceptible to SH-combining reagents (particularly to *o*-iodosobenzoate) after standing for a few days,

probably due to an increase in the amount of denatured proteins.

These substances in the heart-muscle preparation which protected the enzyme from small amounts of arsenical and oxidizing agent were quite ineffective in protecting against the same amount of the mercurial. On the other hand, the enzyme was more susceptible to relatively larger amounts of the arsenical and *o*-iodosobenzoate than to the corresponding amount of mercurial. These findings indicate that the mercurial was much less selective in its action than the other two compounds. The arsenical was the most selective agent of the three.

(3) *Component of the succinic oxidase system inhibited by the sulphydryl reagents.* In Fig. 4 the effect of different amounts of *p*-aminophenylarsenoxide on the activity of both the complete succinic oxidase system and on the succinic dehydrogenase

Fig. 4. Effect of different amounts of *p*-aminophenylarsenoxide on the succinic oxidase system $\circ$, and on succinic dehydrogenase $\bullet$. Reaction carried out at 37° for 60 min.

portion is shown. The fact that the two curves coincide means that the component of the succinic oxidase system which is limiting the activity of the system after treatment with the arsenical is the succinic dehydrogenase, and that the primary action of the arsenical is on the dehydrogenase.

The oxidizing agents, *o*-iodosobenzoate and GSSG, on the other hand, have a greater effect on the complete system than on the succinic dehydrogenase (Figs. 5 and 6). Cupric chloride, which may act primarily as an oxidizing agent, also has a much stronger action on the complete system than on the dehydrogenase. Hellerman, Perkins & Clark (1933) and Mapson (1946) have shown that Cu^{++} does not rapidly inactivate urease, whereas Cu^+ is strongly inhibitory. Hopkins (1929) found that GSH reacted with Cu^{++} by first reducing it to Cu^+ , which then combined with unreacted GSH. It is possible that the same type of reaction occurs with the succinic oxidase system; the first action of the Cu^{++} may be an oxidative reaction on some component of the

system, leading to inactivation of the succinic oxidase system, and this is followed by combination of the Cu^+ formed with the SH groups of the succinic dehydrogenase.

Fig. 5. Effect of different amounts of iodosobenzoate on the activity of succinic oxidase $\circ$, and of the succinic dehydrogenase $\bullet$. Reaction carried out at 37° for 60 min.

Fig. 6. Rate of inactivation of succinic oxidase ($\circ$, curve A) and succinic dehydrogenase ($\bullet$, curve B) by GSSG (20 μequiv./ml. heart-muscle preparation). Temp. 37° .

Since Hellerman *et al.* (1933) found that Cu^{++} catalysed the aerobic destruction of urease, it was of interest to determine whether it had the same effect on the succinic oxidase system. The figures in Table I show that this is not the case; Cu^{++} had the same inhibitory effect whether it was tested in the presence or in the absence of air.

The effect of different amounts of *p*-chloromercuribenzoate on the succinic oxidase and succinic dehydrogenase activities is shown in Fig. 7. The Thunberg method of measuring succinic dehydrogenase activity was employed, since potassium cyanide, which is used in the manometric method to inhibit cytochrome oxidase, reverses the inhibition of the dehydrogenase (see p. 137). The mercurial, like the oxidizing agents but unlike the arsenical, inhibits the complete succinic oxidase system more than the dehydrogenase.

Table 1. *Effect of cupric ions on the succinic oxidase system and on succinic dehydrogenase*

(Heart-muscle preparation (1 ml.) + 0.4 ml. CuCl_2 were either shaken in a manometer flask in the presence of air (aerobic) or kept *in vacuo* (CuCl_2 added after evacuation) in a Thunberg tube (anaerobic) for 15 min. at 37° . Concentration of Cu^{++} : $7.2 \times 10^{-5} \text{M}$. 3.6 ml. 0.18M-phosphate buffer were then added and 0.2 ml. samples taken for measurement of enzyme activities. Control without Cu^{++} treated in same way.)

	Succinic oxidase activity ($\mu\text{l. O}_2/10 \text{ min.}$)		Succinic dehydrogenase activity ($\mu\text{l. O}_2/10 \text{ min.}$)	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Control	112	110	41	40
In presence of Cu^{++}	46	44	29	27
Inhibition (%)	59	60	29	34

Fig. 7. Effect of different amounts of *p*-chloromercuribenzoate on the activity of succinic oxidase $\circ$, and of the succinic dehydrogenase $\bullet$. Reaction carried out at 37° for 15 min.

The effect of the arsenical, the mercurial and *o*-iodosobenzoate on the cytochrome oxidase activity is shown in Table 2. Only the mercurial had an appreciable effect on the cytochrome oxidase. The insensitivity of cytochrome oxidase to arsenicals and oxidizing agents is in agreement with the findings of Hopkins *et al.* (1938) with GSSG and Barron & Singer (1945) with arsenicals.

Table 2. *Effect of sulphydryl-combining agents on the cytochrome oxidase of heart-muscle preparation*

(Heart-muscle preparation treated with inhibitors for 1 hr. at 37° . General procedure: see Methods. Note that the cytochrome oxidase activity measured was the true activity at infinite cytochrome *c* concentration, measured by the method of Slater (1949*b*).)

Inhibitor	Inhibition (%)
<i>p</i> -Chloromercuribenzoate (1.25 $\mu\text{equiv.}$)	29
<i>o</i> -Iodosobenzoate (1.0 $\mu\text{equiv.}$)	5
<i>p</i> -Aminophenylarsenoxide (0.5 $\mu\text{equiv.}$)	5

B. Reactivation

The inhibition produced by treatment with *p*-aminophenylarsenoxide was not reversed when the concentration of the inhibitor was reduced by diluting the reaction mixture (Table 3), nor even by precipitating the enzyme with acid and washing away the unreacted inhibitor. Similarly, the inhibition by *o*-iodosobenzoate was not reversed by the latter treatment (Table 4). After treatment with *p*-chloromercuribenzoate also, the enzyme was not reactivated by dilution.

Table 3. *Effect of dilution of heart-muscle preparation, after treatment with p-aminophenylarsenoxide, on the degree of inhibition*

(Inhibited: 1 ml. heart-muscle preparation + 0.5 ml. 0.0004N-*p*-aminophenylarsenoxide kept at 37° for 1 hr.; 3.5 ml. 0.18M-phosphate buffer added and different volumes pipetted into manometer flasks for measurement of succinic oxidase activity in the usual manner. Control: 1 ml. heart-muscle preparation + 0.5 ml. water treated similarly.)

Final dilution of heart-muscle preparation in manometer flasks	O_2 uptake ($\mu\text{l./10 min.}$)		Inhibition (%)
	Control	Inhibited	
1:82	61	35	43
1:41	118	69	42
1:24	187	112	40
1:16	231	141	39

Table 4. *Inhibition of succinic oxidase by o-iodosobenzoate; attempt to reverse inhibition by washing enzyme with water*

(A, 1 ml. heart-muscle preparation treated with *o*-iodosobenzoate (total volume = 1.1 ml.) for 30 min. at 16° ; 3.9 ml. 0.15M-phosphate buffer added. B, 1 ml. heart-muscle preparation treated with *o*-iodosobenzoate as A. Cooled to 0° and 4 ml. 0.01N-acetic acid added, centrifuged, supernatant fluid removed, residue stirred with 2 ml. water, centrifuged and residue suspended in 5 ml. 0.15M-phosphate buffer. 0.2 ml. samples taken for measurement of enzyme activity. Control tests showed that untreated enzyme could be precipitated with acid, washed and resuspended in phosphate buffer in this manner without loss of activity.)

<i>o</i> -Iodosobenzoate ($\mu\text{equiv./ml. heart-muscle preparation}$)	Inhibition (%)	
	A	B
2	71	78
4	85	83

The enzyme can, however, be reactivated by treatment with SH compounds. It was found, in agreement with Potter & Dubois (1943) and Ames & Elvehjem (1944) and in disagreement with Barron & Singer (1945), that complete reactivation of the succinic oxidase system could not be effected by adding the thiol to the enzyme preparation in the presence of air, since the thiol was oxidized by the

enzyme preparation and this oxidation was accompanied by inactivation of the succinic oxidase system. Ames & Elvehjem (1944) believed that this inhibition, caused by cysteine and GSH, was due to the action of cystine and GSSG formed by oxidation of the thiol compounds. It was found in the present study, however, that this could not be the complete explanation since the inhibition caused by GSH is much greater than that resulting from treatment with the equivalent concentration of GSSG. This observation was the starting point of the investigation of reducing agents on the succinic oxidase system which is described elsewhere (Slater, 1949*c*). It was found that GSH had no effect on the enzyme under completely anaerobic conditions, and that, even in the presence of air, the succinic dehydrogenase was much less susceptible than the complete succinic oxidase system to the thiol. Thus, in order to study the reversal of the inhibition, it is necessary to use Hopkins & Morgan's (1938) procedure of treating the enzyme with the thiol under anaerobic conditions. When using dispersions, it is difficult to remove the thiol from the enzyme quickly after the treatment before the measurement of the enzyme activity. This difficulty was overcome by treating a high concentration of enzyme with the thiol under anaerobic conditions and quickly diluting the suspension about 50 times before measuring the enzyme activity. The final very low concentration of the thiol was then without appreciable effect on the enzyme. In some experiments, such a high concentration of thiol was necessary to reactivate the enzyme that the thiol concentration, even after this 50-fold dilution, was appreciable and caused a small inactivation of the enzyme. In these experiments, the extent of the inactivation was determined in a separate control experiment and taken into account when calculating the degree of reactivation of the enzyme (see Fig. 8).

In the presence of air, the relative effectiveness of different thiols in reversing the inhibition caused by SH-combining reagents will depend, not only on the relative affinity of the thiol and the enzyme for the inhibitor, but also on the rate of oxidation of the thiol. The latter factor must be taken into account when considering the finding of Barron & Kalnitsky (1947) that several dithiols were more effective than BAL in reversing the inhibition of the succinic oxidase system caused by various heavy metals, since these authors worked under aerobic conditions and Barron, Miller & Kalnitsky (1947) had previously shown that these other dithiols were oxidized more slowly than BAL.

(1) *p*-Aminophenylarsenoxide

(a) *Rate of reactivation.* The heart-muscle preparation, after treatment with 0.5 μ equiv. of *p*-aminophenylarsenoxide per ml. of heart-muscle

preparation for 30 min. at 37°, was reactivated by treatment with BAL for various times before dilution. The results, which are given in Fig. 8, show that the reactivation was quite rapid. It should be noted that the times of contact with BAL refer only to the time before dilution. It seems likely, from the

Fig. 8. Rate of reactivation of succinic dehydrogenase, after inhibition by *p*-aminophenylarsenoxide, by treatment with BAL under anaerobic conditions; equiv. BAL/equiv. *p*-aminophenylarsenoxide = 3.76. Mixture *A*, 12 ml. heart-muscle preparation + 6 ml. water kept at 37° for 30 min.; 2 ml. *A* diluted with 4.4 ml. 0.18M-phosphate buffer, and succinic dehydrogenase activity of 0.2 ml. of diluted solution determined; value *a*. Mixture *B*, 12 ml. heart-muscle preparation + 6 ml. 0.0005M-*p*-aminophenylarsenoxide kept at 37° for 30 min.; 2 ml. *B* diluted with 4.4 ml. 0.18M-phosphate buffer, and succinic dehydrogenase activity of 0.2 ml. diluted solution determined; value *b*. Samples of *A* (2 ml.) placed in Thunberg tubes, containing 0.25 ml. 0.005M-BAL in hollow stoppers. Evacuated, contents of Thunberg tubes mixed and reacted for various times before rapidly diluting with 4.4 ml. 0.18M-phosphate buffer; 0.2 ml. of diluted solutions taken immediately for measurement of succinic dehydrogenase activity; value *c*. Samples of *B* (2 ml.) treated in exactly the same manner; value *d*. Percentage inactivation = $100(a-b)/a = 88$; percentage reactivation = $100(ad/c-b)/(a-b)$.

shape of the curve, that some reaction took place either during or after dilution and the curve should not be taken to represent the true rate of reactivation. It can be seen, however, by comparison with Fig. 1, obtained under the same conditions, that the rate of reactivation was considerably greater than the rate of inactivation.

(b) *Reactivation by different concentrations of BAL and GSH.* The effect of different concentrations of BAL on the degree of reactivation is shown in Fig. 9, which includes measurement both of succinic dehydrogenase and of the complete succinic oxidase system. The succinic dehydrogenase and the complete system were reactivated to about the same extent. Under the conditions of this experiment, the reactivation was not quite complete, amounting to

about 80% of full reactivation. This may be because, during the necessary manipulations, the enzyme-arsenical compound was allowed to stand for over 1 hr. before the addition of the BAL. Barron & Singer (1945) found that complete reactivation of

the maximum reactivation obtained. When a similar experiment was carried out in an open test tube, BAL equivalent to 2.5 times the amount of arsenical reactivated to the extent of only 36 %, even allowing for the inactivating effect of BAL itself. These findings may be compared with those of Barron, Miller, Bartlett, Meyer & Singer (1947), who found that BAL equivalent to 2.5 times the concentration of lewisite reactivated the succinic oxidase system by 83 %, while double this amount completely reactivated the system. The amount of BAL required to reverse the inhibition of the succinic dehydrogenase by *p*-aminophenylarsenoxide is considerably less than that found by Stocken, Thompson & Whittaker (1947) to be necessary to reactivate the pyruvic oxidase system after the action of various therapeutic arsenicals.

GSH was much less effective than BAL in reversing the inhibition; thus, GSH equivalent to 1.5 times the concentration of arsenical produced no measurable reactivation, while 7.5 times the equivalent concentration caused 36% reactivation. This result is similar to that obtained by Barron, Miller, Bartlett, Meyer & Singer (1947), who found that GSH equal to 5 times the lewisite used reversed the inhibition by 23 %.

Fig. 9. Reactivation of succinic dehydrogenase and succinic oxidase, after treatment with *p*-aminophenylarsenoxide (0.5 μ equiv./ml. heart-muscle preparation), by treatment with various concentrations of BAL under anaerobic conditions. ●, succinic oxidase; ○, succinic dehydrogenase. Procedure as Fig. 8; reactivation time, 30 min.; inactivation, 85%.

the enzyme inhibited by *p*-chloromercuribenzoate occurred only when the thiol (GSH) was added soon after the mercurial and suggested that the enzyme whose SH groups are combined is readily denatured on standing. The most interesting feature of Fig. 9 is the small amount of BAL required to produce the maximum reactivation, viz. about twice the amount of BAL equivalent to the arsenical added; BAL exactly equivalent to the arsenical produced 80 % of

(2) *o*-Iodosobenzoate

The experiments reported in Table 5 show that much greater amounts of thiol are required to reverse the inhibition caused by *o*-iodosobenzoate. BAL equivalent to 10 times the amount of oxidizing agent reactivated the succinic dehydrogenase by 61 %, while BAL equivalent to 50 times the amount of iodosobenzoate brought about nearly complete reactivation. GSH was rather less effective than

Table 5. *Reactivation of succinic dehydrogenase and of succinic oxidase after inhibition by o-iodosobenzoate*

(Exp. 1: 5 ml. heart-muscle preparation treated with 5 ml. 0.001 M *o*-iodosobenzoate for 30 min. at 37°; 2 ml. samples pipetted into Thunberg tubes containing BAL or GSH in hollow stoppers. Contents mixed after evacuation of tubes. After 30 min. at 37°, tube opened, contents quickly diluted to 5 ml. with phosphate buffer, 0.2 ml. samples pipetted into manometer flasks and quickly diluted with buffer. Exp. 2: Thunberg tubes contained 1 ml. heart-muscle preparation and 0.3 ml. 0.001 M *o*-iodosobenzoate in main compartments and BAL or GSH in hollow stoppers. Evacuated, allowed to react for 30 min. at 37°, then mixed and allowed to stand a further 30 min. at 37°. Subsequent treatment as in Exp. 1. Percentage reactivation calculated as in Fig. 8.)

μ equiv. <i>o</i> -iodosobenzoate/ml. heart-muscle preparation	Inhibition (%)		Reactivating agent	Ratio of BAL or GSH (equiv.): iodosobenzoate (equiv.)	Reactivation (%)	
	Succinic oxidase	Succinic dehydrogenase			Succinic oxidase	Succinic dehydrogenase
			Exp. 1			
2.0	—	57	BAL	1.5	—	12
			BAL	5	—	29
			GSH	1.5	—	0
			Exp. 2			
0.6	96	89	BAL	10.5	26	61
			BAL	50	54	92
			GSH	50	32	70

BAL. The inhibition of the complete system was much less readily reversible than that of the succinic dehydrogenase.

(3) Oxidized glutathione

Owing to the necessity of using very high concentrations of GSSG to inhibit the enzyme, it was not possible to use a large excess of thiol to reactivate the enzyme. In the experiment shown in full in Table 6, BAL, equivalent to twice the concentration

Table 6. Reactivation of succinic dehydrogenase and succinic oxidase after treatment with oxidized glutathione

(A, 1 ml. heart-muscle preparation kept at 37° for 1 hr., diluted to 5 ml. and succinic oxidase and succinic dehydrogenase determined on 0.2 ml. samples. B, 1 ml. heart-muscle preparation + 0.2 ml. 0.05M-GSSG (prepared by aerobic oxidation of neutralized 0.1M-GSH) kept at 37° for 1 hr. Then as A. C, 1 ml. heart-muscle preparation in main tube and 0.2 ml. 0.1M-BAL in hollow stopper. Contents mixed after evacuation and kept at 37° for 30 min. Then as A. D, 1 ml. heart-muscle preparation in main tube and 0.2 ml. 0.1M-BAL in hollow stopper. 0.2 ml. 0.05M-GSSG added to heart-muscle preparation; kept at 37° for 1 hr. Then evacuated, contents of hollow stopper mixed with those of main tube and kept at 37° for 30 min. Then as A.)

Exp.	Succinic oxidase (μ l. O ₂ /10 min.)	Succinic dehydrogenase (μ l. O ₂ /20 min.)
A	101.7	62
B	46.5	41.1
C	78.9	55.2
D	16.6	53.6

Inhibition by GSSG (%)

$$\frac{A-B}{A} \times 100 \quad 56 \quad 36$$

Reactivation by BAL (%)

$$\frac{AD/C-B}{A-B} \times 100 \quad -45 \quad +91$$

of GSSG used to inhibit the enzyme, completely reactivated the succinic dehydrogenase, but did not reactivate the complete succinic oxidase system. In fact, the inhibition of the latter increased during the treatment with BAL. It is impossible to compare the relative ease of reversibility after treatment with *o*-iodosobenzoate and GSSG, since the amount of the latter used was so much greater than that of the former that the concentration of BAL present in the experiment with GSSG was greater than in the experiment with *o*-iodosobenzoate, although the relative concentrations of reactivating agent to inhibitor were much greater in the latter experiment. Further information on the reversibility of the enzyme system after treatment with GSSG could probably be obtained if the enzyme was sedimented by high speed centrifugation and washed free from GSSG before adding the reactivating agent.

Hopkins *et al.* (1938) treated the washed muscle with GSSG under anaerobic conditions. It was found in the present study that the inhibitions of both the complete succinic oxidase system and of succinic dehydrogenase and the reactivations by BAL were the same, whether the enzyme was treated with the GSSG in the presence or in the absence of air.

Inhibition by GSSG was not reversed by 0.01M-cyanide, the concentration used in the measurement of the succinic dehydrogenase by the manometric method, with methylene blue as carrier. This was shown by the fact that the degree of inhibition was the same whether the succinic dehydrogenase activity was measured by this manometric or by the Thunberg method, in which cyanide is not used.

(4) *p*-Chloromercuribenzoate

Heart-muscle preparation, treated with sufficient *p*-chloromercuribenzoate to inhibit completely the oxidation of succinate by methylene blue in the absence of potassium cyanide, was reactivated by 80% by the addition of 0.01M-cyanide. Strangely, the inhibition was not reversed if cyanide was added to the Barcroft flask after the succinate.

After complete inhibition by 1.5 μ equiv. of *p*-chloromercuribenzoate per ml. of enzyme preparation, the complete succinic oxidase system was reactivated by 31% with an amount of GSH equivalent to the mercurial used and by 65% with 5 times as much GSH. The succinic dehydrogenase was reactivated to the same extent as the complete system. The enzyme was also readily reactivated (73%) by adding 0.13% denatured globin to the manometric flask in which the enzymic activity was measured.* In marked contrast, however, the enzyme was not in the slightest degree reactivated by BAL in an amount equivalent to 5 times the amount of mercurial used.

C. Spectroscopic observations

A normal cytochrome spectrum was obtained when sodium dithionite (Na₂S₂O₄) was added to heart-muscle preparation treated with sufficient *p*-aminophenylarsenoxide, *o*-iodosobenzoate or *p*-chloromercuribenzoate to inhibit completely the activity of the succinic oxidase system. The bands did not appear, however, when succinate was used instead of Na₂S₂O₄, showing the absence of an active succinic dehydrogenase. When denatured globin was added to the tube containing succinate and the heart-muscle preparation treated with the mercurial, the

* Under the same conditions, the enzyme treated with one third as much arsenical (i.e. 0.5 μ equiv./ml. of heart-muscle preparation) was reactivated by 25% by treatment with globin. The succinic dehydrogenase and the complete succinic oxidase system were reactivated to the same extent.

bands of the cytochromes immediately became visible. Thus the spectroscopic observations are in agreement with the manometric experiments.

DISCUSSION

Inactivation of succinic dehydrogenase by sulphhydryl-combining reagents and reactivation by sulphhydryl-containing compounds

Under the conditions of the experiments described above, the reaction between the enzyme and the arsenical or *o*-iodosobenzoate was slow. The slowness of the reaction must be considered when comparing the activity of different arsenicals. Barron, Miller, Bartlett, Meyer & Singer (1947) found that lewisite was much more effective than other organic arsenicals or inorganic arsenite in inhibiting the succinic oxidase system. If all arsenicals combine with the same groups on the enzyme to form a difficultly dissociable complex, one would expect all arsenicals to be equally effective, if they are allowed to react with the pure enzyme for a sufficient length of time. The differences found by Barron, Miller, Bartlett, Meyer & Singer may be due to differences in the rates of combination, not in the capacity for combination, or to differences in the relative rates of combination of the arsenical with the succinic dehydrogenase compared with the rate of combination with other proteins which are present in the enzyme preparation and which protect the enzyme from inactivation. These points require further investigation. It should be noted that the present finding, that the arsenical reacts slowly with the enzyme, is not in agreement with Barron & Singer (1945), who found that the inhibition was complete after 6 min. Many of the findings of the present communication do not agree with those of Barron and his co-workers. This may be due to the different method employed in studying the inhibition and perhaps also to the different enzyme preparation used (Barron & Singer (1945) used a preparation made by treating washed skeletal muscle in a Waring blender with phosphate buffer; it was not purified by acid precipitation). The concentration of the enzyme during treatment with the SH-combining reagents in the present studies was about 100 times that used by Barron & Singer (1945).

The finding that GSSG reacts with the enzyme very slowly, and that high concentrations and long incubation times are necessary to obtain an appreciable inhibition, is in agreement with the original observations of Hopkins *et al.* (1938). This is probably due to the difficulty of penetration of the large molecule of GSSG (mol. wt. 612) to the site of the inhibition. Keilin & Hartree (1940) found that disulphides such as tetraethyldithiocarbamyl disulphide and tetrathionate, whose molecular structure resembles that of pyrophosphate which is probably

a competitive inhibitor of succinic dehydrogenase, are much more powerful inhibitors than GSSG, possibly because they can penetrate more readily to the active centre of the enzyme which may be the site of the inhibition.

It is customary to express the effectiveness of an inhibitor of an enzyme in terms of the concentration of the reagent which produces a certain inhibition. This implies that the degree of inhibition is, in fact, determined by the concentration of the inhibitor when the enzyme activity is measured and not by the concentration of the enzyme. This is the case, when the inhibitor enters into a loose easily reversible combination with the enzyme and the inhibition is reversed by dilution. The inhibitors, with which we are concerned in this paper, do not, however, form such an easily reversible compound, but take part in a chemical reaction which proceeds practically to completion, and the compound formed is not dissociated by subsequent lowering of the concentration of the inhibitor by dilution. The degree of inhibition depends then, not on the final concentration of the inhibitor in the enzyme solution when the activity is measured, but on the ratio of the concentration of the inhibitor to that of the enzyme provided that sufficient time is allowed for the reaction to proceed to completion. The actual concentrations of the reactants will, of course, affect the rate at which this point is reached, and become more important than the ratio of the concentrations when the reaction is very slow, for example, when GSSG is the inhibitor.

If the effect of *o*-iodosobenzoate is to oxidize SH groups in the enzyme molecule to S.S, it is not surprising that removal of the inhibitor should have no effect on the inactivated enzyme. It is surprising, however, that, after treatment with the arsenical, the enzyme cannot be reactivated by removing the untreated arsenical and washing with water. It is generally believed that the reaction of the enzyme with the arsenical is

Enzyme Arsenical Enzyme-arsenical compound

If this is the case, one would expect that removal of the arsenical would cause some dissociation of the inactive enzyme-arsenical compound, but this does not appear to occur. No reactivation was obtained by diluting a partially inactivated enzyme preparation 55-fold even after keeping it 3 hr. before measurement of the succinic oxidase activity. The equilibrium of the above reaction must lie far to the right. In this respect, succinic dehydrogenase resembles keratine; Stocken & Thompson (1946*a*) found that 85% of the arsenic combined with the keratine remained after precipitation at pH 4.6 and washing.

p-Chloromercuribenzoate differs from the other agents used in several important respects, viz. (a) it inactivates the enzyme more rapidly; (b) it does not react with other substances in the enzyme preparation before attacking the enzyme; (c) its action is readily reversed by GSH or denatured globin, but not by BAL; the action of the arsenical is, on the other hand, reversed much more effectively by the dithiol than by the monothiol or globin, and BAL is also rather more effective than GSH in reversing the effect of *o*-iodosobenzoate.

These differences are probably all connected with the fact that the mercurial combines with only one SH group thus,

while the arsenical combines with two such groups (either on the same or on different enzyme molecules) as shown in reaction (1) and the oxidizing agent also requires two SH groups to form a disulphide

It is readily understandable that the reaction with one SH group might be more rapid than that with two SH groups. The second point of difference between the mercurial and the other inhibitors probably follows from this difference in the rates of the reaction, if it is assumed that the enzyme preparation contains substances which, unlike the enzyme, are readily attacked by both types of inhibitor. The third point of difference can be explained by assuming that the mercurial readily combines with monothiois, but not with dithiois like BAL. Arsenicals, on the other hand, are known from the work of Stocken & Thompson (1946*b*), extended by Whittaker (1947), to combine much more easily with dithiois, with which they can form stable rings containing the arsenic and the sulphur. GSH and BAL do not differ so markedly in their capacity to reverse inhibition by iodosobenzoate, and the greater activity of BAL in this case may be due to the stronger reducing properties of this compound. Denatured globin behaves, in respect of its ability to reverse inhibition by the mercurial and the arsenical, like a monothiol. Mirsky & Anson (1936) have shown that denatured globin, like other denatured proteins, contains a number of reactive SH groups.

It is interesting to note that Barron & Kalnitsky (1947) have found that the inhibiting action on the succinic oxidase system of mercuric chloride, which would be expected to react more readily with dithiois (it gives an insoluble precipitate with BAL) than with monothiois, is reversed much more readily by dithiois than by monothiois.

From the point of view of the position of the final equilibria, the reactivation of the enzyme inhibited by arsenicals can be explained by competition between the BAL and the enzyme for the arsenical according to the equations.

However, such an explanation introduces a kinetic difficulty. It has already been shown that the reaction between the enzyme and the arsenical is slow and that the position of the equilibrium in reaction (4) is far towards the right. It must follow that the reverse reaction (4) is still slower than the forward; consequently no matter how fast the reaction between BAL and the arsenical, the rate of dissociation of the enzyme-arsenical compound would be the reaction limiting the reactivation of the enzyme, which would therefore be very slow. However, contrary to this conclusion, it was found that the reactivation was much more rapid than the inactivation.

This suggests that the above equations do not correctly express the reactions involved. Two possible modifications are as follows:

(a) The process limiting the rate of inactivation is not the actual combination between the enzyme and the arsenical, but the rate of diffusion of the arsenical to the reactive groups of the enzyme. Thus the actual velocities of the forward and reverse reactions (4) may be much greater than indicated by the slowness of the inactivation. According to this view, the relatively rapid reactivation is due to the ease of penetration of BAL to the site of the inhibition, and to the fact that the arsenical-BAL compound diffuses more rapidly than the arsenical. This explanation is supported by the ability of BAL to penetrate rapidly into tissues and thereby combine with lewisite some time after contact of the tissue with the lewisite (Stocken & Thompson, 1946*b*), and by the finding of Peters & Stocken (1947) that the compound between BAL and mapharside (which is very similar to the arsenical used in the present investigation) is more toxic to rats than the arsenical itself, presumably owing to readier penetration of the compound to sites which are dangerous to the animals.

(b) The reactivation is not due to competition between the enzyme and the BAL for the arsenical formed by dissociation of the enzyme-arsenical compound, but essentially irreversible reactions are involved, both in inactivation and reactivation.

Inactivation

Reactivation

Is succinic dehydrogenase a monothiol or a dithiol?

Dithiols were introduced as antidotes in arsenical poisoning by Peters *et al.* (1945). Stocken & Thompson (1946*b*) found that the inhibition of the pyruvic oxidase system by arsenicals was completely reversed by dithiols, while monothiol were ineffective, and suggested that this was due to the fact that the arsenical combines with two essential SH groups, closely situated in the enzyme molecule, so as to form a relatively stable cyclic structure. Since the dithiol BAL is also much more effective than GSH in reversing the inhibition of succinic dehydrogenase by *p*-aminophenylarsenoxide, it might appear justifiable to conclude that the succinic dehydrogenase molecule also contains two closely situated SH groups.

Before considering this matter, it should be noted that there are important differences between the pyruvic and succinic enzymes with regard to inhibition by arsenicals and reversal by thiols, as has been pointed out by Thompson (1948). Thus, Stocken & Thompson (1946*b*) have shown that monothiol are completely ineffective in preventing or reversing the action of arsenicals on the pyruvic enzyme, whereas Barron, Miller, Bartlett, Meyer & Singer (1947) and the present investigation have shown that GSH is able to reverse partially the inhibition of the succinic enzyme, although it is much less effective than BAL. In addition, it has been shown by Peters, Sinclair & Thompson (1946) that in brain preparations containing both enzymes, the pyruvic is much more susceptible than the succinic enzyme to arsenicals.

It does not necessarily follow from the greater effectiveness of dithiols compared with monothiol in reversing the inhibition by arsenicals that the enzyme is, itself, a dithiol. The weakness of this type of argument is shown by the fact that if applied to the findings with the arsenical it would be concluded that the succinic enzyme was a dithiol, while if applied to the experiments with the mercurial, the conclusion drawn would be that the enzyme was a monothiol. As it is the same enzyme in both cases, the different results obtained must be a property of the inhibitor, not of the enzyme. Since Stocken & Thompson (1946*b*) have shown that dithiols form more stable compounds with the trivalent arsenicals

than do monothiol, it would be expected that the dithiols would be more satisfactory reactivating agents, regardless of the nature of the combination between the arsenical and the enzyme.

Thus it is not permissible to conclude, from the experiments on the reactivation of the enzyme inhibited by arsenicals, that the enzyme contains two SH groups which react with the same arsenic atom. The other findings, in fact, without definitely settling the matter, suggest that it is more likely that the enzyme contains only one SH group necessary for activity, since it is difficult to reconcile otherwise the great ease of inhibition by the mercurial with the failure of BAL to reactivate after such inhibition, which shows that the mercurial does not readily react with compounds containing two closely situated SH groups. The finding that BAL in an amount equivalent to the amount of arsenical combined in the enzyme almost completely reactivated the enzyme, showing that the arsenical forms a very much firmer union with the simple dithiol than with the enzyme, is also in agreement with this view. If the enzyme contains only one essential SH group, it is probable that the arsenical and oxidizing agents combine with two SH groups from different molecules, only one of which may be the group on the enzyme necessary for its activity.

Components of the succinic oxidase system inhibited by SH-combining reagents

It has been shown that the inhibition of the complete succinic oxidase system by the arsenical can be accounted for by the inhibition of the succinic dehydrogenase. The simplest explanation of the action of arsenicals is that they combine with two SH groups, either both in the same succinic dehydrogenase molecule or on different molecules, and it would be expected that SH groups capable of reacting in this way would be oxidized by oxidizing agents to disulphides. However, the fact that the complete succinic oxidase system is affected to a greater extent than the succinic dehydrogenase shows that the latter is not the only point of attack by oxidizing agents on the system. The curve shown in Fig. 2 indicates that two reactions are proceeding, even at room temperature, and the further oxidation might proceed even more readily at 38°. The other part of the system which is attacked can also be reactivated by treatment with thiols, but with much greater difficulty than is the succinic dehydrogenase. The fact that the action of an oxidizing agent can be reversed by treatment with SH compounds is often taken as an indication that the oxidizing agent has attacked SH groups. However, this does not necessarily follow. Other groupings besides SH groups might be oxidized by oxidizing agents and reduced by subsequent treatment with reducing agents. It seems preferable to consider that SH

groups are attacked only when the enzyme is inhibited by a variety of agents, viz. alkylating agents—such as iodoacetate or chloroacetophenone—arsenicals and mercaptide-forming agents. In this case, the other portion of the system is not attacked by arsenicals. It has been shown that the cytochrome oxidase activity is not affected by oxidizing agents. It seems possible then, that the oxidizing agents affect, in addition to the succinic dehydrogenase, the component of the succinic oxidase system which links the dehydrogenase to cytochrome *c*, and, as is discussed elsewhere (Slater, 1949*c*), is destroyed by coupled oxidation with BAL or GSH. However, the possibility that the oxidizing agents, in addition to their effect on SH groups, have the non-specific type of effect on the system discussed by Keilin & Hartree (1949) and Slater (1949*d*) cannot be excluded.

It is interesting that treatment of the enzyme with GSH, in the absence of air but in the presence of GSSG, increased the inhibition of the succinic oxidase system while the succinic dehydrogenase was completely reactivated. It is possible that GSSG, in the high concentration used in this experiment, may be able to replace oxygen in promoting the destruction by BAL of the component of the system mentioned in the preceding paragraph. However, this point requires further clarification.

It is somewhat surprising that the enzyme, inhibited by oxidizing agents, is not reactivated by potassium cyanide (0.01*M*), since Walker (1925) showed that cyanide reduces disulphides. Possibly, higher concentrations of cyanide would be effective.

The effect of *p*-chloromercuribenzoate is rather complicated. It is clear that it attacks the complete system more readily than the succinic dehydrogenase portion. This is in agreement with the work of Cook & Perisutti (1947) and of Cook, Kreke, McDevitt & Bartlett (1946) on the effect of phenylmercuric nitrate on the succinic oxidase system, although with this reagent these workers found a greater difference between the two inhibitions. *p*-Chloromercuribenzoate inhibited cytochrome oxidase, but this is insufficient to account for the greater effect on the complete succinic oxidase system than on the succinic dehydrogenase, since the cytochrome oxidase is not the limiting factor in the succinic oxidase system in the heart-muscle preparation. Cook *et al.* (1946) also found an inhibition of the cytochrome oxidase activity with phenylmercuric nitrate. They found, in addition, that other enzymes, viz. lactic dehydrogenase and catalase, which do not usually react with SH-combining reagents, were inhibited by this compound. This suggests that these mercurials have a non-specific effect on the entire succinic oxidase system as well as combining with SH groups in the dehydrogenase.

The function of the sulphydryl groups in succinic dehydrogenase

Since succinate or competitive inhibitors of succinic dehydrogenase (which are assumed to combine with the enzyme at the same point as succinate) protect the enzyme from the action of SH combining reagents, Hopkins & Morgan (1938) suggested that the SH group is at or near the point where succinate attaches itself to the enzyme. However, since succinate was unable to reduce succinic dehydrogenase treated with GSSG, these authors concluded that oxidation of SH to S.S did not play any part in the catalysis of the oxidation of succinate. Potter & Dubois (1943), on the other hand, have ascribed such a catalytic function to the SH group, which is presumed to mediate the transfer of electrons from succinate to cytochrome oxidase, by oscillating between the EnSH and EnS. forms. They suggest that the SH group lies between two 'carbonyl affinity groups', which form hydrogen bonds with the carbonyl oxygen atoms of succinic acid. By means of atomic models, Potter & Dubois were able to show that the combination of the type they envisage would result in the hydrogen atoms of succinate being directed towards the SH group, while the hydrogen atoms of malonate would be directed away, thus explaining the fact that malonate, although combining with the enzyme, is not oxidized. Although this theory of Potter & Dubois satisfactorily explains some of the properties of the enzyme, a further examination to see if it can explain the specificity of the enzyme and competitive inhibition by oxaloacetate and pyrophosphate seems to be required.

Unpublished experiments of the author have raised some doubt whether it is even permissible to conclude from the experiments of Hopkins & Morgan (1938) and Potter & Dubois (1943) that the SH group is near the point where the succinate is attached to the enzyme molecule. It was found that succinate and competitive inhibitors will protect the succinic oxidase system from destruction by BAL, although the point of attack on the succinic oxidase system is not even on the succinic dehydrogenase, but on a completely separate component. This suggests that the attachment of succinate or a competitive inhibitor to the succinic dehydrogenase has an effect extending beyond the succinic dehydrogenase molecule itself. It is known that the separate components of the succinic oxidase system must be closely associated with one another, so that each component is easily accessible to those with which it reacts.

SUMMARY

1. Inhibition of the succinic oxidase system by *p*-aminophenylarsenoxide, *o*-iodosobenzoate and oxidized glutathione is a slow reaction; *p*-chloromercuribenzoate reacts much more rapidly.

2. The heart-muscle preparation contained substances which protected the succinic oxidase system from small amounts of *p*-aminophenylarsenoxide and *o*-iodosobenzoate, but not of *p*-chloromercuribenzoate.

3. *p*-Aminophenylarsenoxide acts on the succinic oxidase system by inhibiting the succinic dehydrogenase. Oxidizing agents (*o*-iodosobenzoate, oxidized glutathione and cupric salts) have a more pronounced effect on the succinic oxidase system than on the succinic dehydrogenase, possibly by oxidizing the factor linking succinic dehydrogenase with cytochrome oxidase. *p*-Chloromercuribenzoate also affects the succinic oxidase system more than the succinic dehydrogenase; in addition, it has some effect on the cytochrome oxidase. It is likely that inhibition of the succinic oxidase system by *p*-chloromercuribenzoate is partly due to a non-specific effect on the entire succinic oxidase system.

4. After treatment with sulphhydryl-combining reagents, the enzyme was not reactivated by dilution or by precipitation of the enzyme and washing away the excess inhibitor.

5. After inhibition by *p*-aminophenylarsenoxide, the enzyme was rapidly reactivated by small concentrations of BAL. Reduced glutathione was less effective and denatured globin only slightly effective as reactivating agents. The succinic dehydrogenase and the succinic oxidase system were reactivated to the same extent.

6. After inhibition by iodosobenzoate, the succinic dehydrogenase was almost completely reactivated by high concentrations of BAL. Reduced glutathione was slightly less effective. The succinic oxidase

system was less readily reactivated than the succinic dehydrogenase.

7. BAL readily reactivated the succinic dehydrogenase after partial inhibition by oxidized glutathione, but increased the inhibition of the succinic oxidase system. Cyanide did not reactivate the enzyme inhibited by oxidized glutathione.

8. Succinic dehydrogenase inhibited by *p*-chloromercuribenzoate was largely reactivated by 0.01 M-cyanide. The succinic oxidase system, after treatment with the mercury compound, was readily reactivated by reduced glutathione and denatured globin, but not by BAL.

9. It is suggested that the action of these inhibitors on the succinic oxidase system be expressed in terms of the amount of inhibitor required to react with a definite amount of the enzyme preparation, not by the concentration of inhibitor required to produce a certain effect.

10. The differences between *p*-chloromercuribenzoate and the other inhibitors can partly be explained by the fact that this compound reacts with one sulphhydryl group, while the other compounds require two sulphhydryl groups.

11. The available evidence is not sufficient to decide definitely whether succinic dehydrogenase contains one or two sulphhydryl groups which are necessary for its activity, although one is the more likely.

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