5. Evidence is produced suggesting that cytochrome *b* is involved in the catalysis of the reduction of methylene blue by succinate.

6. The enzyme preparations are colloidal solutions of particles, which are probably derived from some subcellular structure in the tissue.

7. The fact that the molar concentration of copper in the heart-muscle preparation is of the same order of magnitude as that of the haematin

compounds suggests that a copper-protein compound might have some catalytic function in the preparation.

8. The Q_{O_2} (at 37°) of the cytochrome *c* in the heart-muscle preparation is 38,000, i.e. about half the value in yeast.

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The Action of Inhibitors on the System of Enzymes which Catalyse the Aerobic Oxidation of Succinate

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As has been discussed in the previous paper (Slater, 1949a), the enzyme preparations used in the investigation of the succinic oxidase system are colloidal solutions of particles, which are probably derived from some subcellular structure in the tissue. The oxidation of succinate by molecular oxygen requires the co-operation of a number of electron or hydrogen carriers, which appear to be firmly attached to these particles. It is obvious that any one carrier will be unable to react with the carriers before and after it in the reaction chain unless the three are closely situated in or on the particle. Thus, the maintenance of the structure of the particle, which keeps these carriers in the correct spatial relationship to one another, is of paramount importance for the activity of the succinic oxidase system, as has been pointed out by Keilin & Hartree (1940, 1949).

Substances which combine with specific groups in the enzyme molecule, thereby inhibiting the reaction catalysed by the enzyme, are widely used for studying the properties of enzymes. When dealing with a complex system of the type of the succinic oxidase system, the possibility must be considered that the inhibitor does not combine with any specific

groups on the enzyme molecule, but acts non-specifically on the enzyme system as a whole, by affecting the properties of the particles in the solution in such a way as to impair the mutual accessibility of the components of the system. Thus, before inhibitors can profitably be used to obtain information about the components of the succinic oxidase system, the characteristics of the action of inhibitors of this latter type must be determined.

METHODS

The methods of obtaining the enzyme preparations and of measuring enzymic activities have already been described (Slater, 1949a). Except where otherwise stated, the succinic oxidase activity was measured in the presence of added cytochrome *c* (4×10^{-5} M). All activities are expressed as Q_{O_2} ($\mu\text{l. O}_2/\text{mg. fat-free dry wt./hr.}$). Most of the measurements of succinic dehydrogenase activity were made by the manometric method used in the previous paper, but in some cases the Thunberg procedure was used.

Denatured globin (kindly supplied by Dr E. F. Hartree) had been prepared by the method of Keilin & Hartree (1947).

$\text{Ca}_3(\text{PO}_4)_2$ gel was prepared according to the method of Keilin & Hartree (1938).

RESULTS

Effect of ageing

The effect of keeping a preparation of heart muscle at 4° for several weeks on the activities of the succinic dehydrogenase and of the complete succinic oxidase system is shown in Fig. 1. It must be

Fig. 1. Effect of storage of heart-muscle preparation at 4° on the activities of the succinic oxidase and of succinic dehydrogenase; A, succinic oxidase activity, expressed as Q_{O_2} ; B, succinic dehydrogenase activity, determined by the Thunberg method and expressed as q_{MB} , which is in arbitrary units and cannot be compared with the Q_{O_2} .

emphasized that this preparation was not a normal one; probably too much acid was added during the preparation, and consequently its initial succinic oxidase activity was only about one quarter that of a normal preparation. However, the figures are presented because they show very clearly how fluctuations in the activity of the succinic oxidase system are not reflected by the succinic dehydrogenase activities. Usually, the activity of the succinic oxidase system declines after about a week's storage, at which point there is heavy bacterial contamination, while the succinic dehydrogenase activity declines more slowly. This might be due either to the destruction of a component of the succinic oxidase system not required for the succinic dehydrogenase or to changes in the physical properties of the particles. In the present case, however, the increase in the succinic oxidase activity between the first and second week, without change in the succinic dehydrogenase activity of the preparation, must be due to a physical factor. Such an increase in the activity of the succinic oxidase system after 5 days was quite unusual, but an increase during the first day, without a concomitant increase in the succinic dehydrogenase

activity, was quite common. Elliott & Grieg (1938) also found a spontaneous increase in the succinic oxidase activity of a tissue homogenate after standing in the cold for several hours, and attributed this to further dispersion of the components of the succinic oxidase system.

A preparation which was suspended in about one tenth of its volume of M-phosphate buffer and glycerol (final concentration, 50 %) did not show any obvious signs of bacterial contamination after a year at 4°. However, its succinic oxidase activity declined, after a few weeks' storage, at about the same rate as that of a normal preparation which was heavily contaminated after about a week. Its succinic dehydrogenase activity, on the other hand, remained constant for at least 3 months.

Effect of phosphate concentration

The effect of phosphate concentration on the activity of the succinic oxidase system and on the succinic dehydrogenase activity of a heart-muscle preparation is shown in Fig. 2. It can be seen that the

Fig. 2. Effect of phosphate concentration on the activity of the succinic oxidase system and of succinic dehydrogenase. A, succinic oxidase system; B, succinic dehydrogenase.

concentration of phosphate buffer is quite critical for the activity of the succinic oxidase system, but has little effect on the succinic dehydrogenase activity. The optimal concentration of phosphate buffer for succinic oxidase activity was 0.11 M (cf. Keilin & Hartree, 1949, who found that 0.15 M was the optimal phosphate concentration). Readings obtained with concentrations of phosphate lower than about 0.03 M were somewhat erratic.

If the action of phosphate is physical and affects

the mutual accessibility of the components of the succinic oxidase system, one would expect, since cytochrome *c* is one of these components, that the effect of phosphate would be greater if the succinic oxidase activity were measured in the absence of added cytochrome *c* than in its presence. For example, Keilin & Hartree (1940) showed that certain treatments (e.g. alternate freezing and thawing in liquid air) of the heart-muscle preparation, while not affecting the amount of cytochrome *c* in the heart-muscle preparation, did affect its accessibility to the remainder of the succinic oxidase system, so that the activity measured in the absence of added cytochrome *c* was greatly reduced, although the activity in the presence of added cytochrome *c* was not affected. More recently, Keilin & Hartree (1949) have shown that denatured globin can reverse the effect of various physical treatments of the heart-muscle preparation. Table 1 shows the influence of added cytochrome *c* and globin, both separately and together, on the effect of phosphate on the succinic oxidase activity.

Table 1. *Effect of denatured globin and cytochrome c on the effect of phosphate on the succinic oxidase activity of heart-muscle preparation*

	Succinic oxidase activity (Q_{O_2})			
	Nil	Nil	0.13%	0.13%
Globin added ...	Nil	$4 \times 10^{-5} M$	Nil	$4 \times 10^{-5} M$
Cyt. <i>c</i> added ...	Nil	$4 \times 10^{-5} M$	Nil	$4 \times 10^{-5} M$
Phosphate conc. (M):				
A. 0.138	467	604	520	695
B. 0.028	124	349	595	716
B/A	0.27	0.58	1.14	1.03
C. 0.372	323	359	345	414
C/A	0.69	0.65	0.66	0.60

It is apparent from Table 1, first, that the inhibiting effect of low phosphate concentrations is, in the absence of globin, considerably less in the presence than in the absence of cytochrome *c*, and, secondly, in agreement with Keilin & Hartree (1949), that globin completely abolishes the effect of low phosphate concentrations. There is, therefore, reason to believe that the inhibiting action of low phosphate concentrations is due to an effect on the particles of the enzyme preparation, which causes an impaired mutual accessibility of the components of the succinic oxidase system. The inhibiting effect of high phosphate concentrations is not, however, affected by either globin or cytochrome *c*. It is shown in another paper (Slater, 1949b) that high phosphate concentrations have a true and very strong inhibiting action on the cytochrome oxidase. It is possible that the inhibition of the succinic oxidase system by high phosphate concentrations is due to this effect on cytochrome oxidase, which is a part of the succinic oxidase system. Denatured globin has no effect on this inhibition of cytochrome oxidase, nor, of course, has cytochrome *c*, which is the actual substrate of

the enzyme. The variations in cytochrome oxidase activity also found with low phosphate concentrations probably play no part in the effect of such concentrations on the succinic oxidase system, since, in dilute phosphate, the cytochrome oxidase activity is so much higher than that of the succinic oxidase system.

The succinic oxidase activity of the kidney preparation used in the present investigation was affected by the phosphate concentration in much the same way as that of the heart muscle. In this respect, as in others (Slater 1949a), this kidney preparation differed from that of Keilin & Hartree (1949), who found that the activity was optimal at low phosphate concentrations. This difference is probably due to the larger amounts of denatured proteins in the Keilin & Hartree (1949) preparation, since the heart-muscle preparation behaves like their kidney preparation if denatured proteins are added.

Effect of surface-active compounds

Straub (1942), by dispersing a heart-muscle preparation with bile salts, followed by ammonium sulphate fractionation, obtained a preparation which possessed an active succinic dehydrogenase and cytochrome oxidase, but which was unable to oxidize succinate aerobically. He found that the succinic oxidase activity was restored when he added a preparation made by heating heart-muscle preparation at pH 9.0 to 55° for 15 min., which treatment completely destroyed the succinic dehydrogenase. He believed that this second preparation contained a factor, the 'SC factor', which linked the succinic dehydrogenase to the cytochrome system, and which was split off the enzyme by the action of bile salts. The experiments of Straub (1942) were repeated, four preparations being made as follows:

(1) *Phosphate extract*. Washed minced heart muscle was ground for 2 hr. in a mechanical mortar with sand and an equal volume of 0.1 M-phosphate buffer, pH 7.3, and centrifuged for 20 min. at 2000 rev./min. The supernatant was used.

(2) *Cholate preparation*. The method followed was exactly that described by Straub (1942). To 200 ml. of the phosphate extract, 50 ml. 10% sodium cholate, followed by 43.7 g. $(NH_4)_2SO_4$, were added. After standing overnight at 4°, the precipitate was centrifuged off and discarded. The supernatant was treated with 125 g. $(NH_4)_2SO_4/l.$ and the solution centrifuged. The sticky precipitate, which collected at the top of the centrifuge tube, was dissolved in 30 ml. 0.1 M-phosphate buffer to give a turbid solution containing 42.6 mg. fat-free dry material/ml.

(3) *SC factor preparation*. This was also prepared exactly as described by Straub (1942). The phosphate extract (50 ml.) was brought to pH 9 by the addition of *N*-NaOH and heated at 55° for 15 min. The mixture was cooled and neutralized with *N*-acetic acid. Weight of the fat-free dried material = 14.4 mg./ml.

(4) *Keilin & Hartree (1947) preparation*. This was prepared from the phosphate extract by the method of Keilin & Hartree (1947). Weight of fat-free dried material = 32.3 mg./ml.

The succinic dehydrogenase and succinic oxidase activities of the preparations are compared in Table 2. The cholate preparation had no succinic oxidase activity and its succinic dehydrogenase activity was only about one fifth that of the phosphate extract and one sixth that of the Keilin & Hartree (1947) preparation. The properties of both

Table 2. *Activity of Straub and Keilin & Hartree preparations*

(Activities based on the weight of the fat-free dried substance.)

Preparation	Q_{O_2}	
	Succinic oxidase	Succinic dehydrogenase
Phosphate extract	330	150
Cholate preparation	1	33
SC factor	0	1
Keilin-Hartree	510	200

the cholate and the SC factor preparations changed on standing for a few days. When both were freshly prepared, the SC factor had a considerable activating effect on the succinic dehydrogenase of the cholate preparation, and also restored the succinic oxidase activity to a small extent (Table 3). After standing

Table 3. *Effect of addition of SC factor to cholate preparation on activity of latter, using fresh preparations*

(Activities expressed in terms of weight of fat-free dried cholate preparation.)

SC factor added (mg./mg. cholate preparation)	Q_{O_2}	
	Succinic oxidase	Succinic dehydrogenase
0	1	33
0.8	2	72
1.7	14	—

Table 4. *Effect of addition of SC factor to cholate preparation on activity of latter, after keeping both preparations at 4° for 2 days*

(Activities expressed in terms of weight of fat-free dried cholate preparation.)

SC factor added (mg./mg. cholate preparation)	Q_{O_2}	
	Succinic dehydrogenase	Succinic oxidase
0	41	2
1.7	48	6
3.4	50	20
6.8	43	29

2 days at 4°, the succinic dehydrogenase activity of the cholate preparation had increased somewhat and the SC factor preparation was less effective in increasing this activity (Table 4). It should be noted that this behaviour of the cholate and SC factor preparations on storage is the exact reverse of that

found by Keilin & Hartree (1949), who found that the activity of the SC factor—obtained by a method slightly different from that of Straub (1942)—increased and the dehydrogenase activity of the cholate preparation decreased after standing a few days. Since the activity of these preparations depends on physical factors which are little understood, it is not surprising that such unpredictable variations should occur. The fact that treatment with bile salts affects, not only the complete succinic oxidase system, but also the succinic dehydrogenase, and that the SC factor preparation reactivates both, indicates that the bile salts do not act specifically on one component of the system, but have a general effect on the whole enzyme system; it is not surprising that its effect on the more complex complete succinic oxidase system should be greater than on the succinic dehydrogenase. Straub's (1942) SC factor must act by reversing this general effect, and not by supplying a missing factor, since it is difficult to see how a factor which operates between succinic dehydrogenase and cytochrome oxidase could re-activate the succinic dehydrogenase.

This conclusion agrees with that of Keilin & Hartree (1949), who showed that the SC factor is not a specific reactivator of the succinic oxidase system treated with bile salts, since they found that it could be replaced by calcium phosphate, which removed the bile salts from the particles. It seems, therefore, that the action of bile salts is due not to the removal of a specific factor, but to a physical effect on the particles of the heart-muscle preparation. Such an effect is quite obvious to the eye, since the cloudy colloidal solution of heart muscle is considerably clarified by the addition of bile salts. Bile salts are strong denaturing agents and the strong shading between the *b* and *c* bands observed spectroscopically after the addition of sodium dithionite ($Na_2S_2O_4$) to the cholate preparation is probably due to denatured haematin compounds. The intensities of the *c* and *b* bands were lower than in the heart-muscle preparation, although the *a* band was normal.

The action of another surface-active agent, viz. the haemolytic substance which has been isolated by Laser & Friedmann (1945) from a number of tissues, has also been examined. This substance, which was kindly provided by Dr Laser, is a long-chain unsaturated fatty acid. Its effects are shown in Table 5, which summarizes three separate experiments, Exp. 1 with one heart-muscle preparation and Exps. 2 and 3 with a different preparation. Exps. 1 and 2 show that low concentrations of the haemolytic substance practically completely inhibit the succinic oxidase system without having any effect on the succinic dehydrogenase. The two heart-muscle preparations differed in their susceptibility to the inhibitor. The effect of the addition of cytochrome *c* on the degree of inhibition of the succinic oxidase system was

Table 5. *Effect of haemolytic substance (HS) on the succinic oxidase system*

	Inhibition (%)			
	Succinic oxidase system		Succinic dehydrogenase	Cytochrome oxidase
	Without cyt. c	With cyt. c ($6 \times 10^{-5} \text{ M}$)		
Exp. 1 (0.012 ml. heart-muscle preparation/ml.):				
HS($1 \times 10^{-4} \text{ M}$)	—	78	0	—
HS($1 \times 10^{-4} \text{ M}$) + 2 mg. $\text{Ca}_3(\text{PO}_4)_2/\text{ml.}^*$	—	52	—	—
HS($1 \times 10^{-4} \text{ M}$) + 0.2 ml. 'SC factor'/ml.*	—	6	—	—
Exp. 2 (0.012 ml. heart-muscle preparation/ml.):				
HS($0.5 \times 10^{-4} \text{ M}$)	10	11	—	—
HS($1 \times 10^{-4} \text{ M}$)	26	23	0	—
HS($1.75 \times 10^{-4} \text{ M}$)	60	44	—	—
HS($2.5 \times 10^{-4} \text{ M}$)	98	91	1	—
HS($5 \times 10^{-4} \text{ M}$)	100	100	—	—
Exp. 3 (0.006 ml. heart-muscle preparation/ml.):				
HS($1.25 \times 10^{-4} \text{ M}$)	97	97	26	31
HS($2.5 \times 10^{-4} \text{ M}$)	—	—	—	72
HS($4.5 \times 10^{-4} \text{ M}$)	—	—	42	—

* The calculated percentage inhibition has been corrected for the stimulating effect of calcium phosphate gel and 'SC factor' preparation on untreated heart-muscle preparation and for the slight residual activity of the 'SC factor' preparation.

studied in Exp. 2, the results, which are plotted in Fig. 3, showing that the inhibition is greater in the absence of added cytochrome *c* than in its presence. The S shape of the curves in Fig. 3 is probably due

Fig. 3. Effect of adding cytochrome *c* ($6 \times 10^{-5} \text{ M}$) on the inhibition of the complete succinic oxidase system by Laser's haemolytic substance (HS); A, percentage inhibition of system, measured in the absence of added cytochrome *c*; B, percentage inhibition of system, measured in the presence of added cytochrome *c*.

to the protective action, at low concentrations of the inhibitor, of other substances (e.g. denatured proteins) in the enzyme preparation. This is probably also the reason for the finding in Exp. 3 that when the concentration of the enzyme preparation was halved it became much more susceptible to the inhibitor. In Exp. 3, it was found that succinic dehydrogenase and cytochrome oxidase were both inhibited to a certain extent by a concentration of inhibitor sufficient completely to inactivate the complete succinic oxidase system. It is apparent from Exp. 3 that the order of increasing susceptibility to

the inhibitor is succinic dehydrogenase, cytochrome oxidase, complete succinic oxidase system.

Both calcium phosphate gel and Straub's (1942) SC factor were able to reactivate the succinic oxidase system after treatment with the haemolytic substance; reactivation by the SC factor preparation was complete (Exp. 1). It seems very likely that this haemolytic substance acts on the succinic oxidase system in the same manner as bile salts.

DISCUSSION

In considering the action of inhibitors on the succinic oxidase system, it is advisable to distinguish between those substances which act specifically on one component of the system (e.g. malonate on succinic dehydrogenase, cyanide on cytochrome oxidase) and those which act non-specifically on the enzyme system as a whole, by affecting the mutual accessibility of the components of the system, on the particles of the enzyme preparation (cf. Keilin & Hartree, 1949). Examples of this non-specific type of action are adsorption of the inhibitor on the particle, dispersal by surface-active substances, and aggregation. Such treatments must have a more drastic action on the complete succinic oxidase system (involving a number of components) than on those reactions which depend upon only some of the components of the system, for example the reduction of methylene blue by succinate. Thus, it has been invariably found that, with these types of inhibitors, the complete succinic oxidase system is much more susceptible than is the succinic dehydrogenase; in fact, concentrations sufficient to inhibit completely the succinic oxidase system often have no effect on the succinic dehydrogenase, which

require higher concentrations in order to detect any inhibition. The susceptibility of the cytochrome oxidase to the haemolytic substance is intermediate between that of the succinic dehydrogenase and the complete system.

Inhibitors which act in this non-specific manner possess certain characteristics which will usually enable them to be distinguished from the more specific inhibitors, viz. (1) concentrations which are necessary to inhibit completely the succinic oxidase system will affect the cytochrome oxidase to a certain extent, while higher concentrations will have a strong effect on the cytochrome oxidase, and, perhaps, some effect on the succinic dehydrogenase; (2) the inhibition of the complete succinic oxidase system is lower when the activity of the latter is measured in the presence of cytochrome *c* than when it is measured in its absence; (3) the inhibition is often reversed by the addition of substances such as calcium phosphate gel or denatured proteins, which either remove the inhibitor from the particles by absorption (as is probably the case with surface-active compounds) or, in some unknown way, are able to restore the mutual accessibility of the components of succinic oxidase system on the particles after the latter have been affected by physical agents (for example, effect of globin on the effect of low phosphate concentrations).

The more specific inhibitors will, generally, act on only one component of the system, will be unaffected by the presence or absence of cytochrome *c* (unless, of course, the inhibitor acts specifically on the cytochrome *c*; no such inhibitor is, however, known) and will be unaffected by the addition, after the inhibitor has acted on the enzyme, of substances such as denatured proteins or calcium phosphate gel. Denatured globin can, however, reverse the action of substances which combine with thiol groups (Slater, 1949*c*).

If all these points are considered, together with the general physical and chemical properties of an

inhibitor, it should be possible, in most cases, to determine whether the inhibitor has a general non-specific effect on the system or is acting specifically on a single component of the system. A particular application of this method of distinguishing between the two types of inhibition will be found in the following paper (Slater, 1949*d*).

SUMMARY

1. During storage of a particular heart-muscle preparation at 4° for several weeks, the activity of the succinic oxidase system at first increased, then decreased, while the succinic dehydrogenase activity remained constant. It is believed that this is due to an alteration of the physical properties of the particles of the enzyme preparation.

2. The concentration of the phosphate buffer has a profound effect on the activity of the succinic oxidase system, but has little action on the succinic dehydrogenase. The inhibitory action of low phosphate concentrations was greater when the activity was measured without the addition of cytochrome *c*; it was completely prevented by denatured globin.

3. Straub's (1942) claim to have removed, by the action of bile salts, a component of the succinic oxidase system, which can be restored by the addition of a heart-muscle extract, heated under alkaline conditions, has not been confirmed. Bile salts have a general physical effect on the particles of the enzyme preparation.

4. A haemolytic substance had the same effect as bile salts on the system.

5. Inhibition of the succinic oxidase system by non-specific agents possesses certain characteristics, which distinguishes this inhibition from that induced by agents which combine specifically with a component of the system.

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