Inhibition by Antimycin A of Succinic Oxidase in Heart-muscle Preparations

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Antimycin A, an antibiotic prepared in crystalline form by Dunshee, Leben, Keitt & Strong (1949), was found by Ahmad, Schneider & Strong (1950) to have a powerful inhibitory effect on cellular respiration, as exemplified by the succinic oxidase system. Potter & Reif (1952) reported that the compound had a similar inhibitory effect on the system catalysing the oxidation of reduced diphosphopyridine nucleotide, although the existence of an antimycin-insensitive pathway for the oxidation of this substance was also noted, and was subsequently investigated more fully (Reif & Potter, 1954). The results of Ahmad *et al.* (1950) were consistent with the view, later put forward by Potter & Reif (1952), that antimycin A inhibited the succinic oxidase system at some intermediate stage, possibly that involving the factor which is destroyed by 2:3-dimercaptopropanol (BAL) in the presence of air (Slater, 1948, 1949*d*), since the inhibitor at concentrations several hundred times greater than that required to inhibit succinic oxidase activity completely had no effect on cytochrome oxidase activity, and only at these very high concentrations had any notable effect on succinic dehydrogenase activity as measured by the Thunberg method with methylene blue. Spectrophotometric studies of the percentage oxidation of components of the cytochrome system in the steady state, in the absence and in the presence of various concentrations of antimycin A, led Chance (1952) to a similar conclusion as to the point of action of the inhibitor. These facts, together with the extreme potency of antimycin A, make the inhibitor a valuable tool in the study of the cytochrome system, and of the phosphorylation accompanying electron transport by this system in mitochondria.

The work to be described arose from a general study of the interaction between 'succinic dehydrogenase' and 'cytochrome oxidase', and has been carried out with heart-muscle preparations of the type described by Keilin & Hartree (1947). For this reason, the sequence of respiratory carriers as set out by Slater (1949*d*), and modified by Bonner (1954), will be used as the basis for discussion, although current work on the nature of the electron-transport system (see, for example, the recent review by Green & Beinert, 1955), on the intramitochondrial system of electron transport (e.g. Chance

& Williams, 1955), and the recent work of Keilin & Hartree (1955), may lead to a revision of views on the number, sequence, interaction and significance of respiratory catalysts. For most of the purposes of this paper the components of the succinic oxidase system will be described as 'succinic dehydrogenase' (corresponding to succinic dehydrogenase and cytochrome *b*), the antimycin-inhibitable factor (corresponding provisionally to the BAL-sensitive factor) and 'cytochrome oxidase' (corresponding to cytochromes *c*, *a* and *a₃*). The term 'succinate-methylene blue reductase' will be used to denote the enzymic activity of the system which is estimated with methylene blue, since the purified soluble succinic dehydrogenase described by Singer & Kearney (1954) does not react with methylene blue.

The studies of Cleland & Slater (1953) indicate that the enzyme-bearing particles in Keilin & Hartree (1947) preparations are fragments of the membranes of sarcosomes originally present in the heart muscle, and that the components of the succinic oxidase system are firmly bound to the sarcosomal membrane. Spectroscopic studies by Slater (1949*b*) and by Chance (1952) suggest that the components of the succinic oxidase system are present in approximately equal amounts in these preparations. The enzyme-bearing particles may perhaps be regarded, therefore, as carriers of a number of 'succinic oxidase assemblies', to use Chance's (1952) expression, each 'assembly' containing one of each of the components of the system. The present work can best be interpreted on the basis of interaction between 'succinic oxidase assemblies'. It seems probable that this interaction takes place only between 'assemblies' within the same particle, and not between those in different particles (see, for example, Slater, 1953). Most of the work to be described is kinetic, and offers no precise answer to the questions of the nature and location of the antimycin-inhibitable factor. A theory describing the mechanism of action of the inhibitor does, however, emerge. An experiment combining the use of BAL and antimycin A suggests that the BAL-sensitive factor may not be the same as the antimycin-inhibitable factor. Parts of this work have already appeared in preliminary form (Thorn, 1955, 1956).

METHODS

Enzyme preparations

The general method of preparation of the enzyme was that of Keilin & Hartree (1947) and Slater (1949*b*). Bonner (1954) has shown that inorganic phosphate affects the interaction between 'succinic dehydrogenase' and 'cytochrome oxidase', and for this reason some of the preparations used were made by Bonner's (1954) method, in which the isoelectric precipitate, after being washed with ice-cold water, is resuspended in 0.2M borate buffer, pH 7.3. These preparations were found to lose their activity fairly rapidly, and they were not used when it was desirable, for reasons of convenience, to have a preparation with an almost constant activity over several days. Furthermore, it was found that when a Keilin & Hartree (1947) preparation was diluted for use with 0.2M borate buffer, pH 7.3, and when the same buffer was used in making up the reaction mixture, the characteristic properties of 'low-phosphate' preparations could be reproduced, although to a slightly less-marked degree. In what follows, the preparation according to Bonner will be termed a 'low-phosphate' preparation, and that according to Keilin & Hartree a 'normal' preparation. Fresh pig hearts were used as the starting material. Enzyme concentrations are expressed in terms of fat-free dry weight (Slater, 1949*b*). The dry weights of all preparations were usually between 20 and 25 mg./ml. The inorganic phosphate contents of the preparations were determined by estimating inorganic phosphate in the trichloroacetic acid supernatant liquids from the dry-weight determinations. The inorganic phosphate contents of low-phosphate preparations were usually between 1.0 and 1.5 $\mu\text{g.}$ of P/mg. dry weight; those of normal preparations were about 50 times greater.

Measurement of enzyme activities

Succinic oxidase activity was determined manometrically in the Warburg apparatus at 37° with air as the gas phase. Borate buffer, pH 7.3, histidine and succinate were contained in the main compartments of the flasks, and the enzyme was added from the side arms after temperature equilibration. Alternatively, in some experiments in which heart-muscle preparation was treated with an inhibitor before addition to the flasks, the enzyme was pipetted into the main compartments of the flasks, and succinate was contained in the side arms and added after temperature equilibration. The total fluid volume was 3.0 ml. Final concentrations were: borate, 0.1M; succinate, 0.025M; histidine, $4.1 \times 10^{-3}\text{M}$.

It should be noted that the concentration of histidine used was less than that recommended by Bonner (1954). It was found that the low-phosphate preparations used in this work had higher activities in the absence of activators, and reached full activity with lower concentrations of activators, than those described by Bonner.

Succinate-methylene blue reductase activity, determined manometrically, was taken as a measure of 'succinic dehydrogenase' activity. The method was similar to that for measuring succinic oxidase activity, except that in addition to the constituents described, the reaction mixture also contained 0.01M-KCN and 0.001M methylene blue (unless otherwise stated). The enzyme was always contained in the side arm and was tipped after temperature equilibration.

Cytochrome oxidase activity was determined by the procedure recommended by Slater (1949*a*), and was calculated for infinite cytochrome *c* concentration.

Procedure with inhibitors

Antimycin A. Stock solutions of antimycin A in ethanol were made up, containing 400–420 $\mu\text{g.}/25\text{ ml.}$ The stock solution was diluted with ethanol to give solutions one-fifth and one-tenth as concentrated.

Method 1. Measured volumes of not more than 0.06 ml. of dilute solutions of antimycin A were added to the main compartments of the flasks, which contained the other reagents necessary for the measurement of enzyme activity. The same volumes of ethanol were added to the control flasks. The enzyme was contained in the side arms. The final concentrations of ethanol present in the reaction mixture, with this procedure, were not observed to have a significant effect on enzyme activity.

Method 2. Heart-muscle preparation (0.5 ml.) was treated at 0–4° with suitable volumes of 0.2M borate buffer, pH 7.3, and of stock antimycin A solution (not more than 0.13 ml.), so that the final volume of the mixture was 2.5 ml. Appropriate volumes of the mixture were immediately pipetted into the main compartments of the flasks, which already contained all other reagents necessary for the measurement of enzyme activity (succinate in the side arms). To serve as a control, a further quantity of heart-muscle preparation was diluted in a similar way, except that ethanol was substituted for the ethanolic solution of antimycin A. The brief exposure of the enzyme to this concentration of ethanol at low temperature had no significant effect on its activity.

Method 2 has the advantage that in all flasks there is unquestionably the same ratio of antimycin A to heart-muscle preparation, and is of value in experiments in which the effects of other substances on the degree of inhibition by antimycin A are being investigated.

p-Aminophenylarsenoxide. The method described below corresponds closely with that recommended by Slater (1949*e*). Heart-muscle preparation (1 ml.) was incubated in a glass-stoppered tube with a suitable quantity of *p*-aminophenylarsenoxide solution for 60 min. at 37°. At the end of this time the contents of the tube were diluted to 5 ml. with 0.2M borate, pH 7.3, and appropriate volumes of the mixture were pipetted immediately into the main compartments of the flasks, which already contained all other reagents necessary for the measurement of enzyme activity (succinate in the side arms). It was confirmed that the reaction between the enzyme and the arsenical agent was virtually complete after 60 min. at 37°, and preliminary experiments were carried out with each heart-muscle preparation in order to determine the amounts of *p*-aminophenylarsenoxide required to produce the desired degrees of inhibition.

BAL. The method described below corresponds closely with that recommended by Slater (1949*d*). Solutions of BAL (approx. 0.02M) in glass-redistilled water were prepared under nitrogen immediately before use, and 1 ml. quantities were pipetted into the side arms of Warburg flasks. Heart-muscle preparation (1 ml.) was pipetted into the main compartment of each of the flasks, which were then attached to manometers and placed in the bath at 37°. After temperature equilibration the taps were closed and readings were taken. The oxygen consumption was found to be negligible

until the BAL solution was tipped from the side arm. Preliminary experiments were undertaken with each heart-muscle preparation to determine the approximate oxygen consumptions of the mixture of BAL and enzyme, corresponding with the desired degrees of inhibition of succinic oxidase activity. When the appropriate amounts of oxygen had been taken up the flasks were removed from the bath, quickly detached from the manometers, and the contents were diluted to 5 ml. with 0.2M borate, pH 7.3. Suitable volumes of the mixture were immediately pipetted into the main compartments of the flasks, which already contained all other reagents necessary for the measurement of enzyme activity (succinate in the side arms).

Reagents

Glass-redistilled water was used in making up all solutions, and pH values of solutions were checked with a glass electrode.

Antimycin A was kindly given by Professor F. M. Strong. *p-Aminophenylarsenoxide* was kindly given by Professor E. C. Slater.

BAL (2,3-dimercaptopropanol) was the pure compound manufactured by Boots Pure Drug Co., Nottingham.

Cytochrome c was the powder supplied by L. Light and Co., Colnbrook, Bucks. Solutions in water were standardized spectrophotometrically (Potter, 1946).

Borate buffer. That described by Bonner (1954) was used.

Succinate, L-histidine and KCN solutions were as previously described (Thorn, 1953) and were adjusted to pH 7.3–7.4.

RESULTS

General characteristics of inhibition of succinic oxidase by antimycin A

It was desirable first of all to compare the results obtained by the two methods described for studying the inhibition of succinic oxidase by antimycin A. The experiments illustrated in Table 1 show that the

two methods did not give identical results, but certain qualifications must be added. First, in the region of 25–75% inhibition a slight change in the ratio of antimycin A to heart-muscle preparation produces a relatively large effect on the degree of inhibition, whereas in the regions of almost complete or only slight inhibition such a change would produce only a minor effect (this state of affairs is evident in Fig. 1). It is therefore a critical test to attempt this comparison of methods around 50% inhibition. In fact, an error of 4% in the measurement of 0.2 ml. of enzyme solution, or in the measurement of 0.09 ml. of antimycin A solution, for example, would account for a change of up to 20% in the percentage inhibition recorded. Smaller cumulative errors over the whole set of pipetting operations would produce the same effect. Method 1 is more susceptible to such errors. The apparent disagreement between the two methods, as shown in Table 1, is therefore not as serious as might be thought at first sight. Secondly, it can be seen that the ratio of antimycin A to enzyme preparation was increased in the second experiment. Whereas a corresponding increase in the degree of inhibition has been recorded with Method 2, no such increase was observed with Method 1. In fact, Method 1 gave a higher percentage inhibition than Method 2 in the first experiment, and a lower percentage inhibition in the second. The error would appear to be random, and not a systematic error arising from, for example, the different lengths of time during which the enzyme was exposed to the inhibitor before the assay of enzyme activity was performed. In general, therefore, Method 2 was used in preference to Method 1, except in cases where a large number of observations was to be made. As was pointed out in

Table 1. Comparison of methods for studying the degree of inhibition of succinic oxidase by antimycin A

Method 2. The method was exactly as described in the Methods section. Mixtures before addition to flasks contained 12.9 mg. of enzyme and 1.46 μ g. (Expt. 1) or 1.55 μ g. (Expt. 2) of antimycin A in 2.5 ml. Enzyme and inhibitor were in contact for about 20 min. before zero readings were taken in the measurement of succinic oxidase activity. *Method 1.* The principle of the method was as described in the Methods section, but dilute solutions of antimycin A were obtained as described below, instead of in the usual way. The quantities of heart-muscle preparation and of antimycin A which were mixed and diluted to 2.5 ml. in Method 2 were separately diluted to 2.5 ml., the enzyme with 0.2M borate, pH 7.3, and the inhibitor with aqueous ethanol. The diluted enzyme was added to the side arms, and the diluted inhibitor to the main compartments of the flasks, the volumes added being equal to the volume of the mixture added in Method 2. Aqueous ethanol was added to the control flasks. Enzyme and inhibitor were mixed after temperature equilibration, that is, about 3 min. before zero readings were taken. Succinic oxidase activity was measured manometrically at 37°. Constituents of the reaction mixture were: borate, pH 7.3, 0.1M; histidine, 4.1×10^{-3} M; succinate, 0.025M; enzyme and inhibitor as shown; ethanol, 0.06 ml. in Method 1, approx. 0.008 ml. in Method 2; total vol., 3.0 ml.

Expt.	Enzyme (mg.)	Antimycin A (μ g.)	Method	Succinic oxidase activity (μ l. of O_2 /hr.)		Inhibition (%)
				Control	Inhibited	
1	1.03	0.117	1	555	368	33.7
			2	534	396	25.9
2	0.77	0.093	1	375	261	30.4
			2	365	169	53.7

the Methods section, it was regarded as essential to use Method 2 whenever the effect of various conditions or compounds on inhibition by antimycin A was being investigated.

An example of the successful use of Method 2 is given in Table 2, in which is shown the effect of enzyme concentration on inhibition by antimycin A at a constant ratio of antimycin A to enzyme preparation. It is evident that a fourfold change in the enzyme concentration had no effect on the degree of inhibition.

Indeed, this result is implicit in the work of Potter & Reif (1952) who, with rat-liver suspensions, demonstrated direct proportionality between enzyme concentration and the amount of antimycin A necessary to produce 50% inhibition, and used this observation as the basis for what they defined as the 'antimycin titre' of a tissue. The experiments of Potter & Reif (1952) were based on the theoretical approach worked out by Ackermann & Potter (1949), which was designed to distinguish between reversible and irreversible or 'pseudo-irreversible' inhibition. This approach requires experiments in which the enzyme concentration is varied in the absence and in the presence of a fixed amount of antimycin A. If the experiment is repeated with different fixed amounts of antimycin A, a series of curves can be obtained, as shown in Fig. 1. These curves are similar to those already published by Potter & Reif (1952). The four fixed amounts of antimycin A taken were in the ratio 2:3:4:6, and the corresponding amounts of heart-muscle preparation at 50% inhibition were in the ratio 2.0:3.0:4.2:5.7.

Although it would seem that at intermediate degrees of inhibition the degree of inhibition is dependent only on the ratio of antimycin A to

heart-muscle preparation, it is not at once certain that this is the case at 100% inhibition, since the lower parts of the curves are difficult to establish exactly. However, by a simple transformation of the co-ordinates of the experimental points it is possible to superimpose the members of a set of curves of this type, as was shown by Potter & Reif (1952). A transformation, different from that adopted by Potter & Reif, and from which further conclusions may be drawn, is given in a later section (Fig. 3). It is therefore evident that the form of the curves is dependent solely on the ratios of antimycin A to heart-muscle preparation, and it may be concluded that the stoicheiometry between the amounts of antimycin A and of heart-muscle preparation holds good at all degrees of inhibition.

In the series of heart-muscle preparations used in this study, no significant differences have been observed between normal preparations and low-phosphate preparations suitably activated. In preparations in media containing very small quantities of inorganic phosphate, and no other activator, certain complications arise, which are described in a later section. In general, however, 1 mg. of heart-muscle preparation was inhibited 50% by 0.12–0.16 μ g. of antimycin A. The low-phosphate preparations used earlier in the investigation required more antimycin A than the normal preparations used at later stages, within the limits

Table 2. *Effect of enzyme concentration on inhibition of succinic oxidase by antimycin A, at a given ratio of antimycin A to enzyme*

Inhibited preparation: normal heart-muscle preparation was treated with antimycin A by Method 2 (see Methods section). Mixture before addition to flasks contained 12.9 mg. of enzyme and 1.55 μ g. of antimycin A in 2.5 ml. *Control preparation:* treatment as above, except that ethanol was substituted for stock ethanolic solution of antimycin A. Succinic oxidase activity was measured manometrically at 37°. Constituents of reaction mixture were: borate, pH 7.3, 0.1M; histidine, 4.1×10^{-3} M; succinate, 0.025M; heart-muscle preparation as shown; total vol., 3.0 ml.

Enzyme (mg.)	Succinic oxidase activity (μ l. of O ₂ /hr.)		Inhibition (%)
	Control	Inhibited	
0.39	180	89	50.6
0.77	365	169	53.7
1.54	701	343	51.2

Fig. 1. Effect of enzyme concentration on succinic oxidase activity, in the absence and in the presence of fixed amounts of antimycin A. Low-phosphate heart-muscle preparation treated with antimycin A by Method 1 (see Methods section). Succinic oxidase activity was measured manometrically at 37° in the presence of borate, pH 7.3, 0.1M; histidine, 4.1×10^{-3} M; succinate, 0.025M; total vol., 3.0 ml. Quantities of antimycin A: ●, none; ○, 0.067 μ g.; ◐, 0.10 μ g.; ◑, 0.133 μ g.; ◒, 0.20 μ g.

just quoted. No particular significance is attached to this slight difference. If the amount of antimycin A necessary to inhibit a given quantity of heart-muscle preparation by 50% was increased by a half, the inhibition increased virtually to 100%, whereas if the amount of antimycin A was decreased by a half, the inhibition decreased to 5–10%. Taking the average figure of 0.14 $\mu\text{g.}$ as the amount of antimycin A required to inhibit 1 mg. of pig heart-muscle preparation by 50%, the amount required to inhibit completely would be about 0.21 $\mu\text{g.}$, and with 548 as the molecular weight of the inhibitor this represents 0.38×10^{-3} $\mu\text{mole.}$ Slater (1949b) reported that the concentration of cytochrome c in a horse heart-muscle preparation was 0.8 $\mu\text{mole/g.}$, so that 1 mg. of preparation would contain 0.8×10^{-3} $\mu\text{mole.}$ The amount of antimycin A required to inhibit succinic oxidase completely is thus of the same order of magnitude as the amount of a single component of the cytochrome system (assuming all the components are present in equal amounts). The deviation from exact equivalence of these two figures may not be significant, but appears to be similar to that reported by Chance (1952).

*Restoration of activity to preparations
inhibited by antimycin A*

The results of experiments of the type suggested by Ackermann & Potter (1949), and shown in Fig. 1, are of interest from two points of view. The first aspect, discussed in the previous section, is the stoichiometry which evidently governs the interaction between antimycin A and heart-muscle preparation. The second aspect is the sigmoid shape of the curves obtained when enzyme concentration is varied in the presence of a fixed amount of antimycin A. Normally, with irreversible or 'pseudo-irreversible' inhibitors, a straight line, parallel to the line obtained in the absence of inhibitor, is expected. Such a line has been obtained with succinic oxidase preparations, for example, with the inhibitors Cu^{2+} and quinone (Ackermann & Potter, 1949). In these examples, the amount of inhibitor added inactivates completely a quantity of enzyme equal to the intercept on the abscissa, and any excess of enzyme is unaffected, having the same activity as the uninhibited control (i.e. the slope of the line is the same as the slope in the absence of inhibitor). With inhibition by antimycin A, the amount of enzyme in excess of that inhibited completely appears to have an activity greater than that of the uninhibited control (i.e. the 'slope' of the line is greater than the slope in the absence of inhibitor). Such a possibility is unlikely, and the alternative explanation is that a completely inhibited preparation regains activity when treated with an uninhibited preparation. This is illustrated in Table 3. The oxygen consumption which might

be expected in flask 3, calculated by halving the sum of the oxygen consumptions in flasks 1 and 2, is only 274 $\mu\text{l./hr.}$ In fact an oxygen consumption of 447 $\mu\text{l./hr.}$ was obtained. The excess oxygen consumption of 173 $\mu\text{l./hr.}$ must represent some kind of reactivation of previously inhibited succinic oxidase. Furthermore, the oxygen consumption in flask 3 is very close to that in flask 4, confirming that the degree of inhibition observed is dependent solely on the final ratio of antimycin A to heart-muscle preparation. It is also evident that the curves obtained in Fig. 1 by the method of Ackermann & Potter (1949) could also have been produced by adding increasing quantities of uninhibited heart-muscle preparation to appropriate amounts of completely inhibited preparation.

Potter & Reif (1952) suggested that the sigmoid shape of the curves, previously referred to, was understandable in terms of 'an irreversible inhibition of a component that is either originally present in excess or is accompanied by other components that are preferentially titrated by the inhibitor'. In the first alternative, the component present in excess, added with the uninhibited preparation, would provide an electron-transport mechanism for the inhibited as well as the uninhibited succinic oxidase, presumably by interaction between antimycin-bearing and antimycin-free particles. In the second alternative, antimycin A would be removed from the antimycin-bearing particles by some enzymically inert substance in the uninhibited

Table 3. *Restoration of activity to an antimycin-inhibited succinic oxidase preparation by addition to it of uninhibited preparation*

Enzyme A: normal heart-muscle preparation was treated with antimycin A by Method 2 (see Methods section). Mixture before addition to flasks contained 12.4 mg. of enzyme and 2.02 $\mu\text{g.}$ of antimycin A in 2.5 ml. Enzyme B: treatment as for A, but mixture contained 12.4 mg. of enzyme and 1.01 $\mu\text{g.}$ of antimycin A in 2.5 ml. Enzyme C: control treatment as for A, except that ethanol was substituted for stock ethanolic solution of antimycin A. Succinic oxidase activity was measured manometrically at 37°. Constituents of reaction mixture were: borate, pH 7.3, 0.1M; histidine, 4.1×10^{-3} M; succinate, 0.025M; heart-muscle preparation as shown; total vol., 3.0 ml.

Flask	Enzyme	Enzyme (mg.)	Succinic oxidase activity ($\mu\text{l. of O}_2\text{/hr.}$)
1	C	0.99	531
2	A	0.99	16
3	{ C A }	{ 0.495 0.495 }	447
4	B	0.99	459
Average of activities in flasks 1 and 2, to be expected in flask 3			274

preparation, so that reactivation occurred, similar in mechanism to that which was subsequently reported to take place when serum albumin was added to antimycin-inhibited preparations (Reif & Potter, 1953).

The results of Table 3 will now be discussed in more detail, in order to explore possible reasons for the results observed, and to act as a starting point for the experimental approach adopted in the next section. Since the oxygen consumptions in flasks 3 and 4 differed from one another by less than 3 %, the discrepancy may be attributed to experimental error, and it will be assumed that they were both 453 μ l. of O_2 /hr., that is, the average of the observed activities. If, now, the fraction *C* in flask 3 retained the activity it was observed to have in flask 1, it would have contributed 266 μ l. of O_2 /hr. to the total of 453 μ l. of O_2 /hr., leaving 187 μ l. of O_2 /hr. as the contribution of the reactivated fraction *A*. Furthermore, the reactivated fraction *A* might have consisted of uniformly active enzyme, having a Q_{O_2} less than that of fraction *C*, or it might itself have consisted of two fractions, one remaining completely inactive, the other being fully reactivated and having a Q_{O_2} equal to that of fraction *C*. On the other hand, the enzyme in flask 4, which had an activity equal to that of the mixture in flask 3, might be expected to be a homogeneous suspension rather than a heterogeneous collection of fractions of different activities such as those postulated above. It is to be expected that the two mixtures of heart-muscle preparation and antimycin A, present in flasks 3 and 4, both having the same final ratio of enzyme to inhibitor and both possessing the same activity, would have the same distribution of antimycin A. It then follows, either that in flask 3 fractions *C* and *A* became equal in activity by redistribution of antimycin A between them, or that there was an inhomogeneous distribution of antimycin A in flask 4, leading to fractions of unequal activity.

Further experiments, described in the next two sections, were carried out in an attempt to throw some light on this problem.

State of activity of preparations partially inhibited by antimycin A

When a preparation, after treatment with any inhibiting substance, shows diminished activity, the two simplest possibilities are that either a fraction of the enzyme has been inhibited completely, leaving the remainder fully active (for example, the usual case with irreversible inhibition), or that the whole of the enzyme has a uniformly diminished activity. In other words, if the enzyme after treatment has 50 % of its original activity, this may be because half of it has been inhibited completely, leaving the other half fully active, or it may be because the whole of the enzyme is 50 % active.

Putting this concept in terms of elementary enzyme kinetics, the enzyme reaction is first represented in the usual way:

$$V = k'_3[E], \text{ and } K_m = \frac{k_2 + k'_3}{k_1},$$

where *E* represents enzyme, *S* succinate, *ES* the enzyme-succinate complex, and *P* the products of the reaction. k_1 , k_2 and k'_3 are velocity constants, *V* is the velocity of the reaction at infinite succinate concentration (which for the purposes of this discussion will be taken to approximate to the velocity of the reaction at optimum succinate concentration), and K_m is the Michaelis constant. The velocity constant k'_3 is represented with a prime because it has been shown (Slater & Bonner, 1952) that it is not in fact a constant, but varies with the nature and concentration of the hydrogen acceptors. Slater & Bonner were able to demonstrate this because k'_3 was found to be much greater than k_2 , and therefore played a large part in determining the magnitude of K_m .

Now, if half the enzyme is inhibited 100 %, leaving the remainder fully active, *V* will have altered because of a fall in $[E]$, whereas if the whole of the enzyme is inhibited 50 %, *V* will have altered because of a decrease in k'_3 . It is evident that more complex cases will be represented by alterations in *V* due to changes both in $[E]$ and k'_3 simultaneously. A convenient way of deciding whether or not k'_3 has altered is to compare the K_m values of uninhibited and partially inhibited preparations. If K_m of a partially inhibited preparation is the same as that of an uninhibited preparation it may be concluded that k'_3 has not altered, and that the inhibition is due to complete inhibition of a fraction of the enzyme, leaving the remainder fully active. If the K_m of a partially inhibited preparation is less than that of an uninhibited preparation it follows that k'_3 has diminished. Distinction between a fall exclusively in k'_3 and a fall simultaneously in both k'_3 and $[E]$ will be pointed out in connexion with the results of experiments carried out according to the principles described above.

In practice, it was more convenient to determine the ratio K_m/K_i (Thorn, 1953), where K_i is the inhibitory constant of the competitive inhibitor malonate, rather than K_m . Variations in this ratio would be a result of changes in K_m , since K_i is a constant. K_m/K_i was calculated from the degree of inhibition of succinic oxidase activity at one concentration of substrate and inhibitor (method 3 of Thorn, 1953). For each sample of uninhibited or inhibited heart-muscle preparation, however, at least two malonate concentrations were used

(except where it is explicitly stated that one concentration only was used), and the K_m/K_i ratio arrived at was the average of the ratios calculated separately for each malonate concentration. An example of an experiment of this type is shown in Table 4.

This method of approach was used not only with preparations partially inhibited by antimycin A, but also, for comparison, with preparations partially inhibited by treatment with BAL in the presence of air for different periods of time. For further comparison, preparations partially inhibited by *p*-aminophenylarsenoxide were also used, since this inhibitor specifically and irreversibly (under the conditions of these experiments) inhibits succinic dehydrogenase (Slater, 1949*e*), and would be expected to alter $[E]$ rather than k'_3 . Finally, since the variation of K_m/K_i (succinate-malonate) with Q_{O_2} has already been investigated by measuring succinate-methylene blue reductase activity at different methylene blue concentrations (Thorn, 1953), an experiment of this kind was also included.

The results of all these experiments have been collected in Fig. 2, where the K_m/K_i ratios have been plotted against the Q_{O_2} values of the differently treated preparations. In the case of succinate-methylene blue reductase the Q_{O_2} values have been halved in order to correct for the fact that H_2O_2 , and not water, is an end product of the reaction (Slater & Bonner, 1952). The slight scatter of the points in Fig. 2 is certainly to be attributed partly to the fact that different heart-muscle preparations were used during the accumulation of the results, although an attempt was made to achieve reproducibility of

characteristics by using normal preparations rather than the less stable low-phosphate preparations. Nevertheless, it is clear that when the Q_{O_2} was diminished by treatment with various quantities of antimycin A, or with BAL in the presence of air for various periods of time, the K_m/K_i ratio also decreased, and in a manner similar to that observed when the Q_{O_2} was diminished by using different methylene blue concentrations in the measurement of succinate-methylene blue reductase activity. Further, the ordinate intercept was about 3, as was found in previous experiments of this type (Thorn, 1953). These results are in sharp contrast to those obtained when the Q_{O_2} of succinic oxidase was diminished by treatment with different quantities of *p*-aminophenylarsenoxide. In this case the K_m/K_i ratio did not fall correspondingly: in fact it increased slightly. It is not clear why the ratio should increase rather than remain constant, as might have been expected, and this observation may be worth further study.

These experiments demonstrate that if the Q_{O_2} of succinic oxidase is decreased by partial inhibition

Table 4. Determination of the ratio K_m/K_i (succinate-malonate) in a succinic oxidase preparation partially inhibited by antimycin A

Normal heart-muscle preparation was treated with antimycin A by Method 2. Mixture before addition to flasks contained 10.0 mg. of enzyme and 1.18 μ g. of antimycin A in 2.5 ml. This quantity of antimycin A inhibited succinic oxidase activity by 33%. Succinic oxidase activity was measured manometrically at 37°. Constituents of reaction mixture were: borate, pH 7.3, 0.1M; histidine, 4.1×10^{-3} M; succinate, 0.025M; heart-muscle preparation treated as above, 1.0 mg.; malonate as shown; total vol., 3.0 ml.

$$\frac{K_m}{K_i} = \left(\frac{v_0}{v} - 1 \right) \frac{[\text{succinate}]}{[\text{malonate}]}; \text{ see Method 3 of Thorn (1953).}$$

[Malonate] (M $\times 10^3$)	Succinic oxidase activity (μ l. of O_2 /hr.)		K_m/K_i
	Without malonate (v_0)	With malonate (v)	
—	353	—	—
0.333	—	264	25.3
1.33	—	143	27.6
		Average	26.5

Fig. 2. Variation of relative affinity of heart-muscle preparations, treated in various ways, for malonate and succinate (K_m/K_i) with Q_{O_2} . K_m/K_i values were determined as shown in Table 4. Normal heart-muscle preparations were used in all cases. ●, Uninhibited succinic oxidase; ■, uninhibited succinate-methylene blue reductase (concentrations of methylene blue, 2.5×10^{-4} M or 2.5×10^{-3} M); ○, succinic oxidase partially inhibited with antimycin A by Method 2 (cf. Table 3); ●, succinic oxidase partially inhibited by treatment with BAL in air; ●, succinic oxidase partially inhibited by *p*-aminophenylarsenoxide. Each point is an average of K_m/K_i values obtained from determinations with at least two different malonate concentrations, except with succinic oxidase partially inhibited by *p*-aminophenylarsenoxide, when single determinations with one malonate concentration were carried out.

with *p*-aminophenylarsenoxide, the lowered Q_{O_2} is the result of a fall in the amount of active enzyme present $[E]$ and not of a fall in the activity (k'_3) of all the enzyme. On the other hand, the lowered Q_{O_2} obtained after partial inhibition with antimycin A, or with BAL in the presence of air, is the result of a fall exclusively in the activity (k'_3) of all the enzyme present and not of a fall in the amount of active enzyme present $[E]$. If the lowered Q_{O_2} of succinic oxidase were the result of a fall both in $[E]$ and in k'_3 simultaneously, the K_m/K_i ratios would not lie on a straight line with an ordinate intercept of 3 [the value previously found (Thorn, 1953) for K_m/K_i at zero k'_3], but would lie in the space between the two lines shown in Fig. 2, possibly on a straight or a curved line, and with an ordinate intercept which might lie anywhere between 3 and the value for uninhibited succinic oxidase, depending on the complexity of the inhibitory changes taking place.

It should be emphasized that inhibition by antimycin A and by BAL in the presence of air are two entirely different processes. Inhibition by antimycin A appears to be the result of a powerful combination between antimycin A and a factor in the enzyme preparation, which may be reversed under certain conditions, whereas inhibition by treatment with BAL in the presence of air appears to be the result of chemical destruction of a factor in the enzyme preparation, and the effect of this reaction has not been shown to be reversible under any circumstances (Slater, 1949*d*). Antimycin A and BAL have this in common, however, that the part of the succinic oxidase system which they attack does not seem to be involved either in succinate-methylene blue reductase activity or in cytochrome oxidase activity, and that through increasing stages of inhibition k'_3 rather than $[E]$ is correspondingly diminished.

Returning now to a consideration of the results presented in Table 3, it is clear that the enzyme in flask 4 was uniformly diminished in activity, rather than a mixture of fractions possessing different activities. If, as seems probable, the distribution of antimycin A in flasks 3 and 4 was the same, it now follows that some of the antimycin A in combination with fraction A in flask 3 must have been transferred to fraction C, so that both fractions were finally combined with equal quantities of antimycin A and had the same activity. It would seem that fraction C, in process of activating fraction A, itself became partially inhibited. The experiments described in the next section demonstrate this transfer of antimycin A.

Reversibility of combination of antimycin A with a factor in heart-muscle succinic oxidase

It has been postulated in the previous section that an uninhibited heart-muscle preparation (to be

called the 'activating preparation'), on being mixed with an antimycin-inhibited preparation (to be called the 'inhibited preparation'), removes antimycin A from the inhibited preparation, restoring partial activity to it while itself becoming partially inhibited. The principle of the two experiments to be described was to obliterate the activity first of the 'activating preparation', and then of the 'inhibited preparation', by 100 % inhibition with *p*-aminophenylarsenoxide, so that the final activity, after mixing, first of the 'inhibited preparation', and then of the 'activating preparation', could be assessed separately. These two experiments, with suitable controls, are shown in Tables 5 and 6.

In Table 5, the activity of a preparation inhibited by antimycin A was assessed after mixing it with an equal volume of a preparation inhibited by *p*-aminophenylarsenoxide. It is evident, by comparison of flasks 2 and 3 with flask 1, that the oxygen consumptions of the antimycin-inhibited and arsenical-inhibited samples of enzyme were negligible, and that virtually 100 % inhibition had been obtained in each case. On mixing equal amounts of these two samples in flask 4, a considerable succinic oxidase activity was observed, attributable to the sample originally inhibited with antimycin A, since the succinic dehydrogenase in the arsenical-inhibited sample was irreversibly inhibited under the conditions of this experiment. In flask 5, an amount of preparation containing a quantity of active succinic dehydrogenase equal to that in flask 4, but with half the quantity of antimycin A, showed a succinic oxidase activity equal to that in flask 4. This suggested that in flask 4 half the amount of antimycin A, originally combined with the antimycin-inhibited sample, had been transferred to the antimycin-inhibitable factor in the arsenical-inhibited sample. Flask 6 showed that the presence of arsenical-inhibited preparation had no effect on the succinic oxidase activity of an uninhibited preparation mixed with it.

In Table 6, the activity of an uninhibited preparation was assessed after mixing it with an equal volume of a preparation inhibited both by *p*-aminophenylarsenoxide and antimycin A. Flasks 2 and 3 were included in order to show that both treatments to which the doubly inhibited enzyme was subjected were sufficient to bring about virtually 100 % inhibition. On mixing the doubly inhibited enzyme with the control enzyme, in flask 5, it is evident that the activity of the control enzyme was depressed. In flask 6, an amount of preparation containing a quantity of active succinic dehydrogenase equal to that in flask 5, but with half the quantity of antimycin A, showed a succinic oxidase activity equal to that in flask 5. This suggested that in flask 5 half the amount of antimycin A, originally combined with the doubly inhibited sample, had been

transferred to the antimycin-inhibitable factor in the control sample.

As a comment on the reproducibility of these methods, it may be noted that the results presented in Table 3 and in Table 6 were obtained with different heart-muscle preparations and with different stock solutions of antimycin A. By coincidence, the control activities expressed as $\mu\text{l. of O}_2/\text{hr.}$ were the same, and it can be seen that the activities of the partially inhibited mixtures, obtained by similar methods, were within a few per cent of one another.

It is considered that the experiments presented in Tables 5 and 6 together constitute an adequate demonstration of the redistribution of antimycin A equally over all the antimycin-inhibitable factor available when an antimycin-inhibited preparation is mixed with an uninhibited preparation. This process can scarcely be described as reactivation, however, because although the originally inhibited fraction has regained some activity, this activity has been obtained at the expense of some inhibition of the originally uninhibited fraction.

Effect of concentration of antimycin-inhibitable factor on succinic oxidase activity

It has been previously shown that the velocity constant k'_3 for succinic oxidase activity is different from the k'_3 for succinate-methylene blue reductase

Table 5. *Reactivation of an antimycin-inhibited succinic oxidase preparation by a preparation inhibited by p-aminophenylarsenoxide*

Enzyme A: normal heart-muscle preparation (1.0 ml.) was treated with 0.32 $\mu\text{equiv.}$ of *p*-aminophenylarsenoxide as described in Methods section. Mixture before addition to flasks contained 25.6 mg. of enzyme in 5.0 ml. Enzyme B: normal heart-muscle preparation was treated with antimycin A by Method 2 (see Methods section). Mixture before addition to flasks contained 12.8 mg. of enzyme and 2.2 $\mu\text{g.}$ of antimycin A in 2.5 ml. Enzyme C: control treatment as for B, except that ethanol was substituted for stock ethanol solution of antimycin A. Succinic oxidase activity was measured manometrically at 37°. Constituents of reaction mixture were: borate, pH 7.3, 0.1M; histidine, $4.1 \times 10^{-3}\text{M}$; succinate, 0.025M; heart-muscle preparation as shown; total vol., 3.0 ml.

Flask	Enzyme	Inhibitor	Enzyme (mg.)	Succinic oxidase activity ($\mu\text{l. of O}_2/\text{hr.}$)
1	C	(Control)	1.02	440
2	B	Antimycin	1.02	9
3	A	Arsenical	1.02	18
4	{B A}	{Antimycin Arsenical}	{1.02 1.02}	391
5	{B C}	{Antimycin (Control)}	{0.51 0.51}	399
6	{C A}	{(Control) Arsenical}	{1.02 1.02}	435

Table 6. *Inhibition of a succinic oxidase preparation by a preparation inhibited both by p-aminophenylarsenoxide and by antimycin A*

Enzyme A: normal heart-muscle preparation (1.0 ml.) was treated with 0.4 $\mu\text{equiv.}$ of *p*-aminophenylarsenoxide as described in Methods section. Mixture before addition to flasks contained 25.7 mg. of enzyme in 5.0 ml. Enzyme B: normal heart-muscle preparation was treated with antimycin A by Method 2 (see Methods section). Mixture before addition to flasks contained 12.9 mg. of enzyme and 2.04 $\mu\text{g.}$ of antimycin A in 2.5 ml. Enzyme C: normal heart-muscle preparation was treated with *p*-aminophenylarsenoxide exactly as A, but before final dilution it was treated with antimycin A in the same proportion as B. Mixture before addition to flasks contained 25.7 mg. of enzyme and 4.08 $\mu\text{g.}$ of antimycin A in 5.0 ml. Enzyme D: control treatment as for B, except that ethanol was substituted for stock ethanol solution of antimycin A. Succinic oxidase activity was measured manometrically at 37°. Constituents of reaction mixture were: borate, pH 7.3, 0.1M; histidine, $4.1 \times 10^{-3}\text{M}$; succinate, 0.025M; heart-muscle preparation as shown; total vol., 3.0 ml.

Flask	Enzyme	Inhibitor	Enzyme (mg.)	Succinic oxidase activity ($\mu\text{l. of O}_2/\text{hr.}$)
1	D	(Control)	1.03	531
2	A	Arsenical	1.03	23
3	B	Antimycin	1.03	14
4	C	Arsenical and antimycin	1.03	0
5	{C D}	{Arsenical and antimycin (Control)}	{1.03 1.03}	461
6	{B D}	{Antimycin (Control)}	{0.514 0.514}	454

activity (Slater & Bonner, 1952). The same workers also showed that k'_3 could be progressively altered by changing the concentration of methylene blue in the measurement of succinate-methylene blue reductase activity. Since the magnitude of k'_3 depends on the concentration of methylene blue, k'_3 must contain a variable term, related to methylene blue concentration, in addition to a quantity which is a true velocity constant. Extending this argument to the variable k'_3 , which has now been demonstrated in heart-muscle preparations partially inhibited by antimycin (Fig. 2), the variable term in k'_3 would logically be related to the concentration of unbound antimycin-inhibitable factor in these preparations. Following the analogy with methylene blue a step further, it is a necessary corollary that each molecule of antimycin-inhibitable factor is available to 'succinic dehydrogenase' in 'succinic oxidase assemblies' other than that in which it is situated.

Since it is common practice in the study of enzymes to investigate the effect of the concentration of substrates and cofactors on enzyme activity,

an attempt was made to determine the effect of the concentration of available antimycin-inhibitable factor on the succinic oxidase activity of a given amount of heart-muscle preparation. Such an attempt is shown in Fig. 3*a*. The experimental points were obtained by transforming the co-ordinates of the points shown in Fig. 1, and from many other experiments such as those shown in Tables 2, 3, 5 and 6, where the succinic oxidase activity of a partially antimycin-inhibited preparation, with a given ratio of antimycin A to heart-muscle preparation, was known in relation to the succinic oxidase activity of an uninhibited control.

Fig. 3. (*a*) Variation of succinic oxidase activity with decreasing quantities of antimycin A/mg. of heart-muscle preparation. The vertical line is the ordinate constructed at the apparent point of origin of the hyperbolic part of the complex curve. The arrows represent the maximum velocity calculated from the line in (*b*). (*b*) Points on the hyperbolic part of the complex curve in (*a*) plotted according to Lineweaver & Burk (1934). $\times$, Points derived from Fig. 1, low-phosphate preparation treated with antimycin A by Method 1; $\bullet$, points obtained from experiments with a number of normal preparations treated with antimycin A by Method 2 (see Methods section).

The enzyme activities were all expressed as a percentage of the uninhibited control, and the quantities of antimycin A present were calculated, by simple proportion, in relation to 1 mg. of heart-muscle preparation, since, as has been pointed out earlier, the degree of inhibition is determined solely by the ratio of inhibitor to enzyme. Owing to the fact that the form of the curve was the main object of the investigation, it was considered justifiable to reduce the scatter of the points by correcting these quantities of antimycin A, calculated in relation to 1 mg. of heart-muscle preparation, so that 50 % activity occurred at the average value of 0.14 μ g. of antimycin A. It was possible to do this because enough experiments had been carried out with each preparation to enable an estimate to be made of the actual amount of antimycin A required to obtain 50 % activity. Finally, the quantities of antimycin A were plotted on a reversed scale, to represent increasing quantities of available antimycin-inhibitable factor/mg. of preparation.

It is evident that the complex curve, obtained by the above methods, apparently consists of an initial portion which approximates to a straight line, followed by a hyperbola. If the presumed hyperbola is extrapolated to the abscissa, the co-ordinates of the points on this part of the curve can be related to a new abscissa scale, obtained, as shown in Fig. 3, by subtracting the number of μ g. of antimycin A shown on the original abscissa scale from the number of μ g. present at the origin of the hyperbola. When the experimental points, related to the new abscissa scale, are plotted according to Lineweaver & Burk (1934), a straight line is obtained as shown in Fig. 3*b*. It is possible to calculate a theoretical maximum velocity and a quantity analogous to a Michaelis constant from the Lineweaver-Burk plot. The theoretical maximum velocity has been marked in Fig. 3*a*, and proves to be greater than the actual maximum velocity observed in uninhibited heart-muscle preparations. This would be expected, because such preparations do not contain an infinite concentration of antimycin-inhibitable factor. A quantity analogous to the Michaelis constant is not given, since the significance of such a quantity in this context is rather doubtful at present.

The experiments of the previous two sections, together with the results shown in Fig. 3, suggest that the sigmoid curves obtained in Fig. 1 may be the result, not only of a particular characteristic of the action of antimycin A, but also of the kinetic relationship between adjacent components of the succinic oxidase system in this type of enzyme preparation. These topics will be considered in more detail in the Discussion, and in relation to the experiments described in the next section.

Effect of activators and of added cytochrome c on inhibition of succinic oxidase by antimycin A

It has been shown that in the presence of low concentrations of inorganic phosphate, heart-muscle preparations have a greatly impaired succinic oxidase activity (Keilin & Hartree, 1949; Slater, 1949c; Ball & Cooper, 1949; Bonner, 1950, 1954). Activity can be restored to such preparations, not only by optimum concentrations of inorganic phosphate, but also by other substances, notably denatured globin (Keilin & Hartree, 1949; Bonner, 1950), histidine (Ball & Cooper, 1949; Bonner, 1951, 1954) or ethylenediaminetetraacetic acid (Bonner, 1954). Added cytochrome c also has an activating effect on succinic oxidase activity, which Keilin & Hartree (1949) showed to be a specific catalytic effect, rather than the 'mutual accessibility' effect which they put forward to explain the activating effect of denatured globin. Since the results presented above have led to the postulate that the antimycin-inhibitable factor is freely available to 'succinic dehydrogenase' it follows that at this point in the structure of the 'succinic oxidase assembly' there must be some flexibility. Such a point of flexibility might be the point at which the mutual accessibility of adjacent components of the system assumes greatest importance for the activity of the system. Accordingly histidine and cytochrome c, separately and together, were tested for their possible effects on inhibition of succinic oxidase activity by antimycin A in a medium containing a suboptimum amount of inorganic phosphate.

The results of one such experiment are given in Table 7, and it is clear that the addition of either histidine or cytochrome c led simultaneously to an increase in the succinic oxidase activity of the control and to a decrease in the inhibitory effect of antimycin A. In this experiment, contrary to previous experience, the addition of cytochrome c to the preparation already activated by histidine did not lead to a further increase in the succinic oxidase activity of the control. However, a further decrease in the inhibitory effect of antimycin A was observed.

Bonner (1954) has shown that preparations in media deficient in inorganic phosphate can also be activated simply by the addition of more enzyme preparation, i.e. the Q_{O_2} increases with enzyme concentration. This type of activation was also tested for its ability to influence inhibition by antimycin A. In the experiment described in Table 8, the same ratio of antimycin A to heart-muscle preparation was present in the three flasks containing inhibitor, but different concentrations of enzyme were present. It was observed that as the Q_{O_2} increased with increasing enzyme concentrations, so the degree of inhibition by antimycin A

decreased. This behaviour is in marked contrast to the behaviour of fully activated preparations where, as was shown in Table 2, the same degree of inhibition is observed at a given ratio of antimycin A

Table 7. *Effects of cytochrome c and histidine, separately and together, on inhibition of succinic oxidase by antimycin A, in a medium containing a suboptimum concentration of inorganic phosphate*

Inhibited preparation: normal heart-muscle preparation was treated with antimycin A by Method 2 (see Methods section). Mixture before addition to flasks contained 12.2 mg. of enzyme and 1.52 μ g. of antimycin A in 2.5 ml. Control preparation: treatment as above, except that ethanol was substituted for stock ethanol solution of antimycin A. Succinic oxidase activity was measured manometrically at 37°. Constituents of reaction mixture were: borate, pH 7.3, 0.1M; succinate, 0.025M; heart-muscle preparation, 0.97 mg.; inorganic phosphate (introduced with enzyme), 6.8×10^{-4} M; total vol., 3.0 ml. When added, concentration of cytochrome c was 5×10^{-5} M, and concentration of histidine was 4.1×10^{-3} M.

Additions	Succinic oxidase activity (μ l. of O_2 /hr.)		Inhibition (%)
	Control	Inhibited	
None	193	47	75.7
Cytochrome c	266	122	54.2
Histidine	569	353	38.0
Cytochrome c } Histidine	569	394	30.8

Table 8. *Effect of enzyme concentration on inhibition of succinic oxidase by antimycin A, at a given ratio of antimycin A to enzyme, in a medium containing a suboptimum concentration of inorganic phosphate*

Inhibited preparation: normal heart-muscle preparation was treated with antimycin A by Method 2 (see Methods section). Mixture before addition to flasks contained 12.9 mg. of enzyme and 1.30 μ g. of antimycin A in 2.5 ml. Control preparation: treatment as above, except that ethanol was substituted for stock ethanol solution of antimycin A. Succinic oxidase activity measured manometrically at 37°. Constituents of reaction mixture were: borate, pH 7.3, 0.1M; succinate, 0.025M; heart-muscle preparation as shown; inorganic phosphate, 2.16×10^{-3} M; total vol., 3.0 ml. This amount of inorganic phosphate was that unavoidably added with the enzyme preparation when used at the highest concentration. The amounts of inorganic phosphate present at the two lower enzyme concentrations were made equal to that at the highest enzyme concentration by addition of suitable quantities of dilute phosphate buffer.

Enzyme (mg.)	Succinic oxidase activity (μ l. of O_2 /hr.)		Inhibition (%)
	Control	Inhibited	
0.514	62	20	68
1.54	420	186	56
3.08	928	556	40

to heart-muscle preparation, irrespective of enzyme concentration.

It is concluded that activators and cytochrome *c* interact with heart-muscle preparations at a point very close to that at which antimycin A exerts its inhibitory effect, and that their effect in decreasing the inhibition due to antimycin A is not to be attributed to the removal of antimycin A, as was observed by Reif & Potter (1953) with serum albumin, but is a result of improved communication between 'succinic dehydrogenase' and the remainder of the electron-transport system, either by increased 'mutual accessibility' in the case of activators, or perhaps as a kind of 'electron bridge' in the case of cytochrome *c*.

Support is given to this interpretation, in the case of cytochrome *c*, by the experiment illustrated in Fig. 4. The effect of enzyme concentration on succinic oxidase activity in the absence and in the presence of a fixed amount of antimycin A is shown, both in the absence and in the presence of cytochrome *c*. Different ordinate scales have been chosen, so that the activities in the absence of antimycin A appear to be the same whether cytochrome

c was present or not. It appears that the effect of cytochrome *c* is to modify the shape of the rising portion of the curve, rather than to alter the amount of preparation inhibited completely by the quantity of antimycin A used. The results of Reif & Potter (1953) also indicate that antimycin A, unlike the naphthoquinone SN-5949, is not bound by added cytochrome *c*. In terms of a transformation of data, similar to that shown in Fig. 3*a*, this effect of cytochrome *c* would be mainly to alter the characteristics of the hyperbola.

Preliminary studies (Thorn, 1956) of the effect of lack of optimum concentrations of inorganic phosphate or histidine on the characteristics of the hyperbola obtained as shown in Fig. 3*a* suggest that activators affect both the quantity analogous to K_m and the maximum velocity. The quantity analogous to K_m may perhaps be interpreted as an inverse measure of the 'affinity' or 'accessibility' of the antimycin-inhibitable factor to an adjacent component in the electron-transport system. Detailed appraisal of this type of evidence must await further study, which is being pursued at the present time.

Component of the preparation inhibited by antimycin A

Ahmad *et al.* (1950) showed that antimycin A had little effect on succinic dehydrogenase (succinate-methylene blue reductase) and no effect on cytochrome oxidase, although very high concentrations of antimycin A had a definite inhibitory effect on succinic dehydrogenase. These observations have been confirmed in the present study, although no attempt was made to use the very high concentrations of antimycin A reached by Ahmad *et al.* (1950). With a quantity of antimycin A just sufficient to inhibit succinic oxidase activity completely, the inhibition of succinate-methylene blue reductase activity was, in one experiment, 18%, and this degree of inhibition did not increase when the ratio of antimycin A to heart-muscle preparation was increased 2.5 times. An amount of antimycin A double that which was just sufficient to inhibit succinic oxidase activity completely had no effect on cytochrome oxidase activity calculated at infinite cytochrome *c* concentration, and no effect on the catalytic efficiency of the added cytochrome *c*.

On the basis of this kind of observation, and of other features of the effect of antimycin A, Potter & Reif (1952) tentatively suggested that the component of the succinic oxidase system which was inhibitable by antimycin A might be the BAL-sensitive factor (Slater, 1948, 1949*d*). An experiment combining the use of BAL and antimycin A was devised in an attempt to test the possibility that the antimycin-inhibitable factor and the BAL-sensitive factor might be identical.

Fig. 4. Effect of enzyme concentration on succinic oxidase activity, in the absence and in the presence of a fixed amount of antimycin A, carried out in the absence and in the presence of cytochrome *c*. Low-phosphate heart-muscle preparation was treated with antimycin A by Method 1 (see Methods section). Succinic oxidase activity was measured manometrically at 37° in the presence of borate, pH 7.3, 0.1 M; histidine, 4.1×10^{-5} M; succinate, 0.025 M; antimycin A, when added, 0.133 μ g.; cytochrome *c*, when added, 4×10^{-5} M; total vol., 3.0 ml. ○, Cytochrome *c* absent, antimycin A absent; ●, cytochrome *c* present, antimycin A absent; □, cytochrome *c* absent, antimycin A present; ■, cytochrome *c* present, antimycin A present.

It was argued that if the two factors were the same substance, then destruction of the BAL-sensitive factor in a sample of heart-muscle preparation might deprive the preparation of its power to restore activity to an antimycin-inhibited sample. The experiment is shown in Table 9. The enzyme samples in flasks 2 and 3, treated with antimycin A and with BAL respectively, showed negligible activity when compared with an untreated sample in flask 1. On mixing the two inhibited samples together, however, some succinic oxidase activity was restored to the antimycin-inhibited sample, as shown in flask 4. This succinic oxidase activity was slightly less (12%) than the activity obtained with an amount of preparation containing a quantity of potentially active succinic oxidase equal to that added as antimycin-inhibited preparation in flask 4, but with half the quantity of antimycin A (flask 5). However, in flask 6 it is shown that the activity of the control sample was slightly inhibited (10%) by the preparation treated with BAL. The depression of activity in flask 4 compared with flask 5, being similar to the depression of activity in flask 6 compared with flask 1, would be entirely accounted for by some inhibiting substance introduced with the BAL-treated sample of enzyme. It is therefore

concluded that treatment with BAL in the presence of air, for the length of time necessary to inhibit succinic oxidase activity completely, does not impair the capacity of a heart-muscle preparation to combine with antimycin A.

DISCUSSION

General features of inhibition of succinic oxidase by antimycin A

Antimycin A, as has been previously demonstrated by Ahmad *et al.* (1950), inhibits succinic oxidase activity, and the very low concentrations necessary for complete inhibition have little or no effect on succinate-methylene blue reductase activity or on cytochrome oxidase activity. At complete inhibition of succinic oxidase activity the molarity of the inhibitor and the probable molarity of the inhibited factor in the electron-transport system are of the same order of magnitude. This agrees with the findings of Chance (1952), and indicates a very powerful combination between antimycin A and the factor. A result of this powerful combination is the possibility of 'titrating' the antimycin-inhibitable factor in succinic oxidase preparations, as has been demonstrated by Potter & Reif (1952). The same authors have also shown that the degree of inhibition of succinic oxidase activity by antimycin A is a function solely of the ratio of inhibitor to enzyme preparation. These aspects of the action of antimycin A have been confirmed in the present study. Reif & Potter (1953) pointed out that these characteristics of antimycin A lead to its classification as an irreversible or 'pseudo-irreversible' inhibitor, according to the criteria of Ackermann & Potter (1949). At the same time, Reif & Potter (1953) reported that inhibition by antimycin A could be reversed by the addition of serum proteins, and traced this action of serum to the albumin fraction. On the basis of these results antimycin A was classed as a 'pseudo-irreversible' inhibitor. The present work does not alter this conclusion, but emphasizes the ready reversibility of the combination between antimycin A and the factor, since it has been shown that antimycin A rapidly redistributes equally over the available factor when inhibited and uninhibited preparations are mixed together.

The redistribution of antimycin A does not, however, explain the sigmoid type of curves obtained in experiments of the type described by Ackermann & Potter (1949), and illustrated in Fig. 1. It would be expected that, provided that the same amount of factor was bound by antimycin A, straight lines parallel to the line obtained in the absence of inhibitor would result, whatever the distribution of antimycin A. Possible reasons for

Table 9. *Reactivation of an antimycin-inhibited succinic oxidase preparation by a preparation inhibited by BAL in the presence of air*

Enzyme A: normal heart-muscle preparation (1.0 ml.) was treated with BAL (final concn. 0.012M) as described in Methods section. Mixture was shaken at 37° for 30 min., during which time an oxygen uptake of 178 μ l. occurred. Mixture before addition to flasks contained 24.3 mg. of enzyme in 5.0 ml. Enzyme B: normal heart-muscle preparation was treated with antimycin A by Method 2 (see Methods section). Mixture before addition to flasks contained 12.2 mg. of enzyme and 2.1 μ g. of antimycin A in 2.5 ml. Enzyme C: control treatment as for B, except that ethanol was substituted for stock ethanol solution of antimycin A. Succinic oxidase activity measured manometrically at 37°. Constituents of the reaction mixture were: borate, pH 7.3, 0.1M; histidine, 4.1×10^{-3} M; succinate, 0.025M; heart-muscle preparation as shown; total vol., 3.0 ml.

Flask	Enzyme	Inhibitor	Enzyme (mg.)	Succinic oxidase activity (μ l. of O ₂ /hr.)
1	C	(Control)	0.97	562
2	B	Antimycin	0.97	16
3	A	BAL + O ₂	0.97	10
4	{ B A	{ Antimycin BAL + O ₂	{ 0.97 0.97	444
5	{ B C	{ Antimycin (Control)	{ 0.485 0.485	506
6	{ C A	{ (Control) BAL + O ₂	{ 0.97 0.97	506

this sigmoid type of curve will be considered in detail, with special reference to Fig. 3, and to the finding that the depression of activity in partially antimycin-inhibited preparations is the result of a fall in the activity (k'_3) of all the enzyme present, rather than a fall in the amount of active enzyme present ($[E]$).

The normal case of an irreversible or 'pseudo-irreversible' inhibitor would be represented, in a plot such as Fig. 3*a*, by a straight line from zero to 100% activity. Such a line, when transformed to the type of plot shown in Fig. 1, gives a straight line parallel to the line obtained in the absence of inhibitor, as expected. Potter & Reif (1952) suggested that the sigmoid curve might be explained by the presence of another component in the preparation, preferentially titrated by antimycin A. Such a suggestion, although acceptable on qualitative grounds, is unlikely for the following reasons:

(1) If the combination between antimycin A and the competing substance were irreversible, addition of antimycin A to an enzyme preparation in successively greater quantities would, at first, lead to no inhibition at all. Since the combination between antimycin A and the factor has been shown to be reversible, antimycin A would preferentially combine almost exclusively with the competing substance. When, however, the competing substance had combined with antimycin A to its full capacity, inhibition of the factor would then proceed linearly with successively greater additions of antimycin A. The form of curve obtained in this way would not be hyperbolic, but would be similar to the type of curve which Slater (1949*e*) found to describe the effect of increasing quantities of *p*-aminophenylarsenoxide on heart-muscle preparations. [If Slater's (1949*e*) Fig. 4 is viewed upside down, the scales correspond in direction with those of Fig. 3*a* of this paper.]

(2) If the combination between antimycin A and the competing substance were reversible, the quantity of antimycin A added would be partitioned between the competing substance and the factor in a constant ratio, depending on their relative affinities for antimycin A. The partition of antimycin A between competing substance and factor would be independent of the concentration of antimycin A, in a situation analogous to the partition of an enzyme between its substrate and a competitive inhibitor, which is independent of enzyme concentration. Fig. 3*a* would again appear as a straight line from zero to 100% activity, but the scale of antimycin A added/mg. of preparation would not represent the actual amount of antimycin A in combination with the factor. It is worth noting that such a state of affairs might be superimposed on the complex curve in Fig. 3*a*. It is, in fact, impossible to tell from the present data whether there might not be such a competing sub-

stance, the only effect of which would be to alter the abscissa scale without influencing the form of the curve.

(3) If the competing substance were an enzymically inert substance in the preparation, e.g. an inert or denatured protein, it might be expected that the amounts of antimycin A required for 50% inhibition would vary considerably among preparations, or even in the same preparation during ageing. In fact, the constancy of the characteristics of inhibition by antimycin A is one of its striking features. This objection would, of course, be ruled out if the competing substance were a component of the succinic oxidase system itself, but whose catalytic properties were not affected by combination with antimycin A.

Since the presence of a competing substance does not satisfactorily explain the data, the other possibility suggested by Potter & Reif (1952) may be examined, that is to say, that the component inhibited by antimycin A might be in excess. If the component is present in an amount greater than that required for maximum activity, the gradual addition of increasing quantities of antimycin A would produce, at first, no inhibition, and then, a linear response of inhibition to amount of antimycin A. The result would be a curve somewhat like that which would occur in the presence of a competing substance combining irreversibly with antimycin A. The explanation now to be submitted does, however, involve the properties of the electron-transport system, rather than the properties of antimycin A or of a competing substance.

It has been pointed out that there is an analogy between the variable k'_3 observed when different methylene blue concentrations are employed in the estimation of succinate-methylene blue reductase activity, and the variable k'_3 which is observed when different concentrations of antimycin A are present during the estimation of succinic oxidase activity. The variable factor in k'_3 under these latter circumstances was postulated to be related to the available amount of free antimycin-inhibitable factor, and such a concept implies the availability of the factor to 'succinic dehydrogenase' in 'succinic oxidase assemblies' other than that in which it is situated. Thus the antimycin-inhibitable factor is regarded as a freely available hydrogen (electron) acceptor. However, if the antimycin-inhibitable factor is an electron-transport component (as opposed to a grouping which is necessary for the linking together of adjacent electron-transport components), it is clear that it must act not only as a hydrogen (electron) acceptor for 'succinic dehydrogenase' but also as an oxidizable substrate for 'cytochrome oxidase', and the considerations outlined above may alternatively imply the availability of reduced factor to 'cytochrome oxidase' in 'succinic oxidase

assemblies' other than that in which it is situated. Whichever view is taken, however, the concept is the same, namely that the antimycin-inhibitable factor is functioning as a freely available substrate or coenzyme.

It is well known that variation of substrate or coenzyme concentration usually causes a hyperbolic response of enzyme activity, and this effect is explained by the Michaelis-Menten theory in terms of the reversible formation of an enzyme-substrate or enzyme-coenzyme complex, with 'saturation' of the active centres of the enzyme at high concentrations of substrate or coenzyme. The hyperbolic curve can be put into a linear form by the familiar procedures of Lineweaver & Burk (1934).

If, as seems reasonable in the light of the previous discussion, the amount of antimycin A present/mg. of heart-muscle preparation represents an indirect measure of the amount of inhibited or unavailable factor, it follows that the curve shown in Fig. 3*a* describes the enzymic response to increasing quantities of unbound or available factor in 1 mg. of heart-muscle preparation. Although it is evident that the curve as a whole is not a hyperbola, a large section of it is demonstrably so, and this leads to the conclusion that provided that there is sufficient antimycin-inhibitable factor remaining unbound (about 25%), it acts as if it were freely available, confirming the deduction made above from the experiments demonstrating a variable k'_s . However, at low concentrations of factor, there is a straight-line response of enzyme activity to factor concentration, implying no free availability. It would be expected that this line, if extrapolated to zero antimycin A concentration, would reach 100% enzyme activity. Such an extrapolation, shown in Fig. 3*a*, is seen to reach only 50% enzyme activity. The significance of this observation is not clear, but serves to underline the probable complexity of the interaction between components of the electron-transport system in the region of the antimycin-inhibitable factor.

To sum up, an attempt has been made, in this part of the discussion, to show that the curious feature of the inhibition of succinic oxidase by antimycin A, namely the sigmoid curve obtained in experiments of the type recommended by Ackermann & Potter (1949), which prompted the experimental approach described, cannot satisfactorily be explained in terms of the relationship of the inhibitor to the factor with which it combines, or to the presence of competing substances, but is rather to be attributed to the organization and function of the electron-transport system itself. Since our knowledge of these matters is still far from complete it has been necessary to conduct the discussion in general terms and largely on the basis of analogy. An attempt will now be made to relate the concepts

put forward above to known properties of the electron-transport system.

*Functioning of the electron-transport system in
Keilin & Hartree (1947) preparations*

The interaction, for the purposes of electron transport, between adjacent components in a structurally bound system has been a matter for speculation. Chance (1952) and Slater (1953) considered two possibilities. The first is that the components of the system are bound together fairly rigidly into 'succinic oxidase assemblies', each assembly containing one molecule of each component, in such a way that interaction between components in different 'assemblies' is excluded. The second possibility is that the 'assemblies' are less rigidly bound, allowing interaction between components in different 'assemblies' to take place. Slater (1953) quotes spectroscopic evidence which suggests that the second possibility is the more likely. The interpretation offered here, of the variation of succinic oxidase activity in a given amount of heart-muscle preparation with decreasing concentrations of antimycin A, would be a direct demonstration of the second possibility, i.e. that there is some flexibility in the arrangement of the components of the system. At high concentrations of antimycin A, however, an approximately linear response of enzyme activity to concentration of antimycin A is observed, and this linear response may, perhaps, be taken as an indication that there are limits to the flexibility of the structure when only small amounts of the antimycin-inhibitable factor are available.

The term 'free availability', which has been used to describe the relationship of the antimycin-inhibitable factor to an adjacent component of the electron-transport system, must now be commented on. The term as it stands could mean availability to 'assemblies' of the electron-transport system in the same enzyme-bearing particle, or, more widely, to all 'assemblies' of the electron-transport system irrespective of location within particles. The second possibility is considered to be unlikely for the following reasons:

(1) Slater (1949*d*) has shown that interaction does not take place between enzyme-bearing particles inhibited by arsenical, and enzyme-bearing particles inhibited by treatment with BAL in the presence of air, although the two types of particles between them contained all the components of the succinic oxidase system.

(2) Tissières (1954) was unable to demonstrate manometrically an interaction between two types of deficient particles, prepared from various strains of *Neurospora crassa*, which between them contained components capable of bringing about the oxidation of succinate, for the reaction did not take

place at a detectable rate unless added soluble cytochrome *c* was present. More sensitive spectroscopic methods did, however, reveal some interaction.

It is concluded that if interaction takes place between enzyme-bearing particles the proportion of the total electron transport which occurs by this route is negligible. The term 'free availability', as used in this paper, must therefore be taken to apply only within an enzyme-bearing particle.

To return to the response of enzyme activity to factor concentration at high concentrations of factor (low concentrations of antimycin A), the hyperbolic nature of this response is not to be taken as evidence of a true Michaelis-Menten relationship between adjacent components of the succinic oxidase system. Commenting on the variation of cytochrome oxidase activity with soluble cytochrome *c* concentration, Slater (1949*a*) pointed out that a hyperbolic response is not necessarily indicative of such a relationship, and that the hyperbola may be interpreted as a shift in the rate-determining stage of the overall reaction. In the hyperbolic relationship shown here, it can be envisaged that the rate-determining stage at low concentrations of factor might be a reaction involving the factor itself, whereas at high concentrations of factor the rate-determining stage may shift to the reaction between succinate and 'succinic dehydrogenase' or to one of the internal reactions of the 'succinic dehydrogenase'.

It has been repeatedly emphasized (Keilin & Hartree, 1949; Slater, 1949*c*) that full activity of the succinic oxidase system depends on the mutual accessibility of its components, and Bonner (1954) has traced the effect of activators, required for preparations in media containing very little inorganic phosphate, to the step between cytochrome *b* and the BAL-sensitive factor, or that between the BAL-sensitive factor and cytochrome *c*. The fact that activators influence the degree of inhibition of partially antimycin-inhibited preparations places the antimycin-inhibitable factor in the part of the system influenced by activators. It is suggested that the flexibility of 'succinic oxidase assemblies', which is reflected in the free availability of the antimycin-inhibitable factor to an adjacent component of the electron-transport system, is the property which leads to the susceptibility of the system to inactivation at low concentrations of inorganic phosphate. Therefore, the fact that 'succinic oxidase assemblies' appear to interact at this point of the system need not mean that similar interactions can take place at other points of the system. For the purpose of the results presented in this paper it is sufficient to postulate a 'succinic dehydrogenase assembly' flexibly linked to a 'cytochrome oxidase assembly'; each of these assemblies may have a rigid structure. Such a

'succinic dehydrogenase assembly' may perhaps correspond to the succinic dehydrogenase complex of Green, Kohout & Mii (1954). The flexibility of 'succinic oxidase assemblies', otherwise to be regarded as the 'accessibility' of the antimycin-inhibitable factor to an adjacent component of the system, may be amenable to mathematical expression in terms of quantities analogous to Michaelis constants, such as may be derived by the methods illustrated in Fig. 3. The use of antimycin A in this type of experiment might be of value in the investigation of differences between the various forms in which the electron-transport system may be prepared.

The indirect experiment reported in Table 9 suggests that the BAL-sensitive factor and the antimycin-inhibitable factor are not identical. However, the evidence would not be inconsistent with the view that destruction by BAL in the presence of air, and combination with antimycin A, are properties of different chemical groupings of the same substance, and that both groupings are essential for its activity. The solution of this problem requires studies of a more direct kind. It should be borne in mind that the chemical groupings responsible for the properties of sensitivity to the presence of BAL in air and of combination with antimycin A need not be electron-transport catalysts, but may be concerned in the linking of electron-transport components in such a way that they may react together.

Further discussion of the results presented here cannot usefully be undertaken until the components of the electron-transport system have been further clarified, and until more is known of the possible changes which may be brought about in the structure and function of the system during its isolation from the tissues.

SUMMARY

1. With heart-muscle preparations (Keilin & Hartree, 1947), confirmation has been obtained of the general features of the inhibition of succinic oxidase activity by antimycin A, as described by previous workers (Ahmad *et al.* 1950; Potter & Reif, 1952; Chance, 1952).

2. A general method has been described for distinguishing between inhibition as a result of complete loss of activity of a fraction of the enzyme present, leaving the remainder fully active, and inhibition as a result of uniform loss of activity of all the enzyme present.

3. With this general method, it has been shown that antimycin A, and 2:3-dimercaptopropanol (BAL) in the presence of air, bring about a uniform loss of activity of all the enzyme present, whereas *p*-aminophenylarsenoxide brings about the complete

loss of activity of a fraction of the enzyme present, leaving the remainder fully active.

4. By experiments combining the use of antimycin A and *p*-aminophenylarsenoxide it has been shown that antimycin A becomes rapidly redistributed over all the available antimycin-inhibitable factor present when antimycin-inhibited and uninhibited samples of heart-muscle preparation are mixed together. These experiments underline the reversible nature of the combination between antimycin A and the factor (Reif & Potter, 1953).

5. When succinic oxidase activity is plotted against decreasing amounts of antimycin A present/mg. of heart-muscle preparation, a complex curve is obtained, the major part of which is hyperbolic. It is considered probable that the decreasing scale of antimycin A is an indirect measure of increasing concentrations of antimycin-inhibitable factor, and that the factor is relatively freely available, within an enzyme-bearing particle, to an adjacent component of the electron-transport system. This interpretation is in harmony with the fact that antimycin A brings about the uniform loss of activity of all the enzyme present.

6. In preparations partially inactivated in media low in inorganic phosphate, histidine or cytochrome *c* increased the succinic oxidase activity of the control and simultaneously decreased the degree of inhibition by antimycin A. This effect of histidine and of cytochrome *c* was attributed to enhancement of electron transport within the enzyme-bearing particles.

7. A sample of heart-muscle preparation, completely inhibited by treatment with BAL in the presence of air, still retained its capacity to combine with antimycin A.

8. The results have been discussed in relation to the possible structure of the electron-transport system in Keilin & Hartree (1947) preparations.

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Note added in proof. Since this paper was submitted for publication, Green, Mii & Kohout (1955) have reported that the succinate-cytochrome *c* reductase activity of their succinic dehydrogenase complex, while completely antimycin-sensitive, is not affected

by treatment with BAL in the presence of air. The present work supports this indication that the BAL-sensitive and antimycin-sensitive factors are not identical.

REFERENCES

- Ackermann, W. W. & Potter, V. R. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 1.
- Ahmad, K., Schneider, H. G. & Strong, F. M. (1950). *Arch. Biochem.* **28**, 281.
- Ball, E. G. & Cooper, O. (1949). *J. biol. Chem.* **180**, 113.
- Bonner, W. D. jun. (1950). *Nature, Lond.*, **165**, 757.
- Bonner, W. D. jun. (1951). *Biochem. J.* **49**, viii.
- Bonner, W. D. jun. (1954). *Biochem. J.* **56**, 274.
- Chance, B. (1952). *Nature, Lond.*, **169**, 215.
- Chance, B. & Williams, G. R. (1955). *Fed. Proc.* **14**, 190.
- Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
- Dunshee, B. R., Leben, C., Keitt, G. W. & Strong, F. M. (1949). *J. Amer. chem. Soc.* **71**, 2436.
- Green, D. E. & Beinert, H. (1955). *Annu. Rev. Biochem.* **24**, 1.
- Green, D. E., Kohout, P. M. & Mii, S. (1954). *Biochim. biophys. Acta*, **14**, 295.
- Green, D. E., Mii, S. & Kohout, P. M. (1955). *J. biol. Chem.* **217**, 551.
- Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 500.
- Keilin, D. & Hartree, E. F. (1949). *Biochem. J.* **44**, 205.
- Keilin, D. & Hartree, E. F. (1955). *Nature, Lond.*, **176**, 200.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Potter, V. R. (1946). In *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, p. 190. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publ. Co.
- Potter, V. R. & Reif, A. E. (1952). *J. biol. Chem.* **194**, 287.
- Reif, A. E. & Potter, V. R. (1953). *J. biol. Chem.* **205**, 279.
- Reif, A. E. & Potter, V. R. (1954). *Arch. Biochem. Biophys.* **48**, 1.
- Singer, T. P. & Kearney, E. B. (1954). *Biochim. biophys. Acta*, **15**, 151.
- Slater, E. C. (1948). *Nature, Lond.*, **161**, 405.
- Slater, E. C. (1949*a*). *Biochem. J.* **44**, 305.
- Slater, E. C. (1949*b*). *Biochem. J.* **45**, 1.
- Slater, E. C. (1949*c*). *Biochem. J.* **45**, 8.
- Slater, E. C. (1949*d*). *Biochem. J.* **45**, 14.
- Slater, E. C. (1949*e*). *Biochem. J.* **45**, 130.
- Slater, E. C. (1953). *4th Colloquium der Gesellschaft für physiologische Chemie*, p. 64. Berlin: Springer-Verlag.
- Slater, E. C. & Bonner, W. D. jun. (1952). *Biochem. J.* **52**, 185.
- Thorn, M. B. (1953). *Biochem. J.* **54**, 540.
- Thorn, M. B. (1955). *Biochem. J.* **60**, xxxvi.
- Thorn, M. B. (1956). *Biochem. J.* **62**, 12*p*.
- Tissières, A. (1954). *Biochem. J.* **58**, 142.