A Respiratory Catalyst Required for the Reduction of Cytochrome *c* by Cytochrome *b*

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(*Received* 22 *October* 1948)

For the study of the cytochromes, the enzyme system catalysing the aerobic oxidation of succinate (the succinic oxidase system) offers special advantages since, unlike most intermediary metabolites, succinate activated by its dehydrogenase reacts directly with the cytochrome system without the necessity for the intermediary carriers cozymase and diaphorase. The work of Keilin (1929), Keilin & Hartree (1940) and Ball (1938) shows that the transport of hydrogen atoms (or electrons) from succinate to molecular oxygen proceeds through a chain of electron carriers of successively higher oxidation-reduction potential. Four such carriers have been identified spectroscopically, acting in the following manner, the arrows indicating the direction of electron transfer (cyt. = cytochrome):

Succinate → cyt. *b* → cyt. *c* → cyt. *a* → cyt. a_3 (oxidase) → O_2
|
Succinic dehydrogenase

E'_0 at pH 7·3 and 30°:

−0·010* −0·04† 0·262‡ 0·29† ? 0·78 V.

The activity of the complete succinic oxidase system is measured by determining the rate of oxygen uptake in the presence of excess succinate, while the component parts of the system may be studied by the introduction of substances of suitable oxidation-reduction potential which do not require activation by enzymes. Thus the rate of reduction of methylene blue ($E'_0 = +0{\cdot}001$ V., at pH 7·3, 30°; Clark, Cohen & Gibbs, 1925) in the presence of excess succinate is a measure of the succinic dehydrogenase-cytochrome *b* portion of the system, which will henceforth be referred to as 'succinic dehydrogenase'. (It is possible that succinic dehydrogenase is identical with cytochrome *b*. See Bach, Dixon & Zerfas, 1946; Ball, Anfinsen & Cooper, 1947; Slater, 1949*a*.) Similarly, the activity of the cytochrome *a*-cytochrome a_3 portion (henceforth referred to as cytochrome oxidase) may be measured by determining the rate of oxidation, in the presence of excess cytochrome *c*, of a number of substances, such as *p*-phenylenediamine, ascorbic acid and quinol, which rapidly reduce cytochrome *c*.

* Calculated from Borsook & Schott (1931).
† Ball (1938).
‡ Stotz, Sidwell & Hogness (1938).

Several groups of workers have suggested that the aerobic oxidation of succinate requires an additional factor acting between succinic dehydrogenase and cytochrome *c*. This suggestion was based upon the evidence that it was possible, by various treatments, to obtain enzyme preparations which were able to catalyse the reduction of methylene blue by succinate and the oxidation of reducing agents in the presence of cytochrome *c*, and yet were unable to oxidize succinate aerobically. Such preparations can be obtained by treatment with bile salts (Hopkins, Morgan & Lutwak-Mann, 1939; Straub, 1942), repeated isoelectric precipitation or ultracentrifugation (Stern & Melnick, 1939), or by ammonium sulphate fractionation (Stoppani, 1947). Keilin & Hartree (1940) obtained a similar preparation by treatment of the enzyme preparation at pH 5 for 1 hr., but suggested that the inactivation of the succinic oxidase system might be due, not to the destruction or removal of a factor of the type suggested by the other workers, but to an effect on the particles of the enzyme preparation which caused an impaired accessibility of the components of the succinic oxidase system, without appreciably affecting the accessibility of methylene blue or succinate to the dehydrogenase. Stern & Melnick (1939), Straub (1942) and Stoppani (1947) appeared to have obtained strong evidence in support of their view, by restoring the activity of the complete system by the addition of alleged specific factors such as the supernatant fluid from the ultracentrifugation (Stern & Melnick, 1939), an enzyme preparation heated to 55° at pH 9 (Straub, 1942) or a preparation obtained by fractional precipitation with ammonium sulphate (Stoppani, 1947). Keilin & Hartree (1949) have now shown, however, that these supposed specific reactivating fractions could be replaced by such substances as calcium phosphate gel or denatured proteins, which could not possibly be components of the succinic oxidase system. It follows that the enzyme preparations obtained by Stern & Melnick, Straub and Stoppani must have contained all the components of the succinic oxidase system, and that the inactivity must have been due to the loss of the mutual accessibility of these components. The reactivating substances probably act, in some as yet unexplained manner, by restoring this accessibility.

However, Keilin & Hartree (1949) admit that, although their experiments disprove the claims of these workers, they do not disprove the possible existence of such a factor. One method of investigating this possibility is to search for substances which inhibit the complete succinic oxidase system without affecting the activities of the succinic dehydrogenase, cytochrome *c* or cytochrome oxidase, and which do not act non-specifically on the enzyme system as a whole by affecting the particles of the enzyme preparation. In another paper (Slater, 1949*b*), ways of distinguishing between inhibitors which have this latter kind of action and those which act specifically on a component of the system are discussed. The present paper is concerned with the finding that certain reducing agents, in the presence of air, bring about complete inactivation of the succinic oxidase system, without affecting either the succinic dehydrogenase or the cytochrome oxidase. A detailed study of the mechanism of this inactivation showed that it was due to the destruction of a specific component of the succinic oxidase system. This component, which is probably a haematin compound, acts in the system between cytochrome *b* and cytochrome *c*.

A preliminary account of the main findings of this investigation has been given elsewhere (Slater, 1948).

EXPERIMENTAL

Enzyme preparations from horse heart and kidney, obtained by the methods previously described (Slater, 1949*a*), were used as the succinic oxidase system.

Cytochrome c, prepared by the method of Keilin & Hartree (1945*a*), contained 0·34% Fe; *catalase* was prepared by the method of Keilin & Hartree (1945*b*) and *D-amino-acid oxidase* by the method of Negelein & Brömel (1939); *notatin* was obtained (by courtesy of Dr W. F. Short) from *Penicillium notatum*.

Glutathione was prepared by the methods of Hopkins (1929) and of Pirie (1930). It was dissolved and neutralized immediately before use.

2:3-*Dimercaptopropanol* (*BAL*) was kindly supplied by the Department of Biochemistry, Oxford. Aqueous solutions were prepared with glass-distilled water immediately before use.

Enzyme activities were measured according to the methods previously described (Slater, 1949*a*, *c*). All activities are expressed as Q_{O_2} (μl. O_2/mg. fat-free dry wt./hr.).

Copper was determined by the method of McFarlane (1932).

General procedure

The effect of the reducing agent on the enzyme system was most conveniently studied by the following procedure, which will henceforth be referred to as the 'general procedure'. Undiluted enzyme preparation (1 ml.) was pipetted into a Barcroft flask, followed by any other additions to be made and finally by the reducing agent, the total volume (made up with glass-distilled water) being 2 ml. After attaching to a Barcroft manometer the flask was shaken in air at 37° for the required time, then removed from the manometer, and 3 ml. 0·18 M-phosphate buffer added. Samples (0·2 ml.) were pipetted into flasks for the measurement of the enzyme activities, and immediately diluted with the appropriate amount of phosphate buffer. This rapid dilution (about 40-fold) almost completely prevented any further reaction between the reducing agent and the enzyme. Controls treated in exactly the same manner, but using water instead of the reducing agent, were always included and used as the basis for calculating percentage inhibitions.

This procedure, namely reaction at high enzyme and inhibitor concentrations followed by dilution before measuring the enzyme activities, is only valid if the inhibition is not reversed by dilution, as was the case with the inhibitions discussed in this paper.

RESULTS

Effect of reducing agents on the activities of the succinic oxidase system and of succinic dehydrogenase

The effects of equivalent concentrations of ascorbic acid and some thiol compounds on the activities of the succinic oxidase system and of succinic dehydrogenase in the heart-muscle preparation are shown in Table 1. Diethyldithiocarbamate completely inhibited the succinic dehydrogenase activity (cf. Keilin & Hartree, 1940). The other three thiol compounds strongly inhibited the activity of the complete system, but, with the exception of cysteine, had little effect on the succinic dehydrogenase. The degrees of inhibition caused by these three compounds were in the same order as their rates of oxidation in the presence of the heart-muscle pre-

Table 1. *Effect of reducing agents on the activities of the succinic oxidase system and of succinic dehydrogenase in heart-muscle preparation*

('General procedure' (see above); reaction time, 30 min.)

Reducing agent	Concentration (M)	Oxygen uptake in 30 min. (μl.)	Inhibition (%) Succinic oxidase system	Inhibition (%) Succinic dehydrogenase
Ascorbic acid	0·01	47	77	28
Glutathione	0·02	96	78	9
Cysteine	0·02	247	100	79
Diethyldithiocarbamate	0·02	65	100	100
2:3-Dimercaptopropanol (BAL)	0·01	133	98	5

paration. Ascorbic acid inhibited the complete system to about the same extent as glutathione, but had a greater effect on the succinic dehydrogenase. Under the conditions of this experiment, the compound which had the greatest effect on the complete succinic oxidase system with very little effect on succinic dehydrogenase was 2:3-dimercaptopropanol (BAL). Since it was also found that this compound had very little effect on the cytochrome oxidase activity, it fulfilled the requirements for the present study, and the mechanism of its action was further investigated. These observations reconcile the apparently divergent findings of Webb & van Heyningen (1947) and of Barron, Miller & Meyer (1947) on the effect of BAL on the succinic system, since the latter authors, who found an inhibition, measured the activity of the complete succinic oxidase system, while the former authors, who found no effect, measured the succinic dehydrogenase.

Effect of BAL on the activities of the succinic oxidase system and of succinic dehydrogenase

A study of the effect of time of contact of BAL with the heart-muscle preparation before dilution, summarized in Fig. 1, showed that the succinic

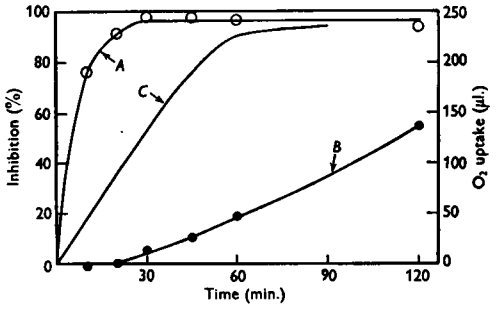


Fig. 1. Inactivation of the succinic oxidase system and succinic dehydrogenase in heart-muscle preparation by shaking in air with 0·0094M-BAL at 37° for various periods of time; 'general procedure' (see p. 15). *A*, inhibition of succinic oxidase system; *B*, inhibition of succinic dehydrogenase; *C*, O_2 uptake by BAL. 0·2 ml. 0·004M-BAL added to flasks used for measurement of control activities, i.e. these flasks contained the same amount of BAL (0·00025M) as test flasks. This amount of BAL inhibited the succinic oxidase activity by 11% and the succinic dehydrogenase by 3%.

oxidase system was inhibited after a few minutes' contact with BAL, and the inhibition was 90% after 20 min. Inhibition of the succinic dehydrogenase, on the other hand, did not commence until about 30 min. after the addition of the BAL to the heart-muscle preparation, i.e. when 57% of the BAL was oxidized. Thereafter, the inhibition of succinic dehydrogenase increased rapidly with time. It was found that shaking the heart-muscle preparation with air for 2 hr. at 37° under the conditions of the experiment, but in the absence of BAL, caused no destruction of the succinic dehydrogenase, and only a 13% loss of the succinic oxidase activity.

It is apparent from these experiments that BAL has two quite distinct effects: (1) a rapid and strong inhibition of the succinic oxidase system, without any effect on the succinic dehydrogenase portion; (2) an inhibition of the succinic dehydrogenase, which does not commence until more than half the BAL is oxidized and increases in velocity as the oxidation of BAL approaches completion.

The possibility that the subsequent addition of KCN in the measurement of the succinic dehydrogenase activity might have reversed an inhibition of succinic dehydrogenase, caused by the BAL, was excluded by an experiment in which the inhibition was measured in two ways, viz. by the manometric method in the presence of KCN, and by the Thunberg method in its absence. The calculated inhibitions were the same by both methods.

The experiment described in Fig. 1 suggested that the effect on succinic dehydrogenase, which did not occur until more than half the BAL was oxidized,

Table 2. *Effect of BAL and oxidized BAL on the succinic oxidase and succinic dehydrogenase activity of heart-muscle preparation*

(*A*. Alkali-treated preparation (1 ml.) + 1 ml. 0·028M-BAL were added to a Barcroft flask which was attached to a manometer and shaken at 37° until the uptake of O_2 had ceased (60 min.). Heart-muscle preparation (1 ml.) was then added to the flask, which was shaken at 37° for a further 30 min.; 2 ml. 0·18M-phosphate buffer were then added and the succinic dehydrogenase and succinic oxidase activities determined on 0·2 ml. samples in the usual way. *B*. This was the same as *A*, except that 1 ml. water replaced the BAL. *C*. Alkali-treated preparation (1 ml.) + 1 ml. 0·028M-BAL + 1 ml. heart-muscle preparation were added to a Barcroft flask which was shaken at 37° for 30 min.; 2 ml. 0·18M-phosphate buffer were then added and the succinic dehydrogenase and succinic oxidase activities determined on 0·2 ml. samples in the usual way. *D*. This was the same as *C*, except that 1 ml. water replaced the BAL.)

	Succinic oxidase activity		Succinic dehydrogenase activity	
	µl./10 min.	Inhibition (%)	µl./10 min.	Inhibition (%)
A	68·6	34	32·1	23
B	104·7	—	41·8	—
C	2·6	98	32·9	20
D	104·2	—	41·0	—

was due to an oxidation product of BAL. Consequently, the effect of oxidized BAL was compared with that of BAL itself. BAL was oxidized by shaking in air with a heart-muscle preparation, treated by the method of Straub (1942) in order to

destroy its succinic dehydrogenase activity, until the absorption of O_2 ceased. The treatment of the heart-muscle preparation consisted of bringing it to pH 9·0, warming at 55° for 15 min. and then cooling and neutralizing. The experiment, described in Table 2, clearly shows that, whereas the inhibition of succinic dehydrogenase activity was about the same when previously untreated heart-muscle preparation was in contact with either completely oxidized BAL (*A*) or with BAL undergoing oxidation (*C*), the inactivation of the succinic oxidase system was much greater when it was present during the oxidation of the BAL. The oxidized BAL produced a slightly greater inhibition of the succinic oxidase system than of the succinic dehydrogenase; other oxidizing agents behave similarly (Slater, 1949*d*). The effect of oxidized BAL on succinic dehydrogenase is probably due to oxidation of SH groups essential for the activity of the dehydrogenase (cf. the inhibition of succinic dehydrogenase by oxidized glutathione, Hopkins & Morgan, 1938).

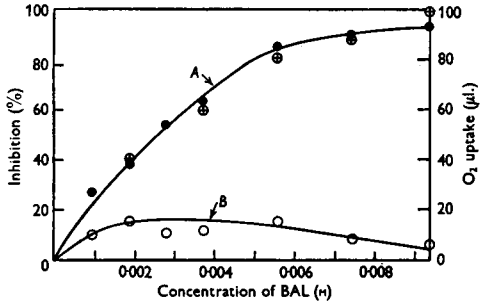


Fig. 2. Inactivation of the succinic oxidase system and of succinic dehydrogenase in heart-muscle preparation by shaking in air with various concentrations of BAL; temp. 39°; reaction time, 20 min.; 'general procedure'. *A*, ● inhibition of succinic oxidase system; *B*, ○ inhibition of succinic dehydrogenase. ⊕ O_2 absorbed by BAL during contact with the heart-muscle preparation (right-hand ordinate scale).

The effect of the concentration of BAL on the inactivation of the succinic oxidase system and of succinic dehydrogenase is shown in Fig. 2. The inactivation of the succinic oxidase system increased with increasing concentration of BAL, following the same curve as the amount of O_2 consumed by the BAL during the period of the experiment (20 min.). The inhibition of succinic dehydrogenase did not, however, increase in the same way; the maximum inhibition was obtained at intermediate concentrations, this effect being more marked when, in other experiments, the time of contact with the BAL was increased. The above findings are understandable in view of the fact that the degree of the inhibition of succinic dehydrogenase is governed by two factors, viz. (1) the concentration of the oxidation product of BAL; (2) the proportion of oxidized BAL to total BAL. With increasing initial concentration of BAL, (1) increases while (2) decreases (see Table 3, the concentration of oxidized BAL after 20 min. can be taken to be approximately proportional to the amount of O_2 consumed during the experiment).

Table 3. *Effect of concentration of BAL on rate of oxygen uptake of BAL in presence of heart muscle and on the percentage of total BAL in the oxidized state after 20 min.*

(Heart-muscle preparation (1 ml.); total volume 2 ml.; temp. 39°; see also Fig. 2.)

BAL concentration (M)	Initial rate of oxidation of BAL (µl. O_2/20 min.)	O_2 absorbed in first 20 min. (µl.)	Percentage of total BAL in oxidized form after 20 min.
0·00186	49	40	92
0·00372	62	60	66
0·00558	85	81	61
0·00774	95	88	51
0·0093	102	99	47

Table 4. *Comparison of effect of BAL on succinic oxidase and succinic dehydrogenase systems in heart-muscle and kidney preparations*

('General procedure', see p. 15; reaction time, 20 min.; values for heart muscle are plotted in Fig. 2.)

BAL concentration (M)	Inhibition (%)			
	Succinic oxidase		Succinic dehydrogenase	
	Heart muscle	Kidney	Heart muscle	Kidney
0·00141	29	63	14	—
0·00382	54	85	16	23
0·0094	93	100	6	12

Table 5. *Effect of BAL under aerobic and anaerobic conditions on the succinic oxidase activity of heart muscle*

(*Anaerobic treatment:* 1 ml. of undiluted heart-muscle preparation + 0·8 ml. water were placed in a Thunberg tube and 0·2 ml. 0·095 M-BAL in the hollow stopper. The air in the Thunberg tube was replaced by O_2-free N_2, and, after the contents of the tube had been mixed with those of the hollow stopper, the tube was heated for 30 min. at 38°. The tube was then cooled in ice, opened to air and 3 ml. cold 0·18 M-phosphate buffer quickly added. After mixing, 0·2 ml. was pipetted into a Barcroft flask and immediately diluted with 2·7 ml. 0·18 M-phosphate buffer. *Aerobic treatment:* same quantities shaken with air at 38° for 30 min., then treated as above. *Control:* treated as 'aerobic treatment', but water replaced BAL. 0·2 ml. 0·004 M-BAL added to Barcroft flask used for measurement of enzymic activity, in order to give same final concentration of BAL as in other flasks.)

	O_2 uptake (µl./10 min.)	Inhibition (%)
Anaerobic treatment	64·4	12
Aerobic treatment	1·2	98
Control	73·1	—

Thus there is a balance between these two opposing factors, with the result that the inhibition is optimal at intermediate concentrations of BAL.

The kidney preparation behaved essentially like heart muscle except that it showed a greater susceptibility to low concentrations of BAL (Table 4).

It was found that while BAL produced practically complete inhibition under aerobic conditions, it produced very little inhibition if its oxidation was prevented by working under anaerobic conditions (Table 5). The slight inhibition under anaerobic conditions probably occurred during the final dilution, in air.

These experiments show that the effect of BAL on the succinic system is not due to BAL itself or to its oxidation product, but to BAL undergoing aerobic oxidation in the presence of the enzyme system. Accordingly, the oxidation of BAL in the presence of the heart-muscle preparation was further studied.

Oxidation of BAL

The following observations (Table 6) showed that H_2O_2 is produced during the aerobic oxidation of BAL in buffer solutions: (1) the total O_2 uptake of BAL in buffer solution was slightly greater than the theoretical uptake, calculated from the equation $2CH_2OH.CHSH.CH_2SH + O_2 \rightarrow$ disulphide compounds $+ 2H_2O$; (2) this extra O_2 was evolved if catalase was added after the solution had ceased to absorb O_2; (3) if catalase was present during the oxidation there was no subsequent evolution of O_2 on the addition of catalase.

ethanol, and since catalase does not do so unless H_2O_2 is formed by some other reaction (Keilin & Hartree, 1945*c*), it follows that H_2O_2 must have been produced during the oxidation of BAL in the presence of the heart muscle-preparation. The necessity for the addition of catalase as well as ethanol, even though the heart-muscle preparation contains sufficient catalase to decompose H_2O_2 immediately, is understandable in view of the finding of Keilin & Hartree (1945*c*) that larger concentrations of catalase are required for the peroxidatic than for the catalatic reaction of catalase. The amount of catalase added was 300 times that present in the heart-muscle preparation.

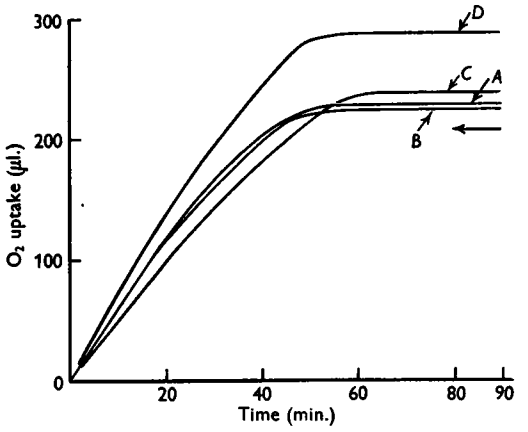


Fig. 3. Effect of catalase and ethanol on the O_2 uptake of BAL + heart-muscle preparation. All flasks contained 1 ml. heart-muscle preparation in a total volume of 2 ml. BAL, 0·0094 M; temp. 37°; pH 7·1. *A*, no addition; *B*, $2{\cdot}6 \times 10^{-6}$ M-catalase; *C*, 40 mg. ethanol; *D*, $2{\cdot}6 \times 10^{-6}$ M-catalase + 40 mg. ethanol. The horizontal arrow indicates the theoretical O_2 uptake for the reaction $2BAL + O_2 \rightarrow BALOx + 2H_2O$. The figures have been corrected for the endogenous respiration (see footnote to Table 6).

Table 6. *Oxygen uptake of BAL under various conditions*

(Enzyme preparation or phosphate buffer (0·05 M, 1 ml.) and BAL (18·5 μM ≡ 207 μl. O_2) in total volume of 2 ml.; temp. 37°; catalase added from dangling tube after O_2 uptake ceased.)

	Initial rate of O_2 uptake (μl./hr.)	Final O_2 uptake (μl.)	O_2 evolved after addition of catalase (μl.)	Net O_2 uptake (μl.)	Net uptake (% theoretical)
Phosphate buffer	121	220	8	212	102
Phosphate buffer + catalase*	—	216	0	216	104
Heart-muscle preparation†	298	236	0	236	114
Kidney preparation	230	224	—	—	—

* $1{\cdot}6 \times 10^{-6}$ M (mol. wt. = 240,000).

† These figures have been corrected for the slight endogenous respiration (probably due to bacterial action) measured after the BAL was completely oxidized. Treatment with the BAL considerably reduced the endogenous respiration.

In the presence of heart-muscle preparation, the O_2 uptake considerably exceeded the theoretical value, but there was no evolution of O_2 on the subsequent addition of catalase. The formation of H_2O_2 during the oxidation of BAL in the presence of heart-muscle preparation was demonstrated by the addition of ethanol and catalase to the system (Fig. 3). Whereas catalase or ethanol alone had little effect on the oxidation of BAL, the rate of O_2 uptake and the final uptake were considerably increased when both ethanol and catalase were added. Since the heart-muscle preparation does not oxidize

The fact that the O_2 uptake of BAL in the presence of heart-muscle preparation was greater than the theoretical value without any accumulation of H_2O_2

at the end of the oxidation must mean that some substance (or substances) in the heart-muscle preparation was oxidized during the oxidation of the BAL.

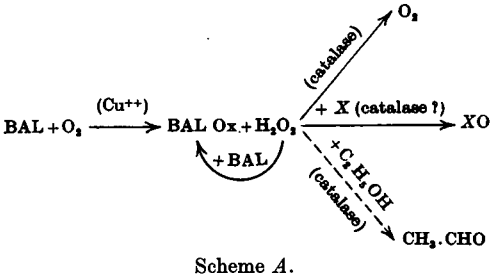
Table 6 shows that the rate of oxidation of BAL was increased 2·5 times by the presence of the heart-muscle preparation, and doubled by the presence of the kidney preparation. Since Barron, Miller & Kalnitsky (1947) have reported that copper catalyses the oxidation of BAL, the possibility that this increased rate of oxidation might be largely due to copper in the heart-muscle preparation was investigated. It was found by direct analysis that the heart-muscle preparation contained sufficient copper ($4{\cdot}2 \times 10^{-5}$ M) to account for its catalytic effect on the oxidation of BAL, provided that this copper was as effective as Cu^{++} ions added to the preparation.

However, it is probable that the copper in the heart-muscle preparations does not arise from accidental contamination with Cu^{++} ions, but is in the form of firmly bound copper-protein compounds; the availability of this copper for the catalytic oxidation of BAL is not known. Further experiments, described below, were designed to give more information of this point.

Mechanism of the effect of BAL on the succinic oxidase system

It has been shown above that (1) BAL strongly inhibits the succinic oxidase system only when it is oxidized by air in the presence of the enzyme preparation; and (2) some substance (or substances) in the heart-muscle preparation is oxidized during the process of oxidation of BAL in the presence of heart-muscle preparation. It seems highly probable that BAL inhibits the succinic oxidase system by causing the oxidation of some substance (X) necessary for the activity of the succinic oxidase system. Two possible schemes (A and B) describing this effect of BAL may be considered.

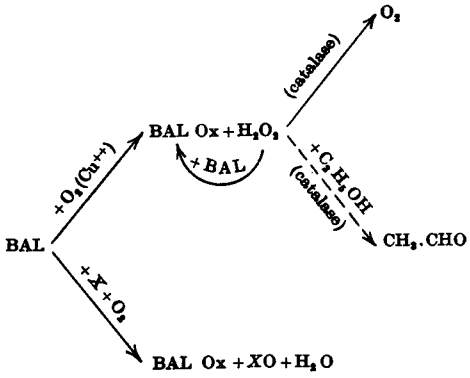
According to the first scheme (A), BAL is oxidized (by copper catalysis) with the formation of oxidized



Scheme *A*.

BAL (BAL Ox) and H_2O_2. The latter may take part in three competing reactions, viz. (1) catalatic destruction by the catalase present in the heart-muscle preparation; (2) oxidation of more BAL; (3) oxidation of the compound (X) as well as of some other substances in the heart-muscle preparation. The latter reaction may be catalysed by catalase. In the presence of ethanol and additional catalase, a fourth reaction (shown by the dotted line in the diagram) is possible.

In the second scheme (B), BAL is oxidized by two competing reactions, one involving copper catalysis as in Scheme A, and the other involving a directly coupled oxidation of BAL with X, in which reaction free H_2O_2, able to react with catalase, is not formed. In this case, as well as in Scheme A, the H_2O_2 formed by the copper-catalysed oxidation might also oxidize other substances in the heart-muscle preparation.



Scheme *B*.

Either of these schemes could explain the destruction of X, the fact that the O_2 uptake of BAL in the presence of heart-muscle preparation exceeds the theoretical, and the extra O_2 uptake in the presence of ethanol and catalase. For the sake of simplicity, a further pathway of oxidation of BAL, viz. through cytochrome c and cytochrome oxidase, has been omitted from both schemes. This reaction could not involve X nor could it contribute to the extra uptake of O_2, since H_2O_2 is not formed during oxidation through cytochrome oxidase. It was calculated from the rate of reduction of the cytochrome c in the heart-muscle preparation by BAL under anaerobic conditions (cf. a similar calculation for ascorbic acid, Slater 1949*c*) that the oxidation of BAL through cytochrome c and cytochrome oxidase could account for no more than one quarter of the O_2 uptake catalysed by heart muscle. BAL reduces cytochrome b, as well as cytochrome c, but the rate of this reduction is so low (time of half reduction about 30 min.) that the rate of oxidation through cytochrome b would be insignificant.

When sufficient extra cytochrome c is added, the oxidation through the cytochrome oxidase system becomes the main pathway (see p. 22).

The experiments, which will now be described, showed that Scheme *A* did not correctly express the reactions involved and that Scheme *B* was probably correct.

(1) *Effect of hydrogen peroxide on the activities of succinic dehydrogenase and the succinic oxidase system in heart muscle.* According to Scheme *B*, the destruction of *X* is caused by a direct reaction with BAL (in the presence of O_2). According to Scheme *A*, however, the destruction of *X* is not caused directly by BAL, but by H_2O_2 produced by the oxidation of BAL. If Scheme *A* is correct, it should be possible to obtain inhibitions of the same type as BAL by means of H_2O_2 produced in a number of different ways. Accordingly, the effects of two primary reactions which produce H_2O_2, viz. D-amino-acid oxidase, with DL-alanine, DL-methionine and D-isoleucine as substrates, and notatin (glucose oxidase) with its substrate glucose, were studied. The results, summarized in Table 7, show that the D-amino-acid oxidase system produced very little inhibition of the succinic oxidase system. The notatin-glucose system, on the other hand, caused some inhibition, but, although the rate of O_2 uptake (and therefore the rate of formation of H_2O_2) was in some experiments less and in others much greater than in the experiments with BAL, the greatest inhibition was only 40%. The degree of inhibition seemed to be independent of the amount of notatin used. These inhibitions of the succinic oxidase system were accompanied by smaller but considerable inhibitions of the succinic dehydrogenase. The reason for the difference between the D-amino-acid oxidase and the notatin-glucose systems is considered below.

(2) *Effect of catalase and ethanol on the inhibition of the succinic oxidase system produced by notatin-glucose and by BAL.* If the effect of BAL on the succinic oxidase system is due to the action on *X* of H_2O_2 produced by the oxidation of BAL, as in Scheme *A*, the addition of catalase and ethanol would be expected to protect *X* from oxidation and the succinic oxidase system from inhibition, the degree of protection depending upon the relative affinity of *X* and the catalase-ethanol system for H_2O_2. Table 8 shows the effect of catalase and ethanol on the inhibition of succinic oxidase produced by notatin-glucose and by BAL.

In the case of the notatin-glucose system, the possible reactions are similar to Scheme *A* and, as would be expected, the addition of ethanol, by com-

Table 7. *Effect of hydrogen peroxide produced by aerobic dehydrogenases and their substrates on the activities of the succinic oxidase system and of succinic dehydrogenase in heart-muscle preparation*

('General procedure', see p. 15); reaction time, 30 min.; D-amino-acid oxidase = A. oxid.)

System producing H_2O_2	Initial rate of O_2 uptake (μl./hr.)	Inhibition (%)	
		Succinic oxidase	Succinic dehydrogenase
A. oxid. (10 mg.) + DL-alanine (3 mg.)*	210	11	—
A. oxid. (50 mg.)* + DL-alanine (3 mg.)	1000	0	0
A. oxid. (10 mg.) + DL-methionine (5 mg.)	480	6	6
A. oxid. (10 mg.) + D-isoleucine (2·4 mg.)	38	14	0
Notatin (5 μg.) + glucose (10 mg.)*	90	38	12
Notatin (10 μg.) + glucose (10 mg.)	190	22	12
Notatin (25 μg.)* + glucose (10 mg.)	415	38	24
Notatin (40 μg.) + glucose (10 mg.)	570	28	17
Notatin (50 μg.) + glucose (10 mg.)	660	40	12
BAL (0·01 M)	315	98	5

* No inhibition by these substances alone.

Table 8. *Effect of catalase and ethanol on the inhibition of the succinic oxidase system produced by notatin-glucose and by BAL*

('General procedure', see p. 15.)

Addition	Inhibition by		
	0·0031 M-BAL, acting for 20 min. (%)	0·0094 M-BAL, acting for 30 min. (%)	Notatin (25 μg.) + glucose (10 mg.), acting for 30 min. (%)
None	53	99	37
0·35 M-Ethanol*	66	—	26
$2·6 \times 10^{-6}$ M-Catalase*	53	97	61
$2·6 \times 10^{-6}$ M-Catalase + 0·35 M-ethanol	59	—	40
$2·6 \times 10^{-6}$ M-Catalase + 0·09 M-ethanol	—	99	—

* No inhibition by these additions, in the absence of BAL or notatin-glucose.

peting with X (this X may not be the same substance as X in Scheme B) for the H_2O_2, gave a definite protection. The addition of catalase alone caused a considerable increase in the inhibition, indicating that the reaction $H_2O_2 + X \rightarrow XO$ is catalysed by catalase, when the latter is added in sufficient concentration. The addition of ethanol as well as catalase reduced the inhibition in the presence of catalase alone by about the same proportion as the reduction of the inhibition by ethanol in the absence of catalase. Neither ethanol nor catalase, nor the two together, had these effects on the inhibition caused by BAL. Thus ethanol, which protected from the notatin-glucose inhibition, slightly increased the BAL inhibition, and catalase, which considerably increased the former, had no effect on the latter. This is strong evidence that BAL does not behave as in Scheme A. The finding is, however, readily understandable if Scheme B correctly formulates the reactions involved. The ethanol added in the presence of catalase reacts with some of the H_2O_2 produced by the copper-catalysed oxidation of BAL. This has the effect, however, only of reducing the extent of the other reactions of H_2O_2 (oxidation of BAL, catalatic destruction), without appreciably affecting the relative amounts of BAL oxidized by the two main pathways suggested, and therefore without appreciably affecting the destruction of X.

(3) *Effect of pyruvate on the inhibition of the succinic oxidase system produced by notatin-glucose and by BAL.* It is known from the work of Holleman (1904) and Negelein & Brömel (1939) that pyruvic acid reacts readily with H_2O_2, as follows:

$$CH_3.CO.COOH + H_2O_2 \rightarrow CH_3.COOH + CO_2 + H_2O.$$

Accordingly pyruvate would be expected to act in the same way as catalase-ethanol, i.e. to have a protective effect if X is oxidized according to Scheme A, and to have no effect if X is oxidized according to Scheme B. The figures in Table 9 show that pyruvate

Table 9. *Effect of pyruvate on the inhibition of succinic oxidase system produced by notatin-glucose and by BAL*

('General procedure', see p. 15.)

	Inhibition (%)		
Pyruvate added ...	Nil	0·02 M	0·1 M
System causing inhibition:			
0·0031 M-BAL, 20 min.	53	—	45
0·0094 M-BAL, 30 min.	98	96	93
Notatin (25 μg.) + glucose (10 mg.), 30 min.	37	—	16

gave considerable protection to the succinic oxidase system against notatin-glucose, but had very little effect on the BAL inhibition, which confirms the conclusions arrived at above. This finding also explains the very small inhibition of succinic oxidase caused by the D-amino-acid oxidase system, since the α-keto acid produced by the oxidation of the amino-acid will react with the H_2O_2 also formed, by a reaction similar to that with pyruvate.

Thus it can be concluded that, although H_2O_2 produced by notatin and glucose does cause some inhibition of the succinic oxidase system, the mechanism of this inhibition differs in several respects from that obtained by treatment with BAL. These observations are to be expected from Scheme B, but cannot be easily explained by Scheme A. There is, however, one possibility which must be considered. Since it is postulated that X is involved in the succinic oxidase system, it is probable that it exists in both an oxidized and a reduced form. In the experiments with BAL, X would be in the reduced form, while in those with notatin-glucose it would be in the oxidized form, and it is possible that reduced X is more susceptible to H_2O_2 than is oxidized X. This explanation was excluded by the experiment which will now be described.

(4) *Effect of cupric ions on the inactivation caused by BAL.* Although copper itself inhibited the succinic oxidase system, this was entirely prevented by BAL, so that the effect of added copper on the inhibition caused by BAL could be studied without any complications arising from the direct effect of copper. Under the conditions of the experiment described in Table 10, $7{\cdot}2 \times 10^{-5}$ M-Cu^{++} trebled the rate of

Table 10. *Effect of cupric ions on the inhibition of the succinic oxidase system produced by BAL*

(Heart-muscle preparation (2 ml.); total volume 2·8 ml. Reaction at 39° with shaking in presence of air, for 15 min.; 7·2 ml. 0·18 M-phosphate buffer added and 0·2 ml. samples used for measurements of enzymic activities.)

	Inhibition (%)	
	Succinic oxidase	Succinic dehydrogenase
0·013 M-BAL	91	13
0·013 M-BAL, $7{\cdot}2 \times 10^{-5}$ M Cu^{++}	66	41

oxidation of BAL in the presence of heart-muscle preparation. The presence of Cu^{++} in this concentration gave considerable protection to the succinic oxidase, while the inhibition of succinic dehydrogenase was increased. This extra inhibition of succinic dehydrogenase is probably partly due to the increased rate of production of H_2O_2 and partly due to the increased rate of production of oxidized BAL. The type of inhibition obtained by treatment with BAL in the presence of Cu^{++} somewhat resembles that obtained by treatment with notatin-glucose. It was found in another experiment, not shown in Table 10, that the addition of pyruvate gave some further protection of the succinic oxidase system in the presence of BAL and Cu^{++} (cf. Table 9).

These results would be expected if Scheme *B* correctly describes the reactions, since the addition of copper would promote the copper-catalysed reaction and spare *X*. They cannot be explained by Scheme *A* even if it is assumed that only reduced *X* is susceptible to H_2O_2; in fact, according to this scheme increased inhibitions might be expected in the presence of Cu^{++}.

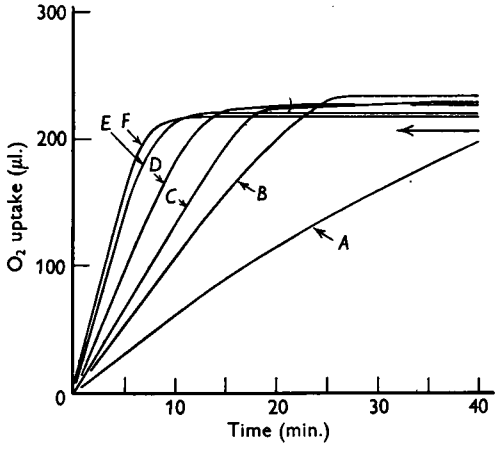


Fig. 4. O_2 uptake of BAL + heart-muscle preparation in the presence of varying amounts of cytochrome *c*. All flasks contained 1 ml. heart-muscle preparation in a total volume of 2 ml. BAL, 0·0094 M; temp. 37°; pH 7·1. *A*, no addition; *B*, $1{\cdot}7 \times 10^{-5}$ M-cytochrome *c*; *C*, $3{\cdot}3 \times 10^{-5}$ M-cytochrome *c*; *D*, $6{\cdot}6 \times 10^{-5}$ M-cytochrome *c*; *E*, $13{\cdot}3 \times 10^{-5}$ M-cytochrome *c*; *F*, $16{\cdot}6 \times 10^{-5}$ M-cytochrome *c*. The horizontal arrow indicates the theoretical O_2 uptake for the reaction $2BAL + O_2 \rightarrow BAL\ Ox + 2H_2O$.

It can be concluded that the inhibition produced by BAL cannot be only, or even mainly, due to the H_2O_2 produced by the oxidation of BAL, but must be caused by some other reaction, probably by the directly coupled oxidation (not involving free H_2O_2) of BAL with some substance or grouping necessary for the activity of the succinic oxidase system.

(5) *The effect of cytochrome* c *on the inhibition of the succinic oxidase system produced by BAL.* As already explained, the rate of reduction of the cytochrome *c* present in the heart-muscle preparation by the BAL is insufficient to allow an appreciable rate of oxidation of the BAL through the cytochrome oxidase system, despite the great activity of the latter. Added cytochrome *c* can, however, be rapidly reduced by the BAL and oxidized by the cytochrome oxidase. Thus, the rate of oxidation of the BAL was greatly increased by the addition of cytochrome *c*, the effect of increasing amounts of which is shown in Figs. 4 and 5. The initial rate of O_2 uptake in the presence of $16{\cdot}6 \times 10^{-5}$ M-cytochrome *c* is about 5 times the rate in the absence of additional cytochrome *c*.

The addition of cytochrome *c* provides a third pathway for the oxidation of BAL, additional to those shown in Scheme *B*. Since H_2O_2 is not formed during the oxidation of BAL by the cytochrome *c*-cytochrome oxidase system, the oxidation of BAL by this pathway will lead to the theoretical O_2 uptake for the reaction $BAL + \frac{1}{2}O_2 \rightarrow BAL\ Ox + H_2O$. Figs. 4 and 5 show, in fact, that with increasing cytochrome *c* concentration, the total O_2 uptake decreases towards this theoretical figure. It would

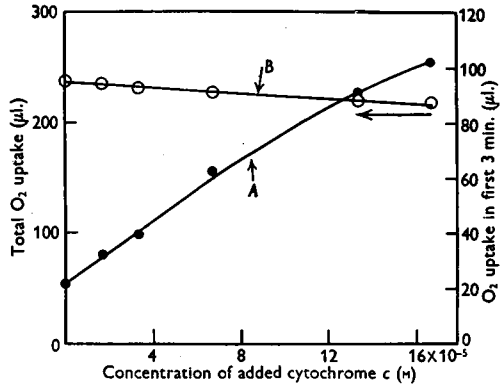


Fig. 5. Effect of concentration of cytochrome *c* on initial rate of oxidation of BAL (uptake of O_2 in first 3 min.) and on total uptake. Experimental conditions as in Fig. 4. *A*, uptake in first 3 min.; *B*, total uptake. The horizontal arrow indicates theoretical O_2 uptake for the reaction $2BAL + O_2 \rightarrow BAL\ Ox + 2H_2O$.

be expected, from these considerations, that the addition of cytochrome *c*, by providing an additional pathway, not involving *X*, for the oxidation of BAL, would protect *X* from destruction. This prediction is fulfilled by the figures in Table 11, which show that

Table 11. *Effect of added cytochrome* c *on the inhibition by BAL of the succinic oxidase system*

('General procedure', see p. 15.)

	Percentage inhibition by		
	0·0031 M-BAL (20 min.)		0·0094 M-BAL (30 min.)
Cytochrome *c* added ($M \times 10^{-5}$)	Succinic oxidase (%)	Succinic dehydrogenase (%)	Succinic oxidase (%)
None	53	13	99
3·3	37	16	—
6·6	19	—	65
16·6	0	4	—

the addition of sufficient cytochrome *c* gave complete protection of the succinic oxidase system against the action of BAL. It should be mentioned at this stage that cytochrome *c* does not reverse the inactivation caused by BAL when it is added after the treatment

with BAL. The fact that cytochrome *c* also prevented the small inhibition of the succinic dehydrogenase occurring under the conditions of the experiment described in Table 11 suggests that the inhibition of succinic dehydrogenase is caused not only by oxidized BAL, but also partly by the H_2O_2 produced by the side reaction in Scheme *B*.

Effect of BAL on the cytochrome oxidase activity

Webb & van Heyningen (1947) showed that treatment with 0·005M-BAL for 15 min. at 37° did not affect the cytochrome oxidase activity, measured by the rate of oxidation of catechol in the presence of excess cytochrome *c*. Since it has been shown elsewhere (Slater, 1949*c*) that catechol is not a suitable reducing agent for this measurement, it was considered necessary to re-investigate the effect of BAL, under the conditions used in the present study, on the cytochrome oxidase activity of heart-muscle preparation. The cytochrome oxidase activity was measured in 0·15M-phosphate, the same concentration as used for the determination of the succinic oxidase activity, although this is not optimal for cytochrome oxidase activity.

Table 12. *Effect of treatment with BAL on the cytochrome oxidase activity of heart-muscle preparation*

('General procedure', see p. 15; 0·0094M-BAL, 30 min.)

Reducing agent	Reducing agent concentration (M)	Phosphate concentration (M)	Concentration of added cytochrome *c* ($M \times 10^5$)	Inhibition of O_2 uptake (%)
Quinol	0·05	0·15	6	11
Ascorbic acid	0·025	0·15	2	13
	0·025	0·15	4	11
	0·025	0·15	6	13
p-Phenylenediamine	0·05	0·15	0	32
	0·05	0·15	1	26
	0·05	0·15	3	19
	0·05	0·15	6	16
	0·05	0·007	6	3
	0·05	0·089	6	10
	0·05	0·144	6	17
	0·05	0·401	6	25
	0·05	0·007	0	20
	0·05	0·089	0	24
	0·05	0·144	0	36
	0·05	0·401	0	18
	0·003	0·15	0	22
	0·0075	0·15	0	31
	0·02	0·15	0	39
	0·05	0·15	0	42

Treatment of either heart-muscle or kidney preparations with BAL caused very little inhibition of the rate of oxidation of quinol or of ascorbic acid in the presence of the enzyme and cytochrome *c*, and this small inhibition was not affected by the concentration of added cytochrome *c* (Table 12). However, when *p*-phenylenediamine was the reducing agent and heart muscle the source of the enzyme, there was a considerable inhibition of the rate of oxidation in the absence of cytochrome *c*, and, although this was decreased by the addition of cytochrome *c*, it was still considerable in the presence of 6×10^{-5}M added cytochrome *c*.

The figures for *p*-phenylenediamine are given graphically in Fig. 6. In Fig. 6*b*, the two lines cross the ordinate at the same point, i.e. at infinite cytochrome *c* concentration the BAL-treated heart-muscle preparation has the same cytochrome oxidase

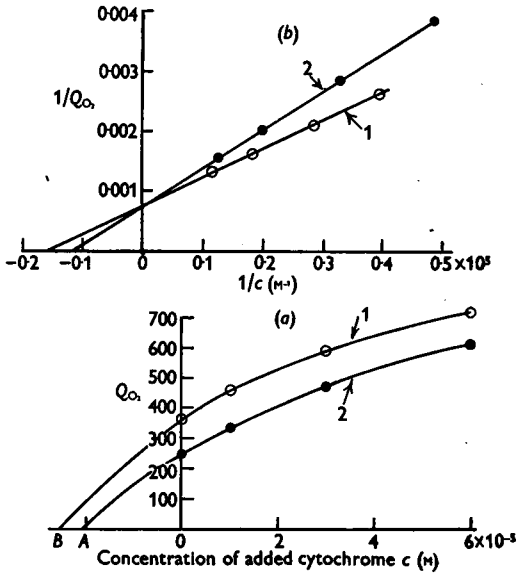


Fig. 6. (*a*) Effect of treatment with BAL on the capacity of heart-muscle preparation to catalyse the oxidation of *p*-phenylenediamine. Heart-muscle preparation treated by 'general procedure' (p. 15) for 30 min. at 38°, with shaking in the presence of air; *p*-phenylenediamine (0·05M); phosphate buffer (0·15M). 1, control; 2, treated with BAL (0·0094M). The curves have been extrapolated to the abscissa by assuming that the points fall on a rectangular hyperbola.

(*b*) Figures in (*a*) plotted according to the procedure of Lineweaver & Burk (1934). The distances *OA* and *OB*, found in (*a*), have been added to the concentration of added cytochrome *c*.

activity as the control. Treatment with BAL has, however, affected the catalytic activity of the added cytochrome *c*: thus if $(\text{cyt. } c)_{\frac{1}{2}}$ is the concentration of cytochrome *c* required for half maximal activity (Slater, 1949*c*), $1/(\text{cyt. } c)_{\frac{1}{2}}$ for the BAL-treated preparation is $0{\cdot}12 \times 10^5 M^{-1}$, compared with $0{\cdot}165 \times 10^5 M^{-1}$ for the control. It has also affected the catalytic activity of the endogenous cytochrome *c*. Thus the extrapolation in Fig. 6*a* shows that the

untreated preparation contains cytochrome *c* equivalent to $2{\cdot}52 \times 10^{-5}$ M added cytochrome *c*, while the endogenous cytochrome *c* of the BAL-treated preparation is equivalent to $2{\cdot}05 \times 10^{-5}$ M added cytochrome *c*; but the added cytochrome *c* is only 0·12/0·165 times as effective in the latter case compared with the control, i.e. the endogenous cytochrome *c* of the BAL-treated preparation is $2{\cdot}05/2{\cdot}52 \times 0{\cdot}12/0{\cdot}165 \times 100 = 59\,\%$ as effective as that of the untreated preparation. The actual amount of cytochrome *c* is unaffected by this treatment (see below).

The finding that BAL affects not the cytochrome oxidase of the heart-muscle preparation, but the catalytic activity of the added and the endogenous cytochrome *c* explains other conclusions drawn from Table 12 and additional experiments, viz.—

(1) The degree of inhibition was decreased by lowering the concentration of enzyme while the concentration of added cytochrome *c* remained constant. Lowering the concentration of enzyme decreases the proportion of the oxidation proceeding through the endogenous cytochrome *c*, since the amount of added cytochrome *c* approaches more closely to that required for maximum activity of the cytochrome oxidase (Slater, 1949*c*).

(2) The degree of inhibition was decreased by lowering the phosphate concentration, which has the same effect as reducing the enzyme concentration (Slater, 1949*c*).

The catalytic activity of the endogenous cytochrome *c* is determined by two factors, viz. (1) the accessibility of *p*-phenylenediamine to this cytochrome *c*, and (2) the accessibility of the cytochrome *c* to the oxidase on the particles of the enzyme preparation. The finding (Table 12) that the degree of inhibition in the absence of added cytochrome *c* was decreased by lowering the concentration of reducing agent shows that it is (2) which is affected by the treatment with BAL. At low *p*-phenylenediamine concentrations, the rate of reduction of cytochrome *c* becomes the limiting factor in both the control and BAL-treated preparations, so that the impaired rate of oxidation of the cytochrome *c* caused by the treatment with the BAL is masked.

Since the effect of BAL on the rate of oxidation of *p*-phenylenediamine is due, primarily, to an effect on the accessibility of the endogenous cytochrome *c* of the heart-muscle preparation to the cytochrome oxidase, it is not surprising that the oxidation of ascorbic acid and quinol are little affected, since the endogenous cytochrome *c* plays only a small part in the oxidation of these substances. The inhibition of the oxidation of *p*-phenylenediamine disappears when sufficient cytochrome *c* is added, but it is interesting to find that the catalytic activity of the added cytochrome *c* is also affected by treatment with BAL. Since this was not the case when other reducing agents were used, it seems that this is due to an effect on the rate of reduction of the added cytochrome *c* (in the vicinity of the oxidase) by *p*-phenylenediamine. This is probably because the *p*-phenylenediamine cannot approach so closely to the cytochrome oxidase after treatment of the heart-muscle preparation with BAL.

Thus the effect of treatment of the heart-muscle preparation with BAL on the rate of oxidation of *p*-phenylenediamine is due to an impairment of the accessibility, both of the endogenous cytochrome *c* and of the *p*-phenylenediamine, to the cytochrome oxidase.

Treatment of the heart-muscle preparation with BAL in the absence of air had no effect on the power of heart-muscle preparation to catalyse the oxidation of *p*-phenylenediamine.

Identification of the BAL-sensitive component of the succinic oxidase system

(1) *Spectroscopic observations.* No bands were observed when a heart-muscle preparation, treated with 0·016 M-BAL for 30 min. at 37° with shaking in air, was placed in a test tube under a microspectroscope. After adding succinate and allowing to stand, the bands of reduced cytochromes $a+a_3$, *b* and *c* all appeared. When the mixture was then shaken for a few seconds, the *b* band remained visible, but the $a+a_3$ and *c* bands disappeared and did not return until the mixture had been standing without shaking for about 10 min. Thus, in the BAL-treated heart-muscle preparation, the reduction of cytochrome *b* and the oxidation of cytochromes *c* and $a+a_3$ were not affected, but the oxidation of cytochrome *b* and the reduction of cytochromes $a+a_3$ and *c* were greatly impaired. The cytochrome *b* could, however, be readily oxidized by methylene blue or by 2:6-dichlorophenolindophenol.

The positions and intensities of the three bands of the reduced cytochromes after the addition of sodium dithionite ($Na_2S_2O_4$) were not affected by treatment with BAL, but the total protohaematin content of the heart-muscle preparation, measured by the intensity of the pyridine haemochromogen band at 550–560 mμ., was reduced by about 20 % by treatment with BAL (Table 13). It follows that some haematin compound, the spectrum of which is not visible in the heart-muscle preparation (Slater, 1949*a*), is destroyed by the treatment with BAL. The amount of haematin compound destroyed is about 30 % of the difference between the cytochrome *b* and total protohaematin contents, as calculated in the latter paper. It is known that BAL has a destructive effect on some haematin compounds. Thus, Barron, Miller & Kalnitsky (1947) have shown that haemoglobin is destroyed by treatment with BAL in the presence of air, and, in the present investigation, it was found that myoglobin was similarly

affected. This reaction is probably essentially the same as that between haemoglobin and another reducing agent, ascorbic acid, which has been studied in detail by Lemberg, Legge & Lockwood (1941). If this is the case, the product of the coupled oxidation of the haematin compound with the BAL would be related to the bile pigments. These pigments have only weak absorption bands, and, considering that the spectrum of the haematin compound is itself not visible, it is not surprising that no such bile pigment could be detected spectroscopically.

Treatment with $Na_2S_2O_4$ and pyridine shifts the $a+a_3$ band from 603–607 mμ. to 580–590 mμ.; this latter band is considerably weaker than the band at 548–560 mμ., and, in view of its proximity to the latter, accurate measurements of its intensity were difficult. It is not known, therefore, whether treatment with BAL leads to any destruction of haematin compounds which yield a pyridine haemochromogen with an absorption band in this position, i.e. those which contain the haem of cytochromes $a+a_3$; but there was no weakening of the $a+a_3$ band obtained with $Na_2S_2O_4$ alone, which is consistent with the finding that the cytochrome oxidase activity was not affected.

The results in Table 13 show that the amount of BAL-labile haematin compound varies from preparation to preparation, but is usually about 14–24%. The figures further show some correlation between the degree of the reduction of intensity of the band and the inhibition of the succinic oxidase system. This is most clearly seen in Exp. 3, in which different concentrations of BAL were employed. Under the conditions of Exp. 2, the BAL was completely oxidized in 25 min. The fact that the reduction in intensity of the band was about the same after 60 min. treatment as after 10 min. shows that oxidized BAL is not responsible for the destruction of the haematin compound. This is supported by the finding that another disulphide, oxidized glutathione, had no appreciable effect on the haematin content. BAL under anaerobic conditions did not cause any destruction of the haematin compound or inhibition of the succinic oxidase system. Notatin-glucose had no measurable effect on the haematin compound.

There was no detectable destruction of the 548–560 mμ. band in the case of the kidney preparation. However, if the BAL-labile haematin compound was present in this preparation in about the same concentration relative to that in heart muscle, as are the cytochromes, this compound would be such a small proportion of the total protohaematin content that its destruction would be hardly detected.

Table 13. *Destruction of haematin compound in heart-muscle preparation by treatment with BAL under various conditions and with oxidized glutathione and notatin-glucose*

(Destruction measured by decrease of intensity of 550–560 mμ. band produced by adding $Na_2S_2O_4$ and pyridine to heart-muscle preparation; 2 ml. heart-muscle preparation in total volume 2·4–2·8 ml. Aerobic treatment in all experiments except for one marked*.)

Exp. no.	Heart-muscle preparation no.	Treatment: Reagent	Treatment: Time (min.)	Destruction of haematin compound (% of total)	Inactivation of succinic oxidase system (%)
1	20	0·0085 M-BAL	15	42	75
2	21	0·0085 M-BAL	10	16	—
		0·0085 M-BAL	20	21	—
		0·0085 M-BAL	30	23	—
		0·0085 M-BAL	60	18	—
3	21	0·0039 M-BAL	15	6	37
		0·0077 M-BAL	15	13	73
		0·0155 M-BAL	15	15	98
		0·031 M-BAL	15	21	99
4	22	0·013 M-BAL	15	16	92
		0·013 M-BAL	15*	1	0
5	22	0·1 M-oxidized glutathione	15	0	47†
		50 μg. notatin, 10 mg. glucose	30	0	12
6	23	0·013 M-BAL	15	24	100
7	27	0·0157 M-BAL	15	14	100
8	29	0·0157 M-BAL	15	9	100

* Anaerobic treatment. † Inhibition of succinic dehydrogenase, 34%.

(2) *Examination of possibility that the inactivation caused by BAL is due to purely physical factors.* Two possible explanations of the effect of BAL on the succinic oxidase system may be considered: (1) Treatment with BAL may so change the structure of the particles of the heart-muscle preparation that, for purely physical reasons, the cytochrome *b* is no

longer accessible to the remainder of the system, but can be oxidized by the small molecule methylene blue. (2) The substance destroyed by the BAL is actually a component of the succinic oxidase system, required to transmit electrons from cytochrome *b* to cytochrome *c*.

The nature of the inactivation of the succinic oxidase system, which has all the characteristics of an actual destruction of a substance, makes the second explanation more likely. In another paper (Slater, 1949*b*), it is shown that inhibitors of the first type, i.e. those which act non-specifically on the enzyme system by affecting the particles of the enzyme preparation, possess certain characteristics which enable them to be distinguished from the more specific inhibitors. The non-specific inhibitors (1) usually inhibit the cytochrome oxidase as well as the succinic oxidase system, (2) affect the succinic oxidase system to a greater extent if cytochrome *c* is not added during the measurement of the enzyme activity, and (3) the inhibition is often reversed by calcium phosphate gel or denatured proteins. The inhibition of the succinic oxidase system by BAL possesses none of these characteristics. The cytochrome oxidase activity is hardly affected; denatured globin, serum proteins or calcium phosphate gel do not reactivate the system after complete inhibition by the BAL and cytochrome *c* does not affect the inhibition, even in the presence of denatured globin (Table 14). It is, therefore, concluded that the second explanation, viz. that BAL destroys a component of the succinic oxidase system required to transmit electrons from cytochrome *b* to cytochrome *c*, is the correct one.

It might appear at first sight that the finding, from experiments with *p*-phenylenediamine, that the accessibility of the endogenous cytochrome *c* to the oxidase is impaired after treatment with BAL, is inconsistent with this conclusion. However, the effect on the accessibility of cytochrome *c* to the oxidase is small compared with the complete inactivation of the succinic oxidase system, and is probably secondary to it. The destruction of the factor necessary for the reduction of cytochrome *c* by cytochrome *b* would make this step in the reaction sequence the rate-determining reaction, so that addition of cytochrome *c*, although it could overcome the minor effect on the oxidation of cytochrome *c*, would have no effect on the degree of inhibition of the succinic oxidase activity.

Other attempts to reactivate the BAL-inactivated system

In addition to the substances already mentioned (viz. denatured globin, serum proteins, calcium phosphate gel and cytochrome *c*), the following were found to be unable to reactivate the enzyme system after treatment with BAL: catalase, Straub's 'SC factor' (Straub, 1942; Keilin & Hartree, 1949; Slater, 1949*b*), aqueous extract of minced horse heart (rich in myoglobin), supernatant from the isoelectric precipitation of heart-muscle extract in phosphate buffer, and heart-muscle preparation treated with sufficient *p*-aminophenylarsenoxide to inhibit completely the succinic dehydrogenase activity. It is shown elsewhere (Slater, 1949*d*) that *p*-aminophenylarsenoxide inhibits the succinic oxidase system by combining with the succinic dehydrogenase; thus, heart-muscle preparation treated with the arsenical will probably still contain the factor linking cytochrome *b* with cytochrome *c*. It is not surprising, however, that a heart-muscle preparation treated with this arsenical was unable to reactivate the enzyme system after treatment with BAL since the

Table 14. *Effect of cytochrome* c (*added after BAL treatment*) *on the degree of inactivation of the succinic oxidase system produced by BAL*

(Exp. 1–3: heart-muscle preparation, treated by 'general procedure', with 0·003 M-BAL for 20 min. Exp. 4: kidney preparation, treated by 'General procedure', with 0·0015 M-BAL for 15 min.)

Exp. no.	Phosphate concentration (M)	Globin concentration (%)	Cytochrome *c* concentration (M × 10^5)	Succinic oxidase activity (μl./10 min.) Control	Succinic oxidase activity (μl./10 min.) BAL-treated	Inhibition (%)
1	0·14	0	0	90	43	52
	0·14	0	4	116	57	51
	0·14	0·13	0	100	59	41
	0·14	0·13	4	132	80	40
2	0·15	0	0	50	18	63
	0·15	0	4	83	30	64
3	0·03	0	0	23·8	3·2	87
	0·03	0	4	36·2	5·4	85
4	0·10	0	0	8·7	4·0	55
	0·10	0	1	19·1	7·6	60
	0·10	0	2	22·2	10·0	55
	0·10	0	6	29·3	8·3	72

inactive system, lacking the factor but containing the succinic dehydrogenase, and the inactive system which has this factor intact but whose succinic dehydrogenase is inhibited, would be on different particles and would be quite inaccessible to one another.

DISCUSSION

There are four possible pathways for the oxidation of BAL in the presence of heart-muscle preparation, viz. by (1) Cu^{++} ions introduced during the preparation or by the buffer solution; (2) copper protein compounds, (3) cytochrome *c* and oxidase, (4) the BAL-labile factor. The actual proportion of the total oxidation passing through the BAL-labile factor, which is the only pathway leading to the destruction of this factor, is not known, but it may be quite small which would explain why relatively large concentrations of BAL are necessary to inactivate the succinic oxidase system.

transfer of electrons from cytochrome *b* to cytochrome *c*.

The relationships between this factor and the cytochromes are shown in Fig. 7, the arrows showing the direction of electron transfer. It can be seen from this diagram that the destruction of the factor would not affect the oxidation of succinate through methylene blue as carrier, nor the oxidation of various reducing agents through cytochrome *c* and cytochrome oxidase, but would completely prevent the transfer of electrons from cytochrome *b* to cytochrome *c* and thence to molecular oxygen. It has been shown previously (Slater, 1948) that treatment with BAL under conditions which caused complete inactivation of the succinic oxidase system, with about 10% inhibition of succinic dehydrogenase, inhibited the activity of the system, measured anaerobically by the rate of reduction of potassium ferricyanide, by 35%. This is probably because potassium ferricyanide, with an oxidation-reduction

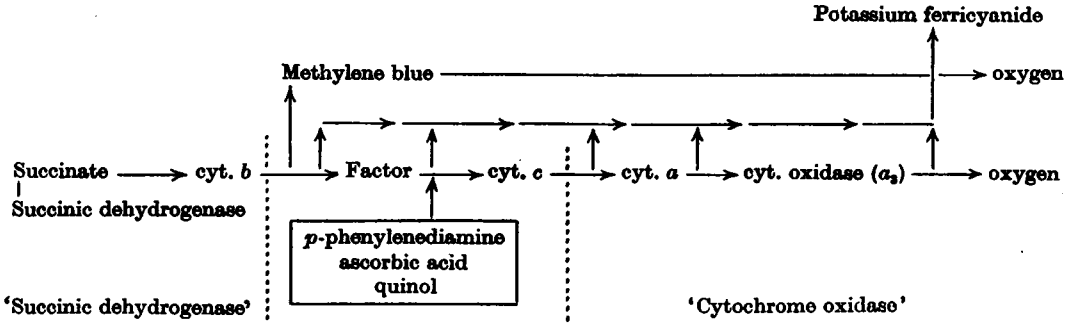


Fig. 7. The components of the succinic oxidase system.

Of the reducing agents mentioned in Table 1, only BAL has been studied intensively. It seems probable however, that the mechanism of the action of the other SH compounds (except diethyldithiocarbamate) and of ascorbic acid on the succinic oxidase system is similar to that of BAL. The larger inhibition of the succinic dehydrogenase found with ascorbic acid compared with BAL may be due to the failure of ascorbic acid ($E'_0 = 0{\cdot}05$ V., at pH 7·3, 30°; Ball, 1937) to protect the SH groups of succinic dehydrogenase from oxidation by H_2O_2. BAL with an E'_0 of $-0{\cdot}15$ V. at pH 7·0 (Barron, Miller & Kalnitsky, 1947) is more favourably situated in this respect. The greater inhibition with cysteine is probably related to the more rapid oxidation of this compound which might be expected to behave in the same way as BAL, if a shorter time of reaction were used.

The chief finding of this investigation is that the primary effect of BAL on the succinic oxidase system is due to the destruction, by directly coupled oxidation, of a factor which is necessary for the potential ($E'_0 = 0{\cdot}41$ V., at pH 7·3) much higher than that of succinate⇌fumarate ($E'_0 = -0{\cdot}01$ V., at pH 7·3) or of cytochrome *b* ($E'_0 = -0{\cdot}04$ V., at pH 7·3), operates in part directly with cytochrome *b* or succinate (activated by the dehydrogenase), and in part through the BAL-labile factor or the cytochromes of higher oxidation-reduction potential (E'_0 of cytochrome $c = +0{\cdot}26$V., of cytochrome $a = +0{\cdot}29$V.). Thus the destruction of the factor causes a partial, but not complete, inhibition of the rate of oxidation of succinate by ferricyanide.

It seems likely that this factor is a haematin compound. The evidence for this is: (1) the inactivation, which occurs only when the reducing agent is added to the enzyme preparation in the presence of air, bears a striking resemblance to the effect of reducing agents on haemoglobin (Lemberg *et al.* 1941); (2) BAL has been shown to destroy haematin itself and haemoglobin (Barron, Miller & Kalnitsky, 1947), myoglobin (this investigation) and the haematin enzymes peroxidase and catalase (Webb & van Heyningen, 1947; Lemberg & Foulkes, 1948),

while glutathione inhibits catalase (Marks, 1936); (3) quantitative measurements of the amount of haematin compounds in the heart-muscle and kidney preparations suggest that these preparations contain haematin compounds, whose spectra are not visible (Slater, 1949*a*); (4) some protohaematin compound is destroyed by treatment of the heart-muscle preparation with BAL, and the destruction of this compound is parallel to the inactivation of the succinic oxidase system.

It is interesting to note that the naturally occurring haematin compounds fall into two groups, according to the nature of their absorption spectra and their susceptibility to reducing agents, viz.—

Group A: peroxidase, catalase and methaemoglobin; these compounds, whether in the ferric or the ferrous state, possess rather weak absorption bands. They are destroyed by reducing agents in the presence of air.

Group B: cytochromes *b*, *c*, *a*, a_3; these compounds possess haemochromogen spectra, i.e. strong bands in the ferrous state, weak bands in the ferric state. They are not affected by reducing agents.

According to this classification, the factor in the succinic oxidase system belongs to Group *A*.

Keilin & Hartree (1949) have suggested that this factor might be related to cytochrome c_1, which, according to Yakushiji & Okuniki (1940), acts between cytochromes *b* and *c* in the succinic oxidase system. However, it has been shown in another study (Slater, 1949*e*) that the evidence for the existence of cytochrome c_1 is unsatisfactory.

The possibility should be considered that the action of the BAL is not on an additional factor, but on cytochrome *b* itself. This could be the case, only if the BAL affects the cytochrome *b* in such a way that its absorption spectrum is unaltered and its reduction is unaffected, but it can no longer be oxidized. This possibility, itself extremely unlikely, is disproved, first, by the evidence that cytochrome *b* is a part of, if not identical with, succinic dehydrogenase (see Slater, 1949*a*) and, secondly, by the observation that, after the treatment with BAL, the cytochrome *b* could still be readily oxidized by dyestuffs.

A consideration of the oxidation-reduction potentials of the known hydrogen and electron carriers reveals that there is a big gap in potential between cytochrome *b* and *c*. The finding of a factor which operates between these two cytochromes narrows this gap. This factor does not account for all the protohaematin found in tissue preparations, and it is possible that some other haematin compound is also involved in the succinic oxidase system.

The question must now be considered whether the factor operates only in the succinic oxidase system or is a catalyst in the main pathway of respiratory catalysis. If cytochrome *b* is a catalyst in the main pathway, it follows that the factor must be a respiratory catalyst concerned in the oxidation of most substrates. If, however, as is possible, cytochrome *b* is concerned only in the oxidation of succinate, the factor may either be in the main pathway between diaphorase and cytochrome *c*, or, like cytochrome *b*, be concerned only with the oxidation of succinate and not with that of those substrates which act through the nicotinamide nucleotides and diaphorase. It is known that pure cytochrome *c* cannot be reduced by pure diaphorase (Lockhart & Potter, 1941). It is not unlikely that the factor required for the reduction of cytochrome *c* by cytochrome *b* is also required, either alone or in conjunction with cytochrome *b*, for the reduction of cytochrome *c* by diaphorase. This can only be decided by further experiments, which are in progress.

Another dehydrogenase which, like succinic dehydrogenase, reacts with the cytochromes without the intervention of the nicotinamide nucleotides or diaphorase, is the lactic dehydrogenase of yeast which is probably identical with cytochrome b_2, a haematin compound, whose spectrum is not visible in yeast, but which was obtained in a concentrated form by Bach *et al.* (1946). These authors showed that the rapid reduction of cytochrome *c* by cytochrome b_2 required an additional factor (*X*), which is removed in the purification and which is not required for the reduction of methylene blue. It is possible that this factor (*X*) is the same as, or closely related to, the factor under consideration. There is, indeed, some evidence in the literature that yeast contains a respiratory catalyst, which is sensitive to thiols (Quastel & Wheatley, 1932; Runnström & Sperber, 1938; Cook & Perisutti, 1947).

p-Phenylenediamine differs from the other reducing agents used in the estimation of cytochrome oxidase activity, first, by being more accessible to the endogenous cytochrome *c* of the enzyme preparation, and, secondly, by being more accessible to added cytochrome *c* in the vicinity of the oxidase (Slater, 1949*c*). The latter, but probably not the former, is affected by treatment with BAL. The reason for this difference in behaviour of *p*-phenylenediamine is unknown. Since treatment with BAL destroys a factor necessary for the reduction of cytochrome *c* by cytochrome *b*, the possibility that this factor catalyses the reduction of cytochrome *c* by *p*-phenylenediamine should be considered. However, the following evidence is strongly against this view: (1) the main reason for the inhibition of the *p*-phenylenediamine oxidation is not an inhibition of the rate of reduction of cytochrome *c* by *p*-phenylenediamine, but an effect on the accessibility of this cytochrome *c* to the oxidase (i.e. on its rate of oxidation); (2) treatment with BAL under conditions which completely inactivated the succinic oxidase system inhibited the rate of oxidation of *p*-phenylenediamine by only 30–40%.

Several workers (see Table 15) have reported inhibitors which affect the complete succinic oxidase system much more markedly than the succinic dehydrogenase and cytochrome oxidase portions. These inhibitors may be of two types, viz. (1) those which react specifically with the factor which links the succinic dehydrogenase to the cytochrome oxidase, and (2) those which act non-specifically on the enzyme system, by affecting the mutual accessibility of the components, on the particles of the enzyme preparation.

Table 15 lists these inhibitors, together with their probable mode of action, which has been investigated thoroughly in the case of only the first three inhibitors. In another paper (Slater, 1949*d*), reasons are given for believing that oxidizing agents and *p*-chloromercuribenzoate act in the manner described; these inhibitors have not, however, been studied in detail. It is impossible to decide, on the basis of the available evidence, the mode of action of fluoride or pyocyanine. It is quite possible that fluoride combines with the BAL-labile factor, with which the present paper is concerned, since there is evidence that this factor is a haematin compound and many haematin compounds combine with fluoride.

Table 15. *Inhibitors of succinic oxidase system which inhibit complete system more than succinic dehydrogenase or the cytochrome oxidase*

Inhibitor	Reference	Probable mechanism of action
Bile salts	Straub (1942), Keilin & Hartree (1949), Slater (1949*b*)	Non-specific
Laser's haemolytic substance (Laser & Friedmann, 1945)	Slater (1949*b*)	Non-specific
Reducing agents + oxygen	Present paper	Destruction of component
Oxidizing agents	Slater (1949*d*)	Destruction of component (?)
p-Chloromercuribenzoate	Slater (1949*d*)	Non-specific (?)
Fluoride	Borei (1945)	?
Pyocyanine	Keilin & Hartree (1940)	?
2-Hydroxy-3-alkyl-1:4-naphthaquinones	Ball *et al.* (1947)	?
4:4′-Dihydroxystilbene	Case & Dickens (1948)	?

Both Ball *et al.* (1947) and Case & Dickens (1948) believe that the compounds which they studied acted on a factor linking the succinic dehydrogenase with the cytochrome oxidase, but the alternative hypothesis has not been investigated by these workers. Indeed, there is reason to believe that the naphthaquinones might act non-specifically, since only hydroxynaphthaquinones with long aliphatic side chains were effective inhibitors. The length of the aliphatic side chain could not appreciably affect the chemical properties of a naphthaquinone, but would profoundly affect its physical properties. The fact that succinic dehydrogenase was inhibited to a certain extent by these compounds is also in agreement with this interpretation (cf. Slater, 1949*b*). Similarly, 4:4′-dihydroxystilbene, the compound studied by Case & Dickens (1948), is not very reactive chemically, but is strongly adsorbed on protein films (Rideal & Shulman, 1939). It is clear that further work is required to elucidate the mechanism of the action of these interesting new inhibitors introduced by Ball *et al.* (1947) and Case & Dickens (1948). Their findings reported to date, like those of Hopkins *et al.* (1939), Stern & Melnick (1939), Straub (1942), and Stoppani (1947) (see p. 14, and Keilin & Hartree, 1949) do not, in themselves, provide any evidence for the existence of a factor between the dehydrogenase and cytochrome oxidase. The reducing agents (in the presence of air) studied in the present paper remain as the only substances known at present to inactivate the factor.

SUMMARY

1. The succinic oxidase system of heart-muscle and kidney preparations is inactivated by treatment with a number of reducing agents in the presence of air. Under certain conditions, many of these reducing agents have little effect on the succinic dehydrogenase.

2. BAL has two quite distinct effects: (*a*) complete inactivation of the succinic oxidase system, without any effect on the succinic dehydrogenase; (*b*) a partial inhibition of succinic dehydrogenase, which does not occur until more than half the BAL has been oxidized. The latter inhibition is probably due to oxidation of SH groups. The former inhibition was studied in detail.

3. Oxidized BAL inhibited the succinic oxidase system much less than BAL, while no inhibition occurred if the enzyme preparation was treated with BAL under anaerobic conditions.

4. H_2O_2 is produced during the oxidation of BAL in buffer solution or in the presence of heart-muscle preparation. Some substance (or substances) is oxidized during treatment of heart-muscle preparation with BAL in the presence of air.

5. H_2O_2 produced by notatin-glucose and by D-amino-acid oxidase and its substrates had a much less pronounced effect on the succinic oxidase system than BAL. Inhibition by these substances was

increased by catalase and decreased by ethanol and pyruvate. Catalase, ethanol and pyruvate had no effect on the inhibition of the system caused by BAL.

6. The addition of copper, which increased the rate of oxidation of BAL, decreased the inactivation of the succinic oxidase system, but increased that of succinic dehydrogenase.

7. It is concluded that the inhibition produced by BAL is not due to the H_2O_2 formed during the oxidation of BAL, but is caused by the directly coupled oxidation of BAL, not involving free H_2O_2, with some substance or grouping necessary for the activity of the succinic oxidase system.

8. The presence of cytochrome *c*, during the treatment of the enzyme with the BAL, prevented the inactivation of the enzyme.

9. Treatment with BAL does not affect the true cytochrome oxidase activity.

10. Treatment with BAL did not destroy the cytochromes; a protohaematin compound, amounting to about 20 % of the total protohaematin content of the heart-muscle preparation, was, however, destroyed by this treatment. There was some correlation between the amount of haematin compound destroyed and the degree of inactivation of the succinic oxidase system when different concentrations of BAL were allowed to act on the enzyme for various times.

11. Treatment with BAL caused an impairment of the oxidation of cytochrome *b* by cytochrome *c*. The cytochrome *b* could, however, be oxidized by methylene blue or dichlorophenolindophenol.

12. Evidence is presented in favour of the view that BAL affects the succinic oxidase system by destroying a component of the succinic oxidase system required for the transmission of electrons between cytochrome *b* and cytochrome *c*.

13. The component of the succinic oxidase system, which is destroyed by the treatment with the BAL, is probably the same as the haematin compound destroyed by the same treatment.

I would like to express my deep appreciation of the interest taken by Prof. D. Keilin, F.R.S., and for his stimulating suggestions throughout the course of this investigation. I would also like to thank Dr E. F. Hartree for supplying the catalase and D-amino-acid oxidase and for many valuable suggestions, and the British Council for a scholarship.

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Addendum (15 *June* 1949)

It has now been found (Slater 1949*f*) that the respiratory catalyst discussed in this paper is also necessary for the oxidation of dihydrocozymase, acting between diaphorase and cytochrome *c* (cytochrome *b* is not required for this oxidation). Thus, the factor links the succinic oxidase and dihydrocozymase oxidase systems and enables the anaerobic oxidation of dihydrocozymase by fumarate.