INTERACTION OF NON-SPECIFIC REDUCING AND OXIDIZING AGENTS WITH THE CYTOCHROME SYSTEM IN HEART-MUSCLE PREPARATIONS

M. B. THORN AND F. L. JACKSON

Department of Biochemistry, St. Thomas's Hospital Medical School, and Department of Bacteriology, King's College Hospital Medical School, London (Great Britain)

(Received November 29th, 1959)

SUMMARY

1. Under anaerobic conditions, 2:3-dimercaptopropanol (BAL), ascorbate and a number of monothiols brought about the reduction of cytochromes *b*, *c* and *a* in KEILIN AND HARTREE heart-muscle preparations. The reduction of cytochrome *b* under these conditions was relatively slow and incomplete, but was made still slower by the addition of antimycin.

2. Under anaerobic conditions, oxidation of reduced cytochromes *b*, *c* and *a* was brought about by hydrogen peroxide in the presence of cyanide, by *o*-iodosobenzoate and the disulphide cystamine. The oxidation of cytochrome *b* under these conditions was relatively fast, but was inhibited by the addition of antimycin.

3. From these experiments it was concluded that antimycin inhibits the respiratory chain between cytochrome *b* and the point of interaction of these reducing and oxidizing agents, tentatively identified as the BAL-sensitive factor.

4. Succinate, but not malonate, protected the succinic oxidase system from inhibition by BAL in the presence of air. Antimycin, but not cyanide, prevented succinate from protecting the BAL-sensitive factor.

5. From these protection experiments it was concluded that the antimycin-sensitive factor lies between succinic dehydrogenase and the BAL-sensitive factor.

6. Under the conditions of the BAL–air inhibition process, the protective effect of succinate was probably to prevent the oxidation of BAL by making the reaction medium essentially anaerobic. When, however, the oxidation of succinate was limited by addition of cyanide, under these conditions, it was found that oxidation of BAL took place to the extent of about one third of that in the presence of cyanide alone. This amount of oxidation of BAL would be expected to lead to substantial inhibition of succinic oxidase activity, but in fact no inhibition was observed.

INTRODUCTION

The reducing agents used in the present work (mono- and dithiols, ascorbate) are described as non-specific because they are not known to be activated by dehydrogenases in heart-muscle particulate preparations (KEILIN-HARTREE). The interaction of oxidizing agents is described as non-specific, either because no enzyme in the

References p. 76.

preparation specifically activates them (*o*-iodosobenzoate, disulphides), or because the enzyme which normally activates them has been inhibited (oxygen and hydrogen peroxide when cytochrome oxidase and catalase are inhibited with HCN).

The interaction of all these substances with the cytochrome system has previously been investigated in different connections. Reducing agents (*e.g.* ascorbate) have been used in the estimation of cytochrome oxidase activity[1], and 2:3-dimercaptopropanol (BAL) in the presence of air has been found to inactivate the succinic oxidase system by destruction of a component between cytochromes *b* and *c*[2]. Oxidizing agents (*e.g.* *o*-iodosobenzoate and disulphides) are generally recognized as inhibitors of sulphydryl enzymes, including succinic dehydrogenase, but SLATER[3] has suggested that oxidizing agents may inhibit the succinic oxidase system between the dehydrogenase and cytochrome *c* as well as the dehydrogenase itself. Oxygen has an inhibitory effect on low-phosphate preparations[4], possibly similar to the effects of the oxidizing agents mentioned, and may also interact, though perhaps indirectly, with the respiratory chain between cytochromes *b* and *c* and so account for the "auto-oxidation" of cytochrome *b*[5]. Spectroscopic observations, now presented, were undertaken in an attempt to discover whether there are common features in the effects of these non-specific reducing and oxidizing agents on the cytochrome system in heart-muscle preparations and also what relationship there might be between the point(s) of action of these substances and the point(s) of action of antimycin and of 2-*n*-heptyl-4-hydroxyquinoline-N-oxide[6] (HOQNO). The latter two substances, like BAL in the presence of air, inhibit the respiratory chain between cytochromes *b* and *c*.

In a previous paper[7], it was inferred that antimycin and BAL in the presence of air inhibited different factors between cytochromes *b* and *c*. At that time it was shown that treatment with BAL in the presence of air did not affect the capacity of a heart-muscle preparation to combine with antimycin. It has now been found that prior combination of the preparation with antimycin does not affect the oxidation of BAL by the preparation, nor has it any protective effect on the BAL-sensitive factor, as shown by a subsequent assay of succinic oxidase activity. This observation permitted a more detailed examination of SLATER'S[3] brief mention of protection of the BAL-sensitive factor by succinate and competitive inhibitors. Such protection would be of particular interest because the factor is considered to be spatially remote from the dehydrogenase. In fact, STOPPANI AND BRIGNONE[8] failed to show any protection of the BAL-sensitive factor by phosphate and fluoride, which inhibit succinic dehydrogenase competitively[9], although they succeeded in demonstrating that phosphate and fluoride protected the dehydrogenase from thiol reagents. This observation is confirmed in the present work, using malonate as competitive inhibitor. Indeed, SLATER[10] has recently acknowledged the fact that competitive inhibitors do not protect the BAL-sensitive factor, and has given an alternative explanation of the experimental results which led to his original contrary claim. The undoubted protective effect of succinate has been further investigated in the present work.

The experiments involving spectroscopic observations of the effects of non-specific reducing and oxidizing agents, and those on protection of the BAL-sensitive factor by succinate, are all consistent with the view that inhibition by antimycin and by BAL in the presence of air are properties associated with different factors between cytochromes *b* and *c*, and that the antimycin-sensitive factor is located between cytochrome *b* and the BAL-sensitive factor.

References p. 76.

MATERIALS AND METHODS

Heart-muscle preparations

Heart-muscle preparations were made essentially according to the method of KEILIN AND HARTREE[11], and were finally suspended either in 0.10 *M* phosphate, pH 7.3, or, after washing the isoelectric precipitate in ice-cold water, in 0.20 *M* borate, pH 7.3.[12].

Reducing and oxidizing agents

All reducing agents were made up in glass-distilled water and, where necessary, adjusted to pH 7.0, immediately before use. BAL was obtained from Boots Pure Drug Co., Nottingham. Cysteamine and cystamine were supplied, in the form of the hydrochlorides, by L. Light & Co., Colnbrook, Bucks. Oxidized glutathione (GSSG) was prepared from glutathione by shaking a neutralized solution in a manometer flask until O_2 consumption ceased[3]. H_2O_2 solutions were prepared by diluting a stock solution, standardized with permanganate, immediately before use.

Spectroscopic experiments

Reducing agents: 1.0 ml of heart-muscle preparation, 25-30 mg fat-free dry weight, was diluted to 2.0 ml with 0.10 *M* phosphate, pH 7.3, and reducing agent solution. Anaerobic experiments were performed in Thunberg tubes, and the reducing agent solution was added from the side arm after evacuating and flushing the tube 4 times with O_2-free N_2. Spectroscopic observations were made by means of a Beck low-dispersion microspectroscope and a 150 c.p. Pointolite.

Oxidizing agents: A procedure similar to that with reducing agents was used, except that about 1 mg of sodium dithionite was added to the heart-muscle preparation before evacuation and treatment with oxidizing agent. This amount of dithionite represented a concentration of less than 3 m*M*, and was considerably less than the concentration of oxidizing agent after its addition from the side arm.

When it was necessary to observe the effect of reducing and oxidizing agents on preparations inhibited with antimycin or with HOQNO, the inhibitors were added as described below, in order to exclude ethanol from the reaction mixture.

BAL–air inhibition experiments

Treatment with BAL was performed as previously described[7], following the procedure of SLATER[2]. When the possible protective effects of succinate and malonate were studied, these compounds were added, with BAL, from the side arms after temperature equilibration. Incubation with BAL was continued for 20 min, unless otherwise stated, and 0.2 ml of diluted reaction mixture was used in the subsequent assay of succinic oxidase activity.

Succinic oxidase activity was determined as previously described[7], except that ethylenediaminetetraacetate (EDTA), 0.5 m*M*, was added in place of histidine. In order to determine the inhibition caused by BAL–air treatment when the enzyme was already completely inhibited with antimycin, fresh heparinized human plasma was added to the medium for assay of succinic oxidase activity. REIF AND POTTER[13] have shown that inhibition by antimycin is reversed by serum albumin.

Addition of antimycin. In the experiments to be described, it was not possible to

References p. 76.

add an ethanol solution of antimycin to heart-muscle preparation, because although contact of the preparation with about 5 % ethanol at 0° for a few minutes (conditions for adding antimycin by method 2 previously described[7]) was not harmful, exposure to this concentration of ethanol at 37° for up to 30 min (conditions for the BAL–air inhibition process) was found to damage the preparation severely. Antimycin was therefore added to the preparation by first measuring the ethanol solution into a manometer flask, then evaporating the ethanol by warming in a stream of air, and finally redissolving the antimycin in the heart-muscle preparation. In a preliminary experiment it was found that on adding a certain quantity of antimycin to a sample of heart-muscle preparation by the method 2 previously described[7], succinic oxidase activity was inhibited 95 %, whereas on adding the same amount of antimycin by evaporation and re-solution at 37° for 10 min, an inhibition of 81 % was observed. This method of adding antimycin therefore appears to be slightly less efficient, and for obtaining complete inhibition it is necessary to use slightly more of the ethanol solution of inhibitor than when adding it directly.

RESULTS

Spectroscopic observations

The following experiments are only roughly quantitative, in that they rely on a purely visual estimate of the relative intensities of the bands of reduced cytochromes *b*, *c* and *a* as viewed with a microspectroscope. This method is, however, adequate for determining the time scale for the sequences of appearance and disappearance of the bands of reduced cytochromes *b*, *c* and *a*, and the effects of antimycin and of HOQNO on these time scales and sequences.

The observations with reducing agents are presented in Table I. The behaviour of the preparation with succinate was first checked. On addition of succinate to the preparation in air all the cytochrome bands rapidly developed, but the b band appeared to develop less strongly (Expt. 1). In the presence of antimycin the b band developed strongly at once, and the c and a bands could only be made to appear transiently on warming (Expt. 2), whereas with HOQNO the c and a bands clearly developed after a short delay at room temperature (Expt. 3). This showed that, at the inhibitor concentrations used, the block produced by HOQNO was less complete than that by antimycin. In anaerobic experiments with BAL as reducing agent, cytochromes *c* and *a* were immediately reduced, and cytochrome *b* was reduced after incubation at 37° for a few minutes. On admitting air and shaking the mixture, all the bands disappeared, but on standing the c and a bands reappeared. The b band developed only under anaerobic conditions (Expt. 4). In the presence of antimycin, reduction of cytochrome *b* by BAL was delayed, but when reduction had occurred, cytochrome *b* was not reoxidized on aeration (Expt. 5). An experiment with HOQNO gave similar results, which were consistent with the less complete block already demonstrated with succinate–succinic dehydrogenase as reducing agent (Expt. 6). Ascorbate readily reduced cytochromes *c* and *a*, but was less effective than BAL in reducing cytochrome *b* (Expt. 7). The monothiols tested all brought about reduction of cytochromes *c* and *a* before the reduction of cytochrome *b*, but the reactions were slow compared with those of BAL (Expts. 8, 9, 10). A further point of interest is that when a freshly-prepared heart-muscle preparation, made in 0.20 *M* borate, was

References p. 76.

incubated under nitrogen at 37° for 20 h, bands of cytochromes *c* and *a* were clearly visible, together with a faint band of cytochrome *b* (Expt. 11). It appears that the preparation contains endogenous non-specific reducing substances, and these may be responsible for its slow oxygen consumption when shaken in Warburg manometers[4].

The observations with oxidizing agents are presented in Table II. The effect of oxygen on a cyanide-inhibited preparation, reduced with dithionite, was first investigated. On swirling the mixture, in air, gently round the walls of the test tube, the band of cytochrome *b* quickly disappeared, showing that excess dithionite was rapidly exhausted. After continuing the exposure to air for some time, the c and a bands also began to fade, until finally the c band disappeared, leaving a trace of the a band (Expt. 12). A similar, but strikingly more rapid, sequence of events was observed using hydrogen peroxide in the presence of cyanide under anaerobic conditions (Expt. 13). In the presence of antimycin the rapid disappearance of the b band was inhibited, that is to say, the c and a bands faded first, the a band being the more stable (Expt. 14). The effect of *o*-iodosobenzoate was the same as that of hydrogen peroxide in the presence of cyanide, but was slower (Expts. 15, 16). It is interesting to note that the disulphide cystamine also brought about oxidation of reduced cytochromes (Expt. 18), although no effect of oxidized glutathione was observed (Expt. 17).

Protection of the BAL-sensitive factor by succinate

It has been pointed out in the Introduction that antimycin has no effect on the oxidation of BAL by heart-muscle preparation, and no protective effect on the BAL-sensitive factor. An experiment demonstrating this will not be described in detail because the results are implicit in the more extended experiments now to be described.

In Table III are presented the results of an experiment which demonstrates the effect of succinate, in the absence and in the presence of antimycin, on the oxidation of BAL by heart-muscle preparation and on consequent changes in succinic oxidase activity. The treatment with BAL inhibited the succinic oxidase activity 76.5% compared with the activity after treatment with succinate alone, but when succinate was present during incubation with BAL there was no loss of activity. In the presence of antimycin, the activity of the enzyme treated with succinate was about 9% less than with succinate alone, because the plasma added did not entirely reverse the inhibition due to antimycin. When the preparation was treated with BAL in the presence of both antimycin and succinate, succinic oxidase activity was inhibited 71.5% compared with the activity after treatment with antimycin and succinate. The results show that succinate protects the BAL-sensitive factor and that antimycin prevents this protective effect.

In Table IV are presented the results of a similar experiment with malonate in place of succinate. It is clear that malonate did not modify the inhibitory effect of the BAL–air treatment, and that the presence of antimycin did not alter the result significantly. This supports the conclusion of STOPPANI AND BRIGNONE[8] that competitive inhibitors do not protect the BAL-sensitive factor (see also, SLATER[10]). In one experiment it was noted that malonate diminished the rate of oxidation of BAL and appeared to protect the BAL-sensitive factor to a slight extent. If, however, the BAL oxidation was allowed to continue until the oxygen consumption equalled that of the control, no protective effect was demonstrated. In the present experiment,

References p. 76.

References p. 76.

TABLE I

REDUCTION OF CYTOCHROMES BY NON-SPECIFIC REAGENTS

1 ml heart-muscle preparation, 25–30 mg fat-free dry wt., was diluted to 2 ml with 0.10 *M* phosphate, pH 7.3, and reducing agent solution. Expts. 4–11 were performed in Thunberg tubes, and the reducing agent solution was added from the side arm after evacuating and flushing the tube 4 times with O_2-free N_2. Spectroscopic observations were made by means of a Beck low-dispersion microspectroscope and a 150 c.p. Pointolite. When antimycin was used, an amount of an ethanol solution of antimycin, sufficient for almost complete inhibition, was first evaporated in the tube in a stream of air. When enzyme was added to the tube and warmed to 37° for 5 min, the antimycin redissolved and combined with the preparation. A similar procedure was used with 2-*n*-heptyl-4-hydroxyquinoline-N-oxide (HOQNO). The amounts of the inhibitors used were: antimycin, 6.8 μg; HOQNO, 20 μg.

Expt.	*Reducing agent*	*Concn. (mM)*	*Inhibitor*	*Conditions*	*Temp.**	*Time scale***	*Relative strength of bands****		
							b	*c*	*a*
1	Succinate	10	None	Aerobic	Room	Immediate	++	+++	+++
2	Succinate	10	Antimycin	Aerobic	Room	Immediate	+++	—	—
					Warm	Fast	+++	++	++
					Room	Fast	+++	—	—
3	Succinate	10	HOQNO	Aerobic	Room	Immediate	+++	—	—
				Allow to stand	Room	Fast	+++	+++	+++
				Shake	Room	Immediate	+++	—	—
4	BAL	12	None	Anaerobic	Room	Immediate	—	+++	+++
				Allow to stand	37°	5 min	++	+++	+++
				Admit air and shake	Room	Immediate	—	—	—
				Allow to stand	Room	Fast	—	++	++
				Re-evacuate	37°	5 min	++	+++	+++
5	BAL	12	Antimycin	Anaerobic	Room	Immediate	—	+++	+++
				Allow to stand	37°	30 min	++	+++	+++
				Admit air and shake	Room	Immediate	++	—	—
6	BAL	12	HOQNO	Anaerobic	Room	Immediate	—	+++	+++
				Allow to stand	37°	5 min	++	+++	+++
				Admit air and shake	Room	Immediate	++	—	—
7	Ascorbate	10	None	Anaerobic	Room	Immediate	—	+++	+++
				Allow to stand	37°	30 min	+	+++	+++
8	Cysteine	25	None	Anaerobic	Room	Immediate	—	—	—
				Allow to stand	37°	1 min	—	++	++
				Allow to stand	37°	25 min	++	+++	+++
9	Glutathione	28	None	Anaerobic	Room	Immediate	—	—	—
				Allow to stand	37°	10 min	—	+++	+++
				Allow to stand	37°	30 min	+	+++	+++
10	Cysteamine	25	None	Anaerobic	Room	Immediate	—	—	—
				Allow to stand	37°	2 min	—	++	++
				Allow to stand	37°	25 min	+	+++	+++

11	None	—	None	Anaerobic	37°	20 h	+	+ + +	+ + +

* Immediately after evacuation the contents of the tubes were colder than room temperature.
** "Immediate" implies within about 5 sec. "Fast" implies within about 15–60 sec.
*** + + + = full reduction, + + = clearly defined, + = faint but definite, ± = faintest perceptible, — = absent.

References p. 76.

TABLE II

OXIDATION OF CYTOCHROMES BY NON-SPECIFIC REAGENTS

Conditions as in Table I, except that about 1 mg of sodium dithionite was added to the heart-muscle preparation before evacuation and treatment with oxidizing agent. This amount of dithionite represents a final concn. of less than 3 m*M*. In Expts. 12–14 a solution of KCN, neutralized to pH 7.3, was added to give a final concn. of 10 m*M*.

Expt.	*Oxidizing agent*	*Concn. (mM)*	*Inhibitor*	*Conditions*	*Temp.**	*Time scale***	*Relative strength of bands****		
							b	*c*	*a*
12	O_2 in presence of cyanide	—	None	Aerobic	Room	Immediate	+ + +	+ + +	+ + +
				Shake	Room	Fast	—	+ + +	+ + +
					Room	30 min	—	+ +	+ +
					Room	45 min	—	—	±
13	H_2O_2 in presence of cyanide	8	None	Anaerobic	Room	Immediate	+ + +	+ + +	+ + +
					37°	Fast	—	+ +	+ + +
					37°	3 min	—	—	+
					37°	5 min	—	—	—
14	H_2O_2 in presence of cyanide	8	Antimycin	Anaerobic	Room	Immediate	+ + +	+ + +	+ + +
					37°	Fast	+ + +	+ +	+ + +
					37°	5 min	+	—	+
					37°	15 min	—	—	—
15	*o*-Iodosobenzoate	7	None	Anaerobic	Room	Immediate	+ + +	+ + +	+ + +
					37°	4 min	—	+ +	+ +
					37°	90 min	—	—	—
16	*o*-Iodosobenzoate	7	Antimycin	Anaerobic	Room	Immediate	+ + +	+ + +	+ + +
					37°	5 min	+ + +	+ +	+ + +
					37°	60 min	—	+	+ +
17	Oxidized glutathione	14	None	Anaerobic	Room	Immediate	+ + +	+ + +	+ + +
					37°	90 min	+ + +	+ + +	+ + +
18	Cystamine	13	None	Anaerobic	Room	Immediate	+ + +	+ + +	+ + +
					37°	60 min	—	+ +	+ +

* Immediately after evacuation the contents of the tubes were colder than room temperature.
** "Immediate" implies within about 5 sec. "Fast" implies within about 15–60 sec.
*** + + + = full reduction, + + = clearly defined, + = faint but definite, ± = faintest perceptible, — = absent.

TABLE III

EFFECT OF SUCCINATE, WITH AND WITHOUT ANTIMYCIN, ON BAL OXIDATION AND INHIBITION OF SUCCINIC OXIDASE

Heart-muscle preparation (BONNER[12]) was treated with BAL by the method of SLATER[2], except that BAL (and/or succinate) was added from the side arm after equilibration for 15 min at 37°. Antimycin was measured into the flasks as a solution in ethanol, the ethanol was evaporated, and the inhibitor redissolved in the heart-muscle preparation during the equilibration period. Composition of the medium: heart-muscle preparation, 21 mg fat-free dry wt.; antimycin, 5.1 μg; BAL, 20 μmoles; succinate, 38 μmoles; in 2.0 ml. After treatment for 20 min, the reaction mixtures were diluted to 5.0 ml with 0.20 M borate, pH 7.3, and 0.2 ml quantities were added to the main compartments of the flasks for succinic oxidase assay. Conditions for succinic oxidase assay: borate, pH 7.3, 0.10 M; succinate (from side arm) 25 mM; ethylenediaminetetraacetate, 0.5 mM; total vol., 3.0 ml; 37°.

Additions	*Oxygen consumption during treatment*			*Succinic oxidase activity after treatment* (Q_{O_2})	*Inhibition by BAL (%)*
	(µl/20 min)	*(µl/h)*	*(apparent Q_{O_2})*		
Succinate	366	1100	52	438	—
BAL	78	234	11	103	76.5
BAL, succinate	359	1080	—	477	0.0
Succinate, antimycin	45	135	6.4	398*	—
BAL, succinate, antimycin	125	375	—	113*	71.5

* The assay medium also contained 0.3 ml of fresh heparinized human plasma to reverse inhibition due to antimycin.

TABLE IV

EFFECT OF MALONATE, WITH AND WITHOUT ANTIMYCIN, ON BAL OXIDATION AND INHIBITION OF SUCCINIC OXIDASE

Conditions as in Table III, except that 3 μmoles malonate were added in place of 38 μmoles succinate.

Additions	*Oxygen consumption during treatment (µl/20 min)*	*Succinic oxidase activity after treatment* (Q_{O_2})	*Inhibition by BAL (%)*
Malonate	—	351	—
BAL	88	60	83.0
BAL, malonate	89	62	82.5
Malonate, antimycin	—	320*	—
BAL, malonate, antimycin	93	40*	87.5

* The assay medium also contained 0.3 ml of fresh heparinized human plasma to reverse inhibition due to antimycin.

malonate, with or without antimycin, had no significant effect on the rate of oxidation of BAL.

The protection of the BAL-sensitive factor by succinate is not likely to be a shielding phenomenon, as postulated by POTTER AND DUBOIS[14] in order to explain protection of succinic dehydrogenase from thiol reagents, since competitive inhibitors should then have a similar action. Possible explanations follow from an inspection of the oxygen consumption figures during the 20-min treatment period, shown in Table III. A very rapid oxygen consumption took place with succinate alone, corresponding to 1100 μl/h, but to an apparent Q_{O_2} of only 52. The limiting factor to oxygen consumption was probably the entry of oxygen into the medium, since the true Q_{O_2} of the preparation with succinate was subsequently shown to be

References p. 76.

438. Where the limiting factor was diffusion of oxygen it is likely that the respiratory chain was largely in the reduced state. The oxygen consumption of the preparation in the presence of BAL corresponded to 234 μl/h, or a Q_{O_2} of 11. This rate of oxygen consumption is not limited by entry of oxygen into the medium, and only a part of the oxidation of BAL is mediated by the cytochrome system[2,15]. Under these conditions the respiratory chain would be largely in the oxidized state, and this is confirmed by the spectroscopic observations in Table I. It was shown that BAL readily reduced cytochromes *c* and *a* anaerobically, but that on aerating the mixture the bands of reduced cytochromes disappeared. It may therefore be postulated that the BAL-sensitive factor is not susceptible to destruction by the BAL–air inhibition process when it is in the reduced state, and that the protective effect of succinate is to maintain the reduced state. Antimycin diminished the oxygen consumption in the presence of succinate to a rate corresponding to 135 μl/h, or a Q_{O_2} of 6.4. This represents an inhibition of succinic oxidase activity of nearly 99 %, and the inhibition by antimycin was now the factor limiting oxygen consumption. When BAL was present together with succinate and antimycin, the observed oxygen consumption equalled the sum of the oxygen consumptions observed separately for BAL and for succinate with antimycin. The function of antimycin in preventing the protection of the BAL-sensitive factor by succinate could therefore be to prevent the reduction of the components of the respiratory chain between the antimycin-sensitive factor and oxygen. On the other hand, when BAL and succinate were both present, it is possible that very little oxidation of BAL in fact took place, since cytochrome oxidase would compete very successfully for the limited supply of oxygen in the reaction medium. An alternative explanation of the results might be that the vigorous oxidation of succinate interfered with the oxidation of BAL, and that antimycin prevented this interference.

In an attempt to distinguish between these alternative explanations, the experiment presented in Table V was performed. The object of this experiment was to inhibit the succinic oxidase system with cyanide, to the point where BAL could be shown to be oxidized simultaneously with succinate, and thus to make it possible to determine whether the oxidation of BAL in the presence of the reduced respiratory chain would lead to inhibition of the BAL-sensitive factor. This experiment was complicated by the fact that WHEELDON[15] had demonstrated that cyanide both inhibited the oxidation of BAL and also exerted a protective effect on the BAL-sensitive factor. It was also necessary to choose a concentration of cyanide which would be sufficiently inhibitory to succinate oxidation in the first part of the experiment but which on dilution would be low enough to permit vigorous oxygen consumption in the subsequent assay of succinic oxidase activity. This requirement was met by using cyanide at a concentration of 0.5 mM during the oxidation of BAL and succinate, which permitted an oxygen consumption in the region of 400 μl/h (apparent $Q_{O_2} = 18$) in the presence of succinate (row 4, column 1, of Table V), while on dilution the concentration of cyanide decreased to 0.01 mM, which inhibited succinic oxidase activity by only about 25 % (row 1, column 4, of Table V). When cyanide was present during the oxidation of BAL, the BAL oxidation was inhibited to a greater extent than would be expected from the data of WHEELDON[15], and to achieve an oxygen consumption of BAL equal to that in the absence of cyanide the incubation was continued for 60 min. The succinic oxidase activity of this sample was then found

References p. 76.

TABLE V

EFFECT OF SUCCINATE IN THE PRESENCE OF CYANIDE ON BAL OXIDATION AND INHIBITION OF SUCCINIC OXIDASE

Conditions similar to those in Table III, but composition of the medium during treatment: heart-muscle preparation, 22 mg fat-free dry wt.; KCN, neutralized to pH 7.3, 0.75 μmole; succinate, 38 μmoles; BAL, 20 μmoles; in 1.5 ml. Treatment with BAL alone was continued for 20 min, but when cyanide was present the incubation was extended to 60 min. After treatment the reaction mixtures were diluted to 5.0 ml with 0.20 *M* borate, pH 7.3, and 0.2-ml quantities were immediately added to the main compartments of the flasks containing the reagents for succinic oxidase assay. Conditions for succinic oxidase assay as in Table III, but untreated control and BAL-treated samples were assayed both in the absence and in the presence of the same concentration of cyanide as was present during assay of those samples which contained cyanide during treatment.

Additions	*Oxygen consumption during treatment*			*Succinic oxidase activity after treatment (Q_{O_2})*	*Inhibition by BAL (%)*
	Total O_2 uptake (μl)	*Time taken (min)*	*O_2 uptake by BAL (μl)*		
Untreated	—	—	—	425 298*	— —
BAL	89	20	89	85 43*	80 86
BAL, cyanide	86	60	86	44	85
Succinate, cyanide	411**	60	—	275	—
BAL, succinate, cyanide	440**	60	29	274	—

* The assay medium also contained 0.01 m*M* cyanide.
** Theoretical O_2 consumption for complete oxidation of succinate added = 420 μl.

to be the same as that of the sample treated with BAL alone, with cyanide added during the succinic oxidase assay, and no protective effect of cyanide was therefore apparent. The reasons for the differences in results between this experiment and that of WHEELDON[15] are not clear. The rate of oxygen consumption of the enzyme incubated with succinate and cyanide diminished after 50 min, and after 60 min the succinate was almost exhausted. At the same time, the oxygen consumption of the sample containing BAL as well as succinate and cyanide was greater by 29 μl, and was 20 μl more than the theoretical quantity for complete oxidation of the succinate present. The succinic oxidase activities of these two samples were therefore determined at the same time as that of the sample treated with BAL and cyanide. It is evident that in the presence of succinate and cyanide, BAL had no inhibitory effect, for the succinic oxidase activity was the same as that in the absence of BAL, and was not far removed from the activity of the untreated control in the presence of the same final concentration of cyanide. In fact, in the region of 0.01 m*M*, small changes in cyanide concentration have a large effect on succinic oxidase activity. This experiment showed that, in the presence of cyanide, succinate interfered with the oxidation of BAL, since the oxygen consumption attributable to BAL was only 29 μl compared with 86 μl in the absence of succinate. Succinate also had a protective effect on the BAL-sensitive factor, however, because the data of SLATER[2], and unpublished experiments of one of us (M.B.T.), show that BAL oxidation equivalent to 29 μl O_2 is expected to inhibit succinic oxidase activity by at least 30 %. An inhibition of this magnitude should have been readily demonstrable under the present conditions.

References p. 76.

DISCUSSION

From the spectroscopic observations described in the first part of this paper it appears simplest to conclude that these reducing and oxidizing agents interact with the electron-transport system at a point between cytochromes *b* and *c* (probably the BAL-sensitive factor), that the BAL-sensitive factor and the antimycin-sensitive factor are not the same, and that the antimycin-sensitive factor is located between cytochrome *b* and the BAL-sensitive factor. It has already been inferred that the BAL- and antimycin-sensitive factors are not the same[7]. The experiments on protection of the BAL-sensitive factor by succinate support the relative spatial location now suggested, as will be discussed later, and at the same time lend weight to our assumption that the site of reduction of the respiratory chain by BAL is in fact the site at which the respiratory chain is inhibited by BAL in the presence of air. The spectroscopic experiments suggest that reduction and oxidation of cytochrome *b* by these non-specific reagents are respectively backward and forward reactions through the antimycin-sensitive factor, and that the oxidation of cytochromes *c* and *a* takes place by a back reaction. The forward reactions are relatively fast (oxidation of cytochrome *b* and reduction of cytochromes *c* and *a*) and the back reactions are relatively slow (reduction of cytochrome *b* and oxidation of cytochromes *c* and *a*), but in general the reactions were slow compared with those brought about by specific substrates. It is implicit in these conclusions that the BAL-sensitive factor can be oxidized and reduced, although this oxidation and reduction may be irrelevant to electron transport under physiological conditions.

The experiments on protection of the BAL-sensitive factor by succinate indicate that this protective effect is probably twofold. The experiment with cyanide suggests that simultaneous oxidation of succinate interferes with the oxidation of BAL, but it also shows that when BAL oxidation proceeds under these conditions, the succinic oxidase system is not inhibited. It is known that the oxidation of BAL by heart-muscle preparation takes place by several pathways, and that the effect of protective agents is usually to enhance the oxidation of BAL by one of the non-inhibitory pathways at the expense of the inhibitory process[2,15]. In the present experiments, the protective effect of succinate may be connected with the maintenance of the respiratory chain in the reduced form, the "coupled oxidation" of BAL and the BAL-sensitive factor being completely suppressed. It would be desirable to investigate this protection phenomenon more closely, and also, in this connection, to study in more detail the overall kinetics of the system when doubly inhibited. Whatever the final explanation of this protection may be, however, the effects of antimycin are clear cut. The fact that antimycin has no effect on BAL oxidation (although a part of the oxidation is brought about by the cytochrome system[2,15]) but, unlike cyanide, can prevent succinate from interfering with the BAL–air inhibition process, suggests that the antimycin-sensitive factor lies between succinic dehydrogenase and the BAL-sensitive factor. This suggested sequence is consistent with the results of the manometric and spectroscopic experiments described here.

The demonstration that oxidizing agents other than oxygen can react with the respiratory chain between the antimycin-sensitive factor and cytochrome *c* suggests that reactions of this kind with endogenous oxidizing agents might play a part in the "auto-oxidation" of cytochrome *b*. A possible connection between the action of

References p. 76.

oxidizing agents and their suggested inhibition[3] of the BAL-sensitive factor remains obscure.

ACKNOWLEDGEMENTS

We gladly acknowledge the helpful collaboration of Miss VALERIE LAWTON, M.Sc., in parts of this work, and one of us (F.L.J.) wishes to thank the Medical Research Council, Great Britain, for a grant.

REFERENCES

1 E. C. SLATER, *Biochem. J.*, 44 (1949) 305.
2 E. C. SLATER, *Biochem. J.*, 45 (1949) 14.
3 E. C. SLATER, *Biochem. J.*, 45 (1949) 130.
4 M. B. THORN, *Biochem. J.*, 69 (1958) 19P.
5 F. L. JACKSON AND J. W. LIGHTBOWN, *Biochem. J.*, 69 (1958) 63.
6 J. W. LIGHTBOWN AND F. L. JACKSON, *Biochem. J.*, 63 (1956) 130.
7 M. B. THORN, *Biochem. J.*, 63 (1956) 420.
8 A. O. M. STOPPANI AND J. A. BRIGNONE, *Biochem. J.*, 64 (1956) 196.
9 E. C. SLATER AND W. D. BONNER JR., *Biochem. J.*, 52 (1952) 185.
10 E. C. SLATER, *Advances in Enzymol.*, 20 (1958) 147.
11 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 41 (1947) 500.
12 W. D. BONNER JR., *Biochem. J.*, 56 (1954) 274.
13 A. E. REIF AND V. R. POTTER, *J. Biol. Chem.*, 205 (1953) 279.
14 V. R. POTTER AND K. P. DUBOIS, *J. Gen. Physiol.*, 26 (1943) 391.
15 L. W. WHEELDON, *Biochim. Biophys. Acta*, 29 (1958) 321.

A CYTOCHROME OF THE *b* GROUP FROM *MICROCOCCUS LYSODEIKTICUS*

F. L. JACKSON AND VALERIE D. LAWTON

Department of Bacteriology, King's College Hospital Medical School, Denmark Hill, London (Great Britain)

(Received December 1st, 1958)

SUMMARY

A method has been devised for separating the cytochrome *b* component of *M. lysodeikticus* from the other cytochromes. The absorption maxima of the reduced component in whole cells, lysates and particulate preparations were at about 560–561 mμ, 525–530 mμ and 429–430 mμ.

The separated cytochrome *b* component was auto-oxidizable, even in the presence of cyanide ($5.0 \cdot 10^{-2}$ *M*). Observations suggest that its potential is lower than that of the bacterial cytochrome *c*.

The cytochrome *b* component could be solubilized by treatment of the particulate preparation with 5 % w/v "Cetrimide" solution, but precipitation of the cytochrome from the solution with ammonium sulphate produced small shifts in the absorption maxima.

References p. 84.