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STUDIES ON THE MECHANISM OF INHIBITION OF THE MITOCHONDRIAL ELECTRON TRANSPORT BY ANTIMYCIN

II. ANTIMYCIN AS AN ALLOSTERIC INHIBITOR

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SUMMARY

1. Pretreatment of sub-mitochondrial particles with cholate results in a change in the curve describing inhibition by antimycin of the succinate–cytochrome *c* reductase from sigmoidal towards linear. This effect of cholate is reversed by partial removal of the cholate by dialysis, either in the absence or presence of antimycin.

2. Treatment with cholate has the same action on the sigmoidal effect curve of antimycin on the reducibility of cytochrome *b*. This is also reversed by dialysis.

3. The effect of antimycin on the displacement to the red of the α-band of ferrocytochrome *b*, measured in the presence of succinate, NADH or reduced ubiquinone Q-2, is also described by a sigmoidal curve that is changed to a linear one by addition of cholate.

4. Linear displacement curves are obtained with menaquinol or $Na_2S_2O_4$.

5. It is proposed that antimycin is an allosteric inhibitor of the respiratory chain. This allosteric effect should be distinguished from the effect of antimycin on the "conformation stability" of Complex III.

INTRODUCTION

The curve describing the inhibition by antimycin of electron transport in sub-mitochondrial particles[1–3] or intact mitochondria[1,4] is strongly sigmoidal. Three explanations may be considered: (i) these preparations contain two antimycin-binding sites, one with a higher affinity for antimycin not concerned in the catalytic activity, and a "catalytic" site with a lower affinity[1]; (ii) an antimycin-sensitive factor necessary for electron transport is either present in large excess[1] or reacts rapidly in comparison with other components of the electron-transport chain[3,4]; (iii) inhibition by antimycin is a co-operative phenomenon similar to that proposed for allosteric inhibitors by MONOD *et al.*[5] (*cf.* ref. 6). In contrast to the behaviour of preparations containing the intact respiratory chain, linear inhibition curves are found

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with fragments of the chain containing the antimycin-sensitive site, *viz.* NADH–cytochrome *c* reductase[7] and Complex III (refs. 8–11).

The present paper describes a further investigation of the conditions in which sigmoidal and linear inhibition curves are obtained. The effect of antimycin on the reducibility of cytochrome *b* (refs. 12–18) was also studied. Preliminary reports of some of these studies have appeared[19–21].

EXPERIMENTS

Effect of cholate on the inhibition curve

Fig. 1 shows the effect of cholate on the inhibition by antimycin of the succinate–cytochrome *c* reductase activity of beef sub-mitochondrial particles (Keilin and Hartree heart-muscle preparation). Pretreatment of the preparation with 4.6% cholate (14 mg cholate per mg protein) resulted in a change of the inhibition curve from sigmoidal to nearly linear*. The activity in the absence of antimycin was little affected by this amount of cholate. Also the amount of antimycin required for almost complete inhibition was unaffected. However, if more detergent per mg protein was present than in the experiment shown in Fig. 1, inactivation occurred with a consequent lowering of the amount of antimycin required for inhibition.

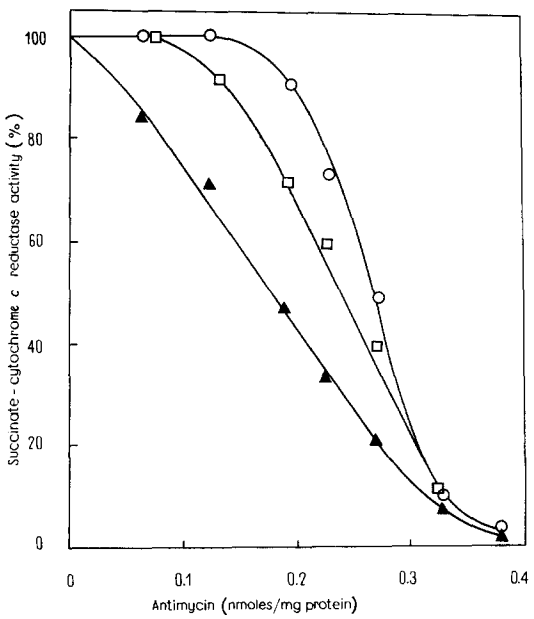

Fig. 1. The effect of cholate on the inhibition by antimycin of the succinate–cytochrome *c* reductase activity of the heart-muscle preparation. ○—○, control preparation (4.3 mg protein per ml); □—□, preparation treated with 7.0 mg cholate per mg protein (26 mg cholate per ml); ▲—▲, preparation treated with 14 mg cholate per mg protein (46 mg cholate per ml). Activities in the absence of antimycin were 0.117, 0.235 and 0.104 unit/min where 1 unit is equal to 1 μmole succinate oxidized per mg protein.

* Because of the very low binding constant of antimycin the curve relating inhibition to total antimycin (bound and free) is practically linear. If it were possible to plot the concentration of free antimycin, the curve would presumably approach a hyperbolic form.

The effect of cholate in transforming the sigmoidal inhibition curve to a nearly linear one is reversed by partial removal of the cholate by dialysis (Fig. 2A). Dialysis does not remove all the cholate and the solution still appears quite clear after 8-h dialysis. However, light-scattering measurements summarized in Fig. 2B show that the return of a sigmoidal inhibition curve is associated with an increased average particle weight.

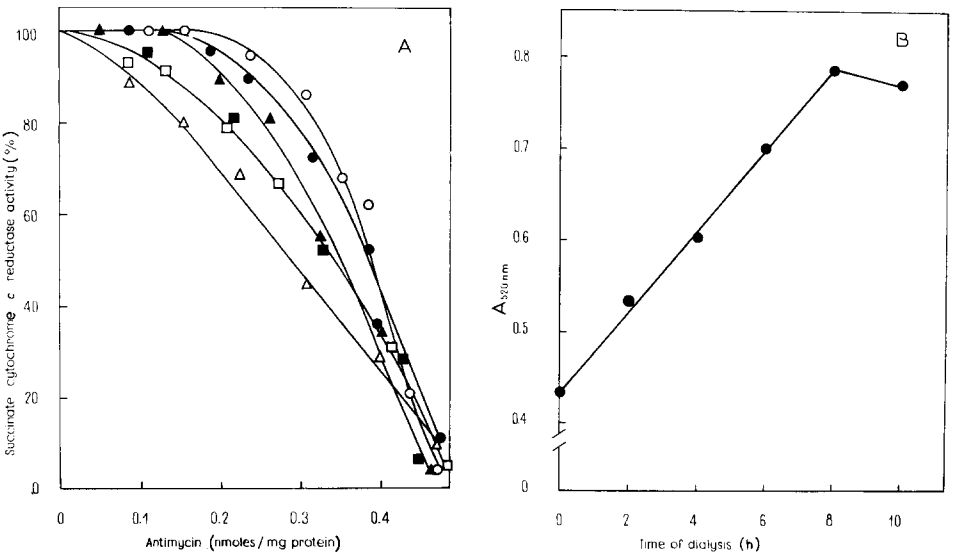

Fig. 2. The effect of dialysis on the antimycin inhibition (A) and light-scattering (B) of heart-muscle preparation treated with cholate (14 mg cholate per mg protein, 53 mg cholate per ml). The dialysis was carried out against 50 vol. of 10 mM Tris–HCl buffer (pH 8.0). Every 2 h samples were taken and assayed for enzymic activity, inhibition by antimycin and light-scattering. A. O—O, control preparation (0.085 unit/min); △—△, preparation treated with cholate not dialysed (0.075 unit/min); □—□, ■—■, ▲—▲ and ●—●, cholate-treated preparation after 2, 4, 6 and 8 h of dialysis, respectively (0.072, 0.074, 0.071 and 0.077 unit/min, respectively). The samples assayed for light-scattering (B) were diluted to 1.2 mg protein per ml.

TABLE I

REACTIVATION OF CHOLATE-TREATED PARTICLES BY REMOVAL OF CHOLATE

Expt. No.	*Heart-muscle preparation (mg/ml)*	*Cholate (mg/ml)*	*Added antimycin (nmoles/mg protein)*	*Activity**	
				Before dialysis	*After dialysis***
1	4.1	56	0	0.193	0.205
			0.17	0.148	0.194
			0.43	0.004	0.008
2	3.8	52	0	0.246	0.213
			0.12	0.158	0.230
			0.19	0.126	0.147
			0.25	0.087	0.068

* The activity is expessed as μmoles cytochrome *c* reduced per min per mg protein.
** For 5 h against 50 vol. of 10 mM Tris–HCl buffer (pH 8.0).

In the experiment illustrated in Fig. 2, the cholate-treated preparation was dialysed and the inhibition curve measured with various amounts of antimycin added subsequent to the dialysis. In another type of experiment, the results of which are given in Table I, antimycin was present during the dialysis. With amounts of antimycin that produced 25 % and 36 % inhibition, respectively, before dialysis, the

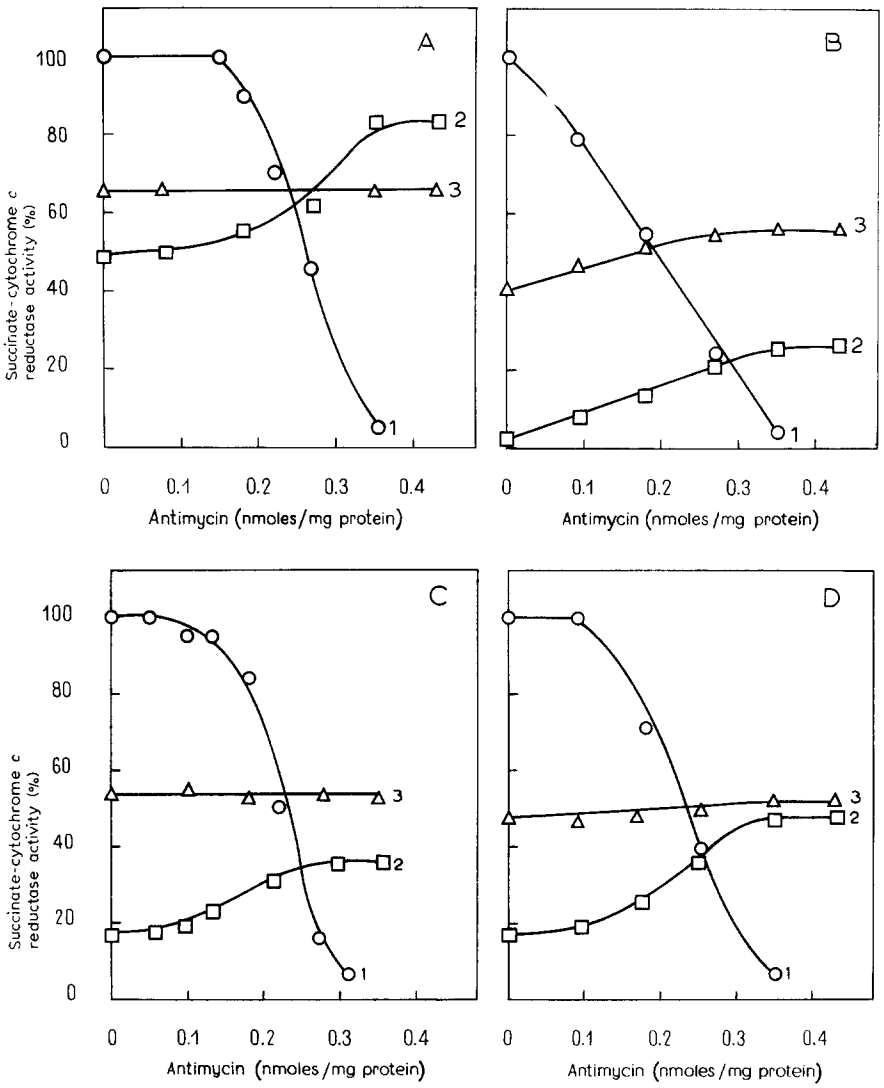

Fig. 3. The effect of antimycin on the reducibility of cytochrome *b* by succinate and menaquinol-o in cholate-treated heart-muscle preparation before and after dialysis. Curve 1, relative activity of succinate–cytochrome *c* reductase; Curve 2, cytochrome *b* reduced by succinate; Curve 3, cytochrome *b* reduced by 1 mM menaquinol-o. As 100%, the amount of cytochrome *b* reduced by dithionite was taken. A. Untreated preparation (0.070 unit/min). B. Preparation treated with 14.2 mg cholate per mg protein (69 mg cholate per ml, 0.068 unit/min). C. Preparation treated with cholate as in B and dialysed for 6 h against 50 vol. of 10 mM Tris–HCl buffer (pH 8.0), 0.065 unit/min. D. Preparation treated with cholate as in B, incubated with antimycin as indicated and dialysed as in C.

inhibition was completely reversed by dialysis, reflecting the transformation from a linear inhibition curve to a sigmoidal. With larger amounts of antimycin, no reversal was obtained, as was to be expected since these amounts of antimycin are sufficient to inhibit even when the inhibition curve is sigmoidal. This shows that the reversal with low amounts of antimycin is not simply due to dialysis of an antimycin–cholate complex (*cf.* also Figs. 3C and 3D).

The sigmoidal inhibition curve was found to be independent of succinate concentration between 0.5 and 10 mM.

Effect of cholate on the interaction between cytochrome b and antimycin

CHANCE[12] showed that antimycin increases both the rate and extent of reduction of cytochrome *b* in heart-muscle preparation and causes a small shift towards the red of the maxima of both the α- and the γ-bands. This has been confirmed by others[13–18].

In agreement with PUMPHREY[14], the effect of antimycin on the reducibility of cytochrome *b* in heart-muscle preparation is also described by a sigmoidal curve (Fig. 3A, Curve 2), and the steep part of the curve covers the same range of antimycin concentrations as in that describing the inhibition of the enzymic activity (Curve 1). Treatment with cholate sufficient almost completely to abolish the sigmoidal inhibition curve of the enzyme activity (Fig. 3B, Curve 1) made the cytochrome *b* non-reducible with succinate (*cf.* ref. 14), in spite of the fact that the succinate–cytochrome *c* reductase activity is unaffected. Antimycin restored the reducibility (Curve 2) somewhat, the activity curve being linear. Dialysis of the cholate-treated preparation either before adding the antimycin (Fig. 3C) or in the presence of the antimycin (Fig. 3D) restored the sigmoidal inhibition curve of the succinate–cytochrome *c* reductase activity and the sigmoidal activation curve of the reducibility of the cytochrome *b*. Reduction by 2-methyl-1,4-naphthohydroquinone (menaquinol-0) was unaffected by antimycin in the untreated (*cf.* ref. 14) or dialysed preparations; it was slightly stimulated in the cholate-treated preparations (*cf.* ref. 14).

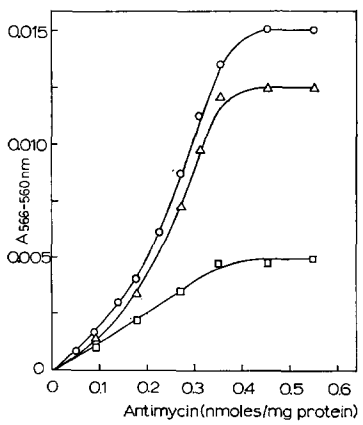

Fig. 4. The effect of cholate on the spectral shift of cytochrome *b* brought about by antimycin. ○—○, untreated preparation; △—△, preparation treated with 7.8 mg cholate per mg protein (25 mg cholate per ml); □—□ preparation treated with 14.4 mg cholate per mg protein (46 mg cholate per ml). The amounts of antimycin indicated were added to the samples after addition of 10 mM succinate. Protein concentration, 3.2 mg/ml.

Fig. 4 shows that the effect of antimycin on the displacement to the red of the α-band of ferrocytochrome *b*, measured in the presence of succinate, is also described by a sigmoidal curve and that the sigmoidicity is again practically abolished by cholate. In agreement with PUMPHREY[14], the magnitude of the red shift is decreased by cholate.

The sigmoidal curve describing the effect of antimycin on the displacement of the α-band was obtained with NADH and QH_2-2 in addition to succinate, but not with menaquinol-0 or $Na_2S_2O_4$ (Fig. 5). PUMPHREY[14] reported a hyperbolic curve with $Na_2S_2O_4$. The maximum displacement was less with QH_2-2 than with succinate or NADH. This is understandable since reduction of cytochrome *b* is also less with QH_2-2 (ref. 14).

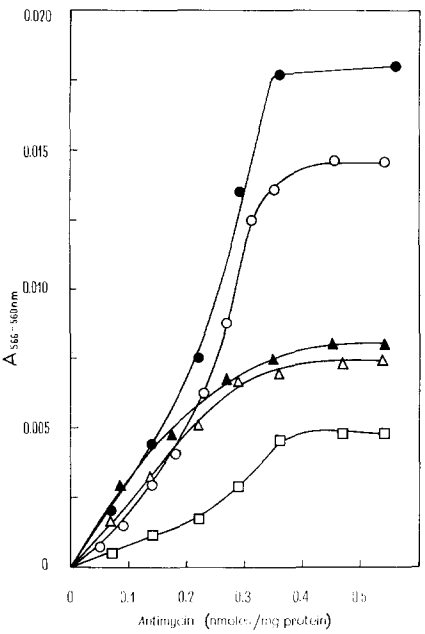

Fig. 5. The effect of substrate on the curve describing the shift by antimycin of the α-band of cytochrome *b*. ●—●, 1 mM NADH; ○—○, 10 mM succinate; □—□, 1 mM QH_2-2; △—△, 1 mM menaquinol-0; ▲—▲, $Na_2S_2O_4$. Antimycin (as indicated) was added to the preparation (3.2 mg protein per ml) treated previously with substrate.

DISCUSSION

The following effects of antimycin on the heart-muscle preparation are described by sigmoidal curves: (1) inhibition of succinate oxidase or succinate–cytochrome *c* reductase; (2) stimulation of reducibility of cytochrome *b*; (3) displacement to red of the α-band of cytochrome *b*. The sigmoidal are replaced by linear curves by treatment with cholate and are restored by removal of the cholate, either before or after addition of antimycin.

Of the three explanations for the sigmoidal inhibition curve considered in INTRODUCTION the first, *i.e.* the presence of a binding site with high affinity not concerned in the enzymic activity, may be eliminated. THORN[3] has already argued against

this explanation. It is not supported by the fact that small amounts of antimycin are extracted by ether to the same extent as larger amounts[11]. Nor does it account for the hyperbolic curves describing the effect of antimycin on the shift of the α-band of cytochrome *b* reduced by menaquinol-o or $Na_2S_2O_4$. Finally the very high slope of the inhibition curve (the slopes of the steep portions of the curves in Fig. 1 of ref. 19 are equivalent to 100 % inhibition with about 0.03 nmole/mg protein) makes this explanation very unlikely, since the concentration of cytochrome c_1 in heart-muscle mitochondria is about 0.25 nmole/mg protein[22]. The second explanation, *viz.* that submitochondrial preparations contain a large excess of, or a rapidly reacting, antimycin-sensitive factor is difficult to reconcile with the fact that sigmoidal antimycin effect curves are obtained with some hydrogen donors but not with others when the effect of antimycin on the reducibility of cytochrome *b* or on the spectral shift is studied.

The specific allosteric model of MONOD *et al.*[5] extended to particulate systems (*cf.* refs. 23, 24) provides a suitable framework for a consideration of the effects of antimycin, but other models could also be used. It is proposed that in particulate preparations, the respiratory chain, or at least the segment involved in the QH_2–cytochrome *c* reductase activity, exists in two enzymically active conformation sites, the R and the T, both oligomeric (or polymeric). The protomer contains two *b* sub-units, one c_1 and maybe others (see ref. 25), and one antimycin-binding site. If under the conditions used the T state is favoured, and antimycin combines more firmly with the R state, a sigmoidal inhibition curve would be expected. This would be replaced by a hyperbolic curve if the polymeric or oligomeric structure is dispersed by cholate, in the same way as urea abolishes allosteric effects in aspartate carbamyltransferase (EC 2.1.3.2)[26]. The linear* inhibition curves obtained with Complex III (refs. 8–11) are also to be expected since this complex appears to be entirely in the form of the protomer[27]. It seems justified then to call antimycin an allosteric inhibitor.

This conclusion is based on the sigmoidal antimycin effect curves found with particulate preparations when physiological electron donors (but not $Na_2S_2O_4$ or menaquinol) are used, and the abolition of the sigmoidal curves on dispersal with cholate. This allosteric effect should be clearly distinguished from its effect on the "conformation stability" of the protomer Complex III, investigated in detail by RIESKE and co-workers[8–10,25], although both effects presumably have a common, but as yet unknown, physical basis. The stabilizing effect of antimycin on the conformation of the protomer is manifested by its inhibition of splitting of Complex III into its components by cholate–ammonium sulphate or guanidine[8,9], its protection against proteolysis of the complex by trypsin[10] and the smaller number of titratable –SH groups in the presence of antimycin[25].

The increased rate and extent of reduction of cytochrome *b* by antimycin[12], which is only seen in non-phosphorylating particulate preparations, is more likely explained by its allosteric effect. Thus, one might imagine that in the oligomer in the T state only one of the two *b* sub-units is reduced during electron transfer from QH_2-2 to cytochrome c_1 (*cf.* refs. 15, 28), but that in the R state both are rapidly reducible. The displacement by antimycin of the absorption peaks of ferrocytochrome *b* may be due to a different hydrophobic environment around the haem in the two states. In

* See footnote, p. 318

this respect it is interesting to recall PUMPHREY's[14] observation that cholate displaces the absorption peak in the opposite direction from antimycin. Cholate has a second effect opposite to that of antimycin, namely it prevents the reduction of cytochrome *b* without, however, inhibiting the succinate–cytochrome *c* reductase activity.

The fact that the same amount of antimycin is required for complete inhibition before and after treatment with cholate can only be explained on the basis of the model proposed if the monomer, obtained by cholate treatment, is entirely in the R form. This is understandable, if T is a constrained state that can only be stabilized in an oligomeric structure. In agreement with this conclusion is the finding reported in the following paper[11] that Complex III binds antimycin much more firmly than particulate preparations. Complex III may be considered as a preparation of the R monomer.

It is of particular interest that the sigmoidal curves relating the effects of antimycin on cytochrome *b* (reducibility and spectral shift) are obtained only with the natural hydrogen donors succinate and NADH or the analogue of a natural donor (QH_2-2). Intermediate protein carriers are most probably involved in these cases, but not with menaquinol-0 (*cf.* ref. 29) or $Na_2S_2O_4$ which can reduce the cytochrome *b* directly. This difference between natural and artificial donors was not found by RIESKE *et al.*[9] in their studies of the effect of reduction on the "conformation stability" of Complex III. Thus a co-operative effect with antimycin is obtained only when interaction between the component enzymes in the multi-enzyme system is of importance. Specifically, we have suggested on the basis of the model of MONOD *et al.*[5] that under the conditions used the T conformation state of the oligomer is favoured.

The possible role of conformational changes in energy conservation has been discussed by BOYER[30], HARRIS *et al.*[31], CHANCE *et al.*[32] and SLATER[21].

METHODS

Beef heart-muscle preparation was prepared by the method of KEILIN AND HARTREE[33], except that the particles were suspended in 50 mM Tris–HCl buffer (pH 8.0) containing 0.66 M sucrose and 1 mM histidine or by SLATER's[34] modification. Protein was determined by the method of CLELAND AND SLATER[35], using egg albumin as standard.

Succinate oxidase and succinate–cytochrome *c* reductase were measured as previously described[6]. Inhibition by antimycin was measured after 5-min preincubation of the preparation with antimycin before adding substrate. Preincubation for 90 min gave the same results as at 5 min.

Antimycin (A, Type III) was obtained from Sigma. The concentration of an ethanolic solution was determined from its absorbance at 320 nm, using an absorption coefficient of $4.8 \cdot 10^6$ $cm^2 \cdot mole^{-1}$ (ref. 36).

Potassium cholate (pH 8.0) was prepared by titration of cholic acid (Mann Research Labs.) with KOH.

Cytochrome *c* was obtained from Sigma or isolated from beef heart[37].

Menaquinol-0 (2-methyl-1,4-naphthohydroquinone) was prepared by reduction of menaquinone-0 with $Na_2S_2O_4$, as described by FIESER[38] and dissolved in ethanol containing 0.1 mM HCl to give a 0.7 M solution. QH_2-2 was prepared by reduction of Q-2 by $Na_2S_2O_4$, as described by RIESKE[39].

Reduction of cytochrome *b* in heart-muscle preparation was determined in a medium containing 0.1 M phosphate buffer (pH 7.0), 10 mM KCN and 1 mg/ml ascorbate (*cf.* ref. 13). Absorption changes corresponding to changes in the redox state of cytochrome *b* were monitored at 563 nm *minus* 575 nm with the Aminco-Chance dual-wavelength recording spectrophotometer. The spectral shift (caused by addition of antimycin) was followed by measuring at 566 nm *minus* 560 nm.

Light-scattering of the heart-muscle preparation treated with cholate was determined at 520 nm.

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