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

I. REVERSAL OF THE INHIBITION BY DIETHYL ETHER EXTRACTION*

ZBIGNIEW KANIUGA, ANDRZEJ GARDAS AND JADWIGA BRYŁA

Department of Biochemistry, Warsaw University, Warsaw (Poland)

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SUMMARY

1. Reversal of antimycin inhibition of succinate oxidation was studied in diethyl ether-extracted heart-muscle preparation and rat-liver mitochondria, respectively.

2. The activity of succinate oxidase in ether-extracted preparations which were not treated with antimycin was completely restored after addition of cytochrome *c*. Under the same conditions the activity of succinate cytochrome *c* reductase was increased 2–3-fold.

3. Antimycin-inhibited activity of succinate oxidase and that of succinate cytochrome *c* reductase was completely restored by extraction of the preparations with diethyl ether providing that soluble cytochrome *c* was added.

4. Sensitivity of the heart-muscle preparations to antimycin was not affected by extraction with diethyl ether.

5. Antimycin which had been isolated from antimycin-treated preparations was not in any way modified and its capacity to inhibit succinate oxidation was preserved.

6. It was found that a complete reactivation of antimycin-inhibited succinate oxidase could be achieved by ether extraction even when some residual antimycin remained in the preparations. This residual antimycin could be removed by extraction with diethyl ether–ethanol solvent mixture but not by repeated extractions with diethyl ether alone.

7. The results suggest that the reversal of antimycin inhibition by diethyl ether extraction of inhibited preparation was due to the removal of the antibiotic from the site which was responsible for the inhibition of the electron transport chain. However, some bound inhibitor remained at another site which did not directly affect the inhibition.

INTRODUCTION

Antimycin has been widely used as an inhibitor of the respiratory chain. The antibiotic is effective even at a very low concentration[2–9]. Its site of action on the

* A preliminary report of this investigation was presented at the 3rd Meeting of the Federation of European Biochemical Societies, Warsaw, 1966 (ref. 1).

respiratory chain has been located between cytochromes *b* and c_1 (refs. 7–9). Two characteristic effects of antimycin have been observed by POTTER AND REIF[2], THORN[6] and ESTABROOK[8]: the inhibition curve displayed a sigmoid shape and the degree of inhibition of succinate oxidase activity by antimycin was dependent solely on the inhibitor:enzyme ratio. The amount of antimycin necessary to produce 50 % inhibition has been defined as the antimycin titer[2]. POTTER AND REIF[2] as well as THORN[6] have assumed that the inhibition of the respiratory chain by antimycin resulted from the titration of one of the components of the chain. Indeed, CHANCE[7] and ESTABROOK[8] have observed that almost stoichiometric quantities of antimycin to cytochrome were capable of inhibiting oxidation in heart-muscle preparations.

The mechanism of the inhibition, as well as the nature of the antimycin-sensitive site, have so far not been elucidated. The experimental approach to this problem has been restricted to measurable aspects of antimycin inhibition: (a) degree of the inhibition, (b) effects of antimycin on the reducibility and spectral properties of cytochrome *b* as first observed by CHANCE[7] and later by others[11–15], (c) specific inhibition by antimycin of the cleavage of Complex III into its component cytochrome[16] and a possible role of non-heme iron in the reaction[17,18], (d) reversal of antimycin inhibition by proteins, *i.e.* serum albumin, as originally reported by REIF AND POTTER[4], and by soluble proteins from chicken-liver mitochondria[19] or by lipophilic compounds like vitamin E (ref. 20) and ubiquinone[21,22], lastly, (e) reversal of antimycin inhibition when uninhibited heart-muscle preparation is added to antimycin-inhibited preparation[6,23].

While studying the effects of diethyl ether on NADH dehydrogenase complex of the heart-muscle preparation[24], we observed that the inhibition of succinate oxidase activity by antimycin could be reversed by diethyl ether extraction providing that cytochrome *c* was subsequently added to the reaction mixture. Thus it appeared that studies of this new type of reversal of antimycin inhibition might provide some clue as to the nature of binding of the inhibitor to the antimycin-sensitive site of the respiratory chain.

The paper reports conditions for reversal of the antimycin inhibition of mitochondrial preparations by diethyl ether extraction. Evidence is also presented that antimycin is bound at least at two sites of the respiratory chain. The first one is responsible for the inhibition of the electron transfer and is visualised by the steep part of the titration curve. Antimycin can be easily removed from this site by extraction with diethyl ether. Antimycin is, however, bound more tightly at the second site and can be removed only by extraction with ether–ethanol solvent mixture. Its presence does not affect restoration of succinate oxidase activity.

MATERIALS AND METHODS

Beef heart-muscle particles were prepared by the method of KEILIN AND HARTREE[25], as modified by SLATER[26]. In the latter method the tissue is disrupted in a Waring blendor and the homogenate fractionated by means of differential centrifugation rather than by precipitation with acid.

Rat-liver mitochondria were prepared in 0.25 M sucrose as described by MYERS AND SLATER[27].

Cytochrome *c* was isolated from beef heart by the procedure of MARGOLIASH[28].

The extinction coefficient of VAN GELDER AND SLATER[29] was employed for calculation of cytochrome *c* concentration.

Yeast hexokinase was prepared according to DARROW AND COLOWICK[30] omitting the final crystallization step.

Antimycin A (Type III) was purchased from Sigma (U.S.A.). Antimycin stock solutions in ethanol (0.6–0.7 μg/ml) were kept at −15°. The concentration was determined from absorbance at 320 mμ applying an absorbance coefficient of $4.8 \cdot 10^6$ $cm^2 \cdot mole^{-1}$ as recommended by STRONG *et al.*[31].

Anaesthesia grade diethyl ether was used throughout the investigation.

Extraction of antimycin from particles and from mitochondria

A suspension of the heart-muscle preparation (2.0 ml) which contained 20–25 mg of protein per ml was incubated for 5 min at room temperature with an amount of antimycin which produced at least 90 % but usually 95 % inhibition of succinate oxidation. Thereafter the preparation was extracted for 15 min with 10 vol. of diethyl ether at room temperature with vigorous shaking. After centrifugation (3 min at 2000 rev./min) the supernatant was decanted and the sediment resuspended in 5 vol. (of the original volume taken for extraction) of 0.1 M phosphate buffer (pH 7.4), which contained 2 mM of EDTA. Residual ether was removed by evacuation of the solution for 10 min with a water pump. The sample was then centrifuged (10 min at 14000 rev./min), the supernatant was discarded and the sediment suspended in phosphate buffer. This suspension was assayed for protein concentration and enzymic activity.

Mitochondria (about 20 mg of protein per ml) were incubated with an amount of antimycin which produced at least 95 % inhibition of succinate oxidation (about 0.05 μg of the antibiotic per mg of protein). Extraction with diethyl ether and removal of the residual ether was performed as described for particles. The ether-extracted mitochondria were suspended in 5 vol. of 0.25 M sucrose solution which contained 1 mM of EDTA.

Measurement of enzyme activities

The succinate oxidase activity of a heart-muscle preparation and of rat-liver mitochondria was determined polarographically at 25° with a Clark electrode according to the procedure of CHAPPELL[32]. The reaction medium for particles contained: 0.1 M phosphate buffer (pH 7.4), 2 mM EDTA, 9 mM succinate, 10 μM cytochrome *c* (where indicated) and 0.5–1 mg of protein in a final volume of 1.5 ml.

The standard reaction medium for ether-extracted rat-liver mitochondria contained: 15 mM KCl, 2 mM EDTA, 50 mM Tris–HCl buffer, 5 mM $MgCl_2$, 30 mM phosphate, 9 mM succinate, 10 μM cytochrome *c* (where indicated) and 0.5–1 mg of mitochondrial protein in a final volume of 1.5 ml. When the oxidase activity was measured in intact mitochondria the reaction medium always contained 0.1 mM ADP, 60 mM glucose and 100–150 Cori units of hexokinase.

Succinate-cytochrome *c* reductase activity was assayed spectrophotometrically by measuring the increase of absorbance of reduced cytochrome *c* at 550 mμ. The reaction medium of 3 ml contained 20 mM succinate, 50 μM cytochrome *c*, 0.1 M phosphate buffer (pH 7.4), 1 mM KCN, 2 mM EDTA and 0.1–0.15 mg of protein.

Protein content was determined by the biuret method according to CLELAND AND SLATER[33] using egg albumin as the standard.

RESULTS

Restoration of succinate oxidase and succinate–cytochrome c activities in ether-extracted preparations

Effect of organic solvents on succinate oxidation by mitochondrial preparations has been studied in several laboratories. It has been established that the extraction of mitochondrial preparations with isooctane[34,35], light petroleum[36], diethyl ether[39] and acetone–water (1:1, v/v) solvent mixture[37,38] produced inactivation of succinate oxidase. The activity could be restored by subsequent addition of cytochrome *c*.

Table I shows the restoration of the activity of succinate oxidase in ether-extracted heart-muscle preparations and in similarly treated rat-liver mitochondria following addition of cytochrome *c* to the reaction mixtures. As seen in Table I, the enzymic activity in ether-extracted preparations was totally missing in the absence of added cytochrome *c*. Subsequent addition of cytochrome *c* resulted in restoration of enzymic activity to a level within ± 10 % of the control in the case of heart-muscle preparation, or 20–35 % greater in the case of rat-liver mitochondria.

TABLE I

RESTORATION OF SUCCINATE OXIDASE ACTIVITY IN DIETHYL ETHER-EXTRACTED PREPARATIONS BY ADDED CYTOCHROME *c*

Preparation	*Activity**			
	No cytochrome c		*With cytochrome c*	
	Control	*Ether-extracted preparation*	*Control*	*Ether-extracted preparation*
Heart-muscle preparation	0.307	0	0.398	0.418
	0.359	0	0.445	0.404
	0.330	0	0.545	0.560
Rat-liver mitochondria	0.235	0.012	0.281	0.341
	0.201	0.015	0.285	0.385
	0.223	0	0.292	0.352
	0.208	0.014	0.249	0.301

* The activity is expressed as μmoles of succinate oxidized per min per mg of protein.

On the other hand, succinate–cytochrome *c* reductase activity was stimulated by extraction of mitochondrial preparations with organic solvents. Thus CRANE *et al.*[35] have observed an activation of the reductase following isooctane extraction of mitochondria while LESTER AND FLEISCHER[37] have observed similar phenomenon after treating mitochondria with acetone–water (1:1, v/v) solvent mixture. A complete reactivation of succinate–cytochrome *c* reductase activity after addition of Tween 80 to the isooctane-extracted preparation has been reported by REDFEARN, PUMPHREY AND FYNN[36]. A 2–3-fold increase in activity of succinate–cytochrome *c* reductase in ether-extracted heart-muscle preparations is presented in Table II.

Restoration of the activity of NADH oxidase and of NADH–cytochrome *c* reductase could not be studied since ether extraction of mitochondrial preparations resulted in a rapid and complete destruction of both activities[24,39] due to a break in

TABLE II

SUCCINATE–CYTOCHROME *c* REDUCTASE ACTIVITY IN DIETHYL ETHER-EXTRACTED HEART-MUSCLE PREPARATION

Prep. No.	*Activity**		*Activation (%)*
	Control	*After extraction*	
1	0.085	0.285	333
2	0.088	0.216	220
3	0.116	0.306	264
4	0.105	0.227	216
5	0.113	0.291	267
6	0.130	0.262	201

* The activity is expressed as μmoles of succinate oxidized per min per mg of protein.

the electron transport chain between flavoprotein and cytochrome *b* (ref. 39). The site of the break in the electron transport has recently been studied by KANIUGA, GARDAS AND JAKUBIAK[24]. Their results seem to indicate that a common site is affected by the action of rotenone, diethyl ether and phospholipase A of *Naja naja* venom.

Reversal of antimycin inhibition by diethyl ether extraction

Once the condition for reactivation of succinate oxidase and of succinate cytochrome *c* reductase had been established, the next stage was to determine conditions (volume of ether, time and temperature of extraction) for the reversal of enzymic inhibition as caused by antimycin. Thus it has been found that antimycin-inhibited oxidation of succinate could be restored by extraction of the preparations with diethyl ether for 15 min at room temperature. Volumes of ether employed were 5-fold and 10-fold for heart-muscle preparation and rat-liver mitochondria, respectively.

The reversal of antimycin inhibition of succinate oxidase of heart-muscle preparation and of that of rat-liver mitochondria is shown in Table III. As seen from the table, the restoration of the enzymic activity was almost complete and usually at 90% level. The same was true for succinate–cytochrome *c* reductase activity (see

TABLE III

REVERSAL OF ANTIMYCIN-INHIBITED SUCCINATE OXIDASE ACTIVITY BY DIETHYL ETHER EXTRACTION

Preparation	*Activity**			*Restoration (%)*
	Control	*Ether-extracted preparation*		
		Uninhibited	*Antimycin-inhibited*	
Heart-muscle preparation	0.445	0.404	0.364	90
	0.545	0.560	0.462	83
	0.314	0.354	0.316	89
Rat-liver mitochondria	0.281	0.341	0.294	87
	0.285	0.385	0.345	90
	0.292	0.352	0.328	93
	0.249	0.301	0.260	87

* The activity is expressed as μmoles of succinate oxidized per min per mg of protein.

Table IV) but in this case, ether-extracted preparations displayed a 2–3-fold increase in activity (see Table II). The restoration of the activity of succinate–cytochrome *c* reductase was in the range of 90 %.

TABLE IV

REVERSAL OF ANTIMYCIN-INHIBITED SUCCINATE–CYTOCHROME *c* REDUCTASE ACTIVITY OF HEART-MUSCLE PREPARATION BY DIETHYL ETHER EXTRACTION

Prep. No.	*Activity after ether extraction**		*Activity restored* (%)
	Uninhibited preparation	*Antimycin-inhibited preparation*	
1	0.285	0.250	88
2	0.216	0.200	92
3	0.306	0.286	93
4	0.227	0.205	90
5	0.291	0.256	88
6	0.262	0.236	90

* The activity is expressed as μmoles of succinate oxidized per min per mg of protein.

Inhibitory properties of antimycin extracted from the preparations

Although the experiments on reactivation of antimycin-inhibited succinate oxidase and succinate–cytochrome *c* reductase showed a full restoration of both activities (see Tables III and IV) nevertheless there were several unexplored questions. (a) Was antimycin extracted from the preparations or merely modified within the preparations? The latter possibility would mean that the inhibitory properties of antimycin towards electron transport were lost with the resulting reactivation of the enzyme systems. This would be reminiscent of the studies of POTTER AND REIF[2] on the modification of antimycin in rat-liver homogenates and a resulting restoration of enzymic activity. (b) Was antimycin extracted from preparations and if so in what amount? (c) Were the inhibitory properties of antimycin preserved when isolated from the preparations? (d) Did the process of ether extraction open an antimycin-insensitive pathway for the reduction of added cytochrome *c* by succinate?

It was shown later (see next section) that an ether-extracted preparation was still completely antimycin-sensitive and a representative experiment, depicted in Fig. 1, answers the first three questions.

Thus the ether extract from antimycin-treated but not from untreated preparations displayed inhibitory properties (Curves 3 and 6, respectively). Treatment of antimycin with ether did not affect the inhibitory properties of the antibiotic (Curve 2). From the shape of Curve 1 it is evident that 50 % inhibition was achieved by the addition of 40 μl of the standard solution of antimycin. A similar degree of inhibition was achieved by the addition of 65 μl of antimycin solution which had been isolated from the preparation (Curve 3). This indicated that some of the inhibitor remained in the preparation. Attempts to remove this residual antimycin by subsequent ether extractions were unsuccessful (Curve 4). The residual antimycin could, however, be removed by extraction with ether–ethanol solvent mixture (Curve 5). The extract thus obtained displayed inhibitory properties identical to the standard solution of antimycin. The results strongly suggest that in spite of the reactivation of both

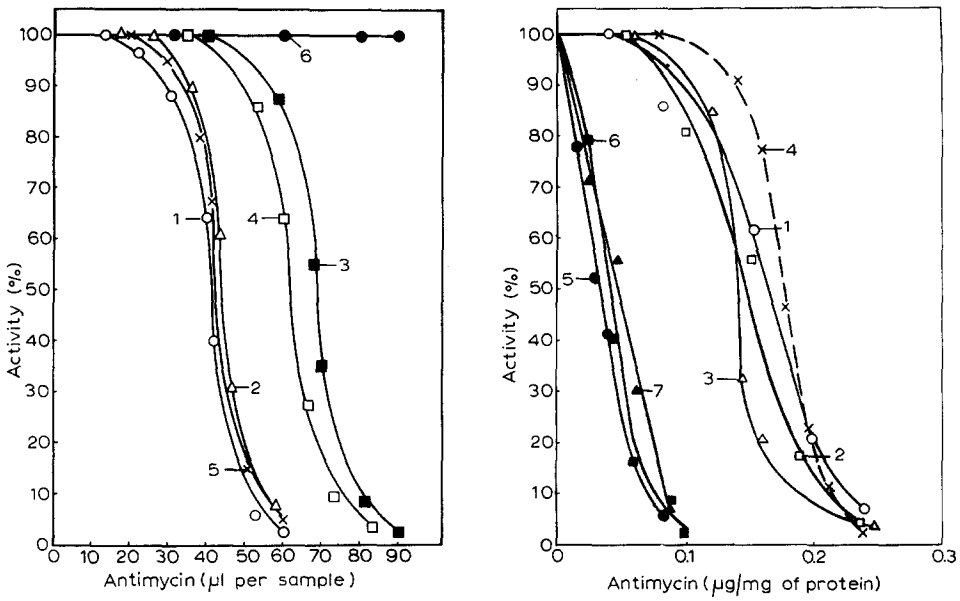

Fig. 1. Titration curves of heart-muscle preparations with (a) standard solution of antimycin, (b) ether-extracted antimycin, (c) antimycin extracted from a preparation with ether, (d) antimycin extracted from a preparation with ether–ethanol solvent mixture. Curve 1 (○—○), standard solution of antimycin (0.463 µg/ml); Curve 2 (△—△), standard solution of antimycin which had been treated with ether. Solution of antimycin which contained 4.63 µg of the antibiotic was stirred with 30 ml of diethyl ether for 35 min. Ether was evaporated and the residue dissolved in 10 ml of ethanol; Curve 3 (■—■), antimycin extracted from antimycin-treated preparation. To 1 ml of the heart-muscle preparation (22 mg of protein per ml) 4.63 µg of antimycin were added. After 5 min of incubation at room temperature, the sample was extracted with 10 vol. of diethyl ether for 15 min and allowed to settle for 2 min. Supernatant ether was decanted and the sediment extracted twice with 10 vol. of ether allowing 5 min for each extraction. Pooled ether extracts were evaporated *in vacuo* and the residue dissolved in 10 ml of ethanol; Curve 4 (□—□), antimycin extracted from antimycin-treated heart-muscle preparation by 5 successive ether extractions. Conditions as for Curve 3, 15 min for the first extraction and 5 min for each successive extraction; Curve 5 (×--×), antimycin extracted from a preparation with ether–ethanol solvent mixture. Incubation with antimycin as above (Curve 3). The preparation was extracted once with 10 vol. of ether for 15 min, the ether was decanted, and the preparation was extracted twice with ether–ethanol (4:1, v/v) solvent mixture. The suspension was allowed to settle, the solvent was decanted and the pooled extracts (including the first ether extract) were evaporated *in vacuo*. The dry residue was dissolved in 10 vol. of ethanol; Curve 6 (●—●), ether extract from antimycin-untreated preparation. An aliquot (1.0 ml) of the heart-muscle preparation (22 mg of protein per ml) was extracted with ether as above (Curve 3). The dry residue was dissolved in 10 ml of ethanol. Succinate–cytochrome *c* reductase activity was assayed.

Fig. 2. The susceptibility towards antimycin of antimycin-treated and -untreated heart-muscle preparation after extraction with diethyl ether. The activity of succinate–cytochrome *c* reductase was determined. Curve 1, preparation not extracted with ether; Curves 2, 3 and 4, antimycin-untreated preparations; Curves 5, 6 and 7, antimycin-treated preparations.

succinate oxidase and succinate–cytochrome *c* reductase, some antimycin remained in the preparation, possibly as a result of a tighter bonding.

Response of antimycin-inhibited and ether-extracted preparation to repeated treatment with the inhibitor

It was assumed that if ether extraction removed from the antimycin-treated preparation only a portion of the inhibitor, then its presence in the preparation would

cause an increase in sensitivity to subsequent additions of antibiotic. The experiment presented in Fig. 2 was undertaken in order to test the validity of this assumption.

As can be seen from Fig. 2, ether extraction alone did not affect the sensitivity of the preparation towards antimycin, however, antimycin-treated and ether-extracted preparations displayed undue sensitivity to subsequent additions of inhibitor. Repeated extraction with ether was found to be ineffective in removing this undue sensitivity to antimycin. Moreover, as can be seen from Fig. 2, removal of antimycin by ether extraction of the preparations was accompanied by a change in the shape of the titration curves, which then resemble the steep part of the titration curve of uninhibited preparation. This is commonly regarded as the antimycin-sensitive site.

DISCUSSION

The degree of inactivation and reactivation of enzymic activity of mitochondrial preparations after extraction with organic solvents depends on the nature of solvent and on the time and temperature of the extraction procedure. Thus CRANE has observed a full recovery of succinate oxidase activity and very often an excess of the activity in electron transport particles extracted with isooctane for 2 h at 0° and subsequently treated with cytochrome *c* (ref. 38). Similar results have been obtained with beef-heart mitochondria which were extracted with the same solvent for 1 h at 18° (ref. 39).

On the other hand, the activity of NADH oxidase was more susceptible to isooctane and even a short extraction of the preparations in the cold produces some changes which were probably responsible for inconsistencies in the published results[34,36]. At room temperature, addition of cytochrome *c* to isooctane-treated preparations did not restore the activity of NADH oxidase[39]. Restoration of succinate oxidase activity by cytochrome *c* in preparations extracted with acetone–water solvent depended upon the ratio of the two solvents[34]. When the ratio was 1:1, the restoration was complete but at higher concentrations of acetone, succinate oxidase was not reactivated. Diethyl ether was a particularly suitable extractant of antimycin since the extraction could be carried out even at room temperature and the restoration of succinate oxidase activity was complete after addition of cytochrome *c* (see Table I). The activity of succinate–cytochrome *c* reductase was increased 2–3-fold under the same conditions (Table II). Similar results have been obtained by CUNNINGHAM, CRANE AND SOTTOCASA[39] with beef-heart mitochondria.

The emergence of the succinate oxidase requirement for cytochrome *c* in ether-extracted preparations did not appear to be a consequence of the extraction of cytochrome *c* but rather due to some physical changes of the respiratory chain which could be induced also by some other treatments, *e.g.* by freezing of the preparation[40] or by incubation with digitonin[41].

Reversal of antimycin inhibition by ether extraction was, prior to our findings, restricted to the effects of added serum albumin[4], of soluble protein from chicken-liver mitochondria[19] or of uninhibited heart-muscle preparation to the antimycin-inhibited preparation[6]. There are also indications that lipophilic compounds are able to reverse antimycin inhibition[20–22]. Recently REDFEARN, WHITTAKER AND BURGOS[23] have demonstrated reactivation of the succinate and NADH oxidase activities of antimycin-treated preparation upon combination with either: (i) acetone; (ii) alkali- or

(iii) rotenone-treated particles. All these reactivation studies are consistent with THORN's[6] suggestion that a reactivation of antimycin-treated particles occurs by a redistribution of the inhibitor. This redistribution would imply that antimycin dissociated from an inhibited particle would have to diffuse through the medium and become attached to the antimycin site on an uninhibited particle or protein. According to the view of REDFEARN, WHITTAKER AND BURGOS[23], if a redistribution of antimycin does take place it must do so by an aggregation or coalescence of the particles.

There are two main differences between reversal of antimycin inhibition by some proteins or lipophilic compounds and by ether extraction as presented in this paper. Firstly, the degree of reactivation in the presence of added proteins depends on two factors: (a) amount of antimycin in the inhibited preparation and (b) the amount of added protein. Secondly, the reactivation is slow and usually incomplete. Contrary, reversal of antimycin inhibition by ethyl ether extraction is fast and complete even when an excess of antimycin was used. Moreover, reversal of antimycin inhibition by ether extraction permitted a new approach to the problem of the mechanism of its action. As can be seen from Fig. 2 there is a substantial change in the shape of titration curves. The typical S-shaped curve for titration with antimycin of uninhibited preparation is transformed to a straight line for the preparation from which inhibitor had been partially extracted by ether. This appears to be caused by some amount of unextracted antimycin present in the preparation which is responsible for increased sensitivity to repeated titer of the preparation with antibiotic. The data presented seems to indicate two antimycin sites with different affinities.

Extraction with ether of antimycin-inhibited preparations resulted in the dissociation of the inhibitor from the site responsible for the inhibition of electron transport. This is revealed as the steep part of the titration curve. On the other hand, antimycin not extracted by ether, which corresponded to the horizontal part of the titration curve, did not directly inhibit electron transport. It seems possible that reversal of antimycin inhibition by some proteins[4,6], by uninhibited heart-muscle preparation when added to antimycin-inhibited preparation[6,23], as well as by lipophilic compounds[20–22], is due to the removal of the antibiotic only from the site revealed as the steep part of the titration curve.

The reversal of antimycin inhibition as caused by extraction with diethyl ether allowed experimental differentiation of the site responsible for electron transport from that which did not directly affect it. Our results might provide a basis for the discussion of the mechanism of action of antimycin along the lines forwarded by GREEN's laboratory[18,42]. Thus we would like to suggest that the bound antimycin, not extracted by ether produced some conformational changes in the protein structure. Once these changes grow to a sufficient extent, a further small addition of the inhibitor (ether-extractable) produces a final conformational change which breaks electron transport. This hypothesis is currently under investigation.

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