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INHIBITION OF ELECTRON TRANSPORT BY ANTIMYCIN A, ALKYL HYDROXY NAPTHOQUINONES AND METAL COORDINATION COMPOUNDS

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Abstract—Inhibition studies of electron transport by antimycin A and its derivatives demonstrated the requirement for available ligand groups and the lipophilic part of the molecule. Inhibition may be caused by the co-ordination of ion in the lipid molecular environment of the electron transport chain. Alkyl hydroxy naphthoquinones may inhibit by a similar mechanism or they may compete with coenzyme Q as their inhibition of succinic dehydrogenase-coenzyme Q indicates. Some conventional metal co-ordination compounds, especially thenoyl trifluoroacetone, are good inhibitors of electron transport but they lack specificity.

INTRODUCTION

THE antibiotic, antimycin A,[1] the dihydrostreptomycin antagonist, 2-heptyl-4-hydroxyquinoline N-oxide,[2–4] and some of the naphthoquinone antimalarials,[5–8] are potent inhibitors of electron transport. Inhibition of the succinate and the reduced diphosphopyridine nucleotide (DPNH) chains by any of these three types of compounds blocks the cytochrome *b* in the reduced state and cytochrome c_1 in the oxidized state.[3–9] This region of electron transport has recently been characterized for the succinate chain as:

succinic dehydrogenase → cytochrome *b*
succinic dehydrogenase → coenzyme Q—cytochrome c_1[10]

This part of the chain is known to contain two lipoproteins and non-heme iron.[11, 12] The site of antimycin A inhibition is between coenzyme Q and cytochrome c_1[13] but the site for the other two inhibitors has not been fully localized. Since these three inhibitors have two structural similarities in common, namely, ligand groups and C_6 to C_9 alkyl side chains, the mechanism of inhibition may involve co-ordination of non-heme iron in a lipid molecular environment. It is the purpose of this paper to report studies of the mechanism of inhibition by antimycin A, by alkyl hydroxy naphthoquinones and by some metal co-ordination compounds.

MATERIALS AND METHODS

Antimycin A and its derivatives were kindly supplied by Prof. F. M. Strong, University of Wisconsin. The 3-alkyl derivatives of 2-hydroxy-1, 4-naphthoquinone were supplied by Prof. L. F. Fieser, Harvard University.

Succinic- and DPNH-cytochrome *c* reductase activities were measured by recording the increasing absorbance at 550 mμ of cytochrome *c* reduction as a function of time using a Bechman DK spectrophotometer. Percentage inhibition by animtycin A and derivatives and by the metal co-ordination compounds was calculated from these recorded reaction rates. The reaction system contained 5×10^{-3} M succinate or DPNH, 1×10^{-3} M cyanide, 1 mg of cytochrome *c* per ml of electron transport particle preparation[14] containing 0·1 mg protein, and 0·1 M phosphate buffer, pH 7·5. Inhibitors were added to the reaction system in small amounts of ethanol, the reaction system was brought to the reaction temperature of 37 °C in 2 min, and the electron transport preparation was added to start the reaction.

A preparation of purified succinic dehydrogenase–coenzyme Q was supplied by D. M. Ziegler. Succinic dehydrogenase–coenzyme Q activity was measured at 37 °C by recording decreasing absorbance at 600 mμ of dichlorophenolindophenol reduction as a function of time. The reaction system contained 4×10^{-3} M succinate, 1×10^{-4} M Versene, 3×10^{-2} M 2 : 6-dichlorophenolindophenol, 5×10^{-2} M phosphate buffer, pH 7·4, and 3 μg (protein) succinic dehydrogenase-coenzyme *Q* preparation. Naphthoquinone and metal co-ordination inhibitors were added either before or during the reaction.

RESULTS AND DISCUSSION

Ligand groups of the known inhibitors

Although the structure of antimycin A (Ia + Ib) has not been established with certainty,[1] the blastomycic acid structure (Ia) is sufficiently known to locate a number of ligand groups which may form chelate rings with iron or other metals. Firstly, the 1-carbonyl group together with the 2-hydroxyl group are a pair of ligands which should have co-ordination properties similar to other analogs of salicyaldehyde or salicylic acid. Secondly, the 2-hydroxyl together with the secondary 3-amide are a pair of ligands somewhat similar to aminophenols. Thirdly, the carboxyl group with its α-secondary amine are a ligand pair similar to amino acids. On the basis of available stability constants,[15–17] the 1-carbonyl-2-hydroxyl groups should be the strongest ligands. Further, the stability of a didentate six-membered metal chelate formed with the 1-carbonyl-2-hydroxyl groups should be increased by the proximity of the other ligand groups. A study of the stability constants of the ligand groups of antimycin A will be very useful, but this must await the completion of structural studies. Antimycin A is known to form a blue-colored co-ordination compound with ferric chloride.

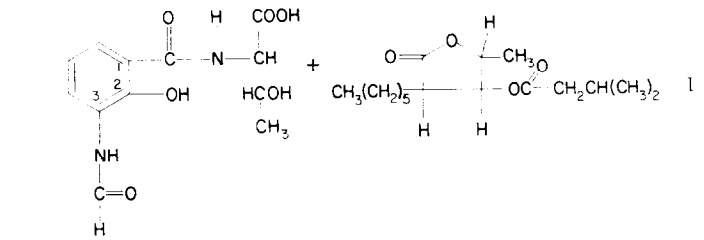
That the 2-hydroxy-3-alkyl naphthoquinones (II) should co-ordinate metals and form chelates may be inferred from information concerning structural analogs. Metal chelates of *o*-hydroxyquinone and 1-hydroxyanthroquinone have been studied in detail.[15, 17] Many dyes containing the *o*-hydroxyquinoid structure, particularly alizarin dyes, are known to co-ordinate iron and other metals.

2-Heptyl-4-hydroxyquinoline-N-oxide (III) has two ligand groups, the hydroxyl and the quinoline nitrogen, which may co-ordinate metals. A co-ordination compound which is colored orange-red is formed when 2-alkyl-4-hydroxyquinoline N-oxides are reacted with ferric chloride.[2]

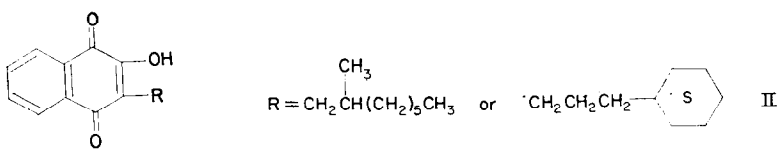
Inhibition of succinate–cytochrome c *reductase activity by antimycin A and its derivatives*

To define the structural requirements of antimycin A as an inhibitor, all currently-

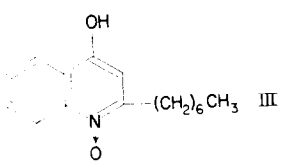
available derivatives were tested, and the results are shown in Table 1. The succinate–cytochrome *c* reductase reaction was chosen as the simplest for evaluating inhibitors that inhibit between cytochromes *b* and c_1. The three fractions of antimycin, A_1, A_2 and A_3, gave 50 per cent inhibition between 0·01 and 0·05 μg per ml. Thus, these three



a b I



II



III

chromatographic fractions, which have very similar physical and chemical properties,[1] have similar biological activity. When the formyl group on the 3-amine is removed, as in deformylated antimycin A_1 hydrochloride, the inhibitory power drops by a factor of 10. Structural considerations given here implicate the phenolic 2-hydroxyl as a metal co-ordinating ligand group. The decreases in inhibition, when this acidic hydroxyl group is substituted, as in antimycin A_3-monomethyl ether and diacetylblastomycin, are most interesting. Conversion of the hydroxyl to the monomethyl ether has a profound effect. The concentration of the ether must be a thousand times greater than antimycin A_3 for equal inhibition. Diacetylblastomycin is one-tenth as inhibitory as blastomycin.

Since both blastomycic acid (Ia) and the structurally similar antimycic acid contain the ligand groups but lack the neutral lipid-soluble gragment (Ib) of antimycin, their lack of strong inhibitory power is an indication of the essentiality of the lipophilic part of antimycin. Blastomycic acid was found to be less than one-thousandth as inhibitory as antimycin. It has an inhibitory power similar to that of the classical metal co-ordination compounds which will be described later in this study. Antimycic acid could not be tested at higher concentrations because it reduced cytochrome *c* directly and thus interfered with the enzymic measurement. The available derivative closest to the neutral fragment (Ib) is 1-methyl-2-hydroxyl-3-*n*-hexyl butyrolactone which was less than one-thousandth as inhibitory as antimycin.

Inhibition of succinic dehydrogenase–coenzyme Q activity by alkyl hydroxy naphthoquinones

Purified succinic dehydrogenase–coenzyme Q was chosen to determine if it contained a site of inhibitions. Poor inhibition was obtained with 2-heptyl-4-hydroxyquinoline N-oxide with 19 per cent inhibition at 5 μg per ml, 57 per cent at 14 μg per ml, and 81 per cent at 35 μg per ml. Since inhibition of overall electron transport does not require such large amounts, we can conclude that the succinic dehydrogenase–coenzyme Q preparation does not contain its site of inhibition. In contrast, 3-alkyl-2-hydroxyl-1 : 4-naphthoquinones proved to be good inhibitors; inhibition by a number

TABLE 1. INHIBITION OF SUCCINATE-CYTOCHROME *c* REDUCTASE ACTIVITY BY ANTIMYCIN A AND DERIVATIVES

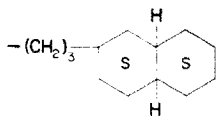
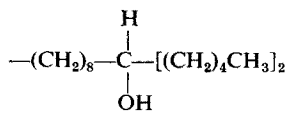
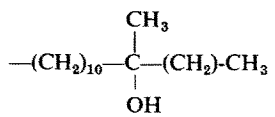
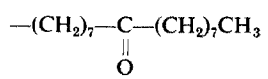
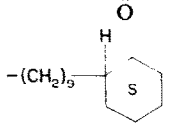
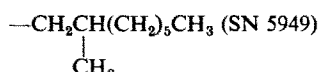
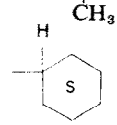
Inhibitor	Concentration (μg/ml)	Inhibition (%)
Antimycin A_1	0·01	5
	0·25	89
	0·25	94
	0·5	100
Antimycin A_{2a}	0·01	47
	0·05	97
Antimycin A_3 (Blastomycin)	0·01	40
	0·05	96
Deformylated antimycin A_1 hydrochloride	0·01	0
	0·05	18
	0·5	91
Antimycin A_3-monomethylether	0·5	0
	5·0	42
	50·0	94
Diacetylblastomycin	0·05	0
	0·25	50
	0·5	90
	5·0	100
Antimycic acid	5·0	20
Blastomycic acid	0·5	0
	5·0	18
	50·0	34
1-Methyl-2-hydroxyl-3-*n*-hexyl butyrolacetone	5·0	0
	50·0	23

Reaction system contained 5×10^{-3} M succinate, 1×10^{-3} M cyanide, 1 mg per ml. Cytochrome *c*, electron transport particle preparation containing 0·1 mg protein, and 0·1 M phosphate buffer, pH 7·5. Percentage inhibition was calculated from reaction rates of cytochrome *c* reduction.

of alkyl derivatives is given in Table 2. Some of these are better inhibitors than SN5949, which has been mainly used[6–8] in research on the electron transport chain. Among the naphthoquinone derivatives tested, the inhibitory power increases with increases in the lipophilic character of the side chain. Polar hydroxyl or carbonyl groups in the middle of long lipophilic side chains do not decrease the comparative

inhibitory power, but the carboxyl group on the C- alkyl does. Among inhibitors of the electron transport system these are the most inhibitory quinones known.

TABLE 2. INHIBITION OF SUCCINIC DEHYDROGENASE-CoQ ACTIVITY BY 3-ALKYL DERIVATIVES OF 2-HYDROXY-1 : 4-NAPHTHOQUINONE

3-Alkyl side chain	Concentration (μg/ml)	Inhibition (%)
	0·5	85
	1	85
	2	94
$-(CH_2)_{12}-CH_3$	0·5	90
	1	90
	2	90
	1	85
	4	100
	1	78
	4	100
	1	73
	1	71
	4	94
$-(CH_2)_9CH_3$	1	63
	4	86
	1	48
	4	92
	1	38
	4	39
$-(CH_2)_9COOH$	1	29
	4	37
	16	68

Reaction system contained 4×10^{-3} M succinate, 1×10^{-4} Versene, 3×10^{-2} M 2 : 6-dichlorophenolindophenol, 5×10^{-2} M phosphate buffer, pH 7·4 and succinic dehydrogenase-coenzyme Q preparation containing 3 μg protein. Percentage inhibition was calculated from reaction rates of indophenol reduction.

Because these naphthoquinones may inhibit at coenzyme Q_{10}, 3-$(CH_2)_3$-decalyl-2-hydroxy-1 : 4-naphthoquinone was tested for competitive inhibition. The results in Table 3 show that it was competitive with externally added coenzyme Q_{10} and also, coenzyme Q_7, coenzyme Q_2 and vitamin K_1. Competition with coenzyme Q_{10} for

reaction with metals may be the mechanism by which these naphthoquinones inhibit the electron transport system. However, this mechanism cannot be definitely established with this system because the competitive effect of coenzyme Q_7, coenzyme Q_2 and vitamin K_1 shows that the competition is not specific.

The fact that vitamin K_1 overcomes the inhibition can be considered as evidence of non-specificity because vitamin K_1, will not replace coenzyme Q_{10} in extracted mitochondria,[18] electron transport particle[19] and cytochrome *c* reductase.[20] Coenzyme Q_{10} and its closely related homologs show good specificity in replacing the coenzyme Q_{10} extracted from these particles. In order to localize the site and to determine the

TABLE 3. COMPETITION OF QUINONES WITH NAPHTHOQUINONE INHIBITOR

Concentration of 3-$(CH_3)_2$-decalyl-2-hydroxy-1 : 4-naphthoquinone (μg/ml)	Quinone	Concentration of quinone (μg/ml)	Inhibition of indophenol reaction (%)
1	CoQ_{10}	0	79
1		4	52
1		8	16
4		8	79
4		28	71
1	CoQ_7	0	77
1		2	61
1		5	50
2		5	67
5		5	81
1	CoQ_2	0	100
1		0·8	0
9		0·8	37
1	Vitamin K_1	0	84
1		1	50
6		2	100
6		10	92

Reaction system and methods are as described for Table 2.

mechanism of alkyl hydroxy naphthoquinone inhibition it will be necessary to study the internal coenzyme Q_{10} of intact mitochondria, as was done with antimycin A inhibition.[13]

When coenzyme Q_{10} and coenzyme Q_2 were added alone to the succinic dehydrogenase–coenzyme Q system, the externally added coenzyme Q_{10}, up to 20 μg per ml, did not increase activity, whereas coenzyme Q_2, at 0·8 μg per ml, increased the rate by 50 per cent. For each naphthoquinone tested and listed in Table 2, 0·8 μg of coenzyme Q_2 per ml overcame the inhibition of 1 μg of naphthoquinone per ml.

Inhibition of electron transport by metal co-ordination compounds

Another approach to defining the physical and chemical requirements for inhibitors of the succinate to cytochrome *c* part of the electron transport chain is to study well-known metal co-ordination compounds which are lipophilic. Eighteen co-ordination compounds were selected[15, 17, 21] and tested in attempts to find powerful inhibitors. Compounds for which complete inhibition data were obtained are compared in Table 4.

The following co-ordination compounds gave essentially no inhibition up to the concentration given: 8×10^{-3} M acetyl acetone, 1×10^{-2} M benzoylacetone, 2×10^{-3} M 8-hydroxyquinoline, 1×10^{-3} M cupferron, 5×10^{-3} M dimethylglyoxine, 3×10^{-3} M dihydroxyethylglycine, 1×10^{-3} M phenylalanine, and 1×10^{-3} M *iso*octylthioglycolate. This showed that these compounds are not strong inhibitors, and they were not tested further. All of the inhibitory compounds (Table 4), except mercaptosuccinic acid, are known to form metal inner complexes which are soluble in lipid solvents,[15, 17] Mercaptosuccinic acid is similar to carboxymethylmercaptosuccinic acid, which is known to chelate iron and copper in lipid solution.[22] The best inhibitors found, 5×10^{-5} M 2-thenoyl trifluoroacetone and 5×10^{-5} M 4 : 7-diphenyl-2 : 9-dimethyl-1 : 10-phenanthroline, can be compared to the three

TABLE 4. INHIBITION OF ELECTRON TRANSPORT SYSTEMS BY METAL CO-ORDINATION COMPOUNDS

Co-ordination compound	Concentration for 50% inhibition ($M \times 10^5$)		
	Succinate–cytochrome *c* reductase	DPNH–cytochrome *c* reductase	Succinic dehydrogenase–coenzyme Q
2-Thenoyltrifluoroacetone	5	200	1
2-Furoyltrifluoroacetone	200	—	10
4 : 7-Diphenyl-2 : 9-dimethyl-1 : 10-phenanthroline	5	20	1
4 : 7Diphenyl-1 : 10-phenanthroline	10	—	1
2 : 9-Dimethyl-1 : 10-phenanthroline	200	—	—
1 : 10-Phenanthroline	200	—	—
Salicyaldehyde	300	—	10
Diphenylthiocarbazone	200	—	—
2 : 2′-Bipyridine	1000	—	—
Mercaptosuccinic acid	200	—	—

Succinate- and DPNH-cytochrome *c* reductase systems and the succinate dehydrogenase coenzyme Q system and the methods of measuring inhibition are given under Methods.

biologically-active inhibitors under consideration. Antimycin A inhibits 50 per cent at 5×10^{-8} M, *n*-heptyl-4-hydroxyquinoline-N-oxide at 5×10^{-7} M[3, 4] and the 2-hydroxy-3-alkyl naphthoquinones at 3×10^{-6} M.[6, 7] Greater inhibition by 2-thenoyl trifluoroacetone than by 2-furoyltrifluoroacetone, can be ascribed to stronger metal co-ordination by the thiophene sulfur than by the furan oxygen; 2-thenoyl trifluoroacetone forms tridentate chelates of great stability.[16, 17] The greater inhibition by the diphenyl phenanthrolines than by the other phenanthrolines appears to be related to their more lipophilic nature.

When the succinate reduction of the cytochromes *a*, *b* and *c*, in 2 mg of electron transport particle per ml, was measured spectrophotometrically in the presence of 1×10^{-4}M or 1×10^{-3} M 2-thenoyl trifluoroacetone or 5×10^{-4} M 4 : 7-diphenyl-1 : 10-phenanthroline, there was no evidence of specific inhibition between cytochromes *b* and c_1, as has been characteristically found for antimycin A, 2-heptyl-4-hydroxyquinoline N-oxide, and the alkylhydroxynaphthoquinones. Rather, there was a general inhibition of the reduction of all three cytochromes.

Some of the better inhibitors were tested for inhibition of the succinic dehydrogenase–coenzyme Q reaction and DPNH–cytochrome *c* reductase activity (Table 4). There was strongest inhibition of the succinic dehydrogenase–coenzyme Q reaction.

Reduction of internal coenzyme Q in mitochondria by succinate was inhibited by 5×10^{-4} M thenoyl trifluoroacetone. Thus, thenoyl trifluoroacetone is a good inhibitor of succinate-catalyzed reductions in the electron transport. Since its mechanism of inhibition must involve metal co-ordination, thenoyl trifluoroacetone should prove useful in future studies of the function of metals in electron transport. The major limitation is that the known co-ordination compounds studied here do not have the specificity of the inhibitors, antimycin A, 2-hydroxyl-3-alkylnaphthoquinones and 2-heptyl-4-hydroxyquinoline-N-oxide. The electron transport particle contains 32 moles of non-heme iron and 4 moles of copper per mole of flavin, and 35 per cent lipid,[11, 12] so there are many sites at which the lipophilic metal co-ordination compounds may inhibit. Their value will be greatest in detailed studies of purified preparations of electron transport chain components in which the number of possible inhibition sites is limited.

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