to a variety of tissue components, as was also found in the course of the present studies. However, the significance of such reactions and the role they play in the carcinogenic process requires to be elucidated since similar combinations of radioactivity were observed with aminofluorenols (Weisburger *et al.*, 1961) which apparently are noncarcinogenic. Nevertheless, there was more radioactivity bound to the liver of male rats which is also the more susceptible organ with N-OH-FAA. Thus, despite the higher reactivity of the intermediate hydroxylamine, a certain selectivity was exhibited. None of this selectivity was apparent in the case of the kidneys, an organ usually not a target of the carcinogenic effect with this compound. In any case, the metabolic behavior of N-OH-FAA noted in these studies bears a closer relationship to its observed biological properties than does that of the precursor FAA. However, it is still somewhat premature to draw cause-and-effect conclusions in terms of carcinogenicity.

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Effect of Antimycin A on Oxidative Phosphorylation with Ferricyanide as Electron Acceptor*

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Antimycin A inhibits respiration of rat liver mitochondria more than 90% when measured in a Warburg-flask-assay system with potassium ferricyanide as electron acceptor, confirming results obtained by continuous spectrophotometric assays. Previous failures to observe inhibition in a Warburg-flask-assay system are due to the destruction and inactivation of antimycin A by potassium ferricyanide in the absence of mitochondria. Antimycin A and potassium ferricyanide undergo a chemical reaction at neutral *p*H to yield products that absorb at longer wavelengths. The reaction rate is dependent on the *p*H of the reaction media and on the concentration of potassium ferricyanide. Antimycin A is bound by rat liver mitochondria in a manner which protects it from destruction by potassium ferricyanide.

Potassium ferricyanide has been used as a terminal electron acceptor in place of oxygen in studies of oxidative phosphorylation by mitochondria and two principally different methods have been employed for the measurement of ferricyanide reduction. In this laboratory (Copenhaver and Lardy, 1952; Maley and Lardy, 1955) and in work by Ernster (1961) the reaction was carried out in Warburg vessels and the ferricyanide reduction was determined by measuring its concentration at the beginning and at the end of the reaction, whereas Pressman (1955) and Estabrook (1961) followed the reduction directly by spectrophotometric methods. With the former method, P:$2e^-$ values between 0.8 and 1.5 for DPN-linked substrates and of about 0.6 for succinate (Copenhaver and Lardy, 1952; Maley and Lardy, 1955; Ernster, 1961) were found. Somewhat higher values were observed

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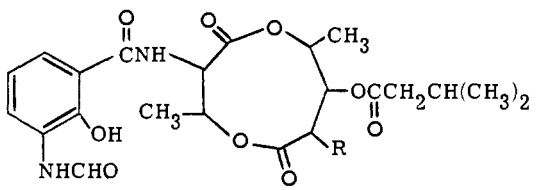
by employing direct spectrophotometric techniques, as P:$2e^-$ ratios of about 2 were reported (Pressman, 1955; Estabrook, 1961) for DPN-linked substrates. The results obtained by both methods seem to indicate two sites of phosphorylation between DPNH and ferricyanide and one site between succinate and ferricyanide. The two methods, however, gave conflicting results with respect to inhibition by antimycin A. The oxidation with either β-hydroxybutyrate (Copenhaver and Lardy, 1952) or succinate (Ernster, 1961) as substrate was completely insensitive to antimycin A in the Warburg-flask assay, whereas Pressman (1955) and Estabrook (1961, 1962) observed more than 90% inhibition with the same substrates by using direct spectrophotometric methods.

Antimycin A is known to be a mixture of at least four components, designated as A_1 to A_4. The structures of A_1 and A_3 have been determined (Van Tamelen *et al.*, 1961) and their formulas are:



R = n-C_6H_{13} Antimycin A_1
R = n-C_4H_9 Antimycin A_3

There have been very few attempts to resolve this discrepancy. The two methods differ in many ways, especially with respect to the concentration and order of addition of the various components. In the Warburg-flask assay a 20- to 30-fold higher ferricyanide concentration is used as compared to the spectrophotometric method. Minakami *et al.* (1962) have shown that, in mitochondrial particles, ferricyanide can accept electrons at more than one site in the electron chain depending on the concentration of the acceptor. It was therefore suggested (Estabrook, 1961) that ferricyanide might act in a similar way in intact mitochondria and that the antimycin A sensitivity would therefore depend on the ferricyanide concentration in the reaction media. This proposal is, however, not in agreement with Pressman's (1955) finding that ferricyanide concentration is not responsible for the divergency of the effectiveness of antimycin A. Ernster (1961) reported an experiment with succinate as substrate, in which the antimycin A sensitivity was found to be dependent on whether or not ATP and phosphate were added to the reaction media. Inhibition was observed in a system with added ATP but in the absence of added phosphate, whereas in the case where phosphate and ATP, or phosphate but no ATP, or neither of the two was added, the system was found to be insensitive to antimycin A. These results are difficult to interpret, and they do not seem to help explain the different inhibitory effects of antimycin A obtained by the Warburg-flask method on one hand and by the spectrophotometric technique on the other, because both assay systems employ phosphate.

The problem of the acceptor site of ferricyanide and of its relation to the inhibition of electron transport by antimycin A was reinvestigated. It has been found that with intact rat liver mitochondria in the Warburg-flask assay antimycin A inhibits ferricyanide reduction more than 90% if the reaction components are added in the right order. Under the conditions used previously (Copenhaver and Lardy, 1952) for the Warburg-flask assay the antimycin A was inactivated by reacting with potassium ferricyanide. Some information concerning the nature of the chemical reaction between antimycin A and potassium ferricyanide has also been obtained.

Experimental

Methods.—All experiments were performed with intact rat liver mitochondria, which were isolated essentially according to the method of Schneider (1948) from male albino rats (Badger Research Corp., Madison, Wisc.). The washed mitochondria were suspended in 1 ml of 0.25 M sucrose per gram of original tissue.

Respiration measurements were performed in Warburg vessels at 30°. The final reaction mixture (main vessels plus side arm) contained 2 mM ATP, 5 mM $MgSO_4$, 17 mM potassium phosphate, *p*H 7.4, 33 m M Tris, *p*H 7.4, 17 mM potassium ferricyanide, 0.3 ml mitochondrial suspension (containing 0.8–1.0 mg nitrogen), 7 mM succinate, 13 mM DL-β-hydroxybutyrate, or 10 mM glutamate. Glutamate was always used as substrate except in the experiments of Table I. All components were added as essentially isotonic solutions and the final volume was made up to 3.0 ml with 0.25 M sucrose. When indicated, 1.1 μg antimycin A (final concentration, 6.7×10^{-7} M) was added in 0.02 ml of 95% ethanol. Control experiments showed that this amount of ethanol had no significant effect by itself. The concentration of the antimycin A solution was determined spectrophotometrically (Van Tamelen *et al.*, 1961). For each experiment, the components of the reaction mixture present in the side arm at the beginning of the reaction will be specified. The flasks were equilibrated in the water bath at 30° for 5 minutes and during this time the air phase was replaced by nitrogen. After the equilibration period the contents of the side arms were tipped into the main vessels and zero-time flasks were removed and the reactions were stopped with 2.0 ml of 10% trichloroacetic acid. The contents of the remaining flasks were deproteinized after a reaction time of 20 minutes. The flask contents were analyzed for ferricyanide (colorimetrically at 420 mμ) and for inorganic phosphate as described earlier (Copenhaver and Lardy, 1952).

Nitrogen was determined by direct nesslerization after complete acid digestion.

Materials.—The sources of the chemicals used are as follows: ATP and hexokinase type IV from Sigma Chemical Co.; Antimycin A from Kyowa Fermentation Industry Co., Ltd., Tokyo (Japan), courtesy Mr. T. Kagawa; Silica Gel G from Merck AG., Darmstadt (Germany). Other chemicals used were of reagent quality.

Results

Data summarized in Table I demonstrate that antimycin A inhibits oxidative phosphorylation and respiration with ferricyanide as electron acceptor if the antibiotic is added to the reaction mixture *after* the mitochondria. When antimycin A was allowed to stand with the reaction mixture for only a few minutes (for time dependence see Table IV) *before* adding the mitochondria no inhibition was observed, confirming the results reported by Copenhaver and Lardy (1952), and by Ernster (1961) who added the components in the same order. Because the kind of substrate (glutamate, β-hydroxybutyrate, or succinate) and the presence of phosphate acceptor were not critical for antimycin A sensitivity, respiration measurements

TABLE I

DEPENDENCE OF ANTIMYCIN INACTIVATION ON THE ORDER OF ADDITION OF MITOCHONDRIA AND ANTIMYCIN TO THE REACTION MIXTURE[a]

Substrate	Order of Addition to Main Vessel	Side Arm	$\Delta K_3Fe(CN)_6$ (μmoles/mg N/20 min)	Δ Phosphate (μmoles/mg N/20 min)	P:2e⁻
Glutamate	Control, no antimycin		11.9		
	(a) Antimycin, (b) mitochondria[b]		11.6		
	(a) Mitochondria, (b) antimycin		1.0		
	Control, no antimycin	Hex/Gluc[c]	26.3	18.0	1.37
	(a) Antimycin, (b) mitochondria[b]	Hex/Gluc[c]	25.4	16.0	1.26
	(a) Mitochondria, (b) antimycin	Hex/Gluc[c]	1.0	0	0
Succinate	Control, no antimycin		22.6		
	(a) Antimycin, (b) mitochondria[b]		21.2		
	(a) Mitochondria, (b) antimycin		1.5		
	Control, no antimycin	Hex/Gluc[c]	18.3	7.7	0.84
	(a) Antimycin, (b) mitochondria[b]	Hex/Gluc[c]	18.6	8.6	0.93
	(a) Mitochondria, (b) antimycin	Hex/Gluc[c]	1.5	0	0
β-OH-Butyrate	Control, no antimycin		12.2		
	(a) Antimycin, (b) mitochondria[b]		13.0		
	(a) Mitochondria, (b) antimycin		0.9		
	Control, no antimycin	Hex/Gluc[c]	16.0	10.7	1.34
	(a) Antimycin, (b) mitochondria[b]	Hex/Gluc[c]	17.3	11.3	1.31
	(a) Mitochondria, (b) antimycin	Hex/Gluc[c]	1.2	0	0

[a] The composition of the reaction medium in Tables I–IV is described in the experimental section. [b] Mitochondria were added 10 minutes after antimycin. [c] Hexokinase type IV, 0.35 mg in 0.2 ml 0.25 M glucose.

with only one substrate (glutamate) are reported in Tables II to IV and hexokinase was omitted. In agreement with Estabrook (1961), no respiratory control could be obtained with succinate as substrate when ferricyanide was the electron acceptor. With glutamate as substrate the phosphate acceptor system enhanced oxidation 120%, and with β-hydroxybutyrate, 30%.

TABLE II

INACTIVATION OF ANTIMYCIN BY $K_3Fe(CN)_6$

Antimycin in Main Vessel	Side Arm	$\Delta K_3Fe(CN)_6$ (μmoles/mg N/20 min)
−	$K_3Fe(CN)_6$[a] (control)	15.6
+	$K_3Fe(CN)_6$	1.0
−	$K_3Fe(CN)_6$ + antimycin	Variable
−	$K_3Fe(CN)_6$ + phosphate[b]	13.4
−	$K_3Fe(CN)_6$ + phosphate + antimycin	11.2
−	$K_3Fe(CN)_6$ + Tris[c]	12.7
−	$K_3Fe(CN)_6$ + Tris + antimycin	10.9

[a] 0.2 ml 0.25 M $K_3Fe(CN)_6$. [b] 0.5 ml 0.1 M potassium phosphate buffer, *p*H 7.4. [c] 0.5 ml 0.1 M Tris buffer, *p*H 7.4.

The first two experiments of Table II demonstrate that inhibition was also obtained when the potassium ferricyanide was added as the last component from the side arm. From these results and from the fact that the order of addition is known not to influence the antimycin A sensitivity under aerobic conditions in the absence of ferricyanide, one must conclude that potassium ferricyanide is responsible for the inactivation of antimycin A. However, under conditions where the antibiotic was incubated with potassium ferricyanide alone only slight and inconsistent inactivation was observed in agreement with earlier findings (Copenhaver and Lardy, 1952). Only when incubation was carried out in the presence of buffers could practically complete inactivation of the antibiotic be obtained as is indicated in Table II.

In order to compare the sensitivity to antimycin A of the ferricyanide system with that found by others,

TABLE III

THE *p*H DEPENDENCE OF THE INACTIVATION OF ANTIMYCIN BY POTASSIUM FERRICYANIDE

*p*H of $K_3Fe(CN)_6$ Soln. in Side Arm[a]	Antimycin in Side Arm	$\Delta K_3Fe(CN)_6$ (μmoles/mg N/20 min)
7.25	−	16.1
7.25	+	16.1
5.80	−	18.4
5.80	+	15.6
4.75	−	18.7
4.75	+	1.5
3.85	−	17.8
3.85	+	1.2

[a] The solutions were prepared by mixing 9 parts of a $K_3Fe(CN)_6$ solution with 1 part of different citric acid–phosphate buffers (Gomori, 1955). The final concentration of the solutions (0.2 ml) in the side arms was always 0.25 M $K_3Fe(CN)_6$. The solutions containing antimycin were left standing for 75 minutes at room temperature.

the antimycin A titer was determined. It was measured by incubating a constant concentration of mitochondria with varying amounts of antimycin A under conditions analogous to those described for experiment 2 in Table II. It was found that 0.3 μg antimycin A/mg nitrogen was sufficient for maximal (over 90%) inhibition, which is in agreement with the value reported by Hemker (1963) for a system under aerobic conditions and with the results of Estabrook (1962) obtained by following respiration spectrophotometrically with ferricyanide as electron acceptor.

Table III demonstrates the strong *p*H dependence of the reaction of potassium ferricyanide with antimycin A. It was also demonstrated that the antibiotic could not be reactivated by adjusting the *p*H of an inactive antimycin A–potassium ferricyanide solution from 7 to 4.

Table IV presents the results of a time study in which antimycin A was incubated in a reaction medium containing 17 mM potassium ferricyanide for various times before the mitochondria were added. The results show that under the conditions described it took 2–3 minutes to inactivate 1.1 μg of antimycin A (2×10^{-9} moles). No gradual increase was observed

TABLE IV
ANTIMYCIN INACTIVATION AS A FUNCTION OF TIME[a]

Time of Incubation of Antimycin with $K_3Fe(CN)_6$ (min)	$\Delta K_3Fe(CN)_6$ (μmoles/mg N/20 min)
Control (no antimycin)	14.5
1	1.1
2	1.1
3	12.1
4	13.4
6	14.1

[a] Warburg flasks with two side arms were used. $K_3Fe(CN)_6$ was in one side arm and mitochondria in the other. Antimycin (1.1 μg) and all the other components were added to the main vessel. The mitochondria were tipped in after 8 minutes of preliminary incubation time, whereas the $K_3Fe(CN)_6$ solution was tipped in from 1 to 6 minutes before the mitochondria were added as indicated in the table. The reaction was carried out at 30°.

because the added 1.1 μg of antibiotic represents a 3- to 4-fold excess for maximal inhibition. It was also found that the time of inactivation was highly dependent on the concentration of potassium ferricyanide. In a reaction medium containing 1.7 mM potassium ferricyanide it took 30–60 minutes and in a 0.85 mM solution 3–4 hours to destroy the same amount of antibiotic.

Ernster (1961) reported that the sensitivity of mitochondria to antimycin A depended on the addition of ATP and the absence of added inorganic phosphate. His reaction conditions were used for the experiments described in Table V. This dependence could not

TABLE V
EFFECT OF PHOSPHATE AND ATP ON ANTIMYCIN SENSITIVITY OF POTASSIUM FERRICYANIDE REDUCTION BY SUCCINATE

Addition (μmoles)		$K_3Fe(CN)_6$ Reduced (μmoles)		
P_i	ATP	No Antimycin	+ Antimycin[a]	+ Antimycin[b]
		9.2 (10.3)[c]	9.1 (10.6)[c]	0.7
60		8.9 (21.9)	10.1 (24.1)	0.5
	10	11.7 (16.5)	10.7 (2.8)	0.5
60	10	20.9 (29.7)	20.2 (28.8)	0.5

[a] Mitochondria added 10 minutes after antimycin. [b] Antimycin added last. [c] Numbers in parentheses are those reported by Ernster (1961). The reaction mixture and the reaction conditions were used as described by Ernster (1961): mitochondria from 300 mg rat liver, 100 μmoles KCl, 40 μmoles glycylglycine buffer, pH 7.5, 16 μmoles $MgCl_2$, 20 μmoles succinate, 30 μmoles $K_3Fe(CN)_6$, 1 μmole KCN, and, when indicated, 1 μg antimycin, in a final volume of 2 ml. Incubation for 20 minutes at 30°.

be confirmed; however, inhibition could always be obtained when antimycin A was added last. It should be noted that some of the relative rates were also different from those reported by Ernster.

Reaction of Antimycin with Potassium Ferricyanide.— In an attempt to obtain some information on the type of reaction antimycin A undergoes with potassium ferricyanide, a few preliminary experiments were carried out. Antimycin A (5 mg) in 0.5 ml of 95% ethanol was added to 50 ml 0.1 M potassium phosphate buffer, pH 7.4, containing 5 mmoles of potassium ferricyanide. On addition to the reaction mixture the antimycin A precipitated as a fine suspension. The reaction mixture was shaken in a 100-ml flask (air as gas phase) for 24 hours at room temperature. The suspension was then extracted twice with benzene and the combined extracts were washed about ten times with water and dried over sodium sulfate, and the solvent was evaporated under reduced pressure. The colored solid products were stirred with 1 ml ethanol which dissolved all but a small amount of highly colored residue. The ethanolic solution retained about 10% of the respiration inhibitory capacity of the starting material. In thin-layer chromatography (solvent system, benzene-ethanol, 4:1) on Silica Gel G, antimycin A formed only one spot (R_F 0.67). In the same solvent system the alcohol-soluble products separated into two major bands. One corresponded to antimycin A and gave a positive phenol test with diazotized benzidine (Randerath, 1963), whereas the other band was a yellow compound (R_F 0.87) which showed no color change on spraying with the coupling agent. A weak green band could also be observed (R_F 0.57). The yellow compound turned green within a few hours on exposure to daylight. In a chromatogram run in the dark, the yellow band was collected from the plate and the silica gel was extracted with ethanol. The spectrum of this extract showed a maximum at 408 mμ with no other maximum down to 260 mμ. The extract contained no ferricyanide.

In a control experiment, antimycin A was shaken with the buffer solution without ferricyanide and 5 mg of crystalline material was obtained from the benzene extract. Biological (Warburg-assay) and spectrophotometric (Van Tamelen *et al.*, 1961) measurements of this crystalline material showed that more than 95% of the antibiotic had been recovered.

DISCUSSION

The results reported demonstrate that antimycin A can be inactivated when it is incubated with potassium ferricyanide in a pH range of 6–7.4 and in the absence of mitochondria. The inactivation rate is a function of the potassium ferricyanide concentration. For the destruction of 1.1 μg of antimycin A it takes only about 3 minutes in a 17 mM potassium ferricyanide solution, whereas about 60 minutes are required in a 1.7 mM and 4 hours in a 0.85 mM solution. When antimycin A is added to the complete reaction mixture including mitochondria, its absorption by the mitochondria is faster than its destruction by 17 mM potassium ferricyanide (Table I); and once the antimycin A is bound to its site of action inside the mitochondria, the electron acceptor is not able to reverse the inhibition (Tables I and II). In view of these facts it becomes clear why no antimycin A inhibition could be detected in the earlier Warburg-flask experiments. In the experiments described by Copenhaver and Lardy (1952), the mitochondria were added as the last component to the reaction mixture. Although not specifically indicated, time in excess of 3 minutes elapsed between the addition of antimycin A and potassium ferricyanide on one hand and mitochondria on the other, thus accounting for complete inactivation of the antibiotic. In the procedures of Pressman (1955) and Estabrook (1961) antimycin A was inhibitory because relatively low concentrations of potassium ferricyanide were employed (0.3–0.95 mM), and also because the antibiotic was given no opportunity to react with the electron acceptor in the absence of mitochondria.

Copenhaver and Lardy (1952) had also investigated the possibility that potassium ferricyanide inactivates antimycin A. Their finding that incubation of antimycin A with potassium ferricyanide did not diminish the ability of the antibiotic to inhibit the oxidation of succinate under aerobic conditions can now be attrib-

uted to the lack of *p*H control during the incubation.

The reason why ATP influenced sensitivity to antimycin A in Ernster's (1961) experiments is not known (*cf.* Table V). It could be that in his experiment, where inhibition was found the reaction components had been added in a different order or that the mitochondria were added before all of the antibiotic had been destroyed.

The chemical reaction of antimycin A with potassium ferricyanide at *p*H 7.4 is of a complex nature, because at least 3 different products could be detected. All the products were colored indicating absorption maxima at higher wavelengths than that of antimycin A, which is known to be at 320 mμ. The structure of antimycin A has been elucidated (*vide supra*) by Van Tamelen *et al.* (1961) and was shown to be a substituted *N*-formyl-*o*-aminophenol. Butenandt *et al.* (1954) found that substituted *o*-aminophenols reacted readily with potassium ferricyanide at *p*H 7 with the formation of the corresponding 3-aminophenoxazones, which also absorb at higher wavelengths than the starting materials. The reaction rate was found to be decreased at lower *p*H values as is also the case with antimycin A (Table III). The limited amounts of antimycin A available to us precluded further studies of the products arising during the reaction with potassium ferricyanide, but a possible relation between this reaction and the one described by Butenandt *et al.* (1954) is obvious.

Ernster (1961) has used the Warburg-flask-assay system with potassium ferricyanide as described by Copenhaver and Lardy (1952) for demonstrating that antimycin A does not inhibit the succinate-linked acetoacetate reduction. His conclusion that the antimycin A site is not involved in this reaction is, however, no longer justified, unless these results can also be obtained under conditions where antimycin A is not inactivated by potassium ferricyanide. It is obvious that not only for intact rat liver mitochondria, but also in other experiments, where antimycin A is used in the presence of potassium ferricyanide, the reaction conditions have to be such as not to allow destruction of the antibiotic by the electron acceptor.

With the present findings the previously existing discrepancies between the spectrophotometric methods and the Warburg-flask-assay system have been eliminated, and potassium ferricyanide can now be used as an even more valuable and meaningful tool for studying partial reactions of oxidative phosphorylation and respiration in mitochondria.

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