In vitro Action of Aureomycin on Oxidative Phosphorylation in Animal Tissues. (19825)

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In a preliminary report(1) from this laboratory, 0.0001 M aureomycin was shown to inhibit strongly the respiration of rat liver homogenates. Loomis(2), using a washed rabbit kidney residue preparation, found that only phosphorylation, but not oxidation, was inhibited by aureomycin at a similar concentration. We have found that the respiration of washed rat liver mitochondria is also inhibited under certain conditions. Using the suspending medium of Kielley and Kielley(3), we also have found that aureomycin had only the effect reported by Loomis. This report is mainly concerned with the conditions and factors which are associated with the inhibition of oxidation of rat liver mitochondria by aureomycin.

Experimental. Normal rat liver mitochondria were prepared according to the method of Schneider(4) using 0.25 M sucrose. The washed sedimented mitochondria were homogenized in 0.154 M KCl to give a suspension containing the equivalent of 1 g of liver per milliliter. All operations were carried out in a refrigerated centrifuge at 0° or in an ice bath. The washed liver mitochondria (500 mg liver equiv. except where noted) were added to Warburg flasks containing the suspending medium as noted in the respective tables and the oxygen consumption was determined. The center well contained 0.2 ml of 2 N NaOH and a 2 sq cm filter paper wick. When inorganic phosphorus was to be determined, the respiration flasks were quickly removed, placed in an ice bath, and the contents transferred to cold centrifuge tubes containing 0.3 ml of 30% trichloroacetic acid. The flasks were rinsed with 1 ml of water and the washings were added to the incubated mitochondrial suspension. The inorganic phosphorus was estimated in the TCA filtrate by the Fiske-SubbaRow method(5). The inorganic phosphorus uptake was determined as the change in inorganic P from the end of the thermal equilibration period (zero time) to the end of the incubation.

Results. The influence of phosphate concentration, ageing of mitochondria, and mag-

TABLE I. Influence of Phosphate Concentration on Oxidative Phosphorylation by Washed Rat Liver Mitochondria.

Addition	10 μm phosphate/flask: Uptake of oxygen in μm	10 μm phosphate/flask: Changes in μm phosphate	10 μm phosphate/flask: Phosphorus-oxygen ratio	30 μm phosphate/flask: Uptake of oxygen in μm	30 μm phosphate/flask: Changes in μm phosphate	30 μm phosphate/flask: Phosphorus-oxygen ratio
Control	3	+ .8		2.5	+ 1.6	
24 μm AMP	3.2	—5.5	1.7	3.8	—10.6	2.8
6 " ATP	5.3	+ .4		4.6	+ 1.4	
24 " AMP + 6 " ATP	3.4	—7.1	2.1	6.3	—10.5	1.7
.4 " A*	3.2	+1.1		1.3	+ 1.4	
24 " AMP + .4 " A	6.5	+1.2		7	— .6	
6 " ATP + .4 " A	1.5	+9.6		2.6	+ 9.3	
24 " AMP + 6 " ATP + .4 " A	5.2	+5.1		4.5	+ 5.3	

Flask contents: 15 μm $MgCl_2$, 120 μm KCl, 0.04 μm Cytochrome C, 30 μm α-ketoglutarate, 300 mg equiv. liver mitochondria, 0.2 ml of 2N NaOH in center well, H_2O to make 3.0 ml. Incubation tem.: 36.6°C. Incubation time: 20 min. after thermal equilibration. All values are means of 4 or more determinations. * A = Aureomycin.

TABLE II. Effect of Aging on the Aureomycin Inhibition of Oxidative Phosphorylation by Rat Liver Mitochondria in the Absence of Added Magnesium.

Aureomycin per flask in μm	Before aging: Uptake of oxygen in μm	Before aging: Changes in μm phosphate	Before aging: Phosphorus-oxygen ratio	After 2 hr aging at 0°C: Uptake of oxygen in μm	After 2 hr aging at 0°C: Changes in μm phosphate	After 2 hr aging at 0°C: Phosphorus-oxygen ratio
.0	6.5	12.8	1.8	6.2	6.4	1
.1	6.1	8.8	1.4	4.5	4.1	.9
.2	4.6	4.5	1	3.4	5	1.5
.4	3.6	3.9	1.1	3.2	3.5	1.1
.8	1.2	2.3	1.9	2.4	3.2	1.3

Flask contents: 125 μm KCl, 30 μm phosphate pH 7.4, 0.04 μm Cytochrome C, 12 μm adenylic acid, 30 μm α-ketoglutarate, 500 mg equiv. liver mitochondria, 0.2 ml 2 N NaOH in center well, H_2O to make 3 ml. Incubation temp., 19.9°C. Incubation time: 30 min. after thermal equilibration.

nesium ion, on the aureomycin inhibition of oxidative phosphorylation are shown in the accompanying tables.

The data in Table I indicate that the rat liver mitochondrial system is capable of considerable phosphorus uptake as well as oxidation when adenylic acid is present. In all cases where aureomycin is present there is but little or no uptake of phosphorus. However, aureomycin inhibits oxygen uptake only when ATP is substituted for AMP. This inhibition of respiration appears to be greater at the lower concentration of inorganic phosphate.

In another experiment the magnesium chloride was omitted from the suspending medium (Table II) and the effect of increasing levels of aureomycin and ageing the mitochondria at 0°C is shown. Here the inhibition of respiration increases with the aureomycin concentration. It should also be noted that the phosphorylating ability of the mitochondria is also reduced by ageing at 0°C, but that the oxidative capacity is little altered. This loss in phosphorylating activity with ageing is similar to that found by Kielley and Kielley (3) in mouse liver mitochondria, aged at 28°C.

When magnesium is added to aged mitochondria the inhibitory effect of aureomycin on respiration is reversed as shown in Table III.

The reversing effect of magnesium on the aureomycin inhibition of respiration is again shown in Table IV. Ageing appears to have little influence on the magnesium reversal of the aureomycin inhibition. In this table it is noted that loss of respiratory activity accompanies ageing. The magnitude of effect of ageing on different preparations varies considerably.

TABLE III. Effect of Magnesium and Aureomycin on Oxidation of α-ketoglutarate by Rat Liver Mitochondria Aged 2½ Hr.

Aureomycin per flask in μm	Oxygen uptake in microatoms, Magnesium per flask in μm: 0	.4	4
.0	6.4	5.1	5.5
.2	4.9	5.5	5.6
.4	3.2	2.2	5.2

Flask contents: 0.04 μm cytochrome C, 12 μm adenylic acid, 125 μm KCl, 30 μm phosphate pH 7.4, 30 μm α-ketoglutaric acid, 500 mg equiv. liver mitochondria aged 2½ hr at 0°C, 0.2 ml 2N NaOH in center well, H_2O to make 3 ml. Incubation temp., 19.9°C. Incubation time, 30 min. after thermal equilibration.

TABLE IV. Influence of Aging on Aureomycin Inhibition of Respiration of Rat Liver Mitochondria in the Presence and Absence of Magnesium.

Additions per flask	Oxygen uptake in microatoms, Aging time at 0°C: 0	1 hr	2 hr
Control	9.2	11.0	4.7
15 μm $MgCl_2$	5.5	7.5	2
.4 " A*	7.7	7.6	0
15 " $MgCl_2$ + .4 μm A	11.9	12.9	3.5

Flask contents: 12 μm adenylic acid, 125 μm KCl, 0.04 μm cytochrome C, 30 μm phosphate pH 7.4, 30 μm α-ketoglutarate, 500 mg equiv. liver mitochondria, 0.2 ml 2N NaOH in center well, H_2O to make 3 ml. Incubation temp., 19.9°C. Incubation time, 30 min. after thermal equilibration.

*A = Aureomycin.

Discussion. It is well established that aureomycin inhibits phosphorylation associated with oxidation of citric acid cycle substrates under certain conditions. Previous reports(2,6) of the *in vitro* action of aureomy-

cin have been based upon systems which were heavily fortified with magnesium, phosphate and fluoride. It has been shown in the present work that the inhibitory effect of aureomycin on oxidation does not appear when high concentrations of magnesium and phosphate are present. In unpublished experiments of this laboratory it has been found that fluoride is also antagonistic to the aureomycin effect on oxidation. It is conjectured that the effect of ageing of mitochondria is to release endogenous magnesium from the centers of enzyme activity into the suspending medium. This would account for the fact that aureomycin is frequently ineffective in reducing the oxygen uptake when fresh mitochondria are used. Aureomycin has been found to form complex metal salts very readily and among them is a very insoluble magnesium salt. Thus if the magnesium concentration of the active centers is low, the addition of aureomycin could be expected to reduce the oxygen uptake by holding the remaining magnesium in an inactive complex. The addition of exogenous magnesium would restore the oxygen uptake. Our studies would seem to support this hypothesis.

In a recent paper(7) on the regulation of the rate of oxidation in rat liver mitochondria Potter and Recknagel examined the relationship of 2, 4-dinitrophenol (DNP) on oxidation and apparent ATP-ase. Their data using DNP, strikingly resembles our results with aureomycin when sufficient magnesium is present in the medium. Aureomycin in a similar system will accelerate the breakdown of ATP, but instead of increasing the oxygen uptake, decreases it or is without effect depending upon the activity of the individual preparation. However, if AMP is substituted for ATP the identical effect found by Potter with DNP and ATP is shown by aureomycin. One would expect that the aureomycin effect on oxidation would be the same using either AMP or ATP, if the only effect of aureomycin were to accelerate the breakdown of ATP. It is believed that this point would bear further investigation. Lardy in a recent review(8) on the influence of inorganic ions on phosphorylating reactions points out that, "although detailed studies have been made of the ion requirements of various oxidizing systems, the requirement for magnesium has been so unquestionably accepted that no studies have been reported on the mode of action." It is evident from the data we have presented that the role of magnesium in oxidative phosphorylation deserves more investigative attention.

It should be emphasized that the inhibitory effects of aureomycin on oxidation and phosphorylation were noted at a lower limit of about 10^{-4} molar. This concentration is quite high when compared to 10^{-8} molar required for inhibition of growth of susceptible microorganisms. Thus, one might argue that these inhibitory *in vitro* effects of aureomycin ae not necessarily related to its antibiotic activity.

Summary. 1. The inhibition of oxidative phosphorylation in animal tissue by aureomycin is confirmed. 2. In the presence of magnesium, low phosphate, and ATP, aureomycin inhibits oxidation by washed rat liver mitochondria. If AMP is substituted for ATP, no inhibition is found. 3. In the absence of magnesium, the inhibitory effect of aureomycin on respiration is proportional to its concentration. Ageing the mitochondria at 0°C appears to accentuate this inhibition. 4. Using aged mitochondria and AMP, the addition of magnesium reverses the aureomycin inhibition of respiration.

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