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Tirandamycin: Inhibition of Oxidative Phosphorylation in Rat Liver Mitochondria

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Tirandamycin inhibits respiration and phosphorylation in rat liver mitochondria. An investigation of individual reaction sequences occurring within the respiratory chain showed that the antibiotic stimulates reduced nicotinamide adenine dinucleotide (NADH)- and succinate-linked coenzyme Q reductase. NADH-linked reduction of tetrazolium salts remains unaffected by tirandamycin. Succinotetrazolium salt reductase is inhibited significantly. Reduction of cytochrome *c* by succinate is blocked by the antibiotic; NADH-cytochrome *c* reductase is inhibited but not completely blocked. Cytochrome *c* oxidase remains unaffected. Mitochondrial difference spectra prepared in the presence of tirandamycin indicate that the reduction of cytochrome *b* is not impaired but no reduction of cytochromes *c* or *a* is apparent. These results indicate that tirandamycin interferes with the respiratory chain at a point beyond the cytochrome *b* and prior to the cytochrome *c* reduction site. Tirandamycin acts also as a potent inhibitor of ribonucleic acid polymerase as discussed in the foregoing paper.

Tirandamycin acts as a very potent inhibitor of ribonucleic acid (RNA) polymerase as discussed in the foregoing paper (7). In addition, it was found that tirandamycin interferes with oxidative phosphorylation in rat liver mitochondria. The antibiotic thus possesses two modes of action. Tirandamycin concentrations of 100 to 200 μg/ml inhibit both processes (RNA polymerization, oxidative phosphorylation) about equally.

MATERIALS AND METHODS

Rat liver mitochondria were isolated from mixed-sex albino rats weighing 150 to 250 g as described by Lardy and Wellmann (2). Mitochondrial protein was determined with biuret reagent by using crystalline bovine albumin as the standard (1). Mitochondrial respiration was measured manometrically. Inorganic phosphate was assayed by the method of Lowry and López (4).

Reduced nicotinamide adenine dinucleotide (NADH)-linked reduction of ferricyanide by mitochondria was followed spectrophotometrically by reading the decrease in optical density (OD) of the reaction mixtures at 420 nm. Mitochondrial reduction of externally added coenzyme Q_{10} was assessed by the method described by Ramasarma and Lester (6). Reduction of tetrazolium salts to formazans was measured as described by Lester and Smith (3). The two tetrazolium salts used were 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) and 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (NBT). Cytochrome *c* reductase and oxidase activities were assessed spectrophotometrically (5, 8). Cytochrome *c* was reduced as described by Smith (8) and had an OD_{550}: OD_{565} ratio of 5. Mitochondrial difference spectra were run at room temperature. The exact experimental conditions are given below.

RESULTS

Mitochondrial oxidation of glutamate, citrate, malate, β-hydroxybutyrate, and succinate. The effects of tirandamycin on mitochondrial respiration and phosphorylation associated with the oxidation of the substrates mentioned above were assessed manometrically. During the oxidation of glutamate, citrate, or malate, significant inhibition of respiration occurred (Table 1). An antibiotic concentration of approximately 100 μg/ml caused 50% inhibition of respiration during glutamate oxidation. The drug concentrations necessary to cause 50% inhibition of respiration during the oxidation of citrate or malate were 70 and 175 μg/ml, respectively. A reasonably strict dose-response relationship was obtained with each of these substrates. Phosphorylation was also significantly inhibited by the antibiotic. A drug concentration of 200 μg/ml

TABLE 1. *Effect of tirandamycin on oxidative phosphorylation in rat liver mitochondria*[a]

Substrate	Tirandamycin	$Q_{O_2}(P)$	Inhibition of respiration	P:O ratio	Uncoupling
	μg/ml		%		%
Glutamate	None	44.1	0	2.8	0
	200	15.1	65.8	0	100
	150	16.6	62.4	2.8	0
	100	22.4	49.3	2.8	0
	50	34.0	23.0	2.9	0
Citrate	None	30.8	0	2.8	0
	200	6.2	79.6	.1	96.5
	150	9.2	69.7	.7	75.0
	100	12.9	57.5	2.3	17.9
	50	17.8	41.3	2.5	10.8
Malate	None	13.5	0	2.1	0
	200	6.2	54.1	0.3	85.8
	150	7.2	46.7	1.1	47.7
	100	7.7	43.0	0.9	57.2
	50	8.9	34.1	1.3	38.1
β-Hydroxy-butyrate	None	18.7	0	2.1	0
	200	17.1	8.6	1.4	33.3
	150	18.4	1.7	2.1	0
	50	19.5	0	2.1	0
Succinate	None	37.7	0	1.9	0
	200	15.6	58.7	0	100
	150	13.4	64.5	.8	57.9
	100	21.8	42.2	1.5	21.1
	50	24.4	35.5	2.0	0

[a] Each vessel contained, in a total volume of 3 ml: 6 μmoles of adenosine triphosphate; 50 μmoles of K-PO_4 buffer (*p*H 7.4); 15 μmoles of $MgSO_4$; 30 μmoles of sodium-L-glutamate; 30 μmoles of sodium citrate; 30 μmoles of sodium malate; 40 μmoles of sodium β-hydroxybutyrate or 20 μmoles of sodium succinate, respectively; and 0.5 ml of mitochondrial suspension (20 mg of protein/ml) in 0.25 M sucrose. Hexokinase (20 μg; Calbiochem) and 50 μmoles of glucose were added from the side arms. Reactions were run for 15 min at 30 C. $Q_{O_2}(P)$ = microliters of O_2 uptake per milligram of mitochondrial protein per hour.

caused complete uncoupling during glutamate oxidation. Lower tirandamycin concentrations had essentially no effect on phosphorylation. With citrate and malate, 200 μg of tirandamycin per ml caused almost complete cessation of phosphorylation (97% for citrate, 86% for malate). In contrast to the observation made with glutamate, lower antibiotic concentrations (50 to 150 μg/ml) also caused significant uncoupling with citrate and malate. During the oxidation of β-hydroxybutyrate which does not proceed via the Krebs cycle, respiration was inhibited by 9% in the presence of 200 μg of antibiotic per ml and 2% by 150 μg/ml. Lower concentrations did not seem to have an effect on respiration. Phosphorylation was inhibited to the extent of 33% in the presence of 200 μg of drug per ml; lower concentrations did not interfere with phosphorylation.

Mitochondrial respiration associated with the oxidation of succinate was inhibited by 50% at an antibiotic concentration of 120 μg/ml.

TABLE 2. *Effect of tirandamycin on mitochondrial NADH oxidation*[a]

Tirandamycin	$Q_{O_2}(P)$	Inhibition
μg/ml		%
None	57.4	0
200	25.6	55.5
150	25.6	55.5
100	28.5	50.4
50	31.5	45.2

[a] Each vessel contained, in a total volume of 3 ml: 6 μmoles of adenosine triphosphate, 50 μmoles of K-PO_4 buffer (*p*H 7.4), 15 μmoles of $MgSO_4$, 20 μmoles of NADH, and 0.5 ml of mitochondrial suspension in 0.25 M sucrose containing 20 mg of protein/ml. Hexokinase (20 μg) and 50 μmoles of glucose were added from the side arms. Reactions were run for 15 min at 30 C. $Q_{O_2}(P)$ = microliters of O_2 uptake per milligram of mitochondrial protein per hour.

TABLE 3. *Effect of tirandamycin on mitochondrial glutamate oxidation in a medium deficient in inorganic phosphate*[a]

Additions	$Q_{O_2}(P)$	Per cent of control
None, control	7.8	100
Tirandamycin (200 μg/ml)	3.2	41.0
DNP	14.7	188.4
DNP + tirandamycin (200 μg, ml)	4.7	60.2
DNP + tirandamycin (100 μg/ml)	7.3	93.5

[a] Each vessel contained, in a total volume of 3 ml: 6 μmoles of adenosine triphosphate, 60 μmoles of Tris-hydrochloride buffer (*p*H 7.4), 15 μmoles of $MgSO_4$, 30 μmoles of sodium glutamate, 0.45 μmole of dinitrophenol (DNP) when applicable, and 0.5 ml of mitochondrial suspension (20 mg of protein/ml) in 0.25 M sucrose. Hexokinase (20 μg) and 50 μmoles of glucose were added from the side arms. Reactions were run for 60 min at 30 C. $Q_{O_2}(P)$ = microliters of O_2 uptake per milligram of mitochondrial protein per hour.

Phosphorylation was completely inhibited by 200 μg of tirandamycin per ml; concentrations of 150 and 100 μg/ml caused 58 and 21% of inhibition, respectively. A 50-μg amount of drug per ml did not affect phosphorylation.

Mitochondrial oxidation of NADH was inhibited to an extent of 45 to 55% by tirandamycin when tested over a concentration range of 50 to 200 μg of antibiotic per ml (Table 2). This indicates that inhibition remained almost constant despite a fourfold variation in antibiotic concentration. In a medium deficient in inorganic phosphate, glutamate oxidation was inhibited by tirandamycin (Table 3). This inhibition was reversed by 2,4-dinitrophenol.

Reduction of tirandamycin by sodium hydrosulfite. Tirandamycin could be reduced. The drug showed no absorption in the visible part of the spectrum. The ultraviolet spectrum showed a strong absorption maximum at approximately 335 nm and inflections at 300 and 250 nm in tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.4 (Fig. 1). On reduction with $Na_2S_2O_4$, the absorption peak showed a hypsochromic shift to approximately 318 nm and the absorptivity increased approximately threefold. No inflections were visible at 200 and 250 nm as observed with the oxidized form.

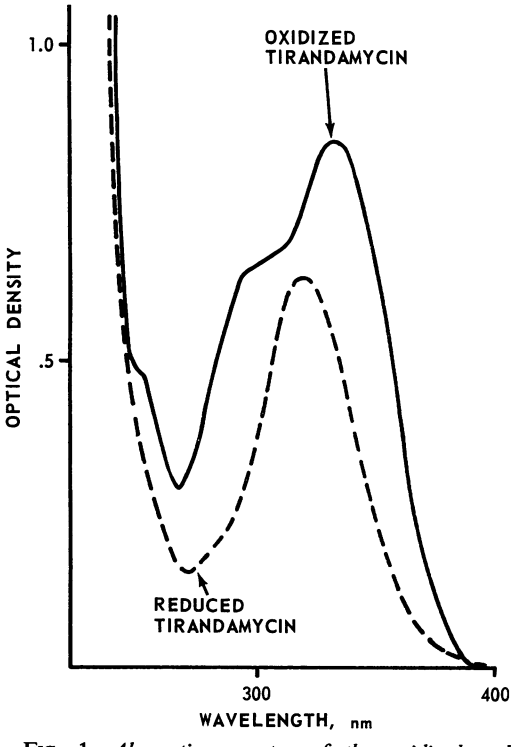

FIG. 1. *Absorption spectra of the oxidized and reduced forms of tirandamycin. The antibiotic was dissolved in 0.05 M Tris-hydrochloride buffer (pH 7.4) to give a concentration of 20 μg/ml for the oxidized form and 5 μg/ml for the reduced form. Reduction was done by adding a few crystals of solid $Na_2S_2O_4$. Both spectra were read against a buffer blank.*

To study further the mode of action of tirandamycin, individual reaction sequences occurring within the respiratory chain were studied in more detail.

Reduction of ferricyanide by NADH. Ferricyanide accepts electrons from the flavoprotein region during the oxidation of NADH without the participation of coenzyme Q or cytochromes. In the presence of 200 μg of tirandamycin per ml, no interference with this reaction was observed (Fig. 2). This indicates that tirandamycin does not interfere with NADH oxidation coupled to ferricyanide.

NADH- or succinate-coenzyme Q reduction. Mitochondrial reduction of externally added coenzyme Q was stimulated in the presence of 200 μg of tirandamycin per ml (Table 4). Stimulation was 47% when the reaction was driven by NADH and 95% with succinate. Thus, neither NADH nor succinate-coenzyme Q reduction is inhibited by the antibiotic under study.

Reduction of tetrazolium salts. Reduction of tetrazolium salts takes place at the flavoprotein site when NADH serves as a substrate and after the coenzyme Q reduction site when succinate serves as an electron donor (3). Tirandamycin

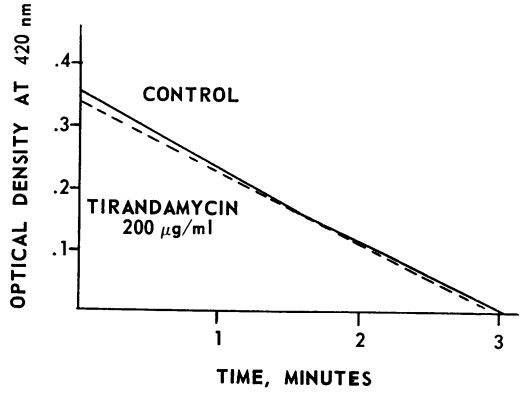

FIG. 2. *NADH-linked reduction of ferricyanide by rat liver mitochondria. The reaction mixtures contained, in a total volume of 3 ml: 150 μmoles of Tris-hydrochloride buffer (pH 7.4), 600 μmoles of sucrose, 600 μg of tirandamycin, 3 μmoles of KCN, 1.5 μmoles of $KFe(CN)_6$, 5 μmoles of NADH, and 0.2 mg of mitochondrial protein. The reaction was started by adding NADH. The optical density was read at 420 nm against a blank cuvette containing all of the reaction mixture components except NADH.*

TABLE 4. *Effect of tirandamycin on NADH or succinate coenzyme Q reductase*[a]

Sample	ΔOD_{270}	Per cent of control
NADH, control	.246	100
NADH + tirandamycin (200 μg/ml)	.363	147.5
Succinate, control	.114	100
Succinate + tirandamycin (200 μg/ml)	.224	196.4

[a] The reaction mixtures contained, in a total volume of 3 ml: 200 μg of coenzyme Q, 100 μmoles of K-PO_4 buffer (*p*H 7.0), 5 μmoles of KCN, 600 μmoles of sucrose, 5 μmoles of NADH or 50 μmoles of sodium succinate, and 1 mg of mitochondrial protein. The reactions were run at room temperature for 30 min and stopped by the addition of 3 ml of 0.1 N $HClO_4$. The samples were neutralized with 1.4 ml of 1 M K-PO_4 buffer (*p*H 7.0) and extracted with 5 ml of cyclohexanol. OD values of the cyclohexanol extracts were read at 270 nm.

did not interfere with the reduction of INT or NBT when NADH served as the electron donor (Table 5). On addition of conenzyme Q in the absence of tirandamycin, reduction of both INT and NBT was stimulated to an extent of 15 to 20% above the control values. This small stimulatory effect of added coenzyme Q was slightly reversed by tirandamycin in the case of INT and fully reversed in the case of NBT. This indicates that tirandamycin interferes only negligibly with the mitochondrial NADH-linked reduction of INT and NBT.

Succinate-linked reduction of INT was stimulated by 44% and succinate-NBT reductase was stimulated by 28% in the presence of coenzyme Q (Table 5). In the absence of coenzyme Q, reduction of both salts was inhibited significantly (INT = 54%, NBT = 36%) by 200 μg of tirandamycin per ml. Upon addition of coenzyme Q, tirandamycin inhibited the reactions somewhat less than in the absence of added coenzyme Q. The actual values were 120% as compared to the control samples with INT and 75% with NBT. Tirandamycin thus appears to interfere with electron transfer at a point beyond the reduction site of coenzyme Q within the respiratory chain.

Cytochrome c reductase. Cytochrome *c* reductase activity in mitochondria was assessed spectrophotometrically by reading the OD of reduced cytochrome *c* at 550 nm. Some reduction of cytochrome *c* occurred in the absence of mitochondria in the NADH-driven test system (Fig. 3). Mitochondrial NADH-linked reduction

TABLE 5. *Effect of tirandamycin on NADH or succino-tetrazolium salt reductase in mitochondria*[a]

Sample	Reaction time	ΔOD_{490} or ΔOD_{530}	Per cent of control
	min		
NADH + INT			
Control	2.5	.637	100
Plus tirandamycin (200 μg/ml)	2.5	.605	94.9
Plus coenzyme Q (0.2 mg/ml)	2.5	.765	120.0
Plus coenzyme Q + triandamycin	2.5	.750	117.7
NADH + NBT			
Control	3	.251	100
Plus Tirandamycin (200 μg/ml)	3	.250	99.6
Plus coenzyme Q (0.2 mg/ml)	3	.294	117.1
Plus coenzyme Q + tirandamycin	3	.242	96.4
Succinate + INT			
Control	5	.421	100
Plus tirandamycin (200 μg/ml)	5	.193	45.8
Plus coenzyme Q (0.2 mg/ml)	5	.605	143.7
Plus coenzyme Q + tirandamycin	5	.507	120.4
Succinate + NBT			
Control	5	.173	100
Plus tirandamycin (200 μg/ml)	5	.111	64.1
Plus coenzyme Q (0.2 mg/ml)	5	.222	128.3
Plus coenzyme Q + tirandamycin	5	.128	73.9

[a] The reaction mixtures contained in a total volume of 1 ml: 40 μmoles of Tris-hydrochloride buffer (*p*H 7.5), 1 μmole of ethylenediamine tetraacetic acid, 3 μmoles of tetrazolium salt, 1 μmole of KCN, 3 μmoles of NADH or 5 μmoles of sodium succinate, and 0.5 mg of mitochondrial protein. The reactions were run at 30 C and stopped by adding 4.7 ml of Triton-sodium formate buffer as described by Lester and Smith (3). The OD values of the samples containing INT were read at 490 nm; those containing NBT were read at 530 nm.

of cytochrome *c* was slightly inhibited by 200 μg of antibiotic per ml (Fig. 3). In the presence of 100 μg of tirandamycin per ml, inhibition was negligible. Antimycin (20 μg/ml) inhibited cytochrome *c* reduction only moderately. Antimycin and tirandamycin (200 μg/ml) combined

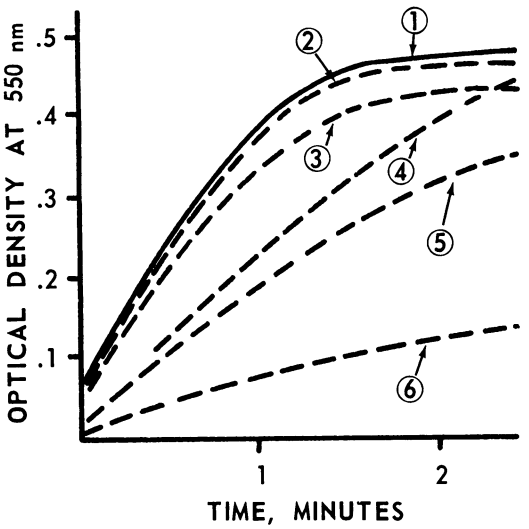

FIG. 3. *NADH-linked reduction of cytochrome c by rat liver mitochondria. The reaction mixtures contained, in a total volume of 3 ml: 150 μmoles of Tris-hydrochloride (pH 7.4), 575 μmoles of sucrose, 60 μg of antimycin A, 3 μmoles of KCN, 1.5 mg of cytochrome c (type III, Sigma), 15 μmoles of NADH, and 0.2 mg of mitochondrial protein. The reactions were started by the addition of NADH. The blanks contained all of the reaction mixture components above except NADH. (1) Control; (2) tirandamycin, 100 μg/ml; (3) tirandamycin, 200 μg/ml; (4) antimycin; (5) antimycin plus tirandamycin, 200 μg/ml; (6) no mitochondria.*

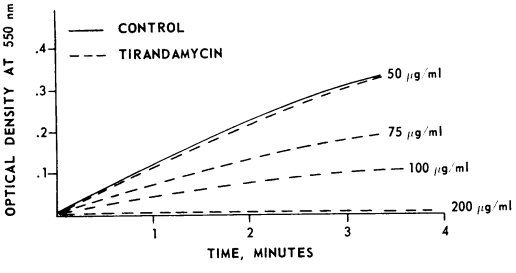

FIG. 4. *Succinate-linked reduction of cytochrome c by rat liver mitochondria. The reaction mixtures contained, in a total volume of 3 ml: 150 μmoles of Tris-hydrochloride (pH 7.4), 600 μmoles of sucrose, 3 μmoles of KCN, 1.5 mg of cytochrome c, 50 μmoles of succinate, and 0.4 mg of mitochondrial protein. The reactions were started by the addition of succinate. The reaction mixtures were read against blank cuvettes containing all of the reaction mixture components except substrate.*

proved more inhibitory than either of these agents alone. This suggests a possible additive effect of antimycin and tirandamycin. The succinate-linked reduction of cytochrome *c* was completely blocked by 200 μg of antibiotic per ml (Fig. 4). Concentrations of 100 and 75 μg/ml caused correspondingly less inhibition and 50 μg/ml had no effect. Succinate-cytochrome *c* reductase was also completely blocked by antimycin (Fig. 5). A combination of antimycin and tirandamycin caused complete cessation of the reaction. Complete inhibition was also observed with tirandamycin concentrations which, in the absence of antimycin, caused only partial inhibition of the system.

Cytochrome c oxidase. Cytochrome *c* oxidation remained unaffected by 200 μg of antibiotic per ml (Fig. 6).

Mitochondrial difference spectra. When either NADH or succinate was used as an electron donor, the control spectra showed distinct absorption peaks at 430 nm characteristic for

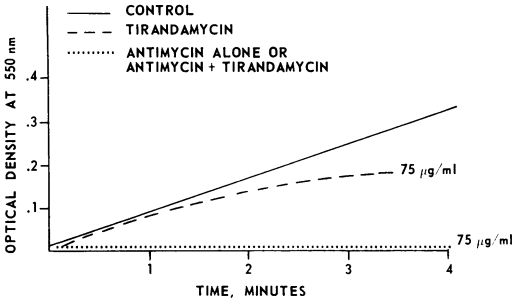

FIG. 5. *Succinate-linked reduction of cytochrome c by rat liver mitochondria in the presence of antimycin. Experimental details are identical with those described in the legend to Fig. 4. Antimycin A was added to yield a concentration of 60 μg/ml.*

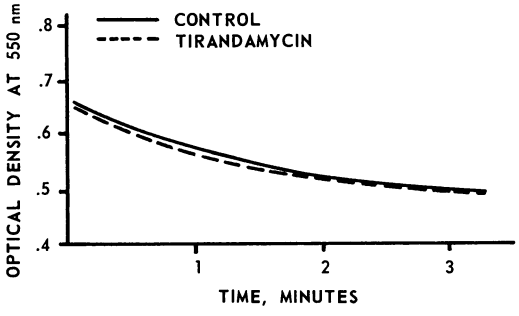

FIG. 6. *Oxidation of reduced cytochrome c by rat liver mitochondria. The reaction mixtures contained, in a total volume of 3 ml: 150 μmoles of Tris-hydrochloride (pH 7.4), 550 μmoles of sucrose, 600 μg of tirandamycin, 0.6 mg of reduced cytochrome c, 3 μmoles of KCN, and 0.5 mg of mitochondrial protein. The reactions were started by the addition of mitochondria. The reaction mixtures were read against blank cuvettes containing all of the reaction mixture components except mitochondria.*

reduced cytochrome *b* (Fig. 7). The 445-nm peaks of the type *a* cytochromes and the 550 nm peaks of the type *c* cytochromes were also clearly discernible with both substrates. The corresponding difference spectra run in the presence of 400 μg of tirandamycin per ml showed a strong absorption peak at 430 nm characteristic for reduced cytochrome *b*. However, the absorption peaks at 445 nm (cytochrome *a*) and 550 nm (cytochrome *c*) were absent, indicating that no reduction of cytochromes *a* or *c* had occurred. The α-bands for reduced cytochromes *b* and *a* were not or were only marginally visible, owing to the low resolution of the spectrophotometer. For comparison, the spectra obtained with antimycin are also shown in Fig. 7. Antimycin interferes with the respiratory chain after the reduction site of cytochrome *b* and prior to the reduction site of cytochrome *c*. The close similarity of the difference spectra obtained with antimycin and tirandamycin suggests that tirandamycin interferes with electron transfer at a point close or identical to the antimycin inhibition site. Difference spectra obtained in the presence of both antibiotics (not shown in the figure) were essentially identical to the ones obtained with antimycin alone.

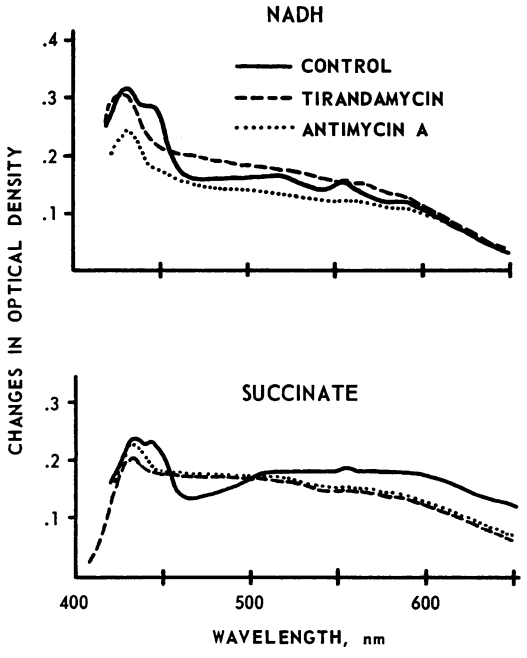

FIG. 7. *Mitochondrial difference spectra with NADH and succinate. The reaction mixtures contained, in a total volume of 3 ml: 200 μmoles of K-PO₄ buffer (pH 7.0), 500 μmoles of sucrose, 37.5 μmoles of NADH or 125 μmoles of succinate, 10 mg of mitochondrial protein, 400 μg of tirandamycin per ml, and 20 μg of antimycin A per ml where applicable. The spectra were read immediately after addition of substrate against a blank cuvette containing all of the reaction mixture ingredients mentioned above except substrate.*

DISCUSSION

Tirandamycin inhibits respiration associated with the oxidation of the nicotinamide adenine dinucleotide-linked substrates glutamate, citrate, and malate. In respect to respiration, reasonably strict dose-response relationships were obtained with each of these substrates. Complete (100%) uncoupling of respiration-associated phosphorylation occurred during glutamate oxidation at a tirandamycin concentration of 200 μg/ml. Lower concentrations did not impair phosphorylation. During the oxidation of citrate and malate, uncoupling was almost complete at 200 μg of antibiotic per ml. However, lower drug concentrations also caused substantial uncoupling. Respiration was less severely inhibited by tirandamycin during the oxidation of the non-Krebs cycle-related substrate β-hydroxybutyrate and uncoupling of phosphorylation was also reduced.

During the oxidation of succinate, respiration was inhibited to a similar extent as observed during the oxidation of glutamate, citrate, or malate. Phosphorylation was completely abolished at an antibiotic concentration of 200 μg/ml; lower concentrations caused correspondingly less inhibition. Fifty micrograms of tirandamycin per ml did not affect phosphorylation. Respiration during NADH oxidation was reduced by approximately 50% at all drug concentrations tested. Under nonphosphorylating conditions, respiration during glutamate oxidation was inhibited by tirandamycin. This inhibition was partially reversed by dinitrophenol.

In general, phosphorylation was inhibited to a larger extent than respiration at a given drug concentration. Tirandamycin thus seems to be more effective as an uncoupling agent than as an inhibitor of respiration.

NADH-tetrazolium salt reductase was only negligibly impaired by tirandamycin regardless of the presence of coenzyme Q. By contrast, succinate-tetrazolium salt reductase was inhibited substantially in the absence of added coenzyme Q but somewhat less in the presence of extraneous coenzyme Q. NADH-linked reduction of cytochrome *c* was partially inhibited by tirandamycin. A combination of antimycin and tirandamycin resulted in a further increase of inhibition, suggesting an additive effect between these two inhibitors. Succinate-cytochrome *c* reductase was completely blocked by tirandamycin. These

results indicate that the specific site of interaction of tirandamycin within the respiratory chain in mitochondria is located beyond the coenzyme Q reduction region and prior to the cytochrome *c* reduction site. Mitochondrial difference spectra indicated that cytochrome *b* reduction was not impaired, but no reduction of cytochromes *c* or *a* was evident. We thus conclude that the antibiotic interferes with electron transfer in the respiratory chain beyond the cytochrome *b* reduction site and prior to the cytochrome *c* reduction site. The similarity of the tirandamycin mitochondrial difference spectra with the spectra obtained with antimycin suggests further that the region of interference is either located close to or at the antimycin inhibition site. The observed additive action of antimycin and tirandamycin in the cytochrome c reduction assay substantiates this conclusion. The reason for the much higher activity of the antibiotic toward some of the succinate-linked reactions is difficult to interpret at this time. It is generally assumed that the electron flow in the respiratory chain for both NADH and succinate follows a common pathway after the flavoprotein region. However, the results described above would suggest slightly different pathways for the two substrates.

It is of interest that tirandamycin inhibits two functionally different cellular synthetic processes such as RNA synthesis and oxidative phosphorylation. One can assume that tirandamycin interacts with two unrelated enzymes or enzyme complexes, namely RNA polymerase and mitochondrial cytochrome reductase. Another possibility to consider would be that active RNA synthesis in mitochondria is an essential prerequisite for the proper functioning of the respiratory chain.

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