THE ACTION OF STREPTOMYCIN

IV. Fatty Acid Oxidation by Mycobacterium tuberculosis, Avian Type

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It has been shown that streptomycin inhibits the *in vitro* growth of *Mycobacterium tuberculosis*, avian type (Youmans and Feldman, 1946), and is effective against avian tuberculosis in chicks (Edison *et al.*, 1948; Solotorovsky, Siegel, Bugie, and Gregory, 1949). The studies reported in this paper were conducted to determine the site of action of the antibiotic on the metabolic pattern of this organism.

Bernheim and Fitzgerald (1947) demonstrated that streptomycin inhibits the oxidation of benzoic acid by a streptomycin-sensitive acid-fast organism designated as *M. tuberculosis* ATCC 607, but not by a streptomycin-resistant derivative of this strain. Later studies (Fitzgerald and Bernheim, 1947, 1948; Fitzgerald *et al.*, 1948) showed a similar streptomycin effect with other nonpathogenic mycobacteria. No such marked streptomycin inhibition was observed on the oxidation of palmitic acid, acetic acid, or various carbohydrates. These authors suggested that streptomycin inhibits the formation of adaptive enzymes for benzoic acid oxidation. Umbreit (1949) and Oginsky, Smith, and Umbreit (1949) have shown that the action of streptomycin on sensitive strains of *Escherichia coli* appears to be on the oxalacetate-pyruvate condensation, and that strains rendered resistant to streptomycin have lost this condensation mechanism (Smith, Oginsky, and Umbreit, 1949).

Fatty acid oxidation by mycobacteria has been demonstrated for *M. tuberculosis*, strain H37R, by Loebel, Shorr, and Richardson (1933) and for several other types of the mycobacteria by Franke and Schillinger (1944) and by Gray (1949). In the oxidation of fatty acids by animal tissue a condensation between oxalacetate and a labile 2-carbon intermediate is apparently involved (Gurin and Crandall, 1948; Breusch, 1948). Previous studies (Oginsky, Smith, and Umbreit, 1949) have shown that a similar condensation is sensitive to streptomycin. Therefore the influence of streptomycin on fatty acid oxidation in *M. tuberculosis*, avian type, was studied.

METHODS

The streptomycin-sensitive culture of *M. tuberculosis*, avian type, strain Kirchberg, has been maintained in Dubos' sorbitan monooleate albumin medium. The resistant strain, designated as MT no. 24, was derived therefrom by repeated subculture in Dubos' medium containing increasing concentrations of streptomycin.[1] The limiting streptomycin concentration permitting growth was 5 μg for

[1] This resistant culture was developed by Mr. Francis J. Gregory.

the sensitive, and 1,000 μg per ml for the resistant, strain. The organisms were grown in Dubos' medium for approximately 7 days and were centrifuged, washed, and suspended in distilled water to give a concentration of 0.5 mg bacterial nitrogen per ml. The cell suspensions were stored in the refrigerator and used within 3 days after harvesting.

The experiments were done by the conventional manometric methods at 37 C in air. One ml of bacterial suspension was added per 3 ml total fluid volume. All experiments were performed with 0.003 M phosphate, pH 7, and substrates were added as the sodium salts at the same pH. The higher (C_{10} to C_{18}) fatty acid solutions were freshly prepared for each experiment, because they were found to be unstable during refrigeration. Streptomycin hydrochloride (Merck) rather than the calcium salt was used in order to avoid any precipitation of the insoluble fatty acid salt. Except when noted, streptomycin was added to yield a final concentration of 33 μg of free base per ml. The antibiotic was added 30 minutes prior to the addition of substrate.

RESULTS

Attempts were made to extend the studies on streptomycin inhibition of the oxalacetate-pyruvate condensation to the streptomycin-sensitive Kirchberg, strain of *M. tuberculosis*, avian type. As shown in table 1, pyruvate, oxalacetate, pyruvate plus oxalacetate, or various other members of the citric acid cycle were oxidized only slightly. All tables and figures are corrected for endogenous respiration. At the concentration used, streptomycin exhibits a slight depressing effect on endogenous respiration. No significant increase in oxygen uptake was obtained by holding the cell suspensions in citrate, *cis*-aconitate, or succinate solutions in the refrigerator for 3 days previous to the determination of oxygen utilization, in an effort to enhance the penetration of the substrate. The other substrates listed in table 1 were not oxidized to any appreciable extent. Without the use of cell-free enzyme preparations, it is difficult to determine whether the low oxygen uptake figures shown in table 1 are due to slow penetration or to the lack of appropriate enzymes. We were unable to repeat the results of Fitzgerald and Bernheim (1947, 1948) on benzoic acid oxidation because the strain of *M. tuberculosis* used in this study did not oxidize benzoic acid at a rate sufficiently high to allow the determination of a significant effect of streptomycin.

The results given above indicated that studies on streptomycin inhibition would require the demonstration of a more active oxidative system, which might be related in fundamental mechanism to the oxalacetate-pyruvate condensation in *E. coli*. Franke and Schillinger (1944) have shown that oxidation of fatty acids is characteristic of the mycobacteria, and that this system is generally more active than those for oxidation of hydroxy-, keto-, or amino acids. Since the mechanism for oxidation of lower fatty acids by animal tissues is considered to involve an oxalacetate-"acetate" condensation followed by oxidation through the citric acid cycle, it was thought that streptomycin might interfere with the oxidation of fatty acids by *M. tuberculosis*, avian type.

Cell suspensions of this organism were therefore allowed to oxidize the even-

numbered saturated fatty acids from C_2 to C_{18}, in the presence and absence of streptomycin. Two μM of substrate were used per cup, since preliminary experiments showed that the use of higher concentrations gave erratic results, because higher concentrations were occasionally oxidized very slowly; this observation is in agreement with the experiments of Grafflin and Green (1948) with kidney homogenates. The data are shown in figure 1. The oxidation of the lower fatty acids (C_2 to C_{10}) was not affected by streptomycin, but that of lauric, palmitic, and stearic acids was partially inhibited. Myristic acid, at the concentration used, produced an inhibitory effect on endogenous respiration. Since the basic problem was on the action of streptomycin, it seemed advisable to explore in detail only the oxidation of the long chain fatty acids. It was thought possible

TABLE 1

Oxidation of various substrates by M. tuberculosis, avian type, Kirchberg strain

SUBSTRATE	AMOUNT ADDED	TOTAL O_2 UPTAKE IN 270 MINUTES	
		Without streptomycin	With streptomycin
	μM	μl	μl
Pyruvate	10	43	41
Oxalacetate	20	50	52
Pyruvate + oxalacetate	10, 20	48	46
cis-Aconitate	10	4	
Citrate	10	3	
α-Keto-glutarate	10	7	
Succinate	10	6	
Fumarate	10	1	
Aspartate	30	24	
Asparagine	30	22	15
Glutamate	10	5	
Lactate	10	35	
Acetate	10	32	41
Glucose	10	20	
Benzoate	10	24	18

that, in this organism, there might be two alternative mechanisms for the oxidation of fatty acids: one for chain length C_2 to C_{10}, and possibly to C_{18}, unaffected by streptomycin, and one for C_{12} to C_{18}, sensitive to streptomycin. Several aspects of the problem were investigated as described below.

Sensitive Strain

Nature of the reaction inhibited. Typical oxidation curves for 2 μM of lauric, palmitic, and stearic acids in the presence and absence of streptomycin are shown in figure 2. The initial rate of oxidation is approximately the same for all three substrates. This rate decreases after a few hours and reaches a constant low level. The level of oxygen uptake and the time at which this decrease occurs depends somewhat upon the individual cell suspension, but mainly on the presence or absence of streptomycin. At 2 μM of substrate, the break in the oxida-

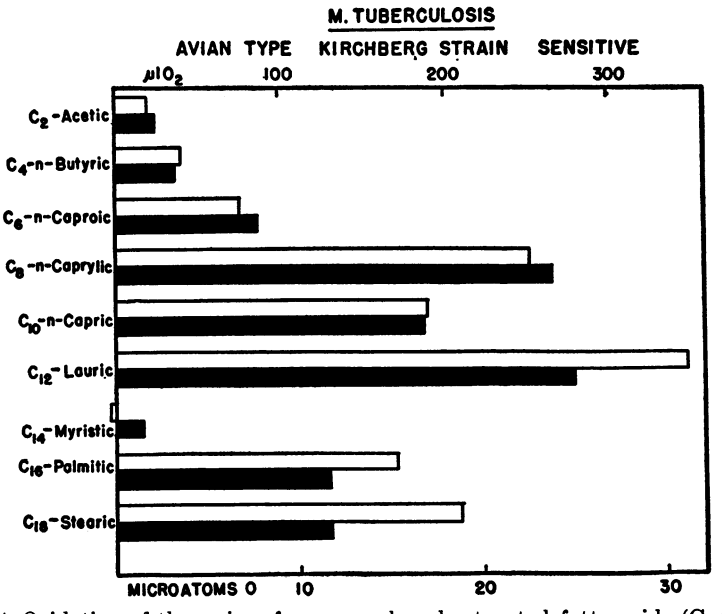

Figure 1. Oxidation of the series of even-numbered saturated fatty acids (C_2 to C_{18}) by *M. tuberculosis*, avian type, Kirchberg strain: total oxygen uptake 270 minutes after substrate addition. One ml cell suspension (equivalent to 0.5 mg nitrogen); 2 μM fatty acid; 100 μg streptomycin added as the hydrochloride (Merck); 0.1 ml M/10 phosphate buffer, pH 7; distilled water to bring total fluid volume to 3 ml. White bars: absence of streptomycin; black bars: presence of streptomycin.

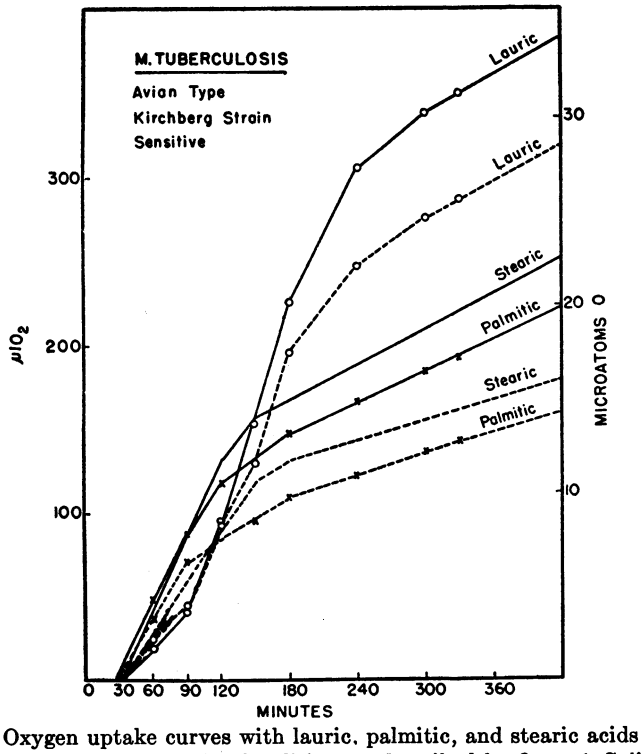

Figure 2. Oxygen uptake curves with lauric, palmitic, and stearic acids for *M. tuberculosis*, avian type, Kirchberg strain. Conditions as described for figure 1. Solid lines: absence of streptomycin; dashed lines: presence of streptomycin.

tion rate occurs between 90 and 180 minutes after the substrate addition. The average level of oxygen uptake at that time in the presence and absence of streptomycin is shown in columns 1 and 2 of table 2. With higher substrate concentrations the oxygen uptake per mole of substrate is essentially the same, although a longer time is required before the break in oxidation rate is observed. Thereafter the cells respiring in streptomycin have a lowered oxidation rate than those respiring in its absence and the two curves become increasingly divergent.

The observation that the rate of oxidation with the higher fatty acids falls off while still far below the level of oxygen uptake required for complete oxidation of the carbon chain to carbon dioxide indicated that further oxidation of the chain was blocked. This could be due to the accumulation of toxic end products or to the formation of a nonoxidizable shorter chain acid. The possibility of toxic end products was eliminated by the observation that if fresh palmitic or stearic acid is added to a cell suspension respiring on the same substrate after the rate

TABLE 2

Comparison of actual and theoretical oxygen uptake with higher fatty acid substrates

SUBSTRATE	ACTUAL OXYGEN UPTAKE (ATOMS/MOLE SUBSTRATE) AT BREAK IN OXIDATION RATE		THEORETICAL OXYGEN UPTAKE (ATOMS/MOLE SUBSTRATE) TO VARIOUS STAGES OF OXIDATION			
	Without streptomycin	With streptomycin	CO_2	C_2 cpds.	$C_{16} + CO_2$	$C_{16} + C_2$ cpds.
	(1)	(2)	(3)	(4)	(5)	(6)
Lauric acid	13.5	10	34	10	—	—
Palmitic acid	4	3	46	14	6	2
Stearic acid	6	4.5	52	16	12	4

decrease has occurred, the newly added fatty acid is oxidized as rapidly as by control cell suspensions. The myristic acid, which would be formed by splitting off two carbons from palmitic, or four carbons from stearic, acid, may be as unsusceptible to oxidation by *M. tuberculosis*, avian type, as is the equivalent amount of myristic acid when added as the primary substrate (see figure 1). It was thought that the rate decrease observed with stearic and palmitic acid might be due to the accumulation of nonoxidizable concentrations of myristic acid. The oxidation of lower concentrations (below 2 μM) of this acid is considerable, however, and is streptomycin-sensitive. Nevertheless, it is possible that the myristic acid added externally is not entirely comparable to that produced inside the cell. A comparison with these acids was therefore made between the actual oxygen uptake figures at the rate break and the theoretical figures for various stages of oxidation, presented in table 2. In the absence of streptomycin, oxygen uptake is greater than could be accounted for by the formation of myristic acid and the 2-carbon fragments, although it does not reach the uptake required for subsequent complete oxidation of the 2-carbon fragments to CO_2. (Compare columns 1, 5, and 6, table 2.) In the presence of streptomycin, oxida-

tion proceeds only slightly beyond that required for the formation of myristic acid and the 2-carbon fragments. (Compare columns 2 and 6, table 2.) Other suggestive evidence that myristic acid is a limiting factor in the oxidation rates of higher fatty acids is the data obtained with *E. coli*, which will be reported later in this paper. Attempts at the separation of myristic, palmitic, and stearic acids by paper chromatography in the hope of identifying them in respiring suspension-substrate mixtures have not been successful. There are, therefore, some indications that the oxidation of palmitic and stearic acids by *M. tuberculosis*, avian type, stops because of the accumulation of myristic acid.

Attempts were made to determine the pathway of fatty acid oxidation in this organism. The general conception of the oxidation of fatty acids by animal tissues involves oxidation at the β-carbon with the formation of 2-carbon intermediates, which either condense to form acetoacetate or condense with oxalacetate yielding citrate (Gurin and Crandall, 1949). These pathways are not mutually exclusive since Floyd, Medes, and Weinhouse (1947), using C^{13}-labeled acetoacetate with rat muscle and brain tissue, have demonstrated the accumula-

TABLE 3

Comparison of Q_{O_2} (N) and Q_{CO_2} (N) during oxidation of palmitic and stearic acids

SUBSTRATE	STREPTOMYCIN	1ST HOUR		3RD HOUR		5TH HOUR	
		Q_{O_2} (N)	Q_{CO_2}(N)	Q_{O_2} (N)	Q_{CO_2} (N)	Q_{O_2} (N)	Q_{CO_2}(N)
Palmitate	−	170	88	60	30	14	14
	+	160	81	42	4	10	2
Stearate	−	184	88	42	20	48	18
	+	126	48	36	16	32	14

tion of labeled citric acid, formed by the condensation with oxalacetate of a 2-carbon intermediate obtained from acetoacetate. Most of the available data on animal tissues refer to the oxidation of fatty acids of lower chain length (C_4 to C_8). Reaction mechanisms applicable to these acids may not hold for acids of greater chain length. Indeed, Kennedy and Lehninger (1949) have reported that acetoacetate is formed by rat liver preparations in appreciable quantities only from the lower fatty acids, and that its formation from higher fatty acids decreases with increasing chain length.

It was, therefore, of interest to determine whether the pathway of oxidation in the acid-fast organism was compatible with the concepts applicable to the oxidation of the lower fatty acids by animal tissues. According to these concepts, the formation of the 2-carbon intermediate from the fatty acid involves oxygen utilization, but no carbon dioxide is released until this intermediate is further metabolized. Since streptomycin was previously found to inhibit the oxalacetate-pyruvate condensation (Oginsky, Smith, and Umbreit, 1949), it might be expected that the inhibition of a similar reaction in fatty acid oxidation would lead to a marked reduction in the evolution of carbon dioxide.

However, the data of table 3 show that, during the early phases of the reaction,

carbon dioxide production from both palmitic and stearic acid was about one-half the oxygen uptake and was not greatly influenced by streptomycin. During the later phases there was a reduction in the evolution of carbon dioxide from palmitic acid by streptomycin, but this effect was not observed with stearic acid. There is no significant inhibition by streptomycin of the carbon dioxide evolution during the early stages of oxidation, as would be expected if the concepts outlined in the previous paragraph were applicable.

Saz (1949) has reported that the tubercle organism oxidizes saturated hydrocarbons. It was considered that the carbon dioxide might possibly have been formed by terminal decarboxylation of the fatty acid, and that subsequent oxidation was on the resulting hydrocarbon chain. However, if the experiments were conducted under nitrogen instead of air, no carbon dioxide was evolved. The hypothesis above does not therefore appear to be valid.

Attempts to implicate acetoacetate or citrate as intermediates in the oxidation of fatty acids by this organism were not successful. Acetoacetate is oxidized slowly, and the rate is not affected by the presence of either oxalacetate or streptomycin. No acetoacetate formation during either caprylic or stearic acid oxidation could be demonstrated by the use of either aniline-citrate (Edson, 1935) or nitroprusside (Folin, 1934) methods. However, small quantities of acetoacetate might have escaped detection. Since citric acid is also slowly oxidized by these bacteria, it was considered that citrate might accumulate as a product of fatty acid, or indirectly of acetoacetate, metabolism. The method of Saffran and Denstedt (1948), which is positive for *cis*- and *trans*-aconitic acids as well as for citric acid, gave completely negative results. Further, attempts to increase the rate of stearic acid oxidation by the addition of oxalacetate, or of aspartic acid or asparagine as sources of oxalacetate, were also not successful, either because these did not penetrate the cell or because the internal supplies were already adequate. Grafflin and Green (1948) and Knox, Noyce, and Auerbach (1948) have found that the presence of members of the citric acid cycle is required for complete fatty acid oxidation. However, it is apparent that so far no positive evidence has been obtained that would support a conception that the oxidation of the higher fatty acids by this organism follows pathways comparable to those involved in the oxidation of the lower fatty acids by animal tissues.

The site of action of streptomycin on the oxidation of higher fatty acids by *M. tuberculosis*, avian type (and, as described later, by *E. coli*), has not been clearly defined by these experiments. In the light of the evidence available, however, the oxidation of the breakdown products of the fatty acids, rather than the oxidation of the chain itself, appears to be the sensitive mechanism. If acetoacetic acid is formed as a product, its oxidation is apparently not involved in streptomycin inhibition. It is possible that the oxidation of a 2-carbon compound, related to, or identical with, "active acetate," is blocked. The possibility of different forms of such a reactive 2-carbon compound has been discussed in a previous paper (Oginsky, Smith, and Umbreit, 1949), and it is possible that the "active acetate" derived from acetate or acetoacetate differs from that derived from higher fatty acids. The action of streptomycin on both the oxalacetate-pyruvate condensation

and the fatty acid oxidation suggests that the same, or closely related, 2-carbon compounds may be involved in both reactions.

Time of streptomycin addition. Earlier experiments with *E. coli* and the oxalacetate-pyruvate condensation (Oginsky, Smith, and Umbreit, 1949) showed that the time of streptomycin addition was an important factor in determining the magnitude of inhibition, since the streptomycin added 30 minutes after the substrate was much less effective than that added 30 minutes before.

In order to determine whether a time factor was involved in the amount of streptomycin inhibition of stearate oxidation by *M. tuberculosis*, avian type, the following experiment was conducted: Thirty-three μg streptomycin per ml were tipped into the Warburg cup 30 minutes before, 30 minutes after, or at the moment when, two μM of stearate were added to the cell suspension. The inhibition obtained after 185 minutes with each procedure is shown in table 4. In contrast to the results with *E. coli* mentioned above, considerable inhibition of the sensitive system occurred even if the streptomycin was added after the substrate. This effect was evident within 30 minutes after the addition of streptomycin.

TABLE 4

Effect of addition time on streptomycin inhibition of stearate oxidation

STREPTOMYCIN (TIME ADDED)	TOTAL O_2 UPTAKE, 185 MIN	% INHIBITION	Q_{O_2} (N) FOURTH HOUR
	μl		
None added	197	0	48
30 minutes before	146	25.9	32
Same time	153	22.3	28
30 minutes after	175	11.3	32

According to the theory discussed in the preceding section, it is the oxidation of the fatty acid chain that produces the substrate for the streptomycin-sensitive reaction, so that in effect the substrate of the sensitive reaction is being added continuously, rather than at zero time. By the fourth hour, the cells in contact with streptomycin all show approximately the same Q_{O_2} (N), regardless of the time of streptomycin addition.

Specificity of inhibitor. Various derivatives of streptomycin obtained from Dr. R. L. Peck of the Research Laboratories of Merck and Company, Inc., were tested for their ability to inhibit the oxidation of stearic acid. These are as follows: streptamine, streptidine, strepturea, dideguanyl-dihydrostreptomycin, dipyridino-dihydrostreptomycin, streptomycin oxime, streptomycin hydroxylamine, and dihydrostreptomycin. The only derivative that showed significant activity was dihydrostreptomycin. This substance, which is as effective *in vitro* and *in vivo* as streptomycin but is less neurotoxic (Edison *et al.*, 1948), exhibited no inhibitory activity until 120 minutes, by which time the streptomycin activity was pronounced. After 120 minutes, however, the oxygen uptake in the presence of either streptomycin or its dihydro derivative proceeded at the same rate. It would appear that dihydrostreptomycin penetrates into these bacteria

more slowly, as it does into rat liver mitochondria (Umbreit and Tonhazy, 1949).

p-Aminosalicylic acid (PAS) has been reported to have bacteriostatic activity *in vitro* against *M. tuberculosis* (Lehmann, 1946; Vennesland, Ebert, and Block, 1948) and to exhibit a similar effect *in vivo* (Lehmann, 1946). Graessle and Pietrowski (1949) found that PAS prevents the *in vitro* development of streptomycin resistance by *M. tuberculosis*, strain H37Rv, in Dubos' sorbitan monooleate albumin medium. It was therefore of interest to determine whether this compound affects the action of streptomycin on fatty acid oxidation by *M. tuberculosis*, avian type. No increased inhibitory effect on oxygen uptake was noted when PAS at a concentration of 1 mg per ml was added to flasks containing 2 μM stearic acid, in the presence or absence of 2.4 μg per ml of streptomycin. This concentration of antibiotic is itself too low for complete inhibition of growth, or for maximum activity on stearic acid oxidation. Actually, oxygen uptake in flasks containing both PAS and streptomycin was slightly higher than in those containing only streptomycin.

Streptomycin inhibition was not prevented by incubating the cells for 30 minutes previous to streptomycin addition with either *meso*-inositol or lipositol, prepared by Woolley's method (Woolley, 1943). A preparation of the latter compound has been reported to antagonize streptomycin inhibition (Rhymer *et al.*, 1947).[2]

Effect of streptomycin concentration. Increasing amounts of streptomycin were added to the cell suspensions 30 minutes before 10 μM of stearic acid were tipped in. The percentage of inhibition varied with the concentration, as shown in figure 3. The addition of 333 μg and 666 μg of streptomycin gave 40 and 44 per cent inhibition, respectively. At the level of streptomycin required to inhibit growth, i.e., 5 μg per ml, inhibition of stearic acid oxidation is pronounced.

Action on other fats and fatty acids. Tuberculostearic and phthioic acids were obtained through the generosity of Dr. R. J. Anderson of the Yale University School of Medicine. Experiments conducted with these acids under the conditions described in the preceding sections showed that tuberculostearic (10-methylstearic) acid oxidation followed the same type of curve as did stearic acid. The oxidation rate of phthioic acid ($C_{28}H_{52}O_2$) remained constant over the 240-minute observation period. Average streptomycin inhibition in two such experiments was 15 per cent with tuberculostearic acid and 31 per cent with phthioic acid.

Crude soybean lecithin[3] was dissolved in water as the sodium salt and tested by the usual procedures. This substrate was oxidized at a rate comparable to that obtained with the fatty acids, and here again streptomycin inhibition of oxidation occurred, equaling 16 per cent after 240 minutes. The lipositol fraction

[2] Since this paper was submitted for publication, Paine and Lipmann (J. Bact., **58**, 547, 1949) have reported the failure of various inositol phospholipids to show antistreptomycin activity when tested with *Escherichia coli* in growth studies.

[3] Provided by Central Soya Company, Decatur, Indiana, and designated "Centrol 4B lecithin."

isolated from this lecithin, for the studies on antagonism to streptomycin activity, was found to be itself oxidizable, and the reaction streptomycin-sensitive.

The unsaturated fatty acids, oleic and linoleic, were found to depress endogenous respiration when 2 μM were added to a bacterial suspension containing 0.5 mg nitrogen. Frank, Lee, and Kibat (1949) have recently reported on studies with *Mycobacterium rubrum*, showing that lower concentrations of oleic acid are more rapidly oxidized than higher ones, and Gray (1949) has reported similar results with *Mycobacterium phlei*. We have, however, made no attempt to extend the studies on streptomycin to the unsaturated fatty acids.

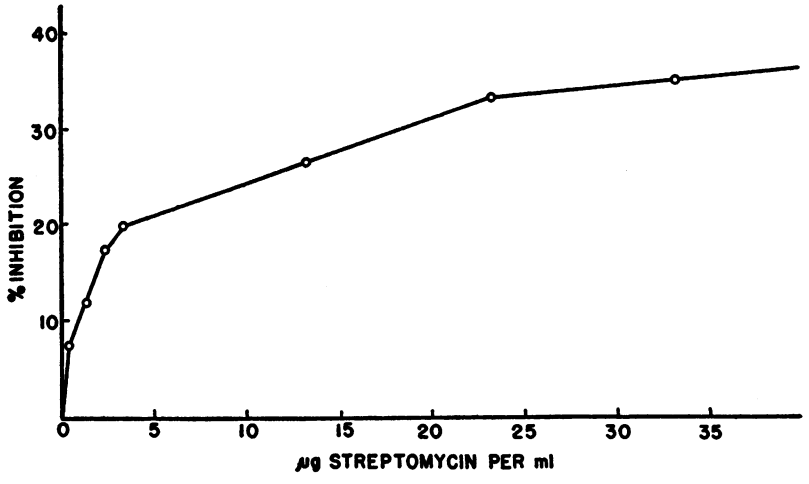

Figure 3. Percentage of inhibition by streptomycin of the oxidation of stearic acid by *M. tuberculosis*, avian type, Kirchberg strain: inhibition observed 300 minutes after substrate addition. One ml cell suspension (equivalent to 0.5 mg nitrogen); 10 μM stearic acid; streptomycin concentrations as noted in figure; 0.1 ml M/10 phosphate buffer, pH 7; distilled water to 3 ml.

Resistant Strain, MT No. 24

These cells were grown and suspensions made by the same procedures described for the sensitive strain, and the manometric methods were also identical. Actually, for many of the experiments discussed above, concurrent tests were run with the resistant organisms. For example, the results that are diagramed in figure 4 were obtained on the same day and with cells of the same age as those of figure 1 for the sensitive strain. It is apparent from examination of the data in figure 4 that the oxidation by resistant cells of the lower fatty acids was not affected by streptomycin. The oxidation of the higher acids was only slightly affected by a concentration of streptomycin (33 μg per ml) sufficient to cause marked inhibition with the sensitive cells. The resistant organisms for this experiment had been grown in the absence of streptomycin, and it was considered that the slight inhibition noted with the higher fatty acids might be due to the reversion of a small number of the cells to the sensitive state.

Accordingly, the resistant cells were grown thereafter in medium containing 100 μg per ml of streptomycin. When the resistant strain was grown in the presence of streptomycin, no inhibitory effect of streptomycin was noted in the manometric experiments. Data obtained with these cells on the oxidation of lauric, palmitic, and stearic acids in the presence and absence of streptomycin are presented in figure 5. These curves show that the oxidation of the three acids is practically the same with or without streptomycin. Similar experiments with tuberculostearic and phthioic acids demonstrated slight streptomycin inhibition

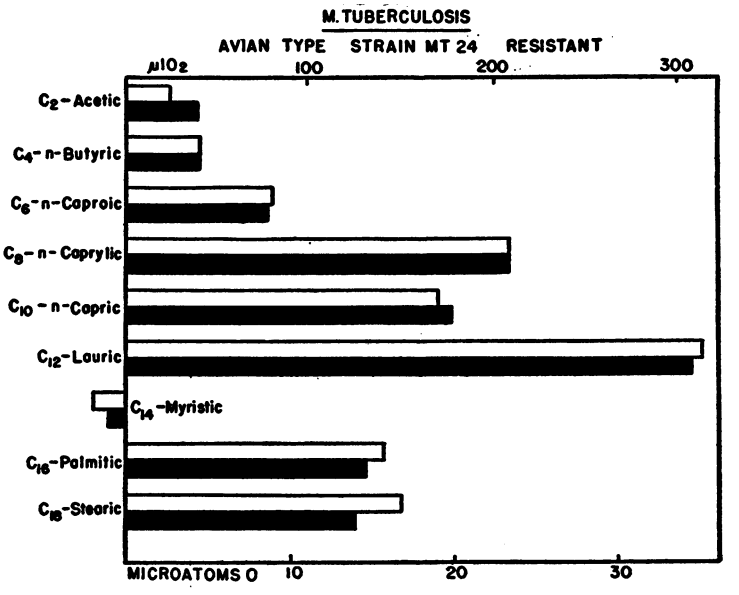

Figure 4. Oxidation of the series of even-numbered saturated fatty acids (C_2 to C_{18}) by *M. tuberculosis*, avian type, MT no. 24, resistant strain: total oxygen uptake 270 minutes after substrate addition. Conditions as described for figure 1. White bars: absence of streptomycin; black bars: presence of streptomycin.

of oxidation with the former and no inhibition with the latter acid. The oxygen uptake curves for resistant *M. tuberculosis*, avian type, with palmitic or stearic acids generally, but not always, lie near the curve of sensitive cells respiring in the presence of streptomycin.

Escherichia coli

E. coli was also found to oxidize stearate. It was therefore of interest to examine the effect of streptomycin on the oxidation of higher fatty acid by this organism.

The "Gratia" strain was employed, and the sensitive and resistant cultures were grown and the suspensions prepared by the methods reported previously (Oginsky, Smith, and Umbreit, 1949). Stearic acid was selected for study. Data obtained with the sensitive and resistant strains in the presence and absence of streptomycin are plotted in figure 6. The sensitive strain oxidized stearic acid after a lag period of about 1 hour, and thereafter the rate continued high for the

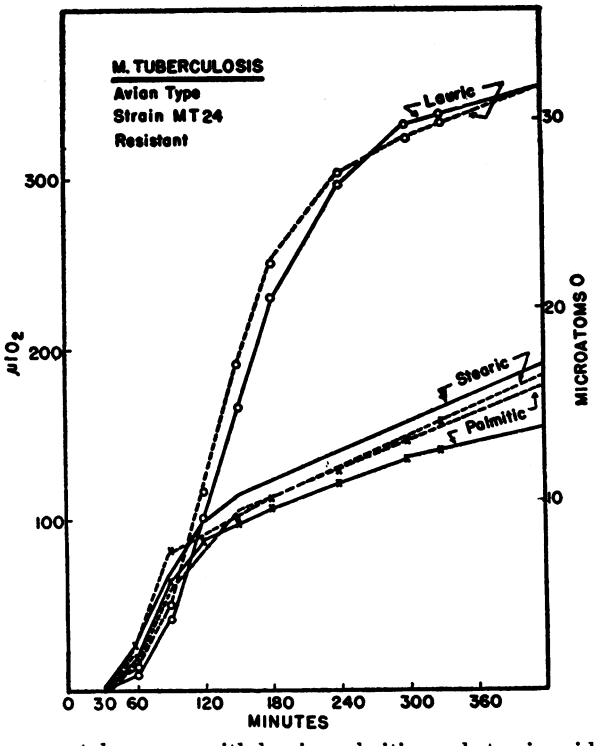

Figure 5. Oxygen uptake curves with lauric, palmitic, and stearic acids for *M. tuberculosis*, avian type, MT no. 24, resistant strain. Conditions as described for figure 1. Solid lines: absence of streptomycin; dashed lines: presence of streptomycin.

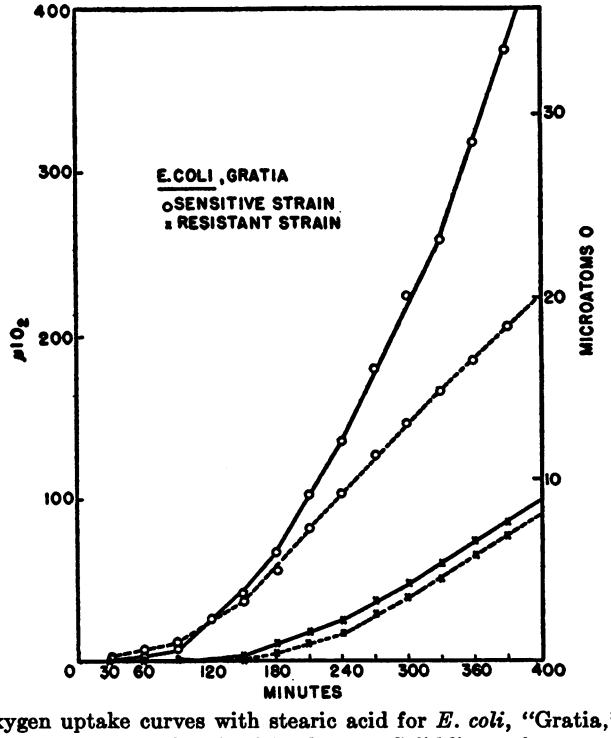

Figure 6. Oxygen uptake curves with stearic acid for *E. coli*, "Gratia," sensitive and resistant strains. Conditions as described for figure 1. Solid lines: absence of streptomycin; dashed lines: presence of streptomycin.

duration of the experiment. In the presence of streptomycin, the lag period was the same, but the rate of oxidation thereafter was lower than in the absence of streptomycin. On the other hand, the resistant strain oxidized the fatty acid only slightly, even after several hours' contact.

No increase in the oxidation rate of sensitive cells could be obtained by the addition of fumarate (as a source of oxalacetate), either at zero time or after oxidation was well advanced, nor did its addition influence the inhibition by streptomycin. Since the stearic acid oxidation appeared to be an adaptive function, according to the form of curve of the reaction (figure 6), the sensitive strain was grown in the usual medium plus 0.01 per cent stearic acid in an attempt to produce stearate-adapted cells. Contrary to expectations, these cells oxidized stearic acid only after a much longer lag period, and their oxygen uptake on this substrate was very low.

TABLE 5

Summary of reactions related to streptomycin

REACTION	STREPTOMYCIN	E. COLI		M. TUBERCULOSIS	
		Sensitive	Resistant	Sensitive	Resistant
Oxalacetate-pyruvate condensation	−	Present	Absent*	Absent*	Absent*
	+	Inhibited	—	—	—
Stearate oxidation	−	Toward completion; lag period	Markedly† depressed	Partial, immediate oxidation	Partial, immediate oxidation
	+	Inhibited	—	Inhibited	Not inhibited

* The term "absent" means that it has not been possible to demonstrate the reaction.

† A slow oxidation after a long lag period is evident, see text.

The oxidation of this fatty acid by *E. coli* follows a much different curve from that by *M. tuberculosis*, avian type, as is evident by a comparison of figures 2 and 6. We have suggested that the low oxidation of the C_{14}-myristic acid may be the reason for the rate decrease of stearic acid oxidation with *M. tuberculosis*, avian type. *E. coli*, which exhibits no such drop in oxygen uptake, was found capable of oxidizing 2 μM myristic acid at a rapid rate. Thus stearic acid oxidation apparently proceeds toward completion in this organism.

COMMENT

Because their meaning is not immediately apparent, the results obtained with resistant and sensitive strains of the two genera studied require additional comment. The salient points established in this and previous papers have been summarized in table 5. In *E. coli*, upon the development of resistance, the sensitive reaction has been essentially eliminated. Since the oxidation of stearate in this organism proceeds toward completion, it is presumably oxidized by a path-

way similar to the citric acid cycle. Previous data have shown that the resistant cell has dispensed with most of the reactions of this cycle. The inability of the resistant strain to oxidize stearate rapidly may be due to the lack of the cycle in these cells. The inhibition of stearate oxidation by streptomycin in the sensitive strain may result from inhibition of the entrance of the products of fatty acid oxidation into the cycle. The lag observed in the oxidation of stearate may be due to the necessity of accumulating the proper intermediates for entrance into the cycle rather than to the formation of adaptive enzymes, especially since growth studies do not show adaptive enzyme formation. The longer lag period and the slow oxidation in the resistant cell might be a reflection of stearate oxidation to the stage of the 2-carbon compound only. The latter substance would not be oxidized because of the lack of the citric acid cycle. In *E. coli*, therefore, the effect of streptomycin and of resistance can be interpreted, insofar as we know the reactions, as the result of inhibiting essentially the same reaction.

On the other hand, with *M. tuberculosis*, avian type, we have been able to obtain no positive evidence for a citric acid cycle. The oxidation of the stearate also proceeds by apparently a different, and unknown, pathway. Its oxidation begins immediately upon addition and does not proceed toward completion. Streptomycin appears to inhibit only the oxidation of the breakdown products of the fatty acid, but not the oxidation of the fatty acid molecule itself. With regard to resistance, the important point is whether or not the resistant strain has eliminated the sensitive reaction. The resistant strain could well be able to oxidize the higher fatty acids but only up to the level of the sensitive cells in the presence of streptomycin. Because of several considerations outlined below, we have been unable to obtain a significantly precise answer to this question. First, not all resistant cell suspensions of *M. tuberculosis*, avian type, have a lowered oxygen uptake with fatty acids, when tested against comparable sensitive cell suspensions. This fact is not of critical importance, however, as the oxidative ability of the suspensions varied somewhat from lot to lot—a variation that has been encountered by other investigators (Zetterberg, 1949)—and no single comparison of batches, even of the same age, may be valid. Second, the resistant strain was grown with streptomycin in later experiments. If sufficient streptomycin had been irreversibly absorbed during growth, the presence of additional streptomycin in the Warburg cup would make no difference in the respiration experiments, and both curves would represent oxygen uptake in the presence of streptomycin. We have not measured bacterial absorption of streptomycin from culture media, since the sensitivity of assay procedures is too low for accurate measurement at the quantities of streptomycin employed. However, even if resistant cells are grown in the absence of streptomycin, their oxidation of higher fatty acids is far less sensitive to inhibition by the antibiotic than is the oxidation by sensitive cells (see figures 1 and 4). Thus, it is so far impossible to decide whether the resistant *M. tuberculosis*, avian type, has eliminated the streptomycin-sensitive reaction. It is is evident that a more detailed knowledge of the reaction mechanism will be required. Particularly, the preparation of cell-free enzymes such as those reported by Geronimus *et al.* (1949) would be useful.

SUMMARY

A study of fatty acid oxidation by *Mycobacterium tuberculosis*, avian type, strain Kirchberg, showed that the oxidation of the higher fatty acids was partially inhibited by streptomycin. The oxidation did not proceed to completion. Streptomycin apparently inhibited the oxidation of the breakdown products of the fatty acids rather than the oxidation of the chain itself. The resistant strain derived from this organism also oxidized the fatty acids, but without comparable streptomycin inhibition.

Similar inhibition by this antibiotic of stearate oxidation by *Escherichia coli* was also observed, although it is evident that the two organisms oxidized this substance somewhat differently. Streptomycin-resistant *E. coli* had lost the major portion of the oxidative mechanism for higher fatty acids.

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