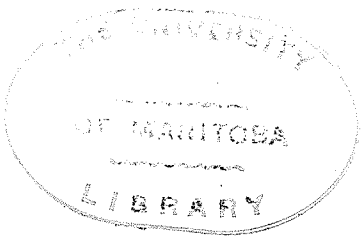
THE MODE OF ACTION
OF
STREPTOMYCIN



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ABSTRACT

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Manometric techniques were used for a direct measurement of the oxygen uptake of resting cell suspensions and cell free extracts of Agrobacterium tumefaciens, Agrobacterium radiobacter and Rhizobium meliloti in the presence of various substrates. The oxygen uptake was stimulated when cells of A. tumefaciens and A. radiobacter were treated with streptomycin. However, no stimulation was observed when cell free extracts were allowed to oxidize the same substrates in the presence of streptomycin. A. radiobacter was found to be impermeable to citrate. It was found that with streptomycin the cells were able to utilize citrate.

Oxidation of various substrates by resting cells of Rhizobium meliloti was stimulated, inhibited or unaffected by streptomycin. Streptomycin has no effect on the oxidation of various carbon sources by cell free extracts. The three organisms showed a slight inhibition in the oxidation of oxaloacetate and oxaloacetate plus pyruvate by cell free extracts in the presence of the antibiotic.

Streptomycin appears to affect the anabolic reactions in A. tumefaciens. The action of streptomycin on R. meliloti is bacteriostatic. A. radiobacter was capable to adapt themselves to the unfavourable environment and continue to reproduce in a normal manner. Antibacterial activity of streptomycin was shown to be greatly influenced by the composition of the medium in which it is acting.

It was concluded that the principle effect of streptomycin on the species studied was an alteration in the nature of the permeability barrier or the character of the permeases present.

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INTRODUCTION

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THE MODE OF ACTION OF STREPTOMYCIN

Streptomycin is an organic base active against many gram negative bacteria and mycobacteria. Many workers agree about its bacteriostatic nature, although under suitable conditions bactericidal actions may be shown. Unlike penicillin, the activity of streptomycin is readily influenced by external stimuli. For example, in an otherwise constant enviroment the activity of streptomycin varies with the concentration, the size of inoculum, the media and the hydrogen ion concentration.

Sodium chloride, sodium sulphate, sodium tartrate and potassium chloride in physiological concentration have been shown to neutralize the activity of streptomycin against S. aureus, P. vulgaris, Sh. dysenteriae, Eberthella typhosa, B. cereus, B. anthrax and B. subtilis (Donovick et al., 1948). The degree of neutralization was directly proportional to the salt concentration. Rhymer and Wallace (1945) have shown that the antibacterial activity of streptomycin is greatly influenced by the composition of the medium. Brain heart infusion and certain peptones, to a limited extent, inhibited the action of streptomycin. Green (1948a, 1948b) stated that glucose and salt concentration greatly influenced the action of streptomycin. Furthermore, Green found that the

addition of either 1% pyruvate or fumarate to nutrient broth permitted growth of E. coli at a concentration of streptomycin that would otherwise have prevented growth.

Compounds such as rhamnose, cellobiose and dulcitol, which are not readily oxidized, reduce the sterilizing effect of streptomycin, whereas maltose, sucrose, inulin, inositol and raffinose enhance its action. Lactose, arabinose and gluconate cause less killing than anticipated. Streptomycin acts more rapidly in nutrient broth than in a synthetic medium. In a synthetic medium cells must manufacture their amino acids, nitrogenous bases and related compounds from inorganic ammonia and glucose, thus reducing the rate of protein synthesis and correspondingly reducing vulnerability of the bacteria to streptomycin (Rosenblum & Bryson, 1953).

Many possible streptomycin mechanisms of action have been described. The most important mechanism follows from many observations that streptomycin affects respiration of sensitive bacteria. Geiger (1947) perhaps was the first to do systemic work on bacterial respiration. The rate of oxidation of glucose, lactate, glycerol, fumarate, malate, oxaloacetate and pyruvate was practically the same in the presence as in the absence of streptomycin. The prior oxidation of fumarate by a strain of E. coli greatly stimulated the oxidation of serine. However, if streptomycin

and serine were added at the same time the rate of oxidation was not increased. Bernheim and Fitzgerald (1947) reported that streptomycin did not inhibit the oxidation of carbohydrates and carbohydrate intermediates. Wight and Burt (1951) observed inhibition of oxidation by E. coli following the addition of serine, aspartate, threonine, glutamate, malate, fumarate, oxaloacetate, pyruvate and acetate.

Umbreit and co-workers (1949) presumed that streptomycin might inhibit the oxaloacetic-pyruvate condensation to a seven carbon compound which does not operate through citrate (Umbreit, 1951). The seven carbon compound was shown to be formed by E. coli in the presence of pyruvate and oxaloacetate. They named it 2 phospho-4-carboxy-adipic acid (Umbreit, 1951). The terminal respiratory processes of E. coli were examined in a direct way by Oginsky, Smith and Umbreit (1949). The cells were aged in a refrigerator for a week to decrease oxaloacetate decarboxylase. These cells oxidize pyruvate to the acetate stage, but oxaloacetate is only slowly oxidized. In the presence of streptomycin this oxidation is not complete but goes only as far as acetate. The authors interpret this as indicating that the antibiotic interferes with oxaloacetate-pyruvate condensation rather than that the metabolism of active acetate is blocked

There are at least five different reactions in which oxaloacetate is involved and which streptomycin modifies.

(1) If streptomycin blocks the terminal reaction of oxaloacetate-pyruvate oxidation, it may also block in the same way other substances, such as succinate and malate proceeding by the same route. This has proved to be so (Oginsky, Smith & Umbreit, 1949).

(2) On aging, E. coli suspensions slowly lose the ability to change oxaloacetate to pyruvate. But these cells were able to oxidize a mixture of oxaloacetate and pyruvate, a reaction which is inhibited by streptomycin.

(3) Although fresh cells of E. coli contain an active decarboxylase which rapidly converts oxaloacetate to pyruvate, there is a marked difference in the effect of streptomycin on the two substances (Oginsky, Smith & Umbreit, 1949).

(4) Samples of vacuum dried cells may fail to oxidize pyruvate. However, addition of fumarate or oxaloacetate to these cells promotes rapid oxidation which is inhibited by streptomycin (Umbreit, 1947).

(5) Based on the observation of Geiger (1947) stimulation of amino acid oxidation by previous

fumarate oxidation is due to oxaloacetate formed as an intermediate which reacts in turn with the keto acid derived from the amino acid. The condensation of oxaloacetate with pyruvate derived from serine and alpha keto butyrate from threonine provides an explanation for the observed phenomenon that streptomycin inhibits the reaction.

Smith, Oginsky and Umbreit (1949) reported an interesting discovery that streptomycin resistant cells do not have the oxaloacetate-pyruvate condensation enzyme. Nor indeed did susceptible strains. Thus, it is probable that strains growing in the presence of streptomycin eliminate the enzyme. No evidence of an oxaloacetate-pyruvate condensation enzyme in Mycobacterium tuberculosis was observed, but an analogous mechanism involving the oxidation of fatty acids was implicated.

Wasserman (1953) observed that streptomycin stimulated the total oxygen consumption of E. coli in oxidizing substances such as glucose, succinate, glutamate, malate and glycerol. Wasserman proposed that streptomycin acted in a same way as dinitrophenol or azide to uncouple assimilatory processes from oxidative phosphorylation. This dinitro-phenol-like effect was demonstrated also by Paine and Clark (1953).

Paine and Clark (1954) showed that streptomycin affects the respiration of resting coliform organisms in one of three ways: it may have no effect, it may inhibit or it may stimulate the uptake of oxygen. Which effect becomes apparent depends on the carbon source on which the organisms have been grown and on the substrate which is being metabolized when exposed to streptomycin. The killing action of streptomycin was related to the metabolic activity of the organisms. These findings do not support the hypothesis that inhibition of terminal oxidation explains the mode of action of streptomycin against bacteria.

Streptomycin appeared to inhibit the oxidation of serine, threonine and a number of tricarboxylic acid cycle intermediates by resting cell suspensions of E. coli. In the same way streptomycin inhibited the aerobic and anaerobic deamination of serinel (Wight & Burk, 1951). Barkulis (1953) reported an inhibitory effect of dihydrostreptomycin on pyruvate fermentation by both resistant and susceptible cells of E. coli. Interference with amino acid metabolism by streptomycin was reported also by Geiger (1947), who found that with sensitive resting cells of E. coli an unidentified intermediate resulting from the oxidation of fumarate (also malate, succinate etc.) was a necessary part of the streptomycin sensitive amino acid metabolizing system.

It has been suggested that streptomycin by inhibiting respiration may interfere with the formation of appropriate adaptive enzymes (Cohen, 1947; Fitzgerald, 1945; Paine & Clark, 1954).

Another probable mechanism of action of streptomycin is the inhibition of diamine oxidise. The diguanadine grouping of streptomycin inhibited a diamine oxidase of Mycobacteria. With streptomycin sensitive strains of Mycobacterium smegmatis this amine oxidizing enzyme was inhibited by streptomycin at concentrations approaching one-millionth molar. With resistant strains inhibition was only partial (Zeller & Owen, 1951). Karlson and Zeller (1951) reported that the reaction between streptomycin and diamine oxidase took place with acid fast organisms as well as with other organisms with pronounced diamine oxidase activity such as P. seruginosa and Micrococcus pyogenes var. aureus.

Henry, Housewright and Berkman (1948) reported that streptomycin did not inhibit catalase, carbonic anhydrase, cytochrome-cytochrome oxidase, succinoxidase, carboxylase, urease or trypsin. This finding prompted the statement that streptomycin either inhibits the enzyme or enzymes involved in carbohydrate metabolism or prevents their formation.

Cohn (1947) described a reaction of streptomycin with certain desoxyribose nucleic acids. The precipitation between thymonucleic acids and streptomycin was caused by

the diguanide component common to both. The opinion was expressed that since nucleic acids are precipitated by streptomycin adaptive enzymes are not formed. Rosenblum and Bryson (1953) proposed the theory that streptomycin combines with some essential metabolite during the process of synthesis, thus destroying the integrity or biologic specificity of the original polymers. The fact that certain salts interfered with the precipitation of desoxyribose nucleic acids by streptomycin prompted Donovick, Bayan, Canales and Pansy (1948) to conclude that the antibiotic mode of action was not due to this mechanism.

The activity of streptomycin and dihydrostreptomycin is decreased under anaerobic conditions. Three suggestions have been offered. Firstly, an altered environment, the direct effect of the incomplete catabolism of the metabolites: this lowers the hydrogen ion concentration and decreases the activity of the antibiotic (Geiger et al., 1946). Secondly, a slower rate of growth: it has been shown that the faster an organism grows the greater its sensitivity. Indeed, there would appear to be a direct relationship (Hurowitz et al., 1955). Thirdly, flocking of oxidative enzyme system of susceptible bacteria. Anaerobes do not possess this system (Bondi, 1946).

Many workers agree that streptomycin is bacteriostatic. Paine and Clark (1953) suggested that streptomycin may merely be fixed in the bacterial cells during the period

of exposure, and death may occur following the initiation of metabolic activity in a suitable culture medium. It is probable that streptomycin renders the cells sterile. The cells are not dead but cannot reproduce. These theories were supported by the finding that the fixing of streptomycin to the susceptible site in the cell with subsequent death of the cell occurred most rapidly in the presence of metabolized substrates and least rapidly with endogenous metabolism. It has been suggested (McElroy, 1947) that bacteriostasis can occur only if inhibition of oxidative mechanisms is the primary cause. It would be necessary to block oxygen consumption completely.

Some knowledge has been obtained about the effect of streptomycin on phosphate metabolism. Donovick et al. (1948) have reported that an increase in phosphate concentration increased the minimal inhibitory dose of streptomycin for growth of Klebseilla pneumoniae. Results obtained by Hurwitz and Rosano (1958) indicate that phosphate metabolism is implicated in some way in the mechanism of the bactericidal action of streptomycin, as well as on oxidative phosphorylation.

Yoshida and Sewag (1958) reported that cells of E. coli grown in a streptomycin-free medium showed an increase in turbidity and, at the same time, a decrease in total phosphate. The decrease in phosphate in RNA was

more striking than in other phosphate fractions. They assumed that the RNA in normal cells is in a form utilizable for cell growth. In constrast, when cells grown in a streptomycin containing medium were transferred to a glucose phosphate-free medium growth did not occur. But the RNA content was the same. It is probable that the RNA in cells grown in streptomycin containing medium are changed into forms non-utilizable for cell growth in a phosphate deficient medium. These workers also found that streptomycin does not inhibit P^{32} incorporation with energy metabolism by normal cells. With streptomycin sensitive phosphate starved cells, however, the phosphate incorporation is strongly inhibited by low concentrations of streptomycin, especially in the RNA fraction. The phosphate incorporation by streptomycin resistant cells is not inhibited even by a high concentration of streptomycin.

Rosenblum and Bryson (1953) reported that with E. coli the rate of growth was inhibited by streptomycin only under favourable growth conditions. They suggested that streptomycin may be inhibiting certain steps in nitrogen metabolism and protein synthesis, rather than inhibiting oxidative process of the cells, as suggested by Oginsky et al., (1949). Gale and Folkes (1953) reported that protein synthesis was not inhibited appreciably by streptomycin. However, streptomycin (100 µg/ml.) greatly inhibited adaptive formation of galactosidase by resting cells of S. aureus (Creaser, 1955).

Stachiewicz and Quastel (1959) reported that in the presence of dihydrostreptomycin M. tuberculosis cells were sensitive to the incorporation of radioactivity from labelled amino acids. Increased concentrations of the drug increased the degree of inhibition but the relationship was not direct. Maximal inhibition occurred at 200 µg/ml. and a further increase of streptomycin showed only a small increase in inhibition. Since dihydrostreptomycin affected the respiration of BCG, the inhibition of incorporation is not the result of an overall diminution of the oxidative processes in the cell. Its action may result from a combination with nucleic acids (Cohen, 1946). Dihydrostreptomycin was reported to inhibit the incorporation of the label into the cell protein from radioactive glucose (Stachiewicz & Quastel, 1959).

The present paper deals with the effect of streptomycin on oxygen consumption of resting cells and their cell extracts in the presence of various substrates. The effect on certain enzyme systems was also studied.

PROCEDURE

PROCEDURE

Effect of Streptomycin on Resting Cell Suspensions

The organisms Agrobacterium tumefaciens, Agrobacterium radiobacter and Rhizobium meliloti employed in these studies were cultured in a medium composed of 2 g. mannitol, 0.5 g. dipotassium phosphate, 0.3 g. magnesium sulphate, 0.2 g. sodium chloride and 1 g. yeast extract per litre.

A tube of mannitol broth was inoculated from a mannitol agar slant culture. After incubation at 25°C. for 24 hours, 1 ml. aliquots were transferred to two one-litre flasks each containing 500 ml. of the same medium. Incubation was at 25°C. for 24 hours on a rotary shaker.

The cell crop was harvested by centrifugation. The clear supernatant culture fluid was poured off and the packed cells resuspended in approximately 50 ml. of 0.85% sodium chloride. The centrifugation process was repeated. The supernatant was again poured off and the entire crop of packed cells resuspended in about 20 ml. of 0.85% sodium chloride. This final suspension represents the resting cell suspension which was then placed on a rotary shaker at 25°C. for 12 hours to reduce endogenous respiration.

The suspension was centrifuged and the supernatant was poured off. The packed cells were resuspended

in about 15 ml. of 0.85% sodium chloride. One millilitre of the cell suspension was added to a Warburg vessel together with 0.5 ml. streptomycin (60 or 120 µg/ml.) or 0.5 ml. of water, and 1 ml. substrate. Two-tenths millilitre sodium hydroxide was added to the centre well to to give a total volume of 2.7 ml. All cell suspensions were tipped into the substrate after an initial equilibration period which usually was ten minutes. The following substances were employed as substrates: oxaloacetic acid, malic acid, fumaric acid, citric acid, isocitric acid, pyruvic acid, succinic acid, alpha keto glutaric acid and mannitol. These substrates were suspended in water at a concentration of 0.0625 g. per 100 ml. and 1 ml. added per vessel to bring the concentration to 0.025%. Each substrate was adjusted to pH 7.

Streptomycin sulphate was suspended in water at a concentration of 300 and 600 µg/ml., and 0.5 ml. added to a Warburg vessel to bring the concentration to 60 and 120 µg. respectively.

Effect of streptomycin on growing cells

The organisms employed in these studies were grown in 50 ml. mannitol medium for 12 hours at 25°C. on a rotary shaker. Each 50 ml. medium was inoculated with a loopful of culture grown in mannitol broth. After incubation for 12 hours, plate cultures were prepared

after which either 60 or 120 µg/ml. streptomycin was added. Further plate cultures were prepared at one hour intervals for four hours. Incubation was for four and eight days at 25°C. At each one-hour interval of exposure to streptomycin, each treated culture was examined microscopically.

In order to determine whether streptomycin inhibits the utilization of mannitol and acids of the Kreb's cycle, bacterial cells grown in salt media A plus 0.025% carbon source in the presence of 60 and 120 µg/ml. streptomycin.

The salt medium A was prepared as follows:

NH_4Cl	.....	1.5 g.
Salts A	.....	1.5 ml.
	$MgSO_4.7H_2O$.....1g.	
	NaCl .....0.05g.	
	$FeSO_4.7H_2O$.....0.05g.	
	$MnSO_4.H_2O$.....0.05g.	
	HCl conc. .....0.1ml.	
	H_2O .....25ml.	
Salts B	.....	1.5 ml.
	K_2HPO_4 .....2.5g.	
	KH_2PO_4 .....2.5g.	
	H_2O .....25ml.	
Water	.....	225 ml.

The medium was adjusted to pH 7.3.

To each of three flasks containing 50 ml. of the above medium 0.025% succinic acid was added. The first flask was used as control. The second flask contained

60 µg/ml. streptomycin. The third flask contained 120 µg/ml. streptomycin. Each medium was then inoculated with a standard loopful of actively growing cells of the species under study. Plate counts were made at 24 hour intervals for 72 hours. The procedure was followed with other sources of carbon.

The carbon sources were prepared in water at a concentration of 0.135 g. per 50 ml. and 1 ml. added per flask to give a concentration of 0.025%. The substrates were adjusted to pH 7.

Since streptomycin did not inhibit the growth of the organisms in the salt media A plus the carbon source the amount of NH_4Cl was reduced from 1.5 g. to 0.5 g. However, this medium still appeared unsatisfactory and a new medium was substituted. This salt medium was prepared as follows:

K_2HPO_4	.....	0.5 g.
$MgSO_4.7H_2O$	.....	0.2 g.
NaCl	.....	0.2 g.
$FeCl_3$	.....	0.02 g.
KNO_3	.....	0.5 g.
Water	.....	1000 ml.

Growth in this case was determined by percentage transmittance.

The procedure was repeated using mannitol yeast extract medium. Growth was estimated by percentage transmittance.

Effect of streptomycin on cell free preparations

Cells were harvested using the same procedure as above. Two grams of packed cells were added to 5 g. of alumina powder which was kept at a temperature of about -10°C. After mixing the cells with the alumina powder, the cells-alumina mixture was wetted with cooled 0.05 M phosphate buffer of pH 7.5 and quickly ground for about eight minutes to a fine paste. The paste was extracted with 4 ml. of phosphate and then centrifuged at 11,000 r.p.m. in a cold centrifuge for 20 minutes. The supernatant was used for subsequent experiments for the determination of enzymatic activity.

To test the cell extract for transaminase activity mixture of amino and keto acids were prepared in distilled water and adjusted to pH 8. The reactants were pipetted in the order listed into 4 inch test tubes.

0.1 ml. of 20 µg. pyridoxal phosphate
0.1 ml. of 10 µM. amino acid (20 µM. if the DL isomer was used)
0.1 ml. of 50 µM. keto acid
0.4 ml. of cell extract
0.1 ml. of streptomycin or distilled water and phosphate buffer of pH 8 to make a total volume of 1.0 ml.

Three controls were included with each series of experiments: one without keto acid, one without enzyme and one with either glutamic or aspartic acid. For inhibition studies the extract and inhibitor were preincubated for one minute before the addition of substrates and pyridoxal phosphate. The system was incubated at 37°C. for two hours. After incubation, 0.1 ml. trichloracetic acid was added to end the reaction. The cultures were centrifuged to remove the precipitated proteins.

The formation of aspartate or glutamate in the incubation mixture was demonstrated by paper chromatography. Five-tenths micro litre of the supernatant was applied to Whatman No. 3 filter paper and chromatographed using n-butanol, acetic acid, water (50:10:40) as a solvent. Amino acid spots were revealed by spraying ninhydrin solution. The amount of the amino acid was roughly found by visual comparison with spots of known amino acid concentrations.

The prepared cell extract was used for manometric studies. One millilitre of cell extract was added to each Warburg flask together with:

KH_2PO_4-HCl buffer, pH 7.2	100 μM.
$MgCl_2$	20 μM.
Adenosine triphosphate	10 μM.
Adenosine diphosphate	2 μM.
Diphosphopyridine nucleotide	3 μM.
Coenzyme A	1 μM.
Thiamine diphosphate	4 μM.
Lipoic acid	4 μM.
Glutathione	10 μM/ml.
Substrate	5 μM.

and streptomycin 120 μg/ml. or distilled water to give a final volume of 3 ml. All cell extracts were tipped into the substrate after an initial equilibration period of five minutes. Oxygen uptake was measured at the end of one hour.

RESULTS

RESULTS

Effect of streptomycin on growing cells

The effect of streptomycin varied widely with the species of the organisms tested. Bacterial counts made on a 12 hour culture of Agrobacterium tumefaciens after an hours exposure to 60 µg/ml. streptomycin and every hour thereafter for four hours revealed a decrease in population. The initial count was 27×10^6 cells per ml. After addition of streptomycin, the population decreased to 17×10^2 cells per ml. When the experiment was repeated, using 120 µg/ml. streptomycin no colonies appeared on the antibiotic free media, even after one hours exposure to streptomycin (Table 1).

The same organisms, inoculated into mannitol yeast extract medium containing 60 and 120 µg/ml. streptomycin respectively, showed no increase in numbers after 120 hours incubation (Table 2).

The inhibition of multiplication as evidenced by absence of growth in mannitol yeast extract medium could be due to either (1) inhibition of division while the cells continued to carry out their metabolic functions and developed into abnormally distorted forms; (2) cessation of division and synthesis entailing death of the

organisms. The first possibility was eliminated by making microscopic examination of the culture after each hour's exposure to streptomycin. No abnormal cells were observed.

It is not possible to say categorically that the cells had already been killed before plating because viability can be determined with certainty only by subculture. The streptomycin may merely be fixed in the bacterial cells during the period of exposure and death may occur following the initiation of metabolic activities concerned with growth when the cells are transferred to a suitable medium. Perhaps streptomycin rendered the cells "sterile", that is, they are not dead but are unable to reproduce.

When a twelve hour culture of Agrobacterium radiobacter was treated with 60µg/ml. streptomycin for four hours, the cell population dropped from 25×10^7 to 24×10^5 cells per ml. as determined by plate count methods with four days incubation at 25°C. There was, however, no significant decrease in cell numbers after the first hours exposure to streptomycin. By this time the very sensitive cells may have been killed. If, however, the plates are incubated for eight days at 25°C. the population increased from the four day count of 24×10^5 to 36×10^5. When the experiment was repeated, this time using 120 µg/ml. streptomycin with an exposure of four hours to the antibiotic,

the number of colonies present in the mannitol yeast extract agar after four days incubation at 25°C. was 21×10^3 cells per ml. as compared to the initial count of 38×10^5 cells per ml. Incubation of the plates for eight days revealed a slight increase in the number of colonies. It is possible that some of the cells were able to recover from the injury received by streptomycin or else the rate of protein synthesis proceeded at such a slow rate that the cell multiplication was prolonged and thus no colonies appeared at the end of the four days incubation period (Table 3).

Agrobacterium radiobacter inoculated into mannitol yeast extract medium containing 60 and 120 µg/ml. streptomycin respectively were able to reproduce although they experienced a 48 hour lag period (Table 4 and Fig. 1). Following the lag period the cells multiplied as rapidly as cells not treated with streptomycin. At the end of five days incubation in broth the numbers of treated and untreated cells as determined by percentage transmittance were approximately equal. The cells thus became adapted to the unfavourable environment and growth proceeded in a normal manner. This may suggest that blocking of one or more enzyme systems by streptomycin may be compensated for by the development of an alternative metabolic pathway.

The action of streptomycin on Rhizobium meliloti appears to be bacteriostatic. Multiplication of R. meliloti ceased in the presence of streptomycin (Table 5) but was not retarded by removal of the organisms to an antibiotic free medium. When the organisms were transferred from a drug containing medium to a drug free medium at one hour intervals they continued to multiply (Table 6).

Antibacterial activity of streptomycin has been shown to be greatly influenced by the composition of the medium in which it is acting (Rosenblum & Bryson, 1953). Basal medium containing NH_4Cl showed a neutralizing effect towards streptomycin. From Tables 7 and 8 it is apparent that the salt greatly reduced the ability of streptomycin to inhibit growth of all strains tested. In each case the effect of streptomycin was dependent upon the concentration of the salt present. A concentration of 0.15% NH_4Cl in the medium completely neutralized the activity of streptomycin. When the medium contained 0.1% NH_4Cl, inhibition appeared only with the highest concentration of streptomycin tested.

There are at least two possible explanations for the neutralization of the streptomycin effect by this salt. There may be a direct action by the salt on streptomycin, producing a complex or this phenomenon may originate as a result of an interaction between the bacteria, the streptomycin and the salt.

Using Salts B medium where 0.05% KNO_3 was substituted for NH_4Cl as sole source of nitrogen, streptomycin inhibited the growth of Agrobacterium tumefaciens at concentrations of both 60 and 120 µg/ml. of medium. Agrobacterium radiobacter experienced a 48 hour lag at 60 and 120 µg/ml. (Table 9). It would thus appear that KNO_3 has little or no effect on streptomycin.

Effect of streptomycin on resting cell suspensions

It has not been possible to obtain a significant streptomycin inhibition of the oxidation of carbohydrates or carbohydrate intermediates by resting cell suspensions susceptible to streptomycin. Streptomycin appears to affect the respiration of resting cells in one of three ways:

(1) it may have no effect

(2) it may inhibit

(3) it may stimulate the uptake of oxygen

The effect which becomes apparent depends upon the organism and upon the carbon source which it metabolizes in the presence of streptomycin.

Resting cell preparations of Agrobacterium tumefaciens were able to oxidize a variety of substrates. However, when 60 µg/ml. streptomycin was present from the beginning of the experiment, it was found that the total oxygen uptake was greater in almost every case (Table 10).

There was, however, a decrease in oxygen uptake when succinate was oxidized.

When 120 µg/ml. streptomycin was present from the beginning of the experiment, it was found that the total oxygen uptake was greater than in the substrates containing no antibiotic. However, the increase in the oxygen uptake was less pronounced using malate, fumarate, oxaloacetate, mixture of pyruvate and oxaloacetate as substrates than in the presence of 60 µg/ml. streptomycin (Fig. 2). Utilization of citrate, α keto glutarate, isocitrate, pyruvate and mannitol appeared to be more complete and more rapid than when 60 µg/ml. streptomycin is present (Fig. 3). It was noted that the stimulation of oxygen uptake was immediate after the cells were exposed to streptomycin.

The stimulatory effect could suggest interference with certain cell wall functions by inhibiting normal synthesis of cell components from the available substrate. The organisms are possibly bringing about the oxidation of the streptomycin or its impurities. The data also suggests that streptomycin stimulated oxidation by uncoupling the assimilatory processes from dissimilation. This would indicate that the oxidation of various substrates proceeded nearly to completion when the organisms were under the influence of the antibiotics.

The effect of streptomycin on the oxidation of a variety of substrates by resting cell suspensions of Agrobacterium radiobacter is similar to that of Agrobacterium tumefaciens. The addition of streptomycin sufficient to make a final concentration of 60 µg/ml. to systems containing substrate resulted in an increase in the total oxygen uptake (Table 11). There was, however, a less pronounced total oxygen uptake during the oxidation of fumarate, malate, and oxaloacetate in the presence of 120 µg/ml. streptomycin. Oxidation of mannitol, pyruvate, citrate, isocitrate, α keto glutarate, oxaloacetate plus pyruvate and succinate showed still further stimulation in the presence of 120 µg/ml. streptomycin. The increased uptake of oxygen was not as rapid as with A. tumefaciens. Only after 30 minutes did the oxygen uptake increase. Perhaps the most significant observation is the effect of streptomycin on the oxidation of citrate. Cells of Agrobacterium radiobacter were found to be initially impermeable to citrate. Treatment of the cells with streptomycin increased their oxygen uptake (Fig. 4).

Streptomycin was noted to have no effect on the total oxygen uptake of Rhizobium meliloti in the presence of α keto glutarate, isocitrate, oxaloacetate plus pyruvate and citrate (Fig. 5), whereas there is an inhibition of oxygen uptake with fumarate, succinate, oxaloacetate and malate as substrates (Fig. 6). However, the oxidation of

pyruvate and mannitol was stimulated by streptomycin (Fig. 7). This effect became more apparent when the concentration of streptomycin was increased to 120 µg/ml. (Table 12). The stimulation did not occur immediately, nor was the stimulation as great as with A. tumefaciens and A. radiobacter.

Effect of streptomycin on cell free extracts

Cell free extracts were able to oxidize the intermediate compounds of the Kreb's cycle. In the presence of 120 µg/ml. streptomycin, no change was observed in the oxidation of pyruvate, cis-aconitate, citrate, isocitrate, α keto glutarate, succinate, fumarate and malate by cell free extracts of the organisms under study. Oxidation of oxaloacetate and oxaloacetate plus pyruvate was, however, slightly inhibited (Tables 13, 14 and 15).

It would appear that streptomycin had no effect on various transamination reactions (Table 16).

TABLES

TABLE 1

EFFECT OF STREPTOMYCIN ON THE GROWTH OF A TWELVE-HOUR SUSPENSION OF *Agrobacterium tumefaciens*

Time after exposure to streptomycin	Streptomycin concentration	Population
0 time	60 µg	27×10^6
1 hour	60 µg	85×10^4
2 hours	60 µg	12×10^4
3 hours	60 µg	36×10^3
4 hours	60 µg	17×10^2
0 time	120 µg	41×10^7
1 hour	120 µg	-
2 hours	120 µg	-
3 hours	120 µg	-
4 hours	120 µg	-

TABLE 2

EFFECT OF STREPTOMYCIN ON THE GROWTH OF

Agrobacterium tumefaciens

IN MANNITOL MEDIA

Time	% Transmittance		
	Streptomycin concentration		
	0 µg.	60 µg.	120 µg
24 hours	80%	100%	100%
48 hours	45%	100%	100%
72 hours	23%	100%	100%
96 hours	13%	100%	100%
120 hours	10%	100%	100%

TABLE 3

EFFECT OF STREPTOMYCIN ON THE GROWTH OF A TWELVE-HOUR SUSPENSION OF *Agrobacterium radiobacter*

Time after exposure to streptomycin	Streptomycin concentration	Population	
		4 days	8 days
0 time	60 µg	25×10^7	26×10^7
1 hour	60 µg	27×10^7	28×10^7
2 hours	60 µg	14×10^6	20×10^6
3 hours	60 µg	26×10^5	38×10^5
4 hours	60 µg	24×10^5	36×10^5
0 time	120 µg	38×10^5	38×10^5
1 hour	120 µg	13×10^4	24×10^4
2 hours	120 µg	55×10^3	70×10^3
3 hours	120 µg	24×10^3	31×10^3
4 hours	120 µg	21×10^3	35×10^3

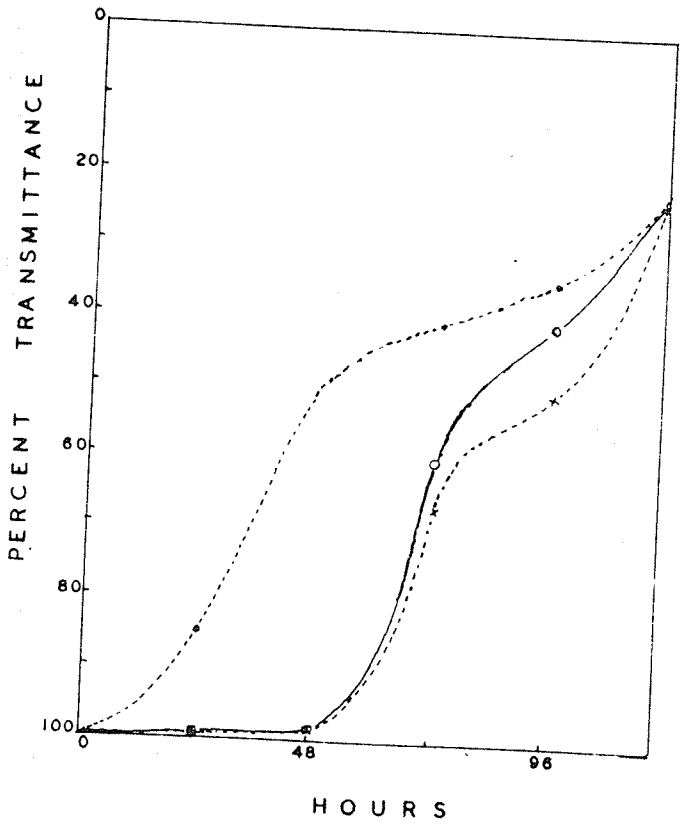

Fig. 1.

Effect of streptomycin on the growth of Agrobacterium radiobacter in mannitol medium

no streptomycin ●------●

60 µg. streptomycin o——o

120 µg. streptomycin x-----x

endogenous ●——●

TABLE 4

EFFECT OF STREPTOMYCIN ON THE GROWTH OF

Agrobacterium radiobacter

IN MANNITOL MEDIA

Time	% Transmittance		
	Streptomycin concentration		
	0 µg	60 µg	120 µg
24 hours	83%	99%	99.5%
48 hours	45%	99%	99%
72 hours	41%	59%	62%
96 hours	35%	38%	49%
120 hours	16%	22%	25%

TABLE 5

EFFECT OF STREPTOMYCIN ON THE GROWTH OF

Rhizobium meliloti

IN MANNITOL MEDIA

Time	% Transmittance		
	Streptomycin concentration		
	0 µg	60 µg	120 µg
24 hours	92%	100%	100%
48 hours	70%	100%	100%
72 hours	50%	100%	100%
96 hours	36%	100%	100%
120 hours	27%	100%	100%

TABLE 6

EFFECT OF STREPTOMYCIN ON THE GROWTH OF A TWELVE-HOUR CULTURE OF *Rhizobium meliloti*

Time after exposure to streptomycin	Streptomycin concentration	Population
0 time	60 µg	89×10^7
1 hour	60 µg	15×10^6
2 hours	60 µg	95×10^5
3 hours	60 µg	84×10^5
4 hours	60 µg	73×10^5
0 time	120 µg	36×10^7
1 hour	120 µg	19×10^7
2 hours	120 µg	15×10^7
3 hours	120 µg	66×10^6
4 hours	120 µg	62×10^6

TABLE 7

EFFECT OF STREPTOMYCIN ON THE GROWTH OF

Agrobacterium tumefaciens

IN BASAL MEDIA A(0.15% NH_4Cl) PLUS VARIOUS SUBSTRATES

Substrates	Time	Population		
		Streptomycin concentration		
		0 µg	60 µg	120 µg
succinate	24 hours	69×10^5	35×10^6	49×10^6
	48 hours	37×10^8	48×10^8	54×10^9
	72 hours	18×10^9	41×10^9	14×10^{10}
pyruvate	24 hours	25×10^6	12×10^6	39×10^4
	48 hours	98×10^7	43×10^7	29×10^6
	72 hours	23×10^{10}	68×10^9	23×10^9

TABLE 8

EFFECT OF STREPTOMYCIN ON THE GROWTH OF *Agrobacterium radiobacter* IN BASAL MEDIA A(0.15% NH_4Cl) PLUS VARIOUS SUBSTRATES

Substrates	Time	Population		
		Streptomycin concentration		
		0 µg	60 µg	120 µg
succinate	24 hours	39×10^6	30×10^6	11×10^6
	48 hours	19×10^8	36×10^7	14×10^7
	72 hours	32×10^{10}	63×10^9	85×10^8
fumarate	24 hours	19×10^7	53×10^6	80×10^6
	48 hours	37×10^{10}	70×10^9	38×10^8
	72 hours	16×10^{11}	25×10^{10}	37×10^9
pyruvate	24 hours	80×10^4	10×10^5	64×10^4
	48 hours	54×10^8	39×10^8	31×10^8
	72 hours	17×10^{11}	20×10^{11}	22×10^{11}

TABLE 9

EFFECT OF STREPTOMYCIN ON THE GROWTH OF *Agrobacterium radiobacter* IN BASAL MEDIA B(0.05% KNO_3) PLUS VARIOUS SUBSTRATES

Substrates	Time	% Transmittance		
		Streptomycin concentration		
		0 µg	60 µg	120 µg
Succinate	24 hours	95%	100%	100%
	48 hours	77%	96%	97%
	72 hours	76%	85%	93%
	96 hours	75%	75%	87%
Malate	24 hours	90%	100%	100%
	48 hours	88%	98%	100%
	72 hours	82%	90%	95%
	96 hours	80%	80%	81%

TABLE 10

EFFECT OF STREPTOMYCIN ON THE OXIDATION OF A VARIETY OF SUBSTRATES BY RESTING CELL SUSPENSIONS OF *Agrobacterium tumefaciens*

Substrate	Q_{O_2}			
	Streptomycin concentration			
	0 µg	60 µg	120 µg	endogenous
mannitol	351	354	415	96
pyruvate	424	450	483	261
oxalo + pyruvate	448.9	498	482	260
citrate	123	129	147	88
isocitrate	367	386	390	270
α keto glutarate	178	189	213	172
succinate	790	762	698	203
fumarate	214	244	226	132
malate	254	284	264	185
oxaloacetate	484	518	500	260

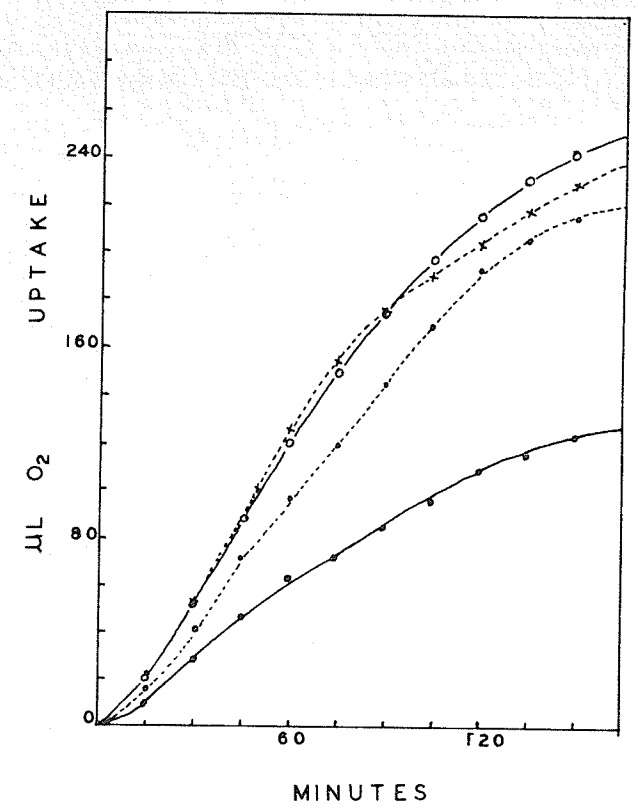

Fig. 2.

Effect of streptomycin on the oxidation of fumarate by resting cells of Agrobacterium tumefaciens

no streptomycin ●-----●

60 µg. streptomycin o——o

120 µg. streptomycin x-----x

endogenous ●——●

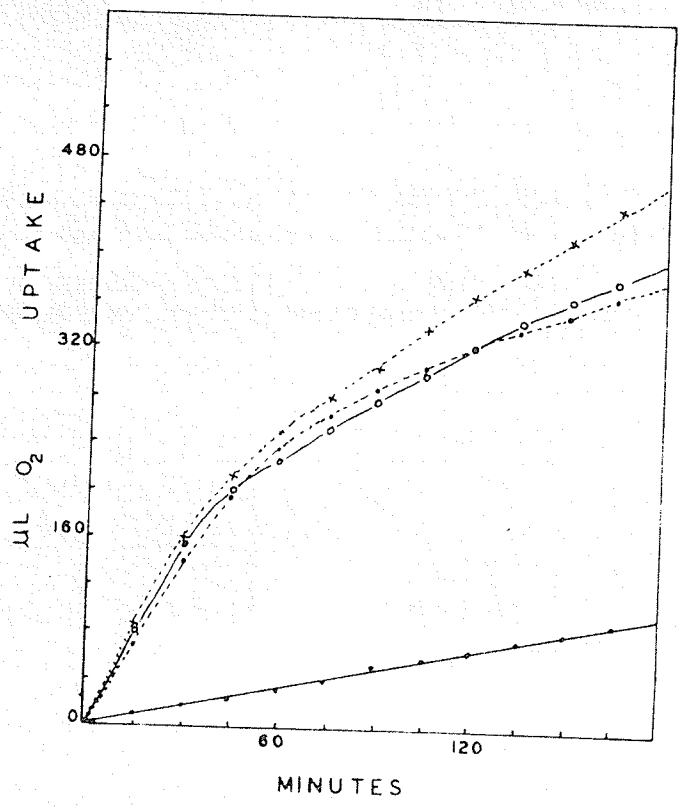

Fig. 3.

Effect of streptomycin on the oxidation of mannitol by resting cells of Agrobacterium tumefaciens

no streptomycin ●------●

60 µg. streptomycin o——o

120 µg. streptomycin x-----x

endogenous ●——●

TABLE 11

EFFECT OF STREPTOMYCIN ON THE OXIDATION OF A VARIETY OF SUBSTRATES BY RESTING CELL SUSPENSIONS OF *Agrobacterium radiobacter*

Substrate	Q_{O_2}			
	Streptomycin concentration			
	0 µg	60 µg	120 µg	endogenous
mannitol	321.34	325.87	333.26	145.78
pyruvate	219.45	242.47	243.53	175.03
oxalo + pyruvate	234.22	248.66	258.87	175.03
citrate	116.88	140.91	149.16	136.78
isocitrate	364.23	371.14	381.08	293.28
α keto glutarate	386.68	481.23	496.52	193.20
succinate	534.04	547.87	553.41	193.20
fumarate	326.38	377.49	322.15	145.78
malate	490.33	502.03	465.00	193.07
oxaloacetate	255.22	268.11	261.61	175.03

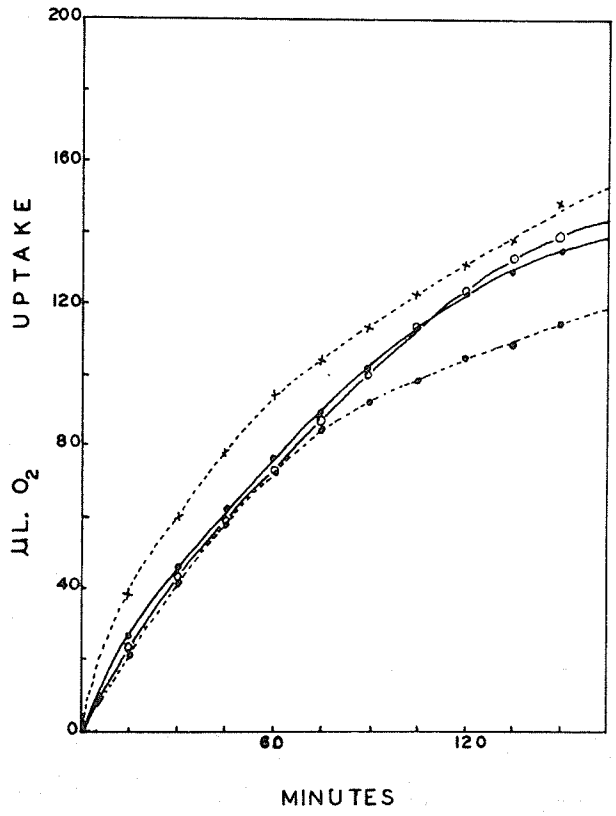

Fig. 4.

Effect of streptomycin on the oxidation of citrate by resting cells of Agrobacterium radiobacter

no streptomycin ●-----●

60 µg. streptomycin o——o

120 µg. streptomycin x-----x

endogenous ●——●

TABLE 12

EFFECT OF STREPTOMYCIN ON THE OXIDATION OF A VARIETY OF SUBSTRATES BY RESTING CELL SUSPENSIONS OF *Rhizobium meliloti*

Substrate	Q_{O_2}			
	Streptomycin concentration			
	0 µg	60 µg	120 µg	endogenous
mannitol	395.32	403.11	425.94	213.69
pyruvate	459.17	497.75	513.87	239.84
oxalo + pyruvate	541.42	538.67	537.13	239.84
citrate	153.05	142.71	144.68	94.00
isocitrate	216.55	218.63	227.62	184.37
α keto glutarate	221.07	225.69	216.02	184.37
succinate	327.92	281.20	269.72	208.70
malate	572.32	494.86	439.77	184.37
oxaloacetate	522.07	469.95	472.19	239.84
fumarate	281.42	247.59	256.01	208.70

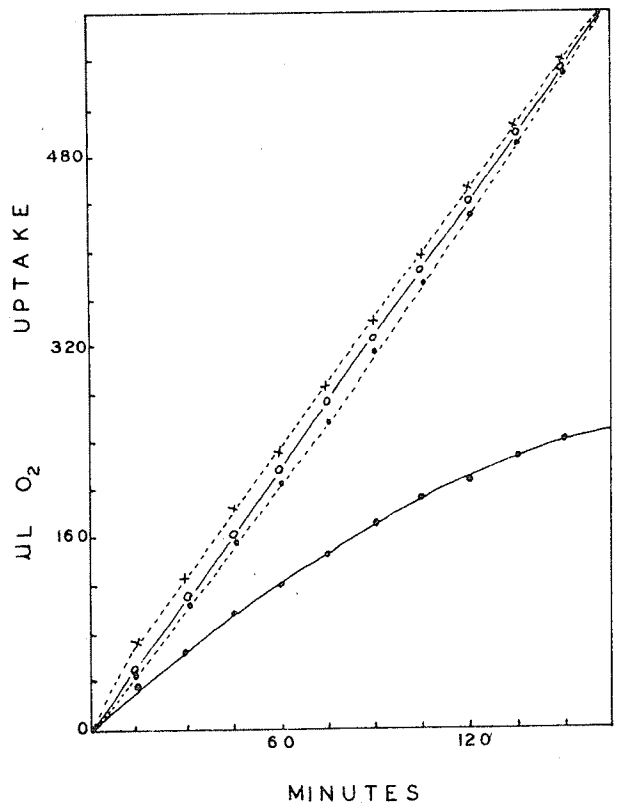

Fig. 5.

Effect of streptomycin on the oxidation of oxaloacetate + pyruvate by resting cells Rhizobium meliloti

no streptomycin	●-----●
60 µg. streptomycin	o——o
120 µg. streptomycin	x-----x
endogenous	●——●

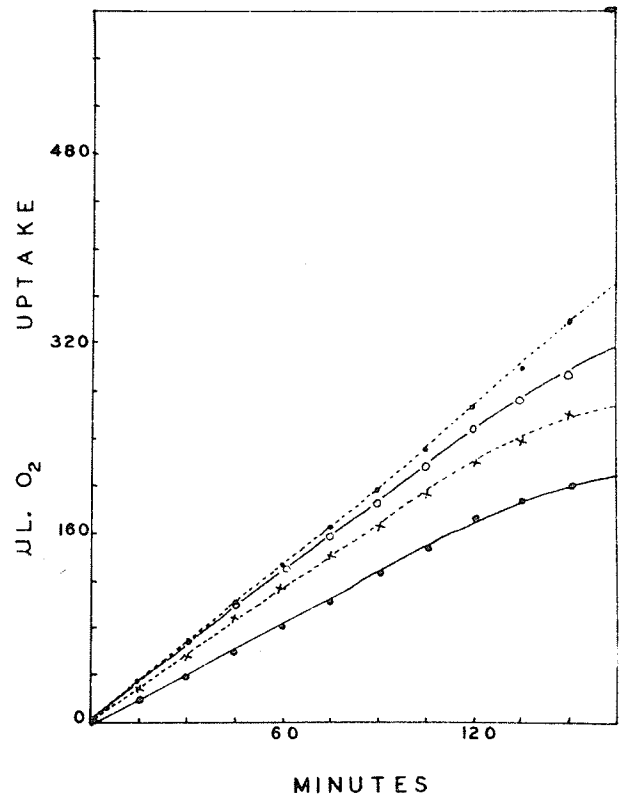

Fig. 6.

Effect of streptomycin on the oxidation of succinate by resting cells of Rhizobium meliloti.

no streptomycin	●------●
60 μg. streptomycin	o——o
120 μg. streptomycin	x-----x
endogenous	●——●

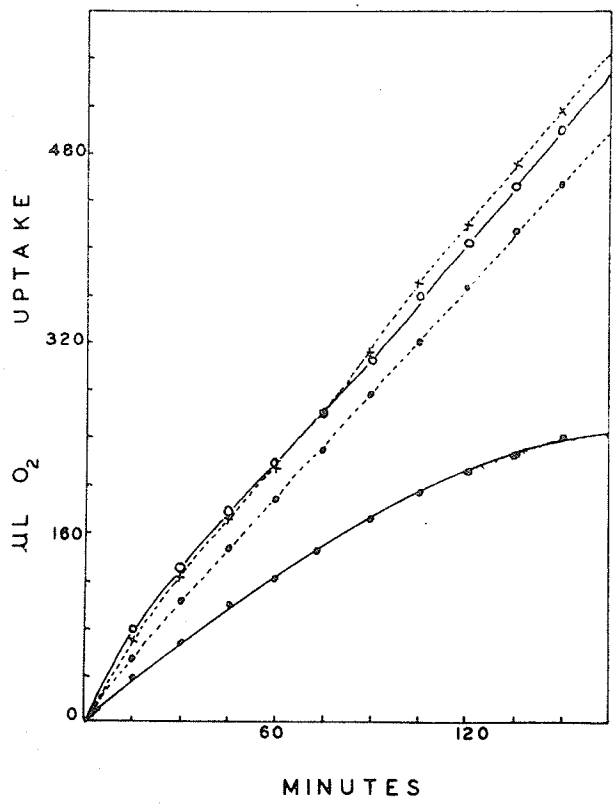

Fig. 7.

Effect of streptomycin on the oxidation of pyruvate by resting cells of *Rhizobium meliloti*

no streptomycin	●-----●
60 µg. streptomycin	o——o
120 µg. streptomycin	x-----x
endogenous	●——●

TABLE 13

EFFECT OF STREPTOMYCIN ON THE OXIDATION OF A VARIETY OF SUBSTRATES BY CELL FREE EXTRACTS OF *Agrobacterium tumefaciens*

Substrate	Q_{O_2}	
	Streptomycin concentration	
	0 µg	120 µg
pyruvate	46.44	44.13
oxalo + pyruvate	53.14	43.76
citrate	86.75	83.12
isocitrate	56.38	50.20
α keto glutarate	46.89	51.72
succinate	51.70	46.44
fumarate	67.90	71.80
malate	55.04	53.67
oxaloacetate	49.86	40.28
cis-aconitate	18.75	21.12

TABLE 14

EFFECT OF STREPTOMYCIN ON THE OXIDATION OF A VARIETY OF SUBSTRATES BY CELL FREE EXTRACTS OF *Agrobacterium radiobacter*

Substrate	Q_{O_2}	
	Streptomycin concentration	
	0 µg	120 µg
pyruvate	36.75	35.72
oxalo + pyruvate	34.18	27.85
cis-aconitate	19.67	21.12
citrate	60.04	59.64
isocitrate	50.84	49.74
α keto glutarate	38.05	37.44
succinate	64.40	68.37
fumarate	66.72	70.07
malate	58.38	55.76
oxaloacetate	45.66	37.73

TABLE 15

EFFECT OF STREPTOMYCIN ON THE OXIDATION OF A VARIETY OF SUBSTRATES BY CELL FREE EXTRACTS OF *Rhizobium meliloti*

Substrate	Q_{O_2}	
	Streptomycin concentration	
	0 µg	120 µg
pyruvate	29.67	28.75
oxalo + pyruvate	31.63	26.73
cis-aconitate	26.96	26.67
citrate	35.38	31.02
isocitrate	49.61	49.05
α keto glutarate	22.66	21.67
succinate	22.83	20.42
fumarate	37.67	38.65
malate	37.15	35.00
oxaloacetate	30.10	26.63

TABLE 16

EFFECT OF STREPTOMYCIN ON TRANSAMINATION REACTIONS

Substrates	Streptomycin concentration	Amount amino acid formed as determined by chromatography		
		A. tumefaciens	A. radiobacter	R. meliloti
aspartic + α keto glutaric	0 µg	+ +	+ +	+ +
	120 µg	+ +	+ +	+ +
alanine + α keto glutaric	0 µg	+	-	-
	120 µg	+	-	-
leucine + α keto glutaric	0 µg	+ + +	+ + +	+ +
	120 µg	+ + +	+ + +	+ +
glutamic + oxaloacetic	0 µg	+ +	+ +	+
	120 µg	+ +	+ +	+
alanine + oxaloacetic	0 µg	+ +	+ +	-
	120 µg	+ +	+ +	-

DISCUSSION

DISCUSSION

During the course of investigations on the mode of action of streptomycin, it was found that the presence of streptomycin increased the rate of oxidation of a variety of substrates by Agrobacterium tumefaciens and Agrobacterium radiobacter. The stimulatory effect suggests that streptomycin is interferring with oxidative assimilation which is similiar to the dinitro-phenol-like effect reported by Paine & Clark (1954) and Wasserman (1953). If the inhibition of the assimilatory process occurs, then the substrate should be more fully oxidized to CO_2 and H_2O, which would explain the increased rate of oxygen uptake. If the uncoupling of oxidative phosphorylation should occur, it would imply that as the energy for multiplication is not available, energy requiring reactions necessary for synthesis are separated from oxidative assimilation. This assumption does not support the hypothesis of inhibition of terminal oxidation as an explanation for the mode of action of streptomycin as proposed by Umbreit (1949).

In 1937 Clifton took the view that substances which increased oxygen uptake interfered with certain cell wall functions by inhibiting normal synthesis of intracellular cell components. The results indicate that streptomycin may have an effect on the cell wall, cell membrane or both. Cells of A. radiobacter were found to

be initially impermeable to citrate. Treatment of the cells with streptomycin increased their oxygen uptake. As mentioned above, streptomycin stimulated the oxidation of various substrates by A. tumefaciens and A. radiobacter. However, oxidation of the same substrates by cell free extracts of either A. tumefaciens or A. radiobacter was not stimulated by streptomycin. In fact, the oxidation of oxaloacetate and a mixture of oxaloacetate and pyruvate was slightly inhibited. This tends to support the hypothesis that the terminal oxidation in inhibited by the presence of streptomycin.

On the basis of the above findings it is concluded that streptomycin affects the permeability of the cell wall or membrane to certain small molecules. This, therefore, does not support the hypothesis that streptomycin uncouples oxidative phosphorylation from assimilation.

There exist, in bacteria, stereospecific permeation systems, functionally specialized and distinct from metabolic enzymes. The entry of organic substrates into bacterial cells maybe mediated by more or less selective permeation systems which connect the intracellular and external world. The increase in respiration of various substrates by resting cells of A. tumefaciens and A. radiobacter in the presence of streptomycin may be due to alteration of the permeases allowing the accumulation of more substrate in the cells and hence increased oxidation.

Cells of A. radiobacter are highly impermeable to citrate. Yet the enzyme active against citrate was extracted from the cells, which when intact are inert towards the same substrate. Although the cells were cryptic towards citrate, they were able to metabolize isocitrate having a similar structure. This substrate was probably oxidized due to the presence of a stereospecific permeation system such as that proposed by Cohen & Monod (1957). On this basis intact cells may have failed to metabolize citrate since citrate permeation sites were not present. Treatment of cells by streptomycin probably altered isocitrate permeases, allowing the structurally related citrate to enter the cell.

The results indicate that the cells of A. tumefaciens were freely permeable to streptomycin as observed by the immediate stimulation of oxidation of various substrates. The effect on A. radiobacter was delayed. At least 30 minutes passed before any great increase in oxygen uptake occurred in the presence of the antibiotic. This difference in permeability to streptomycin maybe due to differences in the composition of either the cell wall, cell membrane, or both.

The oxidation of various substrates by resting cell suspensions of R. meliloti was affected in three ways depending upon the substrate being utilized. The oxygen uptake was inhibited, stimulated or not affected by the

presence of streptomycin. However, the oxidation of the same substrates by cell free extracts was unaffected by streptomycin. Furthermore, it appears that streptomycin may be altering the permeability of the cell barrier. The composition of the cell wall and cell membrane may determine whether or not the antibiotic stimulates, inhibits or has no effect on the respiratory rate. Possibly streptomycin interferes differently with each permeatory system. R. meliloti was similar to A. tumefaciens and A. radiobacter in that there was a slight inhibition of respiration of oxaloacetate and a mixture of oxaloacetate and pyruvate by cell free extracts.

The action of streptomycin on A. tumefaciens appears to affect anabolic reactions. Multiplication not only ceased in the presence of streptomycin, but growth did not occur when the cells were removed from a streptomycin containing medium to an antibiotic free medium. The results indicate that streptomycin affects the permeability barrier. If this is so, it is probably that there is an outward permeability of nucleic and amino acids. This would deprive the cells of nucleic and amino acids necessary for protein synthesis.

Streptomycin apparently affects the permeability barrier of A. radiobacter. Yet, these cells will remain viable after four hours exposure to streptomycin and even reproduce in a streptomycin containing medium after lag

phase of up to 48 hours. It would be expected that if streptomycin damages the permeability barrier the cells would die. However, they seem to have the capacity to adapt themselves to the unfavourable environment and continue to reproduce in a normal manner.

From the results obtained from the oxidation of various substrates by cells of R. meliloti and their extracts, it was concluded that streptomycin alters the permeability barrier or the permeases. The prolonged effect of streptomycin on the cells maybe due to the composition of the cell wall, cell membrane or both. The action of streptomycin upon these cells appears to be bacteriostatic. Growth ceased in the presence of streptomycin, perhaps due to the leakage of some necessary constituents necessary for the synthesis of new protoplasm. However, multiplication was not retarded when the cells were removed from a streptomycin containing medium to a streptomycin free medium. There appears to be a correlation between bacteriostatic and oxygen uptake in the presence of streptomycin. It was observed that the effect of streptomycin on oxygen uptake was less pronounced and less rapid than with A. tumefaciens or A. radiobacter. Thus, the streptomycin damage was less severe, so that when the cells of R. meliloti were transferred to a streptomycin free medium the damage was repaired and the cells continued to multiply.

SUMMARY

SUMMARY

1. The oxidation of a variety of substrates by resting cell suspensions of A. tumefaciens and A. radiobacter was stimulated in the presence of streptomycin. Streptomycin appears to affect the respiration of resting cells of R. meliloti in one of three ways:

(1) it may have no effect

(2) it may inhibit

(3) it may stimulate the uptake of oxygen.

Which effect is to become apparent depends upon the carbon source it is metabolizing in the presence of the antibiotic. Streptomycin enabled the cells of A. radiobacter to oxidize citrate even though they were initially impermeable to this substance.

2. The oxidation of various substrates by cell free extracts was not inhibited by streptomycin. However, streptomycin did tend to slightly inhibit the oxidation of oxaloacetate and oxaloacetate plus pyruvate.

3. The above two observations led to the conclusion that streptomycin altered the permeability barrier or the permeases.

4. Of all the enzymes systems of the Kreb's cycle only the condensing enzyme was slightly inhibited by streptomycin as has been previously reported by Umbreit (1949) in the case of E. coli.

5. Streptomycin appears to affect the anabolic reactions in A. tumefaciens. The action of streptomycin on R. meliloti is bacteriostatic. This was explained on the basis of the effect of streptomycin on the permeability barrier or on the permeases. Cells of A. radiobacter are capable of adapting themselves to the presence of streptomycin.

6. Antibacterial activity of streptomycin has been shown to be greatly influenced by the composition of the medium in which it is acting.

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