STUDIES ON STREPTOMYCIN

III. The Effect of Streptomycin on the Metabolism of Resting Bacteria and on Certain Purified Enzymes

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There has been very little experimental work on the mechanism by which streptomycin (SM) exerts its activity on living cells or the reason for the resistance of some bacteria to its action. Several reports have compared the metabolic activities of strains susceptible and resistant to SM (Graessle and Frost, 1946; Murray *et al.*, 1946; Seligmann and Wassermann, 1947). Some investigators (Benham, 1947; Hirsch and Dosdogru, 1947; Bernheim and Fitzgerald, 1947; Geiger, 1947) have failed to obtain SM inhibition of the oxidation of carbohydrates or carbohydrate intermediates by bacteria susceptible to SM. On the other hand, with certain bacteria SM has been shown to inhibit the oxidation of benzoic acid (Bernheim and Fitzgerald, 1947), amino acid metabolism (Geiger, 1947), and the oxidation of sodium ribonucleate (Krampitz, Green, and Werkman, 1947).

The work reported in this paper is concerned with the effect of SM on (a) certain purified enzyme systems, (b) aerobic and anaerobic oxidations by resting cell suspensions of *Staphylococcus aureus*, *Bacillus cereus*, and *Shigella sonnei*, and (c) the production and utilization of certain intermediate and end products resulting from aerobic metabolism.

METHODS AND MATERIALS

For the experiments with purified enzymes, carboxylase was prepared from dried brewer's yeast (Green, Herbert, and Subrahmanyan, 1941), carbonic anhydrase was prepared from sheep blood (Tauber, 1936), and a rat heart preparation (Stotz and Hastings, 1937) was used for the study of the succinoxidase and cytochrome-cytochrome-oxidase systems. Manometric techniques were employed for measuring the activities of the following enzymes: rabbit blood catalase, carboxylase (Green, Herbert, and Subrahmanyan, 1941), urease (Sizer, 1939), carbonic anhydrase, succinoxidase, and cytochrome-cytochrome-oxidase with hydroquinone as substrate. The method used for measuring tryptic activity was that of Anson (1938). The cytochrome-cytochrome-oxidase system in intact baker's yeast was studied by the spot plate test described by Sevag and Ross (1944). There was ½-hour contact between the enzymes and SM before measurement of

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activity, with the exceptions of urease and trypsin with which the activity was measured immediately after the SM was added.

Strains of *S. aureus*, *B. cereus*, and *S. sonnei*, sensitive to approximately 1 μg SM per ml, were used in the bacterial studies. Variants resistant to 1,000 μg per ml were obtained by serial transfer in broth containing increasing concentrations of SM and were studied concomitantly with the parent-susceptible strains. The *S. aureus* suspensions used were prepared from 18-hour growth in a modified Landy and Dickens (1942) semisynthetic medium at 37 C. The *B. cereus* and *S. sonnei* cells were prepared from 18-hour growth in nutrient broth. All flasks were inoculated from 24-hour agar slants of the cultures. Constant volume Warburg respirometers were used in the manometric experiments. The manometers were shaken through a to-and-fro distance of 3.5 cm at a rate of 110 cycles per minute. All experiments were run in duplicate and were repeated at least once.

For the aerobic experiments the cell suspensions were centrifuged, washed twice with M/60 Sorensen's buffer, pH 7.2, and resuspended in the same buffer to the appropriate turbidity. In the study of endogenous respiration 12 mg (dry weight, *in vacuo* over H_2SO_4) of bacteria were used per flask, and the O_2 consumption was measured over a period of 24 hours at 36.5 C. The respiratory rate of the controls slowly decreased over this time period to about 10 per cent of the initial rate. When substrates were added the cell suspensions were diluted so as to consume approximately 100 to 200 mm^3 O_2 per hour. The Warburg flasks contained a final concentration of M/60 Sorensen's buffer, pH 7.2 in most cases, 0.5 per cent substrate, and 1 to 3 mg cells. In all cases substrates were added as the neutral salts. The inner cup contained 0.2 ml 20 per cent KOH to absorb CO_2. In all manometric experiments the SM was placed in the side arm and added to the system after an equilibration period of approximately 15 minutes in the water bath at 36.5 C. Oxygen consumption was measured over a period of 3 hours.

In one series of aerobic experiments, chemical determinations were made of certain intermediate and end products of metabolism found in the cell environment and derived from various carbohydrate substrates, both in the absence of SM and in the presence of inhibitory concentrations of SM. Substrate concentrations and amounts of cells were chosen in these experiments so that approximately two-thirds of the initial substrate had been utilized by the end of the 3-hour observation period. At the end of the experiments, the cells and supernatants were separated by centrifugation and the chemical tests performed.

Colorimetric methods were used for the determination of glucose (Somogyi, 1945), pyruvate (Bueding and Wortis, 1940), lactate (Barker and Summerson, 1941), and ethanol (Henry *et al.*, 1948). Since even purified SM interfered with the glucose test, the supernatants were always diluted for this determination to reduce the SM concentrations below the level giving interference. Glycerol was determined by a technique developed in this laboratory in which the formaldehyde produced upon splitting the glycerol with periodic acid is determined colorimetrically after reaction with 1,8-dihydroxynaphthalene-3,6-disulfonic acid

(chromotropic acid). Acetate was determined by the steam distillation and titration procedure of Elliott *et al.* (1942). The titration values were converted to acetic acid, although any volatile acid will be determined by this method. The reducing substances in the cells were determined as glucose by the method of Somogyi (1945) following hydrolysis in 3 N HCl at 100 C for 3 hours.

In the anaerobic Warburg experiments the cell suspensions were washed twice with 0.117 per cent $NaHCO_3$ (the concentration necessary to give a pH of 7.2 when equilibrated with the CO_2-N_2 mixture used, 93.5 per cent N_2 + 6.5 per cent CO_2) and resuspended in the same $NaHCO_3$ solution to the appropriate turbidity. The Warburg flasks contained a final concentration of 0.117 per cent $NaHCO_3$, 0.5 per cent substrate, and from 1 to 3 μg cells. CO_2 production was measured over a period of 3 hours.

The cells for the Thunberg experiments were prepared by washing them twice with M/30 phosphate buffer, pH 7.2, and resuspending them in the same buffer. The tubes contained a final concentration of 0.5 per cent substrate (M/50 in the case of amino acids), 0.002 per cent methylene blue, and 100 μg SM per ml in the M/30 phosphate buffer. The cells were placed in the cap. The tubes were evacuated to 20 mm Hg with an ordinary water aspirator for 3 minutes while in a water bath at 40 C. The cells were then mixed with the contents of the tube and reduction time was measured.

The SM preparations used in this work included the following: streptomycin sulfate, Pfizer commercial, lot no. 456; streptomycin hydrochloride, Merck commercial; streptomycin sulfate, Pfizer lot no. S7117A, 830 μg streptomycin base per mg; streptomycin sulfate, Parke, Davis, 800 μg streptomycin base per mg. The dihydrostreptomycin, lot no. 411 × 156, assay 585 μg per mg, was supplied to us by Parke, Davis and Company.

Many of the substrates were kindly supplied by E. S. G. Barron, Department of Medicine, University of Chicago, and by J. F. Owings, Jr., W. N. Nutter, and H. E. Thompson of Camp Detrick, Maryland.

RESULTS

Purified enzyme systems. The activities of rabbit blood catalase, yeast carboxylase, urease, carbonic anhydrase, trypsin, succinoxidase, and the cytochrome-cytochrome-oxidase systems were unaffected by 500 μg SM per ml. The catalase activity of *S. aureus* and the cytochrome-cytochrome-oxidase system of intact baker's yeast were uninhibited by 500 and 1,000 μg SM per ml, respectively.

Endogenous respiration. SM in concentrations up to 2,000 μg per ml had no effect on the rate of endogenous O_2 consumption of *S. aureus* during the 24 hour observation period. The addition of ribonucleic acid (Schwarz Labs., Inc.) to the reaction system had no effect either in the presence or absence of SM.

The endogenous O_2 consumption of the susceptible strain of *B. cereus*, however, was inhibited by even 1 μg SM per ml. The endogenous rate of the resistant strain of *B. cereus* was inhibited only slightly by 100 μg SM per ml. The endogenous R.Q. of the susceptible strain of *B. cereus* was found to be 1.0 in

the absence of SM, indicative of carbohydrate oxidation. In the presence of 10 μg SM per ml, the R.Q. was 1.4, indicating that SM alters the endogenous metabolism.

Aerobic oxidation. In the case of the sensitive strain of *S. aureus*, inhibition of glycerol and lactate oxidations was observed with as little as 1 μg SM per ml, whereas no inhibition occurred with the resistant strain with 100 μg SM per ml (table 1). SM in the concentration of 100 μg SM per ml did not inhibit the oxidation by the sensitive strain of the following substrates: glucose, fructose, ethanol, pyruvate, ascorbate, sodium salt of adenylic acid, glutamate, D-ribose, α-glycerophosphate, glyceraldehyde, dihydroxyacetone, glycerate, and glyoxal. Among the substrates not utilized were succinate, fumarate, butyrate, citrate, acetaldehyde, stearate, glycolate, and glyoxylate. Acetate was utilized oc-

TABLE 1

The effect of streptomycin on the aerobic oxidation of glycerol and lactate by S. aureus

SUBSTRATE (0.5%)	STRAINS	SM	AVERAGE % INHIBITIONS DURING TIME PERIODS (HR) INDICATED			
			0–0.5	0.5–1	1–2	2–3
		μg/ml				
Glycerol	S	1	0	10	14	20
		10	0	15	26	32
		100	0	23	35	42
	R	100	0	0	0	0
		1,000	0	0	0	20
Lactate	S	10	0	7	15	20
		100	0	14	22	25
	R	1,000	0	0	0	0

S = susceptible strain; R = resistant strain.

casionally at a very slow rate; when utilized the oxidation was inhibited completely by 100 μg SM per ml.

With the sensitive strain of *B. cereus*, 1 μg SM per ml produced inhibition of oxidation with all the substrates studied, namely, glycerol, lactate, glucose, pyruvate, ethanol, and acetate. The inhibitions were usually quantitatively greater than those obtained with *S. aureus;* in fact the oxidation of acetate was completely blocked by 1 μg SM per ml. The concentrations of sodium acetate used were 0.054 and 0.027 per cent, both of which gave identical results. Utilization of this substrate began at a slow rate during the second hour and approached a maximum during the third and fourth hours ($QO_2 = 28$). No utilization occurred even at 4 hours when a concentration of 0.5 per cent was used. Appreciably less or no inhibition of the oxidation of these substrates by the resistant strain was produced with 100 μg SM per ml.

In experiments with the sensitive strain of *S. sonnei* inhibition by 10 μg SM

per ml was obtained with glycerol, lactate, pyruvate, succinate, and acetate, but not with ethanol. No inhibition occurred with the resistant strain at a concentration of 100 μg SM per ml.

A comparison between the susceptible and resistant strains of the QO_2 values for each substrate revealed that in some cases there was no significant difference and in the remainder of cases there were as many increased values for the resistant strains as there were decreased values. Furthermore, there was no consistency in these changes between the different organisms.

In almost every instance in which inhibition appeared it did so only after a lapse of time. The inhibition usually increased with time, and the lag period before the appearance of inhibition generally decreased with increasing concentration of SM (cf. table 1). One possible cause for this lag is that time is required for SM to reach the site of action (perhaps a question of cell membrane permeability) or to adsorb onto that site to a sufficient degree to cause the inhibition. Experiments were conducted to test this postulate. SM was incubated for 1 hour with cells of *S. aureus* prior to the addition of substrate (glycerol, lactate). The resultant inhibition appeared at the same time and to the same degree as in experiments in which the SM was added to the cells at the same time as the substrate.

The inhibitions observed apparently are not of the competitive type. Within experimental error the degree of inhibition obtained with several substrates with *B. cereus* and *S. aureus* was the same over a 10-fold range of substrate concentrations.

It was realized that these inhibitions might be a reflection of the bactericidal action of SM. If such is the case, the inhibition-time curve should follow the same course as the viable cell count, time curve. The viable cell count of *B. cereus*, however, remained fairly constant after an initial drop during the first 2 hours' contact with 10 μg SM per ml. As indicated by turbidity measurments, there was no lysis during the course of the experiments. Furthermore, with *S. aureus* and *S. sonnei*, the oxidation of several substrates was not inhibited.

It has been pointed out (Brooks, 1947) that one possible cause for the inhibition of O_2 consumption by inhibitors is an altered permeability of the cell membrane to O_2 or the exogenous substrate. Since inhibitions were observed under anaerobic conditions, it seems unlikely that the inhibitions under aerobic conditions are a reflection of altered permeability to O_2. Altered permeability to exogenous substrate was apparently ruled out by experiments in which it was shown that SM did not affect the permeability of susceptible cells to pyruvate (unpublished data).

It was reasoned that if SM blocks a respiratory chain at a specific point, then the substrate for that particular enzyme should accumulate. Tables 2 and 3 are balance sheets showing the effect of SM on the aerobic carbohydrate metabolism of *B. cereus* and *S. aureus*, respectively.

In the calculation of these tables it was assumed that the endogenous respiration continues uninfluenced by the presence of added substrate, and the O_2 con-

sumption values in the presence of substrate were corrected accordingly. In experiments with acetate as substrate for *B. cereus* SM completely blocked the utilization of acetate (determined by chemical analysis at the beginning and end of the experiments) and reduced the rate of O_2 consumption to that of endogenous controls containing the same SM concentration. Especially with *B. cereus* and

TABLE 2

Balance sheet showing the effect of streptomycin on the aerobic carbohydrate metabolism of B. cereus

SUBSTRATE	SM	% OF THEORETICAL O_2 CONSUMPTION	OVER-ALL % INHIBITION OF O_2 CONSUMPTION	OVER-ALL % INHIBITION OF SUBSTRATE UTILIZATION	R.Q.		RECOVERY AT END OF EXPERIMENT, EXPRESSED AS % C RECOVERED FROM SUBSTRATE USED							GLUCOSE INCREASE IN CELLS EXPRESED AS % C*	TOTAL % RECOVERY
					Theor.	Observed	Glucose	Pyruvate	Lactate	Glycerol	Ethanol	Acetate	CO_2		
	μg/ml														
Glucose	0	37			1.0	1.0		0.46	1.2		0	6.9	37	3.9	50
	1	39		13		1.0		0.23	6.9		0	11	38	4.3	60
	10	43	21	31		1.0		2.1	5.4		0	28	43	1.8	80
Pyruvate	0	50			1.2	1.3	0			0	0	16	52	1.1	69
	1	53	27	30		1.3	0			0	0	26	56	−0.6	81
	10	44	41	34			0			0	0	45	47†	−1.8	90
Lactate	0	55			1.0	1.1	0	0		0		5.8	54	2.5	63
	1	52	10	13		1.1	0	0		0		14	49	0.12	63
	10	47	30	14		1.1	0.62	0		0		21	44	−2.0	64
Glycerol	0	39			0.86	0.72		‡	4.1		0.50	26	33	2.2	66
	1	42	14	16				‡	6.3		0.60	49	34	1.3	91
	10	36	36	32		0.70		‡	8.5		0.85	48	31	0	88
Acetate	0	50			1.0	0.95							43	3.9	47
	1		100	100											
	10		100	100		0.95								−3.2	

Each value is the average of 2 to 6 experiments run in duplicate.

* Change during experiment in total cell-reducing substances not related to endogenous metabolism, determined as glucose, and expressed as percentage of C relative to the total C in the substrate utilized.

† Where an R.Q. value is not given, the R.Q. obtained with the other concentration of SM is assumed.

‡ A violet-red color developed in these determinations unlike the color given by pyruvate. The substance present causing this is unknown.

its relatively high endogenous rate, if this correction is not made certain values in the balance sheets would be changed quantitatively but not qualitatively. Since interpretations are made only from the qualitative nature of these results the validity of this assumption is not critical.

In instances in which an interfering substance was found to be present for a particular analysis and it was not possible to make a correction for this inter-

ference, the analyses were omitted from the data given in tables 2 and 3. Tests for some of the substances in the supernatants from endogenous systems were positive although the amounts found were small. These values were subtracted from those obtained for the supernatants containing initial substrate.

Along with the determinations of reducing substance in the cells at the end of the experiment, an aliquot of cells stored at 4 C during the experiment was analyzed. The total reducing substance in the cells of the endogenous controls at the end of the experiment was usually considerably less than that in the ali-

TABLE 3

Balance sheet showing the effect of streptomycin on the aerobic carbohydrate metabolism of S. aureus

SUBSTRATE	SM	% OF THEORETICAL O_2 CONSUMPTION	OVER-ALL % INHIBITION OF O_2 CONS.	OVER-ALL % INHIBITION OF SUBSTRATE UTILIZATION	R.Q.		RECOVERY AT END OF EXPERIMENT, EXPRESSED AS % C RECOVERED FROM SUBSTRATE USED						GLUCOSE INCREASE IN CELLS, EXPRESSED AS % C*	TOTAL % RECOVERY
					Theor.	Observed	Pyruvate	Lactate	Glycerol	Ethanol	Acetate	CO_2		
	μg/ml													
Glycerol	0	39			0.86	0.73	1.0	1.2		1.6	51	38	1.9	95
	10	36	16	8			1.2	3.2		1.8	57	35†	2.0	100
	100	42	31	36		0.73	1.3	1.5		1.8	63	38	2.7	108
Lactate	0	32			1.0	0.75	5.2		0		35	22	0	62
	10	34	12	16			10		0		49	24	−2.3	81
	100	29	24	19		0.72	16		0		52	19	0	82
Glucose	0	26			1.0	1.0	3.0	5.2			30	27	0.50	66
	100	26	0	0		1.0	3.0	4.5			30	27	0.50	65

Each value is the average of 2 to 6 experiments run in duplicate.

* Change during experiment in total cell-reducing substance not related to endogenous metabolism, determined as glucose, and expressed as percentage of C relative to the total C in the substrate utilized.

† Where an R.Q. value is not given, the R.Q. obtained with the higher concentration of SM is assumed.

quot stored at 4 C. This undoubtedly represents utilization of stored carbohydrate reserves. This was confirmed by the observation of an R.Q. of 1.0 for endogenous O_2 consumption with *B. cereus*. Since in all other calculations it has been assumed that endogenous metabolism is unaffected by the presence of exogenous substrate, the value for the total reducing substance found in the endogenous cells at the end of the experiment was taken as the reference point from which the reducing substance in the cells exposed to exogenous substrate either increased or decreased.

The columns in tables 2 and 3 headed by "% of theoretical O_2 consumption" represent (observed O_2 consumption per theoretical O_2 consumption for complete oxidation of substrate utilized) × 100. This "theoretical O_2 consumption" is seldom realized, and in some reports it has been adequately demonstrated

that a considerable part of the fraction of substrate not oxidized to completion is synthesized to a carbohydrate and retained by the cell. It has been found (Clifton, 1946) that in the presence of appropriate concentrations of certain inhibitors, e.g., azide, the O_2 consumption approaches the theoretical for complete oxidation and the synthesis of the carbohydrate is blocked. As shown in the tables, the percentage of theoretical O_2 consumption did not increase in the presence of SM. These experiments were not allowed to go to completion, and, as seen in the tables, certain substances tested for appeared in increased amounts in the presence of SM. It was considered possible that if these substances (and probably others not tested for) were permitted sufficient time to be metabolized, the percentage of theoretical O_2 consumption might have increased. Several experiments were run with *B. cereus*, using glucose, glycerol, and lactate as substrates, to test this possibility. The experiments were continued at least 1 hour after the substrate was exhausted. The percentage of theoretical was not increased either in the presence of inhibitory or subinhibitory (0.1 μg per ml) concentrations of SM.

The columns headed "Theoretical R.Q." represent R.Q. values for complete oxidation of the substrates to CO_2 and H_2O. The columns headed "Observed R.Q." represent average values for 3-hour experiments, and are not corrected for endogenous respiration. If the corrections are made, the values are changed by a maximum of 5 per cent. Two facts appear upon studying these columns: first, SM in inhibitory concentrations does not affect the R.Q.'s; second, with but two exceptions (glycerol as substrate for both organisms, and lactate as substrate for *S. aureus*), the observed R.Q.'s do not differ significantly from the theoretical values. Since in no instance was a substrate oxidized completely to CO_2 and H_2O, in order for the R.Q. not to change, the total C, H, and O in the other substance or substances formed from the substrate must be in the same or very nearly the same ratio as in the substrate. Failure of the observed R.Q. to conform with the theoretical can only mean the contrary. The oxidation of glycerol by *S. aureus* offered an opportunity to determine whether the observed R.Q. was compatible with the over-all reaction indicated by the chemical analyses for intermediates and end products in a case in which the observed and theoretical R.Q.'s differed, and in which approximately all of the C in the substrate utilized was accounted for. The over-all reaction as indicated by the balance sheet (table 3) gives an R.Q. of 0.72, which agrees very well with the observed R.Q. of 0.73. This close agreement also indicates that at least in this instance the volatile acid determined was acetic.

All the quantities of the various substances tested for in the supernatants at the end of the experiments as well as the reducing substance (determined as glucose) in the cells were converted to C. These C values were then referred to the C which disappeared as the initial substrate. These percentages of C recoveries are listed for the various substrates with and without SM in tables 2 and 3. These values varied somewhat from experiment to experiment but the trends were constant, and since no interpretation is to be made from their exact magnitude, the averages of several experiments are given in the tables.

Acetate was the only substance tested for that was found to be present consistently in higher amounts in the presence of SM than in the absence of SM. This held true for all substrates with both organisms. Acetate also was found to accumulate in increased amounts during the endogenous respiration of the susceptible strain of *B. cereus* in the presence of inhibitory concentrations of SM. As seen in table 2, both the oxidation and utilization of acetate by the susceptible strain of *B. cereus* were completely blocked. In the case of the resistant strain, neither the oxidation nor the utilization of acetate was inhibited by 100 μg SM per ml.

One possible explanation for the inhibition of the oxidation of substrates other than acetate, with *B. cereus*, which was considered was the following: as acetate accumulates the equilibria in the enzymatic chain are shifted until a point is reached at which the over-all rate is decreased. Experiments were run to test this possibility. Acetate in a concentration approximating that recovered at the end of other experiments was added with glucose. The inhibition of O_2 consumption developed at the same time and to the same extent whether or not acetate was present. These results would appear to rule out this possibility.

Examination of the data in the columns headed "Glucose increase in cells, expressed as % C" reveals that with one exception (*S. aureus*, lactate as substrate) the total hydrolyzable reducing substance of the cells increased in the absence of SM. These increases, however, are considerably smaller than those reported for similar experiments with other microorganisms such as yeast (Winzler, 1940). With *B. cereus*, inhibitory concentrations of SM appeared to block this increase partially or completely. With *B. cereus*, therefore, SM apparently blocked the oxidative assimilation of carbohydrate. The percentage of theoretical O_2 consumption did not increase concomitantly because of accumulation of substances such as acetate in the supernatant. With *S. aureus*, however, there was no indication of such a block.

The last columns of the tables show the total percentage of C recovered in the substances tested for (including the CO_2 produced) from the C disappearing as substrate. In only one case (glycerol as substrate for *S. aureus*) was all of the C accounted for. This was to be expected since unquestionably there are many more intermediates formed in the metabolism of these substrates than were tested for. It is quite possible that some of these also accumulate in the presence of SM.

Anaerobic metabolism. Since the multiplication of the three susceptible strains under strictly anaerobic conditions was inhibited by SM, it was of interest to determine whether SM also inhibited the metabolism of any substrates anaerobically. Of the substrates utilized by *S. aureus*, no inhibition was observed in any case. With *B. cereus*, inhibition of the anerobic metabolism of glucose and pyruvate was produced by 1 μg SM per ml. With *S. sonnei*, 1 μg SM per ml inhibited the metabolism of pyruvate. Again when inhibition occurred, usually there was a lag period before it developed. In all cases the resistant strains were unaffected by 100 μg SM per ml.

Differentiation between metabolic CO_2 and acid production (both being meas-

ured as CO_2 liberated under anaerobic conditions) was made in the following three cases (Umbreit *et al.*, 1945): in the oxidation of glucose by *B. cereus* and *S. sonnei* the CO_2 was acid CO_2; in the oxidation of pyruvate by *S. sonnei* approximately 70 per cent of the CO_2 was acid CO_2 and 30 per cent metabolic CO_2 In the latter case the production of both acid and metabolic CO_2 was inhibited by SM.

Using *S. aureus*, the anaerobic metabolism of the following substrates was studied by the methylene blue Thunberg technique: glucose, fructose, ethanol, glycerol, succinate, pyruvate, lactate, acetate, citrate, malate, cis-aconitate, xanthine, stearate, DL-isoleucine, L-leucine, DL-alanine, β-alanine, DL-phenylalanine, DL-valine, L-lysine HCl, DL-lysine diHCl, L-arginine, L-glutamate, DL-methionine, glycine, L-tryptophan, and L-asparagine hydrate. In no case was any inhibition observed; in many instances there was stimulation. In view of the lag period observed before inhibition of respiration develops in the Warburg respirometers, inhibition might not be expected in the Thunberg tube since the reaction was complete within 10 to 20 minutes. Furthermore it was found that, when methylene blue in a concentration that even slightly inhibits oxygen uptake (using a substrate the oxidation of which can be inhibited by SM) is added to the Warburg flasks, as much as 100 μg SM per ml produce no further inhibition. This too might explain why no inhibition was obtained in the Thunberg tube since the controls and the tubes containing SM all contained methylene blue in an inhibiting concentration.

With *B. cereus* the following substrates were studied: glucose, glycerol, lactate, pyruvate, ethanol, acetate, and succinate. There was no inhibition.

Indications that the inhibitions observed are due to SM. Since it is possible that the inhibitions might be produced by impurities in the samples of SM, several different preparations of varying purities were used. The inhibitions were the same with commercial SM-sulfate, commercial SM-hydrochloride, and highly purified SM-sulfate, Pfizer, and SM-sulfate, Parke, Davis. SM inactivated by being boiled 30 minutes in 0.5 N NaOH did not produce inhibition. Dihydrostreptomycin produced the same inhibition of glycerol oxidation by *S. aureus* as SM-sulfate, Pfizer.

In vitro, the maximal activity of SM occurs at approximately pH 7.8, and decreases markedly below pH 7.0 (Wolinsky and Steenken, 1946). A comparison of the pH activity curves of SM on the multiplication of the sensitive strain of *S. aureus* and its aerobic oxidation of glycerol and lactate revealed superimposable patterns.

In correlation with the antagonistic effect of salts on SM inhibition of bacterial multiplication (Berkman, Henry, and Housewright, 1947), salts were found to antagonize the metabolic inhibitions. The phosphate buffer concentration in the respirometers was found to have a direct relation to the degree of inhibition observed. For example, in the oxidation of lactate by *B. cereus*, in M/7.5 buffer no inhibition occurred with 100 μg SM per ml, but in M/120 buffer 50 per cent inhibition occurred with as low as 1.0 μg per ml. Inhibition of the oxidation of pyruvate in M/7.5 phosphate buffer reached 40 per cent with 10 μg SM per ml,

whereas in M/120 buffer inhibition reached 70 per cent with 10 μg per ml. A M/120 phosphate buffer was adequate for maintaining the neutral pH in the respirometers. Salt added to the system after inhibition of respiration had begun did not reverse the inhibition. This might indicate that these inhibitions are irreversible.

DISCUSSION

Numerous quantitative differences were observed in QO_2 values and anaerobic CO_2 production between the susceptible and resistant strains of the three organisms studied. No indication as to the cause for resistance or the mode of action of SM, however, can be derived from these differences since none was consistent among the three organisms. The fact that these changes do not occur consistently in the same direction would indicate that the interpretation of Seligmann and Wassermann (1947) that resistant strains have "damaged" enzyme systems is unwarranted.

The inhibitions of metabolic functions produced by SM seldom reached 100 per cent (excepting acetate as substrate with *B. cereus*), indicating either that some intermediate reaction is only partially blocked or that the step that is blocked is in parallel with others not affected. In every instance studied, acetate (or a similarly volatile acid) accumulated more in the presence of inhibitory concentrations of SM than in its absence. The fact that the oxidation of acetate by the sensitive strain of *B. cereus* was very susceptible to SM might account for the increased accumulation of acetate in this case. With *S. aureus*, however, there is evidence that the greater accumulation in the presence of SM resulted from greater production of acetate in the presence of SM. First, *S. aureus* oxidized acetate at a very low rate. Second, with glucose, a substrate the oxidation of which was not inhibited, more acetate did not accumulate in the presence of SM, although acetate was one of the end products of its metabolism. This increased production of acetate could result from SM blocking an alternate pathway that does not have acetate as an end product.

SM might inhibit the functioning of an enzyme in a carbohydrate reaction chain by combining with it specifically. The lag period usually observed for the development of the inhibition would seem to favor an alternative hypothesis. The lag period may represent the time during which some substance essential for the metabolism is being depleted and not being replenished (other intrepretations were rejected in an earlier section). SM would then be blocking the formation of this essential substance (enzyme? coenzyme? substrate?). The observed inhibitions of oxidative metabolism would thus be an indirect consequence of the primary inhibition.

Whether or not the inhibitions of metabolic functions observed bear a causal relationship to the bacteriostatic action of SM cannot be said from these experiments. Several observations were made which, although not constituting direct evidence for a causal relationship, are, however, compatible with such a hypothesis. These observations are as follows: First, the inhibitions in the susceptible strains were brought about in most cases by concentrations of SM

that just cause bacteriostasis. Second, the same functions in resistant strains were affected appreciably less or not at all by much higher concentrations of SM. Third, the pH-activity curves for SM are very similar in the two cases. Fourth, salts reverse both phenomena. Fifth, SM's of varying purity produced the same degree of inhibitions, indicating that the inhibitions probably were not due to one or more impurities present. This is further evidenced by the failure of SM inactivated by heat and alkali to produce the inhibitions.

SUMMARY

Streptomycin in very high concentrations does not inhibit catalase, carbonic anhydrase, cytochrome-cytochrome-oxidase, succinoxidase, carboxylase, urease, or trypsin.

Streptomycin in concentrations just bacteriostatic noncompetitively inhibits the metabolism of certain carbohydrate intermediates in susceptible strains of *Staphylococcus aureus*, *Bacillus cereus*, and *Shigella sonnei*. Resistant strains of these organisms were little or not at all affected by high concentrations of streptomycin.

It is postulated that streptomycin either inhibits an enzyme or enzymes involved in carbohydrate metabolism or inhibits their formation. This inhibition, in both cases studied in this investigation, resulted in increased accumulation of acetate.

Whether or not the inhibition of metabolic functions observed bears a causal relationship to the bacteriostatic action of streptomycin cannot be concluded from this study, although all the observations made are at least compatible with such a theory.

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