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Streptomycin. IV. Adsorption of Streptomycin by Susceptible and Resistant Bacteria.

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It has been shown in another report[1] that streptomycin (SM) interferes with the carbohydrate metabolism of resting bacteria which are susceptible to the antibiotic, whereas the metabolism of resistant cells is essentially unaffected. Consideration was given to the possibility that this interference with oxidative metabolism might be directly related in a causative way to the inhibition of bacterial multiplication. Assuming this interpretation to be correct, it was recognized that there are at least two possible reasons for the resistance of some bacteria to SM: first, resistant cells may have different metabolic systems containing enzymes insensitive to the drug, or, second, the resistant cell membrane may be impermeable to the antibiotic and thus bar its entrance into the cell. The metabolic systems in the latter case would not have to be qualitatively different from those of sensitive cells.

A corollary of the latter postulate is that the antibiotic exerts is action after passing the cell membrane. The alternative is that the antibiotic exerts its activity at the cell surface. In such a case the resistance of an organism to the antibiotic would not involve permeability of the cell membrane so far as the antibiotic is concerned, but might be associated with the adsorptive capacity of the membrane for the antibiotic.

With these ideas in mind an attempt was made to determine whether or not susceptibility to SM is correlated with either permeability of the cell membrane to SM, or the adsorptive capacity of the cell for SM, and whether its failure to inhibit the carbohydrate metabolism of resistant bacteria results from its inability to gain access to the metabolic enzymes.

Adsorption of streptomycin by bacteria. The experiments reported in this section were run with susceptible and resistant strains (derived from the susceptible strains) of *Staphylococcus aureus, Shigella dysenteriae* Sonne, and *Bacillus cereus*. In each instance the resistant strain was able to grow in the presence of 1000 μg SM/ml and the susceptible strain was inhibited by 1 μg/ml. All cells were grown in Difco brain heart infusion broth with PAB at 37°C for 16 hours on an International shaking machine, after which they were centrifuged, washed once with distilled water and then taken up in the desired volume of distilled water. Wherever NaCl was used it was added in the solid form in quantities sufficient to give the desired concentration. The SM used was streptomycin sulfate, Abbott. All SM assays were done by the method of Loo, *et al.*[2]

In one series of experiments bacterial suspensions having a packed cell volume of 20 to 78% were exposed to varying concentrations of SM, without added salt, with 1 M NaCl at the start, and with 1 M NaCl added at intervals of 3/4 to 2 hours after shaking had begun. Packed cell volumes were measured by centrifuging the cell suspensions in Wintrobe tubes at 4000 R.P.M. for 1 1/2 hours. All exposures of the cells were carried out at 37°C. After 2 to 4 hours exposure to SM the suspensions were centrifuged and the supernatants decanted and assayed for SM. The cells were then resuspended in the same

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TABLE I.
The Combination of Streptomycin with Susceptible and Resistant Strains of *Staph. aureus:* Its Prevention by 1 M NaCl and Poor Elution with Water.

	Packed cell vol. %	Streptomycin μg/ml fluid at 0 time	Streptomycin recovered after shaking at 37°C: 3 hrs μg/ml	3 hrs %	Overnight elution of cells with water μg/ml	Overnight elution %	Total streptomycin recovered, %
Susceptible strain (S) + streptomycin	25	67	12	18	12	18	36
S + 1 M NaCl ¾ hr after shaking started	25	67	55	82	12	18	100
S + 1 M NaCl at start of experiment	25	67	58	87	8	8	95
Resistant strain (R) + streptomycin	23	65	7	11	13	20	31
R + 1 M NaCl ¾ hr after shaking started	23	65	53	82	12	19	101
R + 1 M NaCl at start of experiment	23	65	53	82	8	12	94

TABLE II.
Combination of Streptomycin with Susceptible and Resistant Strains of *Staph. aureus:* Its Prevention and Elution with 1 M NaCl.

	Packed cell vol. %	Streptomycin μg/ml fluid at 0 time	Streptomycin recovered after shaking at 37°C: 4 hrs μg/ml	4 hrs %	Overnight elution of cells with 1 M NaCl μg/ml	Overnight elution %	Total streptomycin recovered, %
Susceptible strain (S) + streptomycin	41	85	35	41	41	48	89
S + 1 M NaCl 2 hrs after shaking started	41	85	75	88	18	21	109
S + 1 M NaCl at start of experiment	41	85	68	80	21	25	105
Resistant strain (R) + streptomycin	38	80	25	31	45	56	87
R + 1 M NaCl 2 hrs after shaking started	38	80	77	96	20	25	121
R + 1 M NaCl at start of experiment	38	80	67	84	20	25	109

TABLE III.
Correlation of the % Cell Suspension of Susceptible and Resistant Strains of *Staph. aureus* with Removal of Streptomycin from Solution.

	Packed cell vol., %	Streptomycin μg/ml fluid at 0 time	Streptomycin recovered after 3 hrs	
			μg/ml	%
Susceptible strain	60	115	21	18
" "	6	49	10	20
" "	0.6	46	32	70
" "	0.06	46	45	99
" "	0.006	46	42	91
Resistant strain	55	98	14	14
" "	5.5	51	9	18
" "	0.5	46	32	70
" "	0.05	46	45	99
" "	0.005	46	40	87

volume of distilled water or 1 M NaCl and placed overnight at 37°C on an International shaking machine. Again, following centrifugation, the supernatants were assayed for SM. Equilibrium between the SM and the bacterial cells in the initial suspension apparently was at least closely approached within 3/4 hour, since the amount of SM removed from solution was constant within experimental error when the time of exposure was varied from 3/4 to 4 hours. Results of typical experiments run under these conditions are shown in Tables I and II. The former table shows that recovery of SM from *Staph. aureus* cells was low in the absence of NaCl and very high in its presence whether the salt was added with the SM or 3/4 hour after the experiment was begun. When water was used to elute these cells overnight practically all the remaining SM was recovered in those instances where the cells had been exposed to salt, while about 65% of the SM was not recovered from those cells not exposed to salt.

The experiment in Table II was run in the same fashion as that in Table I except that in 1 case 1 M NaCl was added to the cells 2 hours after exposure to SM and the cells were eluted overnight with 1 M NaCl instead of with distilled water. Here, using NaCl in the eluting fluid, a great deal more of the SM was recovered from cells not originally exposed to NaCl than when distilled water was used as the eluant. Within experimental error of the test methods used recoveries of SM in the former case always totaled 100%. The totals of some of the recoveries aggregated more than 100%. Presumably this was caused by supernatant fluid not removed by decantation and by fluid remaining in the interstices of the packed cells.

In general it was found that SM was removed from solution to the same extent by both susceptible and resistant strains of the same species of bacteria. The experiments described indicate that considerable amounts of SM are taken up from water solution by heavy suspensions of both resistant and susceptible bacteria.

It seemed of interest to determine the % cell suspension at which the decrease in SM concentration is too small to be detected by the assay method used. These experiments were run by making 5 10-fold dilutions of a heavy suspension of organisms. All the suspensions were exposed to water solutions of SM for 2 to 3 hours at 37°C on an International shaking machine. Table III shows the results of 1 such experiment using susceptible and resistant strains of *Staph. aureus*. It can be seen that as the volume of cells decreased the recovery of SM increased. When the packed cell volume was small (0.05% in this case) recovery was complete within the experimental error of the assay method. The assay method is not sufficiently precise to detect the small decreases in SM concentration resulting from contact with such a relatively small number of cells. With a 60% suspension of cells, on the other hand, there was only 18% recovery of SM. Essen-

tially the same results were obtained with susceptible and resistant strains of the other 2 organisms.

In the presence of bacteria, therefore, SM decreases in concentration in the supernatant. If the cellular membrane is permeable to SM, the latter should distribute itself between the cellular contents and the surrounding medium according to its relative solubility in each of the 2 phases. At a packed cell volume of 0.5%, 30% of the SM disappeared from solution (Table III). If the only cause for this decrease was a greater solubility in the cellular protoplasm, the distribution coefficient would be far from unity, a situation which is very improbable. When 2% NaCl was added with the SM, the cells took up a negligible amount of SM. This indicates that unless salt *per se* blocks entrance of SM into the cell, little or no SM gets in. Such a salt block seems unlikely, since it is difficult to conceive how a distribution coefficient could be so markedly changed by the presence of salt. If the cells were first allowed to take up the SM and then salt added, followed by one elution with water or saline, there was total recovery of the SM in the combined first supernatant and eluate. This is interpreted to mean that little or no SM penetrated the cell membrane.‡ Otherwise one must assume that in the presence of salt the cell excreted SM from the cell against a concentration gradient, which would seem extremely questionable.

All or nearly all of the SM taken up by bacteria would appear, therefore, to be in the adsorbed state at the cell surface. The elution of this SM by salt confirms this hypothesis. SM adsorption and its elution by salt has been observed in inanimate systems,[4] and salts in general antagonize the bacteriostatic action of SM.[5]

Studies on the rate of desorption of SM from bacterial cells and its possible relation to the "persistent" action of SM. Recently reports have begun to appear in the literature indicating a fallacy in the assumption that a certain minimal blood concentration of an antibiotic must be maintained for effective therapy. At least one such report has appeared concerning SM in the treatment of tuberculosis in guinea pigs.[6] Apparently the inhibitory action of the antibiotic can persist after the blood concentration falls below the sensitivity of the test for the antibiotic. If the rate of desorption of SM from bacteria in water is very slow, it was believed that this reported phenomenon of persistent action might be explained as follows: when the blood concentration is high the SM is adsorbed by the bacteria; when the blood level falls to zero the SM adsorbed by the bacteria is still there because of a slow rate of desorption, and it is presumably situated so as to effect an inhibition. The concentration of salts present in body fluids and tissues would not be sufficiently great to prevent completely such an adsorption.

As seen in Table I (and in all other similar experiments) the ratio of SM recovered in the first supernatant to that adsorbed is of the same order of magnitude as the SM recovered in the water eluate to that remaining adsorbed. As previously stated some of the SM in the water eluate undoubtedly originates from the first supernatant. Since this amount cannot be calculated with accuracy, it cannot be determined whether or not these ratios are the same. From these data, therefore, it was impossible to determine the rate of desorption of SM from the bacterial cells in the presence of water. Experiments were designed to investigate further the possibility that the persistent action may be due to a slow rate of desorption of SM from bacterial cells.

‡ It has been reported[3] recently that SM forms a colloidal sol and not a true solution in distilled water. The particle size of the dispersed phase was estimated to be approximately 65 mμ. The fact that SM passes freely through cellophane membrane[4] indicates that these large aggregates must be in equilibrium with particles of much smaller molecular size.

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In one series of experiments, susceptible cells of *B. cereus* B-569 (packed cell volumes of 25 to 30%) were exposed to SM (400 to 1100 μg/ml) in water and in 1 M NaCl for 2 hours at 37°C on an International shaking machine. Aliquots were then placed in cellophane dialysis bags and dialyzed against running water and 1 M NaCl respectively. At intervals during a 48 hour period a set of dialysis bags was removed, the suspensions centrifuged, the supernatants assayed for SM, and the cells resuspended in 1 M NaCl. After 16 hours this salt eluate was also assayed for SM.

In the case of exposure of cells to SM in water followed by dialysis versus water, SM could be recovered in decreasing amounts over the period of 48 hours both in the supernatant and in the NaCl eluate. Approximately 4 times as much SM was recovered in the eluate as in the supernatant at each time interval. In the case of exposure of cells to SM in 1 M NaCl followed by dialysis versus 1 M NaCl solution, either no SM or very minute amounts of SM could be recovered at 24 hours from the supernatant. The fact that in the latter case practically all the SM was lost by dialysis within 24 hours indicates that the SM concentration in the supernatants of cells dialyzed versus water was being maintained by continuous desorption of the SM from the cells in the presence of water. These results confirm the earlier observations on the adsorption of SM by bacteria and show that SM does desorb in the presence of water.

In the above experiments the criterion of adsorption and elution of SM from bacteria was indirectly determined by estimation of the antibiotic in the supernatants. In another series of experiments, adsorption of SM was determined by whether or not the cells were able to multiply, *i.e.*, it was assumed that as long as SM was adsorbed at the critical site or sites of action of SM, the cells would be unable to divide. Cells of the susceptible strain of *B. cereus* B-569 were exposed to concentrations of SM ranging from 10 to 1000 μg/ml for periods of time up to 48 hours. The cells were then counted by the pour plate method in nutrient agar and in nutrient agar containing 1.5% NaCl. The dilutions required to make the plate counts reduced the SM concentrations far below the minimal inhibitory level. Although there was a decided drop in bacterial count after 2 hours exposure to SM, presumably due to its bactericidal action, counts made at intervals over the periods of the experiments were essentially the same in the presence and absence of NaCl. Since in controls this concentration of NaCl completely antagonized the action of 10 μg SM/ml it seems necessary to conclude that, in this case at least, that part of the adsorbed SM responsible for its inhibitory action easily desorbs on exposure to water.

It is possible that SM is adsorbed by one or more constituents of the agar in which the cells with their adsorbed SM were plated out for counting. Such an adsorption by the agar should not affect the affinity between SM and the bacterial cells but might affect the rate of desorption from the cells by quickly binding the SM as it desorbs from the cells and thus keeping the concentration of free SM at a very low level. It does not seem probable that this could be a decisive factor in the desorption of SM from the cells in the agar since the concentration of SM surrounding the cells at the time of plating has been reduced tremendously in the dilution procedure required for making counts and since the actual amount of SM adsorbed per cell is extremely minute. If such an adsorption between agar and SM does not occur, free SM as it desorbs from the bacteria would diffuse into the surrounding medium. Since visible colonies appeared on the plates after approximately the same duration of incubation whether the cells had or had not been exposed to SM, apparently desorption proceeded at a sufficiently rapid rate to permit the cells to begin multiplying almost as soon as untreated cells.

These observations, therefore, are not compatible with the hypothesis that the persistent action of SM *in vivo* is a result of slow desorption of SM from the adsorbed state on bacterial cells.

Summary and Conclusions. Studies with *Staph. aureus*, *S. dysenteriae* Sonne, and *B.*

cereus indicate that streptomycin is adsorbed by bacterial cells, all or nearly all of the adsorption occurring at the cell surface. There was no demonstrable difference in adsorption between susceptible and resistant strains of these organisms. The affinity of streptomycin for bacterial cells is greatly reduced in the presence of NaCl. Evidence is presented indicating that the persistent action of streptomycin *in vivo* after the blood level falls below a detectable value is not related to a slow desorption of streptomycin from the adsorbed state on bacterial cells.

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An Outbreak of Equine Encephalomyelitis, Eastern Type, in Southwestern Louisiana.

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From May to October of 1947 an outbreak of encephalomyelitis occurred among horses and mules in southwestern Louisiana. Thousands were implicated with approximately 3,713 deaths.[1] The disease began in Iberia Parish, spread west to Texas and northwest to DeSoto Parish. Because the eastern type of equine encephalomyelitis virus was recovered from human cases that occurred coincidentally, a preliminary report is submitted.

The eastern equine virus was first isolated from horses in 1933,[2,3] and from children in Massachusetts during 1938.[4,5] Antibodies were found in the serum of an adult in 1939.[6] All cases of the eastern type were thought to be confined to the Atlantic coastline until 1940[7] and 1941[8] when it was recovered from horses in both Alabama and Texas. Subsequently equine cases have been reported in all of the Gulf States;[9] also in Missouri,[9] Michigan,[10] Mexico,[11] Brazil[12] and Panama.[13] Besides the Massachusetts outbreak the only human cases ascribed to the eastern strain were 3 in Texas[14,15] that showed neutralizing antibodies in the serum and 1 in Louisiana from whom the virus was recovered in 1946.[16]

In the present outbreak all human cases except one were in children from 7 months to 15 years of age. The one fatal adult case

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