Uptake of ^{14}C -Streptomycin by *Bacillus megaterium*

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SUMMARY

More than 90 % of the radioactivity taken up by *Bacillus megaterium* growing in medium containing inhibitory concentrations of ^{14}C -streptomycin appeared to be identical with streptomycin, as shown by its behaviour on an ion-exchange resin and by recrystallization with streptomycin derivatives. Streptomycin was not removed from the organisms by washing in growth medium with or without addition of unlabelled streptomycin, or in distilled water. It could be extracted with inorganic acids and trichloroacetic acid, but not by some other commonly used extraction procedures. When the organisms were converted to protoplasts and these lysed and fractionated, up to 99 % of the streptomycin in the organisms was recovered in the material sedimented from the cytoplasmic fraction by centrifugation at 105,000 *g*. Very small amounts of streptomycin were found in the fraction containing protoplast 'ghosts', but these may have represented contaminating cytoplasmic material. This distribution may not, however, be a true indication of the location of streptomycin before fractionation, since a similar distribution was found when streptomycin was added to lysed protoplasts immediately before fractionation. The uptake of streptomycin was decreased at pH values below 7. Uptake depended on continued synthesis of cell material, and environmental conditions which prevented growth and bactericidal action also considerably decreased uptake of streptomycin. The quantity of streptomycin finally taken up was proportional to the concentration of streptomycin in the growth medium, and at the lowest growth-inhibitory concentrations was about 5×10^4 molecules of streptomycin per bacterium.

INTRODUCTION

The previous paper (Hancock, 1962) described investigations of the uptake of ^{14}C -streptomycin by several micro-organisms, and some factors which affected it. *Bacillus megaterium* was chosen for a more detailed investigation since its high sensitivity to streptomycin allowed the use of relatively small quantities of ^{14}C -streptomycin, and because well-established methods for fractionating cells of the organism into morphological constituents (e.g. Weibull, Beckmann & Bergström, 1959) might enable localization of the antibiotic in the cells.

METHODS

Organism, growth and harvesting. *Bacillus megaterium* KM was grown in CGY medium (Hancock, 1962) adjusted to pH 7.2, except in one experiment where the pH value was deliberately changed. The conditions of growth were the same as

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those used earlier for this organism (Hancock, 1961). Streptomycin was added to cultures in the exponential phase of growth when the optical density (at 700 m μ) was 0.6–0.7. The organisms were usually harvested from culture samples of more than 40 ml. volume by centrifugation (6000 g; 5 min.). When rapidity of sampling was necessary, smaller volumes of culture (40 ml. or less) were filtered and washed on Oxoid membrane filters (effective filter diameter 2 cm.); for the equivalent of 10–15 mg. dry wt. organisms this usually took about 30 sec. Except in preliminary experiments (specified in detail) the organisms were washed once in cold CGY medium containing unlabelled streptomycin (100 μ g./ml.).

Radioactivity determinations, mounting of samples and self-absorption corrections were carried out as described by Hancock (1962). Membrane filters were fixed to aluminium planchets with 'Durofix' (The Rawplug Co. Ltd., London, S.W. 7). It was not practicable to determine directly the dry weight of organisms present in filtered samples, but this was calculated when necessary from the optical density (700 m μ) of the culture by using a calibration curve for the organisms. The use of filters is specified in the experimental protocols. Self-absorption corrections for materials in subcellular fractions were assumed to be the same as those for intact organisms.

Isolation of intracellular radioactive compounds. Large quantities (about equiv. to 2 g. dry wt.) of organisms which had grown in the presence of ^{14}C -streptomycin until growth had almost ceased, were harvested and washed in CGY medium; since addition of unlabelled streptomycin during washing did not remove any more radioactivity than CGY medium alone (Table 1), unlabelled streptomycin was not added to the washing medium in these experiments, to avoid any possible dilution of intracellular ^{14}C -streptomycin. The organisms were suspended in 5% (w/v) trichloroacetic acid (TCA) at 5° for 20 min., by which time all the ^{14}C was extracted (Fig. 4, Table 6); the cell residue was centrifuged and washed once with TCA, and the pooled extracts (about 40 ml.) were shaken five times with 50 ml. ether to remove TCA. The remaining extract was carefully neutralized to pH 7 and freeze dried. The total time of exposure to acid conditions was about 90 min. To provide a control on recovery during the isolation procedure, a similar quantity of normal organisms were harvested and a known quantity of ^{14}C -streptomycin added immediately after suspension of the organisms in TCA; the following procedures were carried out identically in experimental and control samples. The dried extracts were taken up in water, and a known quantity (about 10 mg.) of unlabelled carrier streptomycin was added to locate the streptomycin peak in the fractions eluted from an ion-exchange column.

Preparation and fractionation of protoplasts. The procedures were used as described by Hancock (1961). High-speed centrifugations were carried out in the No. 40 rotor of the Spinco centrifuge; values for the centrifugal force are the average values as given in the centrifuge manual.

Streptomycin reineckate was prepared and recrystallized by the procedure of Fried & Wintersteiner (1945) after addition of carrier unlabelled streptomycin. The radioactivity of reineckate samples was corrected for self-absorption from an experimentally determined self-absorption curve. Melting points are uncorrected.

Hydrolysis of streptomycin. A small quantity (about 1 mg.) of ^{14}C -streptomycin

was hydrolysed in 6N-HCl at room temperature for 8 hr.; the solution was freeze dried and redried from a small volume of water. Ribonuclease, deoxyribonuclease and trypsin were recrystallized preparations from L. Light and Co., Colnbrook, Bucks. All other materials and methods were as described by Hancock (1962). Concentrations of streptomycin are expressed throughout in terms of the free base.

Separation of streptomycin on ion-exchange columns. The procedure was based on that of Doery, Mason & Weiss (1950). Amberlite IRC-50 (H) (Analytical grade) was washed with water and converted to the Na form by suspending three times in saturated NaHCO₃ solution. Columns 10 × 1 cm. were prepared and washed with 500 ml. distilled water, and the experimental sample was run through the column at a rate of about 10 ml./hr. The column was washed with 50 ml. distilled water, and eluted with 50 ml. of 0.25N-acetic acid followed by 100 ml. of 0.5N-acetic acid at a flow rate of about 20 ml./hr.; 2.5 ml. fractions were collected. Streptomycin was assayed in the effluent fractions by the maltol method (Boxer, Jelinek & Leghorn, 1947), but with one-quarter of the recommended volumes of sample and reagents.

Streptomycin helianthate was prepared by the procedure used by Karow, Peck, Rosenblum & Woodbury (1952) and recrystallized from warm 30 % (v/v) methanol in water. Precipitates of streptomycin helianthate were separated on Oxoid membrane filters. A self-absorption curve was determined experimentally for streptomycin helianthate and used to correct the observed radioactivity to infinite thinness.

RESULTS

Retention of streptomycin on washing

Under the experimental conditions used, addition of streptomycin to 1 µg./ml. inhibited growth fully after a period of about 1 hr.; at this time the number of viable organisms had fallen to less than 1 % of the initial number. It was found (Table 1) that at various times during this period no significant quantities of

Table 1. *Retention of ¹⁴C-streptomycin on washing*

A number of identical samples were taken at each time after addition of streptomycin (1 µg./ml.), and from the same culture except for the 15 min. samples; the organisms were harvested on membrane filters.

	Period of growth with streptomycin (min.)			
	15	30	45	60
Washing medium	Counts/min./sample			
— (control)	85	39	76	74
Growth medium { × 1	—	36	72	—
{ × 3	89	40	71	73
Growth medium + unlabelled { × 1	—	38	—	—
streptomycin (100 µg./ml.) { × 3	84	39	63	65
NaCl (M) { × 1	—	36	71	—
{ × 2	—	34	—	68
Distilled water	—	—	75	75

streptomycin were removed from the organisms by washing in growth medium, with or without addition of excess unlabelled streptomycin, or by washing in distilled water or in *m*-NaCl. In subsequent experiments, the organisms were routinely washed once in growth medium containing unlabelled streptomycin.

Uptake by growing organisms

At sub-inhibitory concentrations of streptomycin, uptake of streptomycin was parallel to growth of a culture (Fig. 1 A) but at a slightly higher rate. At concentrations which inhibited growth, the uptake followed closely the residual growth of the culture and ceased when growth ceased (Fig. 1 B). It was not possible to detect any

Fig. 1. Uptake of ¹⁴C-streptomycin by *Bacillus megaterium* (●) at a sub-inhibitory concentration (0.05 µg./ml.) (A) and at a growth-inhibitory concentration (1 µg./ml.) (B), and optical density (○). Filtered samples. In one experiment (x), chloramphenicol (200 µg./ml.) was added to a culture at the same time as streptomycin.

separate phases in the uptake curve, such as occur in *Escherichia coli* W (Anand, Davis & Armitage, 1960). When streptomycin was added at concentrations below or above 1 µg./ml., growth was inhibited after a longer or shorter period; when the organisms were harvested as soon as growth had ceased it was found that less streptomycin had been taken up at the lower concentrations of streptomycin (Fig. 2).

In two experiments, radioactivity was taken up from ¹⁴C-streptomycin, which had

been hydrolysed under conditions for liberating the streptidine moiety, at less than 3% of the rate of uptake of the intact antibiotic.

Streptomycin does not cause loss of viability when added to cultures of *Escherichia coli* or *Staphylococcus aureus* under conditions in which the organisms cannot grow (Anand & Davis, 1960; Hancock, 1960). Similar results have been obtained with *Bacillus megaterium* (Hancock, unpublished results), and under these conditions the uptake of streptomycin is much lower than that by growing organisms (Table 2). As in *Escherichia coli* W (Anand *et al.* 1960) a major part of the uptake process therefore depends on growth of the organisms in the presence of streptomycin, as does loss of viability.

Fig. 2

Fig. 2. Amount of ^{14}C -streptomycin finally taken up by *B. megaterium* when growth ceases, from 2 experiments (● and ○). Figures by points indicate the time of harvesting (min.) after addition of streptomycin. Chloramphenicol (100 $\mu\text{g./ml.}$) was added to one set of cultures (x) 10 min. before streptomycin and these were harvested after 30 min.

Fig. 3

Fig. 3. Uptake of ^{14}C -streptomycin by *B. megaterium* (1 $\mu\text{g./ml.}$) following addition of chloramphenicol (100 $\mu\text{g./ml.}$) to samples of a culture at the times indicated by each final point (min. after addition of streptomycin).

Table 2. *Effect of inhibition of growth on uptake of ^{14}C -streptomycin*

Organisms incubated for 45 min. in growth medium under the conditions indicated, and harvested on membrane filters.

Conditions of incubation	Counts/min./sample
37°, air	71
5°, air	7
37°, N_2	18
37°, air, + chloramphenicol (200 $\mu\text{g./ml.}$)	16
37°, air, without glucose	3

Effect of chloramphenicol on uptake of streptomycin

At growth-inhibitory concentrations of chloramphenicol (CAP), uptake of streptomycin, measured after long periods, was considerably decreased (Table 2). Kinetic experiments showed that under these conditions the uptake of streptomycin rapidly reached a plateau and thereafter remained constant (Fig. 1), in a similar way to that with *Escherichia coli* W (Anand *et al.* 1960). The quantity of streptomycin taken up in the presence of CAP also depended on the concentration of streptomycin in the medium (Fig. 2). When CAP was added at successive times during inhibition of growth to samples of a culture containing streptomycin, the uptake of streptomycin was arrested almost completely (Fig. 3).

Effect of pH value

The uptake of streptomycin at subinhibitory concentrations showed a marked dependence on the pH value of the growth medium, under conditions in which the rate of growth was affected only to a small extent (Table 3). This would suggest that the streptomycin cation competed with hydrogen ions at some stage in the uptake process, and is probably the basis of the decrease in activity of streptomycin when the pH values of the growth medium is lowered (Abraham & Duthie, 1946).

Table 3. *Effect of pH on rate of uptake of ¹⁴C-streptomycin*

¹⁴C-streptomycin was added at sub-inhibitory concentration (0.2 µg./ml.) to each culture when the population density was equiv. to 150 µg. dry wt./ml. After 75 min. the organisms were harvested on membrane filters and washed once with growth medium (pH 7.2) containing unlabelled streptomycin (100 µg./ml.).

pH value of growth medium	Final population density (µg. dry wt./ml.)	Counts/min./g. dry wt. organism
5.0	305	250
6.0	405	240
7.0	345	940
7.2	340	1270

Uptake of streptomycin after breakage of the osmotic barrier

Addition of toluene to a culture of *Escherichia coli* W in the presence of CAP results in an increased uptake of streptomycin (Anand *et al.* 1960). The effects on uptake of streptomycin of a number of procedures which break the osmotic barrier of *Bacillus megaterium* was investigated (see Table 4). To avoid complications due to different responses of treated organisms to washing, the organisms or cell residues were not washed in these experiments.

In the presence of CAP, addition of toluene or butanol to a growing culture resulted in increased uptake of streptomycin to values approaching that reached in the absence of CAP; uptake was not, however, increased when the suspension was heated. However, when the organisms were incubated at a higher bacterial concentration, all the ¹⁴C-streptomycin added to the incubation medium was taken up in the presence of cetyltrimethylammonium bromide (CTAB) or toluene, or in boiled suspensions.

Table 4. *Effect of osmotic barrier damage on uptake of ¹⁴C-streptomycin*

¹⁴C-streptomycin (1.5 µg./ml.) was added directly to a growing culture (columns 1, 2 and 3) or after harvesting the organisms and resuspending in growth medium at ten times the original population density (column 4); other additions were then made to the samples as indicated. Each sample (1.8 ml.) in column 4 contained streptomycin at 1.5 µg./ml. (total radioactivity 126 counts/min./sample).

Time with streptomycin (min.)	50		15	
Cell density (µg. dry wt. organisms/ml.)	300	300	300	3000
Chloramphenicol (100 µg./ml.) present	—	+	—	—
Additions	Counts/min./sample			
— (control)	82	21	27	25
Cetyltrimethylammonium bromide (200 µg./ml.)	3	5	8	126
Toluene (4 %, v/v)	53	69	42	123
Butanol (25 %, v/v)	50	60	48	76
Boiled (15 min.)	5	11	7	130

Extraction of streptomycin

The release of streptomycin from washed organisms was investigated by several procedures commonly used to liberate intracellular constituents (Table 5). Breakage of the osmotic barrier alone did not appear to be sufficient to effect release, and the only agents which released streptomycin were acidic solutions; the rate of release by several different acids was found to be similar (Fig. 4). It was later found that there was no detectable breakdown of streptomycin by trichloroacetic acid (TCA) under these conditions, but it is possible that the mineral acids may have caused a small degree of hydrolysis.

Table 5. *Extraction of ¹⁴C-streptomycin*

Organisms were harvested by centrifugation, washed once in growth medium + unlabelled streptomycin (100 µg./ml.) and resuspended in the appropriate medium at a concentration equiv. to about 4 mg. dry wt./ml. The residues were recovered by centrifugation after extraction. Results of two separate experiments.

Extracting agent	¹⁴ C-streptomycin in residue (counts/min.)	
— (control)	38	21
5 % (w/v) trichloroacetic acid 5°, 30 min.	2	3
100°, 20 min.	0	2
0.5 N-perchloric acid 5°, 30 min.	—	0
100°, 20 min.	—	0
Hydrochloric acid at 5° for 30 min. 0.1 N	—	14
N	0	—
— (control)	47	24
Water, 100°, 20 min.	46	25
Ethanol, 75 %, v/v, 60°, 30 min.	45	20
Butanol, 10 %, v/v, 25°, 30 min.	43	—
Toluene, 4 %, v/v, 25°, 30 min.	44	24
Cetyltrimethylammonium bromide 250 µg./ml., 25°, 30 min.	42	25

Identity of intracellular radioactive compounds

All the radioactivity in TCA-extracts of organisms grown with ^{14}C -streptomycin was eluted from an ion-exchange column in precise coincidence with the peak containing streptomycin (Table 6; Fig. 5). A control sample of ^{14}C -streptomycin which had been treated in the same way was also recovered completely, showing that the TCA treatment had not resulted in any breakdown of streptomycin (Table 6). Carrier unlabelled streptomycin was added to samples of the material in the streptomycin peaks from each column, and streptomycin helianthate and reineckate prepared and recrystallized. From the specific activities of the products after recrystallization, it appeared that, within experimental error, all the radioactivity present in the experimental sample was in a form which would recrystallize with these streptomycin derivatives (Table 7).

Fig. 4

Fig. 5

Fig. 4. Release of ^{14}C -streptomycin from washed *Bacillus megaterium* at 5° by 0.5N-HCl ($\times$), $0.25\text{N-perchloric acid}$ ($\bullet$), and 5% (w/v) TCA ($\circ$). Suspension density equiv. to about $6\text{ mg. dry wt. organism/ml.}$

Fig. 5. Elution of radioactivity, extracted with 5% (w/v) TCA, from a cation-exchange column. Radioactivity ($\bullet$) was determined using 1.0 ml. from each eluted fraction; carrier unlabelled streptomycin was estimated as maltol ($\circ$). The broken line represents the elution of radioactivity from a column loaded with acid-hydrolysed ^{14}C -streptomycin; the smaller major peak is streptidine.

Location of streptomycin in cell fractions

The fractionation procedure used resulted in separation of the cell wall, 'ghost' and cytoplasmic fractions (Weibull *et al.* 1959). When organisms which had taken up ^{14}C -streptomycin for short or long periods were fractionated, almost all the radioactivity was recovered in the protoplasts (Table 8). In a few preparations, some radioactivity was lost during conversion of intact organisms to protoplasts, but since this did not occur in most preparations it is thought likely that it was due to a small

Table 6. *Extraction of radioactivity from washed organisms by TCA and recovery with streptomycin in column eluates*

Bacillus megaterium grown with ^{14}C -streptomycin until growth had just ceased, and normal organisms, were extracted with 5 % (w/v) TCA. ^{14}C -streptomycin was added to the control extract immediately on addition of TCA. Each extract was from the equivalent of about 2 g. dry wt. organism, and was loaded onto and eluted from a column of Amberlite IRC-50 as described under Methods.

	Total counts/min. in			
	Control		Experimental	
Cell suspension	2800	4670	2420	2190
Cell residue	0	600	0	0
Loaded onto column	2700	5100	2030	2000
Eluted in streptomycin peak	2890	5120	2270	2160

degree of lysis of protoplasts which sometimes occurred during preparation, rather than indicating location of some streptomycin in the cell-wall fraction which is removed at this stage.

When the 'ghost' fraction, which contains the cytoplasmic membrane of the organisms (Weibull *et al.* 1959) was first separated, about 20 % of the total radioactivity in the organisms was associated with it. On washing in 'lysing medium' (Storck & Wachsman, 1957), almost all of this radioactivity was removed but could be recovered from the washing medium by centrifugation at 105,000 *g* for 4 hr. For this reason, it is thought to represent contamination of the 'ghosts' with cytoplasmic material, since the radioactive material in the cytoplasm can also be sedi-

Table 7. *Recovery of intracellular ^{14}C as streptomycin derivatives*

Unlabelled streptomycin was added to samples of the eluate from an ion-exchange column and the derivatives were formed as described under Methods. Values for the streptomycin content of streptomycin helianthate and reineckate are taken from Kuehl, Peck, Walti & Folkers (1945) and Fried & Wintersteiner (1945), respectively.

Times crystallized	Streptomycin helianthate			Streptomycin reineckate	
	mg. used	Counts/min.	Melting point (°)	mg. used	Counts/min.
1	—	—	—	21.2	66
2	22.8	44	215–220 decomp.	—	—
3	10.8	22	—	5.7	21
4	23.0	46	218–220 decomp.	9.0	28
Final specific activity (counts/min./g. derivative)	2000			3100	
Assumed streptomycin content of derivative (mg./g.)	350			390	
Specific activity, (counts/min./g. streptomycin)	5700			7900	
Carrier streptomycin added (mg.)	73.0			89.5	
Radioactivity in original sample (counts/min.) due to streptomycin, calculated from specific activity of derivative	415			710	
Total radioactivity present in original sample (counts/min.)	450			630	

Table 8. *Distribution of ^{14}C -streptomycin in cell fractions of Bacillus megaterium*

Organisms were harvested from cultures (500–1500 ml.) when inhibition of growth was just complete, at 60–120 min. after addition of ^{14}C -streptomycin (1 or 2 $\mu\text{g.}/\text{ml.}$), except in one experiment (column 1) when harvesting was 10 min. after addition of streptomycin. Results in the last column represent the distribution of radioactivity in one experiment in which ^{14}C -streptomycin was added to protoplasts from normal organisms immediately before lysing.

Time of growth with streptomycin (min.)	...	10	60–120			0
			No. of experi- ments	Mean	Range	
Total radioactivity in cells (counts/min.)		145	—	—	210–1580	—
			% total radioactivity in fraction			
Protoplasts		97	9	87	76–103	100
'Ghosts':						
Unwashed		—	1	22	—	—
Washed in lysing medium		9	5	6	4–8	9
Supernatant cytoplasm		77	5	91	87–96	—
Pellet from cytoplasm 105,000 g, 4 hr.		42	4	79	49–99	87

mented in this way. In all preparations a very small amount of radioactivity remained in the washed 'ghost' fraction; on some occasions the labelling of this fraction was so small that its significance was doubtful, but it was observed repeatedly. It was not removed by washing, with or without unlabelled streptomycin, but could be removed by repeated washing with m-NaCl or by treatment with ribonuclease (Table 9).

Between 87 and 96 % of the radioactivity in the organisms remained in the cytoplasmic fraction after removal of cell-wall fragments and 'ghost' fraction.

Table 9. *Removal of ^{14}C -streptomycin from cell fractions of Bacillus megaterium by washing and enzymic treatments*

Each preparation was from a culture harvested when inhibition of growth by streptomycin (1 or 2 $\mu\text{g.}/\text{ml.}$) was just complete. The 'ghosts' had been washed once in lysing medium before further washing. Enzyme treatments were carried out for 15 hr. at room temperature in 0.05 M-phosphate buffer (pH 7.2) using 100 $\mu\text{g.}$ of each enzyme/ml.

Washing medium	Counts/min./remaining in					
	Ghosts				Pellet after 4 hr. 105,000 g	
— (control)	136	174	86	73	490	460
Distilled water	55	99	85	67	260	—
Distilled water + unlabelled strepto- mycin (100 $\mu\text{g.}/\text{ml.}$)	65	126	—	—	290	—
NaCl (M)	0	0	—	—	—	—
Enzyme treatment						
Trypsin	—	—	—	—	160	22
Ribonuclease	—	—	16	12	74	140

Almost all of this radioactivity could be sedimented by centrifugation at 105,000 *g* in some preparations, but in others only about 50 % was sedimented in this way (Table 8); possible reasons for this variation are discussed later. The labelled material in the sedimentable fraction could be washed, with or without unlabelled streptomycin, with only partial loss of radioactivity; but after digestion with trypsin or ribonuclease most of the radioactivity became non-sedimentable (Table 9). It is known that such high-speed centrifugation sediments most of the ribonucleic acid (RNA) of *Bacillus megaterium* (Weibull, 1953), probably in particulate form as with other organisms (Schachman, Pardee & Stanier, 1952). Since streptomycin forms complexes with nucleic acids (Cohen, 1947), the possibility had to be considered that association of streptomycin with this fraction might be due to fortuitous adsorption following breakage of the organisms, and might not reflect the location of streptomycin before breakage. To investigate this, ^{14}C -streptomycin was added to a suspension of protoplasts from normal organisms immediately after these had been lysed, and the preparation was fractionated in the usual way. In this preparation, the distribution of streptomycin was identical with that in lysates of organisms which had grown with streptomycin (Table 8).

DISCUSSION

All the streptomycin taken up by growing cells of *Bacillus megaterium* at bactericidal concentrations of the antibiotic is bound in such a way that it is not removed by washing the organisms in growth medium, water or sodium chloride, or displaced by unlabelled streptomycin. This situation contrasts with that in resting *Staphylococcus aureus* (Berkman, Henry, Housewright & Henry, 1948), where all streptomycin taken up could be recovered by washing in *m*-NaCl. With *Escherichia coli*, Szybalski & Mashima (1959) found indications that some streptomycin taken up could be removed by washing in saline; and Anand *et al.* (1960) found that almost all the streptomycin taken up could be removed by washing in buffer. Since streptomycin is strongly basic, and the cell surface of many organisms is negatively charged at the pH value of normal growth media (for example, Dubos, 1949), it would perhaps not be surprising if streptomycin were non-specifically adsorbed to some organisms in certain situations, and were displaced competitively by other cations. This would seem to be an important hazard in interpreting any observations about uptake of streptomycin. Breakage of the osmotic barrier is necessary but not sufficient to release bound streptomycin; with the exception of CTAB, only agents having low pH values are effective. This suggests that after breakage of the osmotic barrier, streptomycin must be displaced from its binding site by H^+ ions or by positively charged CTAB ions. Breakage of the osmotic barrier by butanol or toluene can apparently lead to greater accessibility of intracellular binding sites to streptomycin, with consequent increased binding.

The kinetics of uptake of streptomycin in growing and non-growing *Bacillus megaterium* (inhibited by CAP) appear superficially similar to those of the uptake of penicillin by *Staphylococcus aureus* (Rowley, Cooper, Roberts & Lester Smith, 1950), and suggest that a certain quantity of streptomycin-receptor may exist initially in the organisms, more being formed during further growth. Although we were unable to detect two separate phases in the uptake of streptomycin by *B. megaterium*, such

as occur in *Escherichia coli* W (Anand *et al.* 1960), the accuracy of estimation at low values of uptake makes it impossible to exclude such a process completely. The finding that addition of CAP during the later stages of uptake of streptomycin by *B. megaterium* prevents or slows further uptake, suggests that even at these later stages the uptake process is not due solely to diffusion of streptomycin into the organisms, but depends on continued synthesis of some cell constituents.

Death of *Bacillus megaterium* does not appear to follow inevitably the attainment of a certain intracellular concentration of streptomycin, since at lower streptomycin concentrations the organisms were killed after a smaller amount of streptomycin has been taken up, and *vice versa*. Low concentrations of streptomycin within the organism did not, however, necessarily lead to inhibition of growth; the antibiotic was still taken up at sub-inhibitory concentrations. In the presence of CAP and high concentrations of streptomycin, the organisms may take up streptomycin to values approaching those reached in growing organisms at lower concentrations of streptomycin, although in the former case they remain viable whereas in the latter they do not. It is clear therefore that the dependence of killing by streptomycin on the growth of *B. megaterium* cannot be due solely to the dependence of uptake of streptomycin on growth; other factors must also be involved. The maximum amount of streptomycin taken up by *B. megaterium* depends on the concentration of streptomycin in the growth medium, as it does for *Escherichia coli* (Szybalski & Mashima, 1959). Counts showed that 1 mg. dry wt. of *Bacillus megaterium* contained approximately 4×10^8 organisms; the presence of more than one organism per rod made counting difficult and this value is therefore a minimal one. The uptake of streptomycin at the lowest bactericidal concentrations used ($0.25 \mu\text{g./ml.}$) corresponds to not more than about 5×10^4 molecules/bacterium; this value is somewhat lower than the uptake associated with loss of viability in *Escherichia coli* K 12 (Szybalski & Mashima, 1959). It would seem important in considering possible mechanisms of action of streptomycin that at growth-inhibitory concentrations the quantities of streptomycin taken up, between 20 and 200 $\mu\text{g. streptomycin/g. dry wt. organisms}$, are much lower relative to the amount of cellular DNA than the concentrations at which streptomycin forms cross-linked complexes with DNA *in vitro*, for which approximately equal concentrations of streptomycin and DNA are necessary (Cohen, 1947).

The identity of the intracellular radioactive compounds has not been demonstrated in other work with ^{14}C -streptomycin; in the case of penicillin, this antibiotic can only be recovered from cells in an altered form (Schepartz & Johnson, 1956). There seems little doubt that the radioactivity within *Bacillus megaterium* represents unaltered streptomycin; its behaviour on an ion-exchange resin rules out the possibility of any major breakdown of the molecule or of alterations affecting the charge of the guanido or other groups, and it would seem unlikely that the products of any small modifications or substitutions of the molecule would form derivatives crystallizing with streptomycin derivatives in the manner observed.

The variation in the proportion of the total streptomycin found in the fraction containing the ribosomes may reflect the instability of these particles caused by activation of latent hydrolytic enzymes, such as ribonuclease, which they may contain (Elson, 1958). Attempts were made to investigate the distribution of streptomycin among the different sizes of ribosomes by centrifugation in a sucrose density

gradient, but it was not possible to obtain preparations of sufficiently high specific activity. Since the streptomycin in the cytoplasmic fraction was associated with the RNA-containing fraction, that in the 'ghost' fraction may also be associated with cytoplasmic RNA-containing material adhering strongly to the ghosts; its removal by ribonuclease would agree with such a process. Since ^{14}C -streptomycin added to lysed protoplasts was distributed among the cell fractions in the same way as that taken up during growth, at the moment it cannot be excluded that the observed distribution of streptomycin was to some extent an artifact following lysis of protoplasts, and does not represent the state of affairs before breakage. For example, streptomycin might be free in the cytoplasm of the intact organism but prevented from combining with the acidic groups of RNA because these are neutralized with other cations, which are free to diffuse away when the ionic environment is changed by lysis of the protoplast, leaving the RNA free to combine with streptomycin.

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