The Intracellular Accumulation of ^{14}C -streptomycin by *Escherichia coli* strain B in Relation to its Growth-Inhibitory Effect

By MARGOT KOGUT, J. W. LIGHTBOWN AND P. ISAACSON

*National Institute for Medical Research, The Ridgeway,
Mill Hill, London, N.W. 7*

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SUMMARY

The intracellular concentrations of ^{14}C -streptomycin accumulated by *Escherichia coli* strain B during growth in the presence of different extracellular concentrations of the compound have been measured. They increased logarithmically with time at rates proportional to the logarithm of the extracellular concentration; the growth rates declined linearly with time at rates which were also proportional to the extracellular concentrations. Thus the same decrease in growth rate resulted from different intracellular concentrations of streptomycin, according to the conditions of uptake. After removal of extracellular streptomycin, the growth rates remained constant for several hours, during which time 40–60% of the total cell-bound radioactivity was lost. Of the radioactivity lost from the organisms during growth after removal of extracellular streptomycin, 50–80% was recovered from culture filtrates but did not behave as streptomycin with respect to adsorption to charcoal.

INTRODUCTION

In two papers we reported (Kogut, Lightbown & Isaacson, 1965*a, b*) that when aerobic cultures of *Escherichia coli* strain B were taken after addition of dihydrostreptomycin in the early logarithmic phase, and treated to remove extracellular antibiotic before growth has ceased (Hancock, 1962*a*) they continued to grow at exponential but decreased rates for several hours, after which the growth rates increased and gradually returned to those of controls. From the results of study of this inhibited growth in the absence of extracellular antibiotic (by extinction measurements, viable counts, direct microscopic observations) we concluded that the extent of diminution in growth rates of treated populations should reflect the mean dihydrostreptomycin concentration at the sites of inhibition at the time of removal of extracellular antibiotic. We further inferred that this might be only a fraction of the total intracellular dihydrostreptomycin concentration, which might vary within the treated populations. Thus, we postulated that intracellular streptomycin can be in two phases, namely, an inhibitory fraction—presumably bound to the ribosomes, (Flaks, Cox & White, 1962; Spotts & Stanier, 1961; Davies, 1964; Cox, White & Flaks, 1964), and a non-inhibitory 'pool' fraction whose size would differ between different fractions of the population, and which could be transferred to the inhibitory sites according to the availability of these and to the size of the

'pool'. This view implied that the concentrations of streptomycin in these two phases must be governed, at least in part, by independent factors.

In a detailed study of the intracellular accumulation of ^{14}C -streptomycin by a number of micro-organisms, Hancock (1962*a, b*) described some of the factors which govern this, and stated (Hancock, 1962*b*) that 'death of *Bacillus megaterium* does not appear to follow inevitably the attainment of a certain intracellular concentration of streptomycin, since at lower (extracellular) streptomycin concentrations the organisms were killed after a smaller amount of streptomycin had been taken up, and vice versa'. The intracellular concentrations of ^{14}C -streptomycin measured in his experiments, at the time when growth ceased, would have comprised both the phases postulated above. Hancock also reported that in lysed protoplast preparations from *B. megaterium* grown in the presence of ^{14}C -streptomycin, the proportion of radioactivity which sedimented with the ribosomal fraction constituted from 50 to 99% of the total radioactivity in different preparations. However, various extraction procedures designed to break the osmotic barrier and to disrupt different kinds of bonds, did not produce any differential or sequential release of intracellular streptomycin from the organisms. Hancock concluded that 'breakage of the osmotic barrier is necessary but not sufficient to release bound streptomycin'.

In the present work, by using a small amount of ^{14}C -streptomycin of low specific activity, available to us, we have measured its intracellular accumulation from different external concentrations and examined the quantitative relationships between such intracellular concentrations of streptomycin and decreases in growth rates after removal of extracellular antibiotic (Kogut, Lightbown & Isaacson, 1965*a, b*). Parts of this study have been published (Kogut & Lightbown, 1964*a*).

METHODS

Cultures. The organism, *Escherichia coli* strain B, its maintenance, growth media and conditions, the preparation of inocula, procedure for removing extracellular streptomycin and measurements of growth and growth rates were as described by Kogut, Lightbown & Isaacson (1965*a, b*).

Labelled streptomycin and measurement of radioactivity. ^{14}C -streptomycin was the CaCl_2 complex, kindly provided by Dr C. Rosenblum (Merck, Sharpe and Dohme, Rahway, New Jersey, U.S.A.) with a specific activity of $0.054 \mu\text{C}/\text{mg}$. The material, dissolved in 25 ml. of water, was assayed by the plate diffusion technique (Humphrey & Lightbown, 1952) against the International Standard of Streptomycin, and found to contain 700 i.u./ml. Radioactivity was determined in a Nuclear Chicago, Model D 47 ultrathin-window counter, giving background activity of less than 2 c.p.m. The ^{14}C -streptomycin preparation gave $17 \text{ c.p.m.}/\text{i.u.} = 17 \text{ c.p.m.}/\mu\text{g}$. streptomycin base (Humphrey, Lightbown & Mussett, 1959). Non-radioactive streptomycin CaCl_2 complex was obtained from Glaxo Laboratories Ltd. and contained $673 \mu\text{g}$. base/mg.

Intracellular concentrations of ^{14}C -streptomycin were defined and measured as follows. After addition of labelled streptomycin to aerobically growing cultures of *Escherichia coli* B in the early logarithmic phase, samples containing equiv. 1–3 mg. dry wt. organisms were removed at the appropriate times and filtered on a Millipore membrane filter of 0.22μ pore size and 2.9 cm. diameter. As it is known that

streptomycin can be strongly, but non-specifically, adsorbed to bacteria, but can in this form be readily removed by washing in media of high ionic strength (Hancock, 1962*a*; Plotz, Dubin & Davis, 1961), the organisms were washed three times on the filter with a total of one half their original volume of growth medium containing unlabelled streptomycin 50 $\mu\text{g./ml.}$ to remove and exchange any adsorbed radioactivity. We determined in preliminary experiments that no further radioactive material was removed from the organisms by repeating such washing. The retained radioactivity is what we here define as 'intracellular'. The membranes with the organisms were dried in air and fixed to aluminium planchet. A minimum of 1000 counts were obtained from each sample, and the values corrected for background counts. When necessary, adjustment for infinite thinness was made by means of a calibration graph prepared from a culture of *E. coli* B, labelled with ^{14}C -glycine.

RESULTS

The intracellular accumulation of streptomycin and decrease in growth rates

As already reported (Kogut, Lightbown & Isaacson, 1965*a*), the extent of growth inhibition (as % of control rates) produced by dihydrostreptomycin treatment of *Escherichia coli* B under conditions where intracellular accumulation is known to occur (Hancock, 1962*a*) and measured after removal of extracellular antibiotic, is a linear function of the duration of previous aerobic growth in a given concentration of antibiotic. We had suggested that this might directly reflect the intracellular antibiotic accumulation if this were also linear with time. However, intracellular accumulation (in the sense defined above) of radioactive label by aerobically growing cultures of *Escherichia coli* B, after addition of ^{14}C -streptomycin during the early exponential phase of growth was not linear with time. Figure 1 shows the time courses of decrease in growth rates and of intracellular concentrations of labelled streptomycin (c.p.m./mg. dry wt. organisms) after treatment with different external concentrations of the antibiotic. The rate of accumulation apparently increased with duration of growth in the presence of streptomycin, but it also seemed to vary with the external concentration of antibiotic in the medium, and to increase more rapidly with time at the higher external concentrations. Figure 2 shows the relationship between intracellular concentrations of ^{14}C -streptomycin and the % inhibition of growth rates for four different initial concentrations of streptomycin in the medium. Because of the low specific activity of the streptomycin preparation, and the apparently very low intracellular concentrations which were effectively growth-inhibitory under these conditions, it was not possible to measure intracellular concentrations associated with slight decreases in growth rate (by less than 40 %). With the higher external concentrations of antibiotic, there was the additional difficulty of the rapid progress of growth inhibition. However, in all four cases, the relationship between inhibition of growth rates and intracellular concentrations resembled a saturation curve; the intracellular concentration approached asymptotically the value for complete growth inhibition. The shapes of the four curves, for the four external concentrations of streptomycin used, also appear to differ, suggesting that the intracellular concentrations associated with a given decrease in growth rate varied with the extracellular concentrations from which they were accumulated, as suggested by Hancock (1962*b*) for *Bacillus*

Fig. 1. Time course of inhibition of growth rate and of intracellular accumulation of radioactive label, during growth in different concentrations of ^{14}C -streptomycin. (a) % inhibition of growth rate. (b) Cell-bound radioactivity. ^{14}C -streptomycin concentrations in the medium: x—x, 15 $\mu\text{g./ml.}$, two separate experiments; ○—○, 20 $\mu\text{g./ml.}$; Δ—Δ, 30 $\mu\text{g./ml.}$; ■—■, 40 $\mu\text{g./ml.}$.

Fig. 2

Fig. 3

Fig. 2. Reduction in growth rate as a function of intracellular streptomycin concentrations. ^{14}C -streptomycin concentrations in the medium from which intracellular accumulation took place: x—x, 15 $\mu\text{g./ml.}$; ○—○, 20 $\mu\text{g./ml.}$; Δ—Δ, 30 $\mu\text{g./ml.}$; ■—■, 40 $\mu\text{g./ml.}$.

Fig. 3. Double reciprocal plot of data in Fig. 2.

megaterium. These differences are more clearly shown by transforming the data in Fig. 2 to double reciprocal plots, as in Fig. 3. Although it is not possible to evaluate the slopes precisely, because of the difficulties in obtaining a full range of measurements, the figure indicates four different slopes. This would confirm that a given reduction in growth rate can result from different intracellular concentrations of streptomycin, when the extracellular concentrations from which they are accumulated vary.

Loss of intracellular streptomycin during inhibited growth. We have also shown previously (Kogut *et al.* 1965*b*) that after cultures of *Escherichia coli* B were treated as described, periods of exponential growth at decreased rates were followed by recovery of most individuals in such cultures. Such recovery of growth rates was not due to continued uninhibited growth of portions of such treated populations. Further, we inferred from the relationship between time of onset of recovery and inhibition of growth rates that recovery could not be the consequence of a given amount of cell synthesis. At its simplest, it might be due to a time-dependent loss of dihydrostreptomycin from the cells, either by degradation or 'leakage'. In the present study, therefore, we have followed the intracellular concentrations of ^{14}C -streptomycin during the periods of exponential growth at decreased rates, as illustrated in Fig. 4. The cell-bound radioactivity (expressed as c.p.m./mg. dry wt. organisms of original culture at the time of removal of extracellular streptomycin)

Table 1. *Loss of cell-bound radioactivity during exponential growth of Escherichia coli B at decreased rates, after removal of extracellular streptomycin*

Treatment with streptomycin			After removal of extracellular streptomycin				
			% decrease of growth rate	Intracellular concentrations: c.p.m./mg. dry wt. organism at filtration time			% lost
				At filtration	At onset of recovery	Lost during inhibited growth	
Expt.	Concentration ($\mu\text{g./ml.}$)	Time (min.)					
1	15	100	41	1.82	0.91	0.91	50
1	15	115	50	2.11	1.07	1.04	49
1	15	135	56	2.66	1.60	1.06	40
2	15	110	61	3.09	1.47	1.62	52
1	15	155	64	2.64	1.42	1.22	46
2	15	160	77	7.21	—	—	—
3	20	65	49	2.60	1.54	1.06	41
4	20	95	70	4.57	2.17	2.40	51
3	20	87	71	5.54	2.99	2.55	46
3	20	107	87	8.45	3.72	4.73	56
5	20	108	88	8.05	—	—	—
3	20	170	100	15.4	—	—	—
6	30	55	64	4.77	—	—	—
7	30	65	76	6.31	3.41	2.90	46
6	30	75	90	10.90	4.23	6.67	62
7	30	89	96	13.04	5.15	7.89	60
8	40	45	66	7.22	2.89	4.33	60
8	40	55	89	10.60	4.37	6.23	59
8	40	65	98	16.5	—	—	—

decreased at an approximately exponential rate with time. Table 1 shows the intracellular concentrations of streptomycin at the time of removal of extracellular streptomycin and those at the beginning of the recovery phase, in cultures treated with different external concentrations of ^{14}C -streptomycin for various periods, and the resulting decreases in growth rates. The intracellular streptomycin concentrations are referred to dry wt. organisms present at the time of removal of extracellular streptomycin as above, so that the apparent loss of cell-bound antibiotic during growth is genuine and not an artifact due to continued cell synthesis. Although the growth rates and initial intracellular streptomycin concentrations varied widely in the different cultures (over an approximately 9 to 12-fold range), the percentage of the initially-fixed streptomycin which was 'lost' during the periods of inhibited growth by the various cultures varied only between 40 and 60%.

Fig. 4

Fig. 5

Fig. 6

Fig. 4. Intracellular streptomycin concentrations during growth of *E. coli* B, after removal of streptomycin from the medium. Δ — Δ , $\log_{10} E_{500m\mu}$; $\circ$ — $\circ$, intracellular streptomycin as c.p.m./mg. dry wt. organism at the time of removal of extracellular streptomycin.

Fig. 5. Extent of decrease in growth rate of *Escherichia coli* B and cell-bound ^{14}C -streptomycin retained at onset of recovery phase. Symbols refer to ^{14}C -streptomycin concentrations from which accumulation took place: $\times$ — $\times$, 15 $\mu\text{g./ml.}$; $\circ$ — $\circ$, 20 $\mu\text{g./ml.}$; Δ — Δ , 30 $\mu\text{g./ml.}$; $\blacksquare$ — $\blacksquare$, 40 $\mu\text{g./ml.}$

Fig. 6. Extent of decrease in growth rate and cell-bound ^{14}C -streptomycin lost during inhibited growth, before onset of recovery. Symbols refer to ^{14}C -streptomycin concentrations from which accumulation took place: $\times$ — $\times$, 15 $\mu\text{g./ml.}$; $\circ$ — $\circ$, 20 $\mu\text{g./ml.}$; Δ — Δ , 30 $\mu\text{g./ml.}$; $\blacksquare$ — $\blacksquare$, 40 $\mu\text{g./ml.}$

Such losses of cell-bound radioactivity during continued growth in absence of extracellular streptomycin might have occurred by several mechanisms. In the first place, they might have been due to lysis of some individuals in treated populations, more probably those with the greatest intracellular concentrations of antibiotic. A small degree of lysis, say less than 10–15% of the population, would not necessarily be detected by the extinction measurements. However, if such small proportions of the population were to account for 40–60% of the total initial intracellular streptomycin concentrations of the population it would mean that the intracellular concentrations of these organisms would have to be many times higher than the population mean. It would also suggest that the actual concentrations which produced lysis would vary (over a 9 to 12-fold range; see above) with the

total intracellular concentrations. Further, it is difficult to see why such losses, if they occurred by lysis of some individuals with exceptionally high intracellular streptomycin concentrations, should proceed for several hours when the extracellular streptomycin concentrations must be negligible, as compared to those from which accumulation originally took place.

The intracellular streptomycin concentrations, associated with a given decrease in growth rate, vary with the extracellular concentration from which accumulation took place. This suggests that increasing portions of intracellular streptomycin are non-inhibitory when the extracellular concentrations are increased; in other words that intracellular streptomycin contains a non-inhibitory 'pool' fraction. The subsequent loss of cell-bound radioactivity during inhibited growth could, therefore, be from such 'non-inhibitory' antibiotic or from the 'inhibitory' fraction. In the former case, were the losses to represent all of the 'pools' then the streptomycin retained should represent the inhibitory fraction, tightly bound to the sites of inhibition. This reasoning demands that the lowest values for streptomycin retained must represent the maximum values for the 'inhibitory' fraction. Figure 5 shows the cell-bound streptomycin, retained at the onset of recovery (again expressed as c.p.m./mg. dry wt. organism in the original culture) against the % decrease in growth rates. The lowest values, mainly those from experiments where accumulation took place from 15 μ g. streptomycin/ml. culture, give a straight line which goes through the origin and extrapolates to approximately 2.2 c.p.m./mg. dry wt. organism for complete growth inhibition. It could be argued, therefore, that this line represents the minimum 'inhibitory' concentration of intracellular streptomycin. The fact that the values obtained in some of the experiments, where intracellular accumulation occurred from higher external concentrations of streptomycin, diverge from this line, might mean that in these cultures parts of the 'pools' were retained at the times when the final measurements were made (approximately at the time of onset of recovery).

If, on the other hand, the losses of intracellular streptomycin during inhibited growth occurred by breakdown or degradation of the 'inhibitory' fraction of antibiotic, then the maximum amounts lost at onset of recovery would represent minimum values for the concentration of streptomycin initially at the inhibition sites. In general, one would expect that if the 'inhibitory' fraction were gradually lost during growth, the growth rates should show a continuous recovery. Since they do not, but remain exponential for several hours, one might assume that combination with inhibition sites amounts to irreversible inactivation of these, in which case the loss of such an 'inhibitory' fraction need not affect the growth rates. Figure 6 relates the amounts of cell-bound radioactivity lost during inhibited growth until the onset of recovery, to the degree of decrease in growth rates. It indicates that in this case the size of the 'inhibitory' fraction would increase exponentially with increasing inhibitory effect.

Recovery of radioactivity from culture filtrates at the end of inhibited growth

We tried to recover the radioactivity lost from the organisms in the culture filtrates collected at the end of the phase of exponential inhibited growth. Since the total amounts of radioactivity involved were very small, and to gain some idea whether the lost radioactivity still behaved as streptomycin, we treated the culture

filtrates with activated charcoal, and counted the radioactivity adsorbed to this. In a preliminary experiment to test the adsorption of streptomycin to charcoal, a culture of *Escherichia coli* B was grown in the usual medium to the middle of the logarithmic phase (about equiv. 0.8 mg. dry wt. organism/ml.) and after removal of the organisms, streptomycin calcium chloride complex (to 200 μ g. base/ml.) was dissolved in the filtrate. Three 10 ml. portions, each containing 2.0 mg. streptomycin, were treated with 100, 150 and 200 mg. charcoal, respectively. After filtration, the streptomycin in the filtrate was estimated, after appropriate dilution, by the plate diffusion assay (Humphrey & Lightbown, 1952). Table 2 shows the results. Thus, 99 % of the streptomycin in the culture filtrate was adsorbed by 100 times its weight of charcoal.

Table 2. *Adsorption of streptomycin to charcoal from culture filtrate of Escherichia coli B*

Culture filtrate (ml.)	10	10	10
Streptomycin CaCl ₂ added (mg. base)	2	2	2
Charcoal added (mg.)	100	150	200
Weight ratio, charcoal: streptomycin	50	75	100
Streptomycin (μ g.) left in filtrate after removal of charcoal	59.2	24.7	17.9
Streptomycin adsorbed on charcoal (%)	97.1	98.8	99.1

Table 3. *Radioactivity adsorbed to charcoal from filtrates of Escherichia coli B cultures which had been treated with ¹⁴C-streptomycin and continued to grow after removal of extracellular streptomycin*

Filtrate 1		Filtrate 2	
Volume of filtrate (ml.)	19	Volume of filtrate (ml.)	20.5
Maximum expected radioactivity from amounts of cell-bound radioactivity lost		Maximum expected radioactivity from amounts of cell-bound radioactivity lost	
As c.p.m.	20	As c.p.m.	6.1
As μ g. streptomycin	1.18	As μ g. streptomycin	0.86
Charcoal treatment			
	c.p.m. on charcoal		c.p.m. on charcoal
0.2 mg. charcoal, left 20 min. at 20°	1.8	1.0 mg. charcoal, occasional shaking 20 min. at 20°	2.3
+ 1.5 mg. charcoal occasional shaking, 30 min. at 20°	4.63	+ 20 μ g. cold streptomycin, left 18 hr. occasional shaking, + 2.1 mg. charcoal, shaken for 30 min. at 20°	2.65
+ 2.1 mg. charcoal, left with shaking 50 min at 20°	3.08		
Filtrate was lost			
Totals recovered	9.1		4.95

The filtrates from two of the streptomycin-treated cultures, after re-suspension in streptomycin-free media and inhibited growth during which radioactivity was lost from the organisms, were assayed for radioactivity. After storage at -40° they were treated with charcoal and the radioactivity adsorbed to the charcoal counted (Table 3). In this case, up to 80 % of the radioactivity lost from the organisms

during inhibited growth after treatment with ^{14}C -streptomycin, was recovered from the filtrates. However, comparison of the data in Tables 2 and 3 shows that this radioactive material did not behave like streptomycin with respect to adsorption to charcoal. In filtrate 1, a weight ratio of charcoal/streptomycin (estimated on the basis of streptomycin lost from the organisms) of 165, adsorbed only 6.5% of the calculated maximum, and 14% of the total radioactivity which was finally recovered, for which a weight ratio (charcoal/streptomycin) of about 7600 was used. In filtrate 2, the weight ratio of charcoal/streptomycin lost from the organisms was about 8000 and adsorbed 80% of the calculated maximum radioactivity which could have been present. In this experiment unlabelled streptomycin, 20 μg . base, was added to the filtrate after the first charcoal treatment and allowed to stand overnight to facilitate desorption of radioactive material which might have been adsorbed to the glass of the vessel. The possibility that this difference in adsorption to charcoal between the control experiment and the treated samples might have been due to differences in the culture filtrates from which adsorption took place cannot be excluded. Although in the control experiment a filtrate from *Escherichia coli* B which had grown in the same medium for about the same number of generations was used, this culture had not grown in presence of streptomycin. It is possible that there was a difference in the metabolism and/or excreted products as between streptomycin-treated and control cultures which affected the adsorption of streptomycin from such filtrates to charcoal. The alternative view that the radioactive material lost from the organisms was not in the form of free streptomycin, but some degradation product or complex with other cell-constituents seems equally likely. The concentrations were too low to allow biological assay.

DISCUSSION

This work was undertaken to test the inference from other observations (Kogut *et al.* 1965*a, b*) and from a review of the literature (Kogut & Lightbown, 1964*b*) that intracellular streptomycin and dihydrostreptomycin can be in two phases, an 'inhibitory fraction' tightly bound to the sites of inhibition and a 'non-inhibitory pool fraction', and that the uptake into these two fractions must be governed, at least in part, by independent factors. None of the earlier studies on the intracellular accumulation of streptomycin by several bacterial species, as well as algae (Pramer 1956; Litwak & Pramer, 1957; Szybalski & Mashima, 1959; Hancock, 1962*a, b*; Hurwitz & Rosano, 1962) dealt with rates of uptake from different external concentrations and quantitative comparisons between intracellular concentrations and antibiotic effects. However, Hurwitz & Rosano (1962) proposed that 'binding' or uptake of streptomycin by *Escherichia coli* B can be in three phases; (1) adsorption which is rapid, reversible and not related to the antibiotic action; (2) a second phase, claimed to occur 'prior to killing' and presumably before cessation of growth (see Kogut *et al.*, 1965*b*, for discussion of the relation between so-called 'killing' and growth inhibition) which was thought to be the 'effective' intracellular concentration; (3) a third phase which was thought to occur after the organisms were rendered non-viable, but which was shown to be different in character from the uptake of streptomycin exhibited by organisms previously killed by heat or toluene treatment.

We have sought to exclude the first, adsorption, phase by washing procedures,

and have defined as 'intracellular' the radioactivity accumulated during growth in presence of ^{14}C -streptomycin which cannot be removed from the organisms by washing in growth medium containing excess of unlabelled streptomycin, as first proposed by Hancock (1962*a*). Whilst the present paper was in preparation, Dr R. Hancock (personal communication) reported on a 'non-exchangeable fraction of bound streptomycin in *Escherichia coli*'. His use of ditritiostreptomycin of much higher specific activity than the ^{14}C -streptomycin used by us, provided a more sensitive system for the study of the early stages of streptomycin uptake. Dr Hancock's uptake curve for *Escherichia coli* ML 35, from ^3H -streptomycin 25 $\mu\text{g.}/\text{ml.}$ extending over 25–30 min. is very different from ours. Although his washing experiment showed that 50 $\text{m}\mu\text{g.}$ ^3H -streptomycin/mg. dry wt. organism was retained after two washings, (hence defined as the 'non-exchangeable fraction of bound streptomycin') the uptake curve showed that this amount was found within the first few minutes, after which the curve became approximately linear. In our experiments, the cultures might also have contained such a relatively small rapidly bound fraction (presumably residual adsorbed streptomycin) and Hancock's uptake curve would approximately fit ours for 30 $\mu\text{g.}$ streptomycin/ml. external concentration (Fig. 1).

Most of our 'intracellular accumulation', however, overlaps the phases 2 and 3 of Hurwitz & Rosano (1962). Like these authors, we found that intracellular accumulation was not linear with duration of exposure to streptomycin, but we also found that it was not a linear function of the degree of decrease in growth rate. This degree of decrease in growth rate (i.e. the inhibitory effect) was, however, linearly related to the duration of growth in a given streptomycin concentration and the slopes of these linear rates were proportional to the logarithms of the external streptomycin concentrations. We previously inferred that the 'inhibitory effect' must be a direct result of the 'effective concentration' of inhibitor, namely, its concentration at inhibition sites. This inference involves the assumption that the number or concentration of inhibition sites is the same as in controls. It would perhaps be more legitimate to say that the residual growth rates, which must of course also be a linear function of the duration of treatment, reflect the lack of combination between inhibitory sites and streptomycin. The fact that the same residual growth rate can exist with different intracellular streptomycin concentrations, and the fact that, after removal of extracellular streptomycin, the residual growth rates remain constant for several hours, must mean that under all these conditions the concentration of uncombined sites is constant. We have inferred that a non-inhibitory 'pool' fraction (possibly phase 3 of Hurwitz & Rosano, 1962) is accumulated more or less simultaneously with an 'inhibitory' streptomycin fraction which combines with the inhibition sites (presumably the ribosomes), but at different rates. By equating 'non-inhibitory' with 'pool' material, we are alluding to its possible function; the term 'pool' does not exclude combination with some cellular constituents. The proportion of intracellular streptomycin in these two fractions would accordingly vary with the duration of exposure and with the extracellular streptomycin concentrations from which accumulation takes place. After removal of extracellular streptomycin, transfer from the 'pool' to newly synthesized sites (ribosomes) must proceed at rates which would keep the concentration of uncombined sites constant until the onset of recovery. If the total con-

centration of inhibition or combining sites were constant and the same as in controls, this would mean that such transfer was not a simple equilibrium process. However, the total concentration of inhibitory or combining sites need not be constant and the same as in controls, when cultures are exposed to streptomycin. Several workers (Erdős & Ullmann, 1959; Flaks *et al.* 1962; Hancock, personal communication; Dubin, Hancock & Davis, 1963; Hahn *et al.* 1962) have reported that synthesis of some RNA fractions is stimulated on addition of streptomycin to growing cultures; the evidence is conflicting as to which RNA components are affected. Our observations would fit the hypothesis that on intracellular uptake of streptomycin, the synthesis of RNA material, including ribosomes or precursors of ribosomes, is stimulated, so that the concentrations of streptomycin in combination with this material increase logarithmically with time, whilst the concentration of uncombined active ribosomes would decrease linearly with time during growth in streptomycin-containing medium. In this case, after removal of extracellular streptomycin, the further synthesis of ribosomes might be at rates governed by the combined streptomycin fraction, with transfer occurring from this to the new sites, but without liberation of active sites. The rates of transfer could then be proportional to the concentration of streptomycin in the 'pool' or combined fraction. The constancy of growth rates after removal of extracellular streptomycin might also be explained if the synthesis of 'active' ribosomes were strictly dependent on the presence or functioning of active ribosomes. In this case, no transfer from any 'pool' or other sites would be involved.

The loss of cell-bound radioactivity after removal of extracellular streptomycin could provide a reasonable explanation of the recovery phenomenon. If only active ribosomes were replicated and no transfer from 'pool' fractions took place, recovery might occur if some ribosomes were re-activated or synthesized from liberated precursors, by loss of cell-bound streptomycin. Such 're-activation' would have to occur only occasionally, since about 50% of the intracellular radioactivity can be lost from the organisms before the recovery of growth rates becomes apparent. However, if the constancy of growth rates after removal of extracellular streptomycin involves transfer of intracellular antibiotic from 'pool' or 'combined' fractions to newly synthesized ribosomes, recovery could start when rate of transfer, due to depletion of this fraction, became slower than synthesis of new sites.

Several workers have estimated the 'minimum lethal' or 'growth-inhibitory' concentration of streptomycin inside organisms. This was reported to be 3×10^5 molecules/organism for *Escherichia coli* B (Hurwitz & Rosano, 1962), 5×10^4 molecules/organism for *Bacillus megaterium* (Hancock, 1962*b*) and 2×10^4 molecules/organism for *E. coli*, ML 35 (Dr R. Hancock, personal communication). We have obtained minimum values of streptomycin retained by *E. coli* B at the beginning of recovery, which extrapolate to 3×10^4 molecules/organism at complete growth inhibition (2.2 c.p.m./mg. dry wt. = 0.13 μ g. streptomycin/ 3×10^9 organisms). These values could amount to a few molecules/ribosome; 1–2 molecules of streptomycin/ribosome will inhibit polypeptide synthesis *in vitro* by ribosomal preparations from a sensitive strain of *E. coli* (Flaks *et al.* 1962). Since the ribosome content of bacteria has been shown to vary with growth rate (Kjeldgaard & Kurland, 1963; Rossett, Monier & Julien, 1964), the 'effective inhibitory' concentrations of streptomycin/organisms, namely those required to inactivate enough ribosomes to

stop growth, might well vary with growth conditions. We suggest that they may also vary because the synthesis of ribosomes might vary with streptomycin treatment.

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