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THE SEQUENCE OF SOME EFFECTS OF STREPTOMYCIN IN *ESCHERICHIA COLI*

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SUMMARY

The effects of streptomycin on a number of biochemical parameters in growing cultures of *Escherichia coli* have been studied. Using low concentrations of drug and radioactive tracer techniques we have timed these effects relative to each other and to the killing action of the drug. The earlier effects noted were: first, an immediate (primary) uptake of streptomycin; next, an acceleration of K efflux and a transient stimulation of net RNA synthesis; and then, inhibition of protein synthesis and loss of viability. Later effects were: a further (secondary) uptake of streptomycin, increased permeability to a β-galactoside, impairment of respiration, inhibition of RNA and DNA synthesis and RNA breakdown and nucleotide excretion. The present findings cannot be integrated into a definitive theory on the action of streptomycin, but they do emphasize the importance of effects on membrane integrity and on the synthesis of RNA and protein.

INTRODUCTION

In recent years extensive work on the mechanism of action of streptomycin has revealed a multiplicity of effects on growing bacterial cultures. These include: a diphasic uptake of [^{14}C]streptomycin[1]; an inhibition of protein synthesis, which is known to precede inhibition of RNA, DNA, and lipid synthesis[2-4]; decreased respiration[5-7]; degradation of RNA (see ref. 8); and impaired membrane integrity[1,3,9,10]. With the aim of eventually understanding how these effects are causally related to each other and to the lethal action of streptomycin in *Escherichia coli* we have carefully correlated them, in time, with each other as well as with celi death. In the course of this work, we observed a previously undetected early stimulation of RNA synthesis.

To facilitate the detection of small changes we have made extensive use of radioactive tracers, and to separate the various effects in time we have used relatively low concentrations of streptomycin. Since with these concentrations there was some day-to-day variability in the time of onset of drug action (although not in the relative sequence of events), we have measured several parameters simultaneously in each experiment.

Abbreviations: ONPG, *o*-nitrophenyl-β-D-galactoside; K$T_{1/2}$, half-time of K efflux.

MATERIALS AND METHODS

Bacteria

Strain ML-35 of *E. coli*, a mutant constitutive for β-galactosidase (EC 3.2.1.22) but lacking the β-galactoside transport system, was used. This strain was the gift of Dr. J. MONOD.

Media and growth conditions

For convenience in working with ^{42}K a low-K modification of medium A of DAVIS AND MINGIOLI[11] was used. It had the following composition (g/liter): Na_2HPO_4, 5.7; NaH_2PO_4, 2.7; $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 0.048; plus a specified concentration of KCl. This medium is referred to as $A'K_x$ where x represents the millimolar concentration of K. Unless otherwise stated, glucose (0.2 %) was added as carbon source. The initial pH of the medium was 7.0 and this value decreased by approx. 0.1 unit during the course of the experiments.

Cells were maintained with monthly transfer on tryptic digest agar (tryptic digest 1.5 %, agar 2 %). Inocula were grown with aeration overnight in medium $A'K_{0.3}$ with limiting (0.1 %) glucose, and the following morning were diluted into 20–40 vol. of fresh medium. When the uptake of radioactive precursors was to be followed, this medium was supplemented with non-radioactive precursors at levels high enough to prevent substantial depletion during the course of an experiment. For convenience, a standard supplement of non-radioactive precursors was often used, even when the uptake of each of the compounds was not studied. Unless otherwise indicated, the concentrations (μg/ml) used were: leucine, 25; cytosine, 20; uracil, 10; and guanine, 30. Streptomycin was not added until at least 2 generations of exponential growth had taken place. Unless otherwise stated cultures were grown at 37° in test tubes bubbled with filtered air.

Turbidity

Cell mass was estimated by determining the absorbancy of cultures at 490 mμ in a Lumetron colorimeter in tubes of 18 mm inside diameter. For exponentially growing cultures readings were proportional to cell dry weight up to an absorbancy of 0.3. A reading of 0.1 was equivalent to 0.09 mg dry weight and $1.4 \cdot 10^8$ viable cells per ml.

Viable cell count

For viable counts dilutions were made with 0.9 % NaCl, and duplicate samples of the final dilution were mixed in a petri dish with approx. 15 ml of melted tryptic digest agar at 45°. Colonies were counted after incubation overnight at 37°.

Total cell count

Cell number was determined by means of a Coulter Counter (Coulter Electronics, Chicago) fitted with a 10-μ aperture[12]. Dilutions were made in 0.9 % NaCl (previously passed through a 0.45-μ pore size Millipore filter), and samples of the final dilution were counted in triplicate.

Chemicals

Unless otherwise indicated chemicals are non-radioactive; concentrations and

specific activities of nucleic acid bases and streptomycin refer to the free base throughout. Guanine, cytosine, and uracil were obtained from the California Corporation for Biochemical Research and L-leucine from Nutritional Biochemicals Corporation. Streptomycin sulfate was obtained from Squibb and Company.

L-[1-^{14}C]Leucine (specific activity 190 μC/mg) and [2-^{14}C]uracil (17.9 μC/mg) were purchased from New England Nuclear Corporation. Uniformly labeled [^{14}C]-streptomycin (0.54 μC/mg) was the generous gift of Dr. C. ROSENBLUM of Merck and Company. ^{42}K (Isoserve Corporation, Cambridge, Mass.; initial specific activity approx. 300 μC/mg) was the gift of Dr. S. SCHULTZ and the Biophysical Laboratory of Harvard Medical School.

Oxygen consumption

Oxygen consumption was determined in 2 ways. (a) The cells from a 40-ml sample of culture were collected on a Millipore filter (0.45 μ pore size, 47 mm diameter) and washed and resuspended in fresh medium without streptomycin. Oxygen consumption, with glucose as substrate, was then followed for 30 min in a Warburg respirometer as previously described[7]. (b) For more rapid determinations, a polarographic method was used. A closed chamber of 13 ml capacity was filled with a sample taken directly from the culture and stirred magnetically. The oxygen tension was recorded by means of a Clark oxygen electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio) connected to a pen recorder. The initial rate of removal of dissolved oxygen was constant and could be determined accurately over periods of 1–2 min.

All results are expressed as μl of oxygen consumed per mg dry weight of cells per hour (Q_{O_2}).

Radioactive counting procedures

Material in solution or suspension was plated directly on an aluminum planchet; for material collected on a membrane filter the filter was glued to a planchet for counting. All samples were dried under a lamp and were counted in a Nuclear Chicago thin-window gas-flow counter. Appropriate corrections were made for self-absorption, radioactive decay of ^{42}K, and changes in counter efficiency.

Membrane studies

Two parameters were used as indicators of changes in the integrity of the cell membrane: K efflux and the hydrolytic activity of a cryptic mutant towards the synthetic β-galactoside, ONPG. These parameters depend on rates of passage through the cell membrane: K outward, and ONPG inward. Both can be measured over relatively short intervals (approx. 8 min for K, 2 min for ONPG) in small samples of culture; they thus lend themselves more readily to quantitative kinetic studies than the previously used parameters of nucleotide and amino acid leakage[3]. Furthermore, the use of K and ONPG obviates some of the ambiguity of interpretation intrinsic in the use of metabolic intermediates (*cf.* ref. 8). In addition, K efflux appears to be the most sensitive of the parameters thus far studied.

β-Galactosidase activity: Intact cells of strain ML-35 are cryptic, *i.e.*, they show only a small percentage of the β-galactosidase activity of cells whose permeability

barriers have been extensively damaged by toluene treatment*. Decrease in crypticity (increased β-galactosidase activity) is therefore a convenient measure of membrane damage (*cf.* ref. 10). Since most of the enzyme activity resulting from streptomycin treatment was found to be retained by the cells rather than released to the medium, and since the results of the assay were not altered by the presence of streptomycin (at the concentration used)[13], the assay was performed on samples of whole culture. A modification of a published method was used[14]: 1.2 ml of culture was added to 0.6 ml of $3 \cdot 10^{-3}$ M ONPG; after 2 min at 37° 1.2 ml of 1 M Na_2CO_3 was added and the absorbancy at 420 mμ was read in a Klett colorimeter. Activity is expressed as percentage of the activity of a parallel sample of toluenized cells, prepared by shaking a 1/12 dilution of culture with 1/40 vol. of toluene for 15 min at 37°.

Potassium measurements: ^{42}K was used to measure both (a) the K content of cells and (b) the rate of K efflux from cells transferred from labelled to unlabelled medium. In each case, cells were equilibrated with ^{42}K before use by growth for 2 or more generations in ^{42}K-containing medium.

(a) Cellular ^{42}K was determined by either of two methods. In one a sample of culture was passed through a Millipore filter (0.45 μ pore size, 24 mm diameter) and the cellular ^{42}K was calculated from the difference between the radioactivity of filtrate and that of unfiltered culture. Alternatively, the cells were collected on the filter and washed three times with 2 ml of either medium $A'K_0$ or 7.5 % sucrose at room temperature, and the filter was counted. Under conditions where both methods were suitable (*e.g.*, in medium $A'^{42}K_{0.3}$ at an absorbancy of 0.3 in which equilibrated cells contain about 45 % of the total ^{42}K) the two procedures gave similar results. When both ^{14}C and ^{42}K were present in the same sample ^{42}K was determined as the difference between counts before and after its decay (half-life 12.4 h).

(b) For efflux determinations two procedures were used.

Method A (essentially as previously described[9]): Cells were equilibrated with ^{42}K as described above and then were exposed to streptomycin in the same medium. At intervals before and after addition of the drug, samples were removed, and the cells were collected on Millipore filters. The filters were then submerged in non-radioactive pre-warmed medium and their surfaces were gently scraped. In this manner cells could be homogeneously resuspended in fresh medium within 2 min. The cells were then incubated further at 37° with aeration, and cellular ^{42}K was determined at short intervals by the difference between the whole suspension and successive filtered samples. The loss of radioactivity from the transferred cells was approximately exponential over the period of measurement (8–15 min), and the half-time of efflux ($KT_{½}$) was determined graphically.

Method B: For more continuous monitoring of K efflux, cells, equilibrated with ^{42}K as above, were filtered and resuspended in non-radioactive medium only once, at the onset of the experiment. An entire culture was passed through a Millipore filter (0.45 μ pore size, 47 mm diameter), and the cells were resuspended in pre-warmed non-radioactive medium with or without streptomycin and incubated further at 37° with aeration. Cellular ^{42}K was then determined at intervals in washed cells. Although

* In growing cultures this percentage varied from 0.5 to 5 % from day to day, but was constant within a single experiment. The variation appeared to be due to the sporadic emergence of less cryptic mutants during growth in medium $A'K_{0.3}$. After overnight growth in medium A (see ref. 11) the activity, even when measured in medium $A'K_{0.3}$, was always less than 1 %.

the loss of ^{42}K from cells deviated significantly from the exponential after 15 min, this method gave the same initial $KT_{1/2}$ as method A and was useful for detecting differences in efflux for as long as 40 min.

The value for $KT_{1/2}$ in untreated cells depended on the nature of the growth medium. Values of 14–16 min were obtained in medium $A'K_1$, 9–10 min in medium $A'K_{1/2}$ supplemented (as in the experiment of Table II) with leucine, cytosine, and uracil, and 7–9 min in a low phosphate medium[9]. The reasons for these differences are not known. However, the effect of streptomycin on the efflux was similar in all three media.

Protein and nucleic acid synthesis

To detect early changes in rates of macromolecule synthesis [^{14}C]leucine and [^{14}C]uracil were added at the same time as streptomycin, and their incorporation into protein and nucleic acid, respectively, were measured. Radioactivity was determined either in trichloroacetic acid precipitates (*cf.* ref. 15) or in whole cells. Although the latter measurement includes the "trichloroacetic acid-soluble" pool, the kinetics of the streptomycin-induced changes were the same by each method. This would indicate that changes in pool size paralleled changes in biosynthetic rates or were negligible relative to the amount of radioactivity incorporated into macromolecules. Direct measurements of pool size were insufficiently precise to distinguish between these possibilities.

The incorporation data were plotted against a time scale chosen to yield linear curves for a culture in balanced exponential growth; this is a scale proportional to the increase in turbidity of such a culture, measured from the time of addition of radioactivity*. After the short time required to saturate intracellular pools such curves were in fact linear for the control cultures.

When ^{42}K and ^{14}C were measured in the same cells, samples were filtered and washed as described in the previous section, and the cells were counted before and after decay of the ^{42}K.

To measure incorporation of radioactivity from [^{14}C]uracil into both total nucleic acid and DNA, samples of culture were added to 2 vol. of ice-cold 7.5 % trichloroacetic acid and also to 2 vol. of 1 N KOH. The alkaline samples were held at 37° for 1 h to hydrolyze the RNA**, neutralized with HCl, brought to a trichloroacetic acid concentration of 5 %, and chilled in ice. Both sets of precipitates were collected on filters and washed three times with 2 ml of cold 5 % trichloroacetic acid.

When simultaneous incorporation of [^{14}C]leucine into protein and [^{14}C]uracil into nucleic acid was followed, duplicate samples were added to 2 vol. of ice-cold 7.5 % trichloroacetic acid. After one set had been held at 95° for 30 min ("hot trichloroacetic acid" treatment), the precipitates were collected and washed as before. Incorpo-

* Expressed mathematically, the abscissa scale is proportional to (exp. (ln 2/T) t − 1), where T is the doubling time of the culture and t is the time after the addition of radioactive precursors. For balanced exponential growth (neglecting the time of passage through precursor pools), the incorporated radioactivity should increase linearly with this function and should be zero at zero time.

** This modification of the SCHMIDT–THANNHAUSER procedure[16] yielded reasonably reproducible results. The trichloroacetic acid-precipitable radioactivity incorporated from [^{14}C]uracil was constant for replicate samples treated with alkali for periods varying from 0.5 to 3 h, and the alkali-hydrolyzable fraction amounted to 86–88 % of the total trichloroacetic acid-precipitable radioactivity in growing cultures.

ration into protein was taken as the counts in the hot-trichloroacetic acid precipitate and incorporation into nucleic acid as the difference between this value and the counts in the cold precipitate.

Non-radioactive precursors at 4 times their concentration in growth medium were added to the KOH and the trichloroacetic acid solutions to minimize adsorption of radioactivity to filters.

Cytosine was added to cultures in an attempt to suppress incorporation of radioactivity from [^{14}C]uracil into DNA (see ref. 17). However, this suppression was not observed under the conditions of our experiments.

RNA breakdown

In medium A'$K_{0.3}$ the release of nucleotides from streptomycin-treated cells parallels the depolymerization of preformed RNA (see ref. 18). Hence, in the absence of added bases the 260-mμ absorbancy of the medium could be used to follow RNA breakdown.

Streptomycin uptake

The uptake of [^{14}C]streptomycin was measured by collecting cells on membrane filters and washing them three times with 2 ml 7.5% sucrose. This procedure was found to be equivalent to the earlier one of washing the cells with water[19]. The maintenance of osmotic strength with sucrose prevents the loss of cellular K that would otherwise occur[9,20], so that ^{42}K and ^{14}C can be determined on the same samples.

RESULTS

Timing of effects on viability, turbidity, K efflux, crypticity, respiration, and nucleic acid stability

In several experiments the following parameters were measured in the same growing culture at intervals before and after addition of streptomycin: viable count; turbidity; K efflux; crypticity for β-galactosidase; Q_{O_2}; and 260-mμ absorbancy of culture filtrates. Concentrations of streptomycin between 20 and 30 μg/ml proved suitable, in the media employed, for producing effects that were readily detectable but still well spread out in time.

Fig. 1 summarizes one of two virtually identical experiments with streptomycin at 20 μg/ml. The earliest change observed was acceleration of K efflux, starting about 15 min after addition of the drug. Since this measurement (made by method A) requires a number of minutes, it should be noted in particular that the efflux from cells resuspended at 20 min, and followed (in the absence of streptomycin) for the next 10 min, was significantly increased ($KT_{1/2}$ decreased from 15 to 10 min). In contrast, at the end of this measurement (30 min) none of the other parameters had yet been measurably affected except viability, which had just begun to fall.

At about 40 min accessibility of ONPG to β-galactosidase began to increase and Q_{O_2} to decrease, the rate of turbidity increase fell off from the exponential, and the rate of excretion of 260-mμ-absorbing material began to rise. These effects thus appear to be fairly late ones, and their timing relative to each other has not been studied extensively.

However, the effect on Q_{O_2} was investigated further. Measurements made in

a Warburg respirometer (as in this experiment) require about 30 min; they were therefore made on cells resuspended in the absence of streptomycin, to prevent continuing drug action. Thus an early reversible effect might have been missed. To explore this possibility additional experiments were done in which Q_{O_2} was followed using an oxygen electrode. With this method, measurements require 2 min or less, and frequent determinations can be made directly on samples of whole culture. Fig. 2 summarizes such an experiment, in which we also followed viability, turbidity, and β-galactosidase activity. Although there was a longer lag period than in the experiment of Fig. 1, the sequence of events was similar, in that Q_{O_2} started to fall slightly after the onset of killing and at about the same time as the onset of effects on crypticity and on turbidity. In addition, at 90 min, when viability had decreased by 95 %, Q_{O_2} had decreased by only 17 %. We conclude that streptomycin has no early irreversible or reversible effect on respiration in this system.

Correlation of streptomycin uptake with effects on K efflux and viability

The uptake of [^{14}C]streptomycin was studied in conjunction with viability and K efflux (Fig. 3). As previously reported[1,19] there was an initial virtually instantaneous uptake (the "primary uptake") followed by a 30-min "plateau" period and then by a gradually increasing "secondary" uptake.

K efflux was determined in this experiment by method B, in which ^{42}K-labelled untreated cells are transferred to non-radioactive medium, with or without strepto-

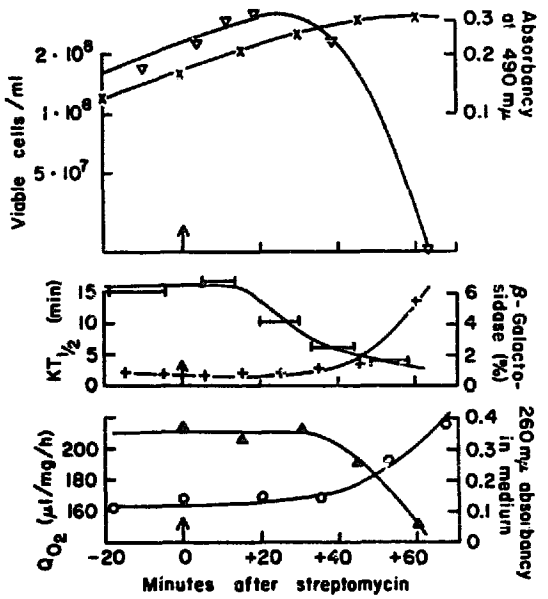

Fig. 1. Effects of streptomycin on viability, turbidity, K efflux, crypticity, Q_{O_2}, and nucleotide excretion. Cells were grown in a shaking flask in medium A'^{42}K$_{0.1}$. Streptomycin, 20 μg/ml, was added at zero time (arrows). Measurements were made as described in the text. K efflux was determined by method A, and Q_{O_2} by the Warburg method (a). Viable cells (▽—▽); turbidity (×—×); $KT_{½}$ (⊢⊣) (the length of each bar indicates the period over which the efflux was measured); β-galactosidase activity (+—+); Q_{O_2} (▲—▲); 260-mμ absorbancy of medium (○—○).

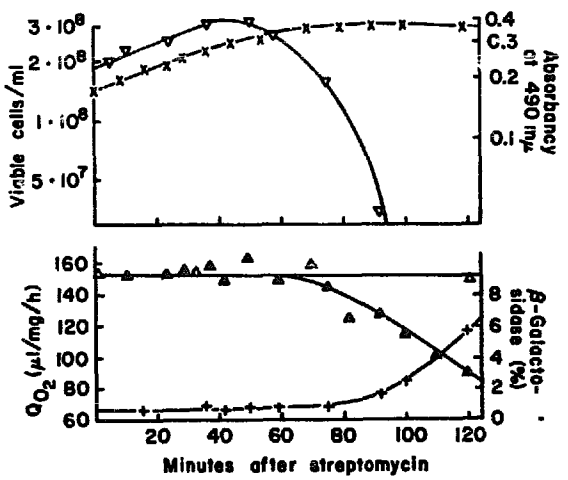

Fig. 2. Effects of streptomycin on viability, turbidity, crypticity, and Q_{O_2}. Cells were grown and treated with streptomycin as in Fig. 1, except that non-radioactive K was used. Measurements were made as described in the text. Q_{O_2} was followed polarographically (method b) in the treated culture and in a parallel control culture. Streptomycin-treated culture: viable cells (▽—▽); turbidity (×—×); β-galactosidase activity (+—+); Q_{O_2} (▲—▲). Control culture: Q_{O_2} (△—△).

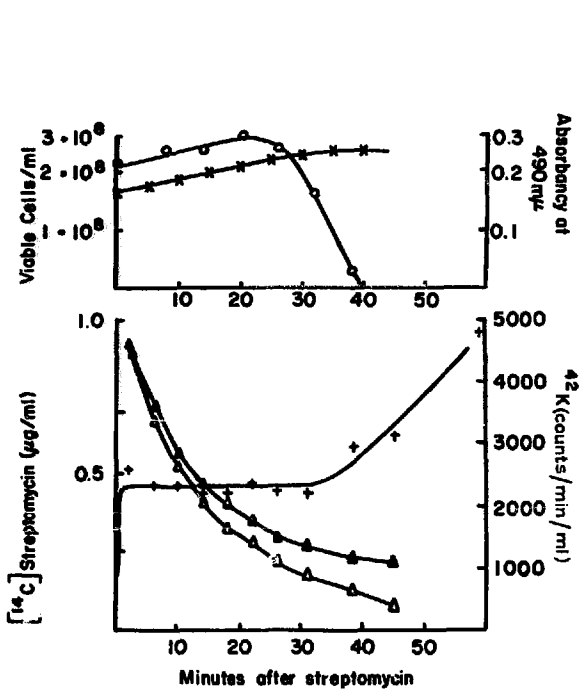

Fig. 3. Streptomycin uptake and effects on viability, turbidity, and K efflux. Cells were grown in medium A′$^{42}K_{0.3}$ supplemented with leucine, cytosine, and uracil. At a turbidity of 0.25 the cells were transferred to non-radioactive medium for measurement of K efflux by method B. They were subdivided into 2 tubes containing prewarmed medium A′K_1 with the same supplements plus, in one tube, 30 μg/ml of [^{14}C]streptomycin at half the stock specific activity. Measurements were made as described in the text. Streptomycin-treated culture: viable cells (O—O); turbidity (×—×); ^{42}K content (Δ—Δ); [^{14}C]streptomycin uptake (+—+). Control culture: ^{42}K content (▲—▲).

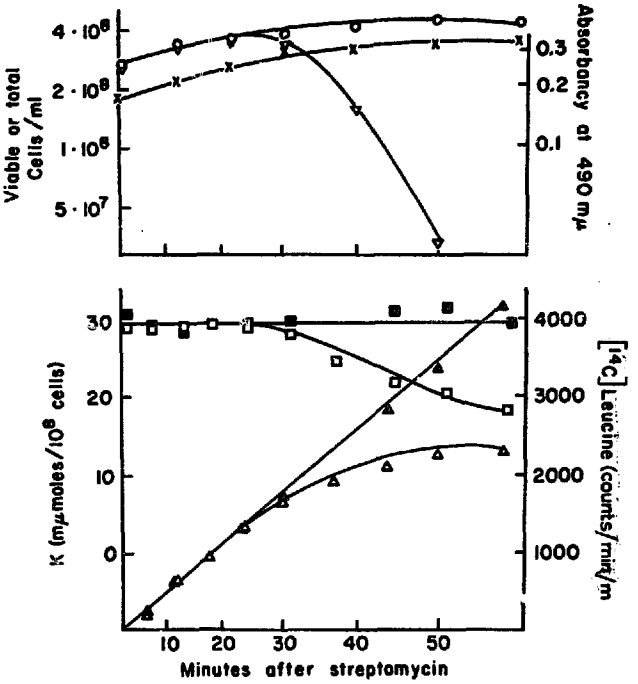

Fig. 4. Effects of streptomycin on cell number, viability, turbidity, K content, and [^{14}C]leucine uptake. Cells were grown in medium A′$^{42}K_{0.3}$ supplemented with leucine, 50 μg/ml. The culture was subdivided at zero time into two tubes containing (in a negligible volume) [^{14}C]leucine to give a concentration of 0.1 μg/ml; one tube also contained streptomycin to give a concentration of 20 μg/ml. Measurements were made as described in the text, except that the precision of the viable counts was increased by using duplicate sets of dilutions. [^{14}C]Leucine incorporation was measured in cold trichloroacetic acid precipitates. The time scale was made proportional to the increase in turbidity of the control culture (doubling time 49 min), as described in the text. Upper panel (streptomycin-treated culture only): total cell number (O—O); viable cells (▽—▽); turbidity (×—×). Lower panel (both cultures): K content, control (■—■), streptomycin (□—□); [^{14}C]leucine, control (▲—▲), streptomycin (△—△).

mycin, at the onset of the experiment. The streptomycin-treated culture showed a marked acceleration of efflux (Fig. 3), just as had been observed with method A (Fig. 1). This began well before the secondary uptake of the drug and slightly before the onset of loss of viability.

It should be noted that during the plateau period there was a constant amount of bound streptomycin per ml of culture rather than per mg of cells as reported earlier[1,19]. The present data seem the more reliable, because the lower concentration of drug resulted in a longer plateau period, during which turbidity increased by 65 % without an increase in bound streptomycin. It appears that growth during this period is unaccompanied by the formation of new streptomycin-binding sites.

Correlation of effects on protein synthesis with effects on K efflux and viability

We next studied the effects of streptomycin on the synthesis of various macromolecules, with the purpose of timing of these effects relative to each other as well

as to loss of viability and to altered K metabolism (chosen as the earliest indicator of impaired membrane integrity).

A close correlation was regularly found between loss of viability and slowing of protein synthesis as measured by [^{14}C]leucine incorporation. Fig. 4 presents data from a representative experiment. Such data allow one to estimate for the treated culture the instantaneous rate of leucine incorporation per viable cell at any time. As shown in Table I (columns A and B) the rate remained relatively constant through a 10-fold fall in viability. This finding suggests that, to a first approximation, all viable cells were synthesizing protein at the normal rate, and the death of an individual cell was associated with abrupt cessation of protein synthesis.

TABLE I

RELATIONS AMONG VIABLE COUNT, CELL NUMBER, RATE OF PROTEIN SYNTHESIS, AND TURBIDITY IN A STREPTOMYCIN-TREATED CULTURE

The data are from the experiment of Fig. 4. The rate of [^{14}C]leucine incorporation was estimated as the slope of the curve of leucine incorporation per ml of culture *vs.* time (plotted on linear coordinates). This figure was then divided by the corresponding figure of column A to give the rate of incorporation per 10^8 viable cells (column B).

Time after streptomycin (min)	*Viable cells per ml ($\times 10^{-8}$) (A)*	*Rate of [^{14}C]leucine incorporation (counts/min) per 10^8 viable cells (B)*	*Total cells per ml ($\times 10^{-8}$) (C)*	*Turbidity (absorbancy at 490 mμ) per 10^8 total cells (D)*
−33	1.97	—	1.80	0.072
− 3	2.47	—	2.62	0.073
+12	3.11	12	3.26	0.068
22	3.46	11	3.46	0.076
31	3.28	9	3.68	0.079
40	1.52	11	4.00	0.079
50	0.31	15	4.22	0.079

Implicit in this reasoning is the assumption that the viable count accurately reflected the mass of viable cells per unit volume of culture and was not significantly distorted by cell aggregation or by gross changes in cell size. Total cell counts in this experiment supported this assumption, as the turbidity per cell increased only slightly during the course of treatment* (Fig. 4 and Table I, columns C and D). One would expect this parameter to be, if anything, less altered in the viable than in the dead cells.

Intracellular K was also followed in the experiment of Fig. 4. Detectable K depletion began at about the same time as did killing and slowing of protein synthesis and thus lagged somewhat behind increased K efflux (by comparison with the data of Figs. 1 and 3). This result is to be expected in view of the continuing unidirectional influx[9], which tends to obscure the initial depleting effects of increased efflux.

Correlation of effects on nucleic acid synthesis with effects on other parameters

Studies on nucleic acid synthesis yielded an unexpected result. There was an early stimulation by streptomycin of [^{14}C]uracil incorporation, which lasted for a

* At later times this parameter decreases, as the cell number remains constant in the face of the decreasing turbidity associated with large-scale RNA degradation[8,21].

number of minutes before the eventual inhibition of nucleic acid synthesis. The stimulation was somewhat variable in magnitude but was consistently detected. That it was an effect on RNA and not DNA is shown in Fig. 5. The rate of incorporation of radioactivity into DNA was unaffected for a period of 30 min during which incorporation into total nucleic acid was stimulated by up to 30%. Later, the rates of incorporation into both the DNA and the total nucleic acid of the treated culture fell as all biosynthetic processes were inhibited.

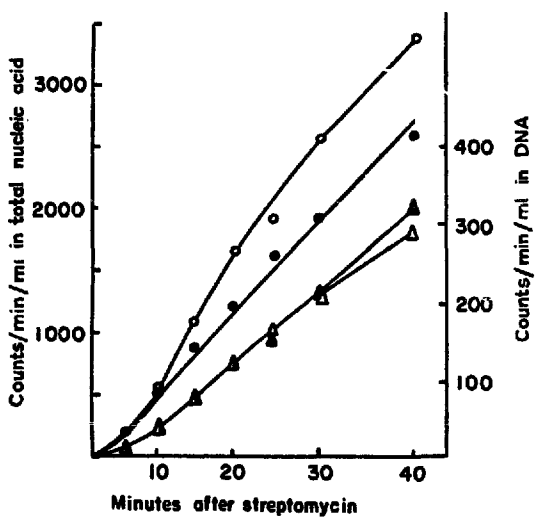

Fig. 5. Effects of streptomycin on DNA and total nucleic acid synthesis. Cells were grown in medium A'$K_{0.3}$ supplemented with guanine, leucine, uracil, and cytosine. At a turbidity of 0.23 (zero time), the culture was diluted 5:7 into 2 tubes containing prewarmed medium with the same supplements and in addition [^{14}C]uracil to give a concentration of 2 μg/ml. One tube also contained streptomycin to give a concentration of 25 μg/ml. Incorporation of radioactivity into total nucleic acid and into DNA was measured in trichloroacetic acid precipitates as described in the text. The scale of the abscissa was determined as for Fig. 4 (doubling time of control culture, 44 min). The turbidity of the two cultures began to differ detectably at about 30 min. [^{14}C]Uracil in total nucleic acid, control (●—●), streptomycin (○—○); [^{14}C]uracil in DNA, control (▲—▲), streptomycin (△—△).

The stimulation of incorporation into RNA cannot be due to preferential utilization of exogenous over endogenous precursors, since this mechanism should affect DNA as well as RNA. Furthermore, similar results (to be presented in detail elsewhere) were obtained using [^{14}C]guanine and [^{32}P]phosphate. These latter findings also exclude the possibility that the increased uracil incorporation might be due to synthesis

TABLE II

EFFECTS OF STREPTOMYCIN ON NUCLEIC ACID AND PROTEIN SYNTHESIS

Cells grown as in Fig. 5 were subdivided at a turbidity of 0.21 (zero time) into 2 tubes containing (in a negligible volume) [^{14}C]leucine and [^{14}C]uracil to give concentrations of 0.05 and 2 μg/ml respectively. One tube contained streptomycin to give a concentration of 25 μg/ml. Incorporation into trichloroacetic acid precipitates was measured as described in the text. No effect on turbidity was noted up to 25 min; killing began at about 15 min.

Time after streptomycin (min)	*[^{14}C]Uracil incorporation (counts/min/ml)*		*[^{14}C]Leucine incorporation (counts/min/ml)*	
	Control	*Streptomycin*	*Control*	*Streptomycin*
4	130	117	87	91
7	272	277	148	148
10	484	523	222	222
13	639	729	301	301
16.3	878	1110	412	372
19.5	1200	1590	460	406
24	1680	2030	580	434
29	2050	2490	730	534
34	2630	2880	990	580
40	3420	3160	1120	600

of a relatively uracil-rich RNA. It therefore appears that streptomycin transiently increases the rate of accumulation of one or more RNA fractions.

The RNA stimulation preceded the slowing of protein synthesis, as is shown in Table II. This experiment is representative of three such experiments, in which four successive samples were obtained before the slowing of protein synthesis became detectable (*i.e.*, before the leucine incorporation of the control exceeded that of the treated culture by as much as 2 %). In each of these experiments the last two or three of these samples showed a stimulation of uracil incorporation of more than 5 %.

We were unable, however, to separate in time the onset of this stimulation from that of increased K efflux (see Table III). It thus appears that the effect on RNA begins about as early as the first change we have been able to detect in membrane integrity.

TABLE III

EFFECTS OF STREPTOMYCIN ON K EFFLUX AND NUCLEIC ACID SYNTHESIS

Cells were grown as in Fig. 3. At a turbidity of 0.16 the cells were transferred to non-radioactive medium for measurement of K efflux by method B. Equal numbers of cells were added to 2 tubes (zero time) containing 20 ml of prewarmed medium $A'K_1$ supplemented with leucine and cytosine as before, plus [^{14}C]uracil, 5.5 μg/ml at 1/10 the stock specific activity. One tube contained in addition streptomycin, 30 μg/ml. Measurements were made on washed cells as described in the text. Effects on viability and turbidity became detectable at about 15 and 25 min respectively.

Time after streptomycin (min)	*^{42}K in cells (counts/min/ml)*		*[^{14}C]Uracil in cells (counts/min/ml)*	
	Control	*Streptomycin*	*Control*	*Streptomycin*
1	6070	6100	18	16
4	4780	4750	38	36
8	3580	3530	75	77
12	2840	2580	128	137
16	2310	1900	177	194
20	1890	1395	232	265
24	1630	980	297	332
28	1390	760	345	401
32	1100	450	415	470
36	1000	340	451	502

DISCUSSION

The sequence of events observed in streptomycin-treated cells in these experiments can be summarized as follows: first, the instantaneous primary uptake of the drug; next, after a lag, stimulation of net RNA synthesis and acceleration of K efflux; next, loss of viability and inhibition of protein synthesis; and finally, secondary uptake of the drug, increase in permeability to ONPG, impaired respiration, decreases in RNA and DNA synthesis, and RNA breakdown with excretion of nucleotides. At present these effects cannot be integrated in a unitary theory on the action of streptomycin, but certain comments are in order.

The primary uptake of [^{14}C]streptomycin has characteristics of an electrostatic interaction: it occurs rapidly at 0° as well as at 37° (see ref. 1) and is in large part rapidly reversible by salts[1,19,22,*]. There is also evidence that the binding occurs

* In this regard, the binding differs markedly from that in *Bacillus megaterium*[23]. The basis for this difference is unknown.

largely at the outer surface of the cell: the electrophoretic mobility of bacteria is immediately affected by addition of streptomycin[24], and the amount of drug bound is rapidly and markedly increased by damaging the permeability barrier of the cell with toluene[1].

These considerations, together with the following, suggest that the effect on K metabolism involves a direct interaction between streptomycin and the cell membrane: the effect begins quite early in the plateau period; it is not the result of simple displacement of intracellular K by the cationic drug[9]; and it cannot be due to gross changes in cell surface: volume ratio in view of the relative constancy of the ratio of turbidity to cell number (Table I). We suggest that adsorbed streptomycin causes local distortions in the growing membrane. Abnormal channels soon develop which initially allow K to escape and eventually become large enough for the passage of molecules such as ONPG and nucleotides. Support for such a model can be found in studies by CHRISTIAN[20] on the effects of exposing bacteria to hypotonic solutions. In these studies, K loss could be observed at a lesser degree of osmotic shock than that required to produce nucleotide loss.

The secondary uptake of streptomycin may similarly occur *via* enlarged channels in what had been an impermeable membrane. Another possibility is suggested by the fact that the uptake of streptomycin is extremely sensitive to salts[19]. The extensive K depletion observed at the time of the secondary uptake appears to be accompanied by depletion of Mg (see ref. 9) and presumably of other cations which normally serve to neutralize fixed intracellular anions (such as those of nucleic acids). The secondary uptake may thus be an indirect effect of membrane damage, resulting from changes in the intracellular ionic milieu rather than from the initiation of penetration through the membrane. This hypothesis makes unnecessary the assumption that the undamaged membrane is completely impermeable to streptomycin. In fact, the effects on RNA and protein synthesis, which start during the plateau period, are most readily explained in terms of the early penetration of at least a small amount of drug into the cytoplasm.

The stimulation of incorporation of [^{14}C]uracil is the most novel, while the least explored, of the effects we have observed. It has been reported that streptomycin-treated cells accumulate a slowly sedimenting RNA fraction[4,25,*], a portion of which has properties of messenger RNA[27]. Our early stimulation may be due to the accumulation of such an RNA before inhibition of RNA synthesis becomes the dominant process. In this event, the accumulation may reflect decreased breakdown, especially in view of the report that messenger-RNA synthesis is unaffected by streptomycin[4].

Alternatively, the streptomycin-RNA may resemble chloramphenicol-RNA in being largely ribosomal in base composition[28]. However, the accumulation of chloramphenicol-RNA can be explained by current theories on the regulation of RNA synthesis, which postulate that intracellular amino acids act as inducers of RNA synthesis[17,29]. Accelerated synthesis would result from expansion of the amino acid pool consequent to the slowing of protein synthesis (which occurs immediately on exposure to chloramphenicol). In contrast, the stimulation of RNA synthesis by streptomycin clearly precedes the slowing of protein synthesis, and thus one cannot

* These authors did not report stimulation of overall synthesis, but the effect is often small and transient; in fact, published data from a previous study in this laboratory[26] do show a small stimulation, which was overlooked.

apply the same explanation. More detailed studies on streptomycin-RNA are in progress.

Our findings on inhibition of protein synthesis are compatible with either a direct effect of streptomycin on the protein-synthesizing system, as has been shown in a cell-free system[27,33], or with an indirect effect, associated with membrane damage. In this connection it is pertinent that streptomycin-treated cells, even after virtual cessation of protein synthesis (Fig. 4), did not exhibit as extensive K depletion as that required for comparable slowing of protein synthesis in a mutant of *E. coli* lacking the K-transport system[31,32]. However, one cannot exclude the possibility that depletion of other factors contributes to the effect of streptomycin on protein synthesis.

The kinetics of the inhibition of protein synthesis suggest, as stated above, that this effect is an all-or-none one in the individual cell and is closely related to cell death. However, we have not tested this correlation under a variety of conditions. WHITE AND FLAKS[4] have reported cessation of protein synthesis before the onset of killing, under conditions that produced earlier killing than in our experiments. On the other hand, HURWITZ *et al.*[33], using lower concentrations of streptomycin, reported a bacteriostatic effect coinciding with the initial inhibition of protein synthesis; it is possible that this "bacteriostasis" represented a temporary balance between cell death and cell division.

In the present studies the inhibition of respiration started slightly later (relative to effects on turbidity) than in previous studies using *Staphylococcus aureus*[7] and *Bacillus subtilis*[34]. However, it appears that at least in *E. coli* damage to the respiratory system does not play a primary role in the lethal action of the drug.

The late occurrence of large-scale depolymerization of RNA supports the earlier inference[8] that this too is a secondary effect.

In summary, the present findings tend to focus attention on changes in membrane integrity and in RNA and protein synthesis as early effects of streptomycin in *E. coli*. It is not clear whether the membrane damage is itself sufficient to cause the changes in macromolecule synthesis, whether it merely permits such changes by facilitating entry of streptomycin, whether it is entirely unrelated to the other effects, or whether cell membrane and macromolecule-synthesizing systems have a common, streptomycin-sensitive component. The irreversible (hence lethal) event also remains undefined. It is evident that more remains to be done on this problem.

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REFERENCES

1 N. ANAND, B. D. DAVIS AND A. K. ARMITAGE, *Nature*, 185 (1960) 23.
2 F. E. HAHN AND J. CIAK, *Bacteriol. Proc.*, (1959) 131.
3 N. ANAND AND B. D. DAVIS, *Nature*, 185 (1960) 22.
4 J. R. WHITE AND J. G. FLAKS, *Federation Proc.*, 21 (1962) 412.
5 R. J. HENRY, R. D. HOUSEWRIGHT AND S. BERKMAN, *J. Bacteriol.*, 57 (1949) 447.
6 F. L. JACKSON, *Nature*, 181 (1958) 281.

[7] R. HANCOCK, *J. Gen. Microbiol.*, 23 (1960) 179.
[8] D. T. DUBIN AND B. D. DAVIS, *Biochim. Biophys. Acta*, 55 (1962) 793.
[9] D. T. DUBIN AND B. D. DAVIS, *Biochim. Biophys. Acta*, 52 (1961) 400.
[10] T. D. BROCK, *Antimicrobial Agents Ann.*, 297 (1961).
[11] B. D. DAVIS AND E. S. MINGIOLI, *J. Bacteriol.*, 60 (1950) 17.
[12] H. E. KUBITSCHEK, *Nature*, 182 (1958) 234.
[13] D. S. FEINGOLD, unpublished observations.
[14] J. LEDERBERG, *J. Bacteriol.*, 60 (1950) 381.
[15] D. ROODYN AND G. MANDEL, *Biochim. Biophys. Acta*, 41 (1961) 80.
[16] G. SCHMIDT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
[17] C. G. KURLAND AND O. MAALØE, *J. Mol. Biol.*, 4 (1962) 193.
[18] D. T. DUBIN, unpublished observations.
[19] P. H. PLOTZ, D. T. DUBIN AND B. D. DAVIS, *Nature*, 191 (1961) 1324.
[20] J. H. B. CHRISTIAN, *Australian J. Biol. Sci.*, 11 (1958) 538.
[21] D. S. FEINGOLD AND B. D. DAVIS, *Biochim. Biophys. Acta*, 55 (1962) 787.
[22] S. BERKMAN, R. J. HENRY, B. D. HOUSEWRIGHT AND J. HENRY, *Proc. Soc. Exptl. Biol. Med.*, 68 (1948) 65.
[23] R. HANCOCK, *J. Gen. Microbiol.*, 28 (1962) 493.
[24] K. MCQUILLEN, *Biochim. Biophys. Acta*, 7 (1951) 54.
[25] N. R. EATON AND R. CAFFREY, *J. Bacteriol.*, 81 (1961) 918.
[26] N. ANAND AND B. D DAVIS, in *Proc. Symp. Proteins, Chem. Res. Comm. Soc. Biol. Chem., India*, Wesley Press, Mysore, 1961, p. 100.
[27] J. G. FLAKS, E. C. COX AND J. R. WHITE, *Biochem. Biophys. Res. Commun.*, 7 (1962) 385.
[28] A. B. PARDEE AND L. S. PRESTIDGE, *J. Bacteriol.*, 71 (1956) 677.
[29] G. STENT AND S. BRENNER, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 2005.
[30] J. SPEYER, P. LENGYEL AND C. BASILIO, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 684.
[31] H. L. ENNIS AND M. LUBIN, *Biochim. Biophys. Acta*, 50 (1961) 399.
[32] M. LUBIN, personal communication.
[33] C. HURWITZ, C. L. ROSANO AND J. LANDAU, *J. Bacteriol.*, 83 (1962) 1210.
[34] J. LIGHTBOWN, personal communication.