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Phenotypic suppression by streptomycin-adenylate and bluensomycin-adenylate*

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1. INTRODUCTION

Attempts to define the functional group(s) responsible for the mode of action of the aminoglycoside antibiotics have utilized structure–activity relationships. This approach involves testing natural or synthetic analogues of the drug molecule for biological activity. If the analogue is as active as the parent molecule, the altered region of the analogue is considered unimportant for the drug's mode of action. An increasing volume of evidence supports the idea that misreading (as defined originally *in vitro* to mean substitution of one amino acid for another in the translation of an mRNA) and inhibition of protein synthesis caused by streptomycin (Sm) are probably two distinct expressions of the drug's action (reviewed by Davis, 1968). Clearly, Sm analogues which are biologically active in one of these areas but not in the other (reviewed by Davies, 1967) would be of considerable importance to this model.

The recent discovery of R (Resistance) factor-coded, drug-inactivating enzymes and the characterization of their products have provided a new group of naturally synthesized aminoglycoside analogues which can be used in such structure–activity studies. For example, R factor enzyme-inactivated aminoglycosides have been tested in *in vitro* systems which measure the inhibition of cell-free protein synthesis as well as misreading. To date, such studies have been performed with altered forms of both kanamycin (Km) (Hori & Umezawa, 1967) and Sm (Yamada, Tipper & Davies, 1968; Harwood & Smith, 1969). *In vitro* observations with two such analogues proved to be of particular interest. In order to test Sm-adenylate and bluensomycin (Blu)-adenylate, the products of an R factor ('RE 130') enzyme-catalysed inactivation, we had employed the poly U-directed, cell-free protein-synthesizing system (Harwood & Smith, 1969). In the presence of 10 μg Sm/ml, the levels of both misreading ([^{14}C]isoleucine incorporation) and inhibition of polyphenylalanine synthesis obtained when *E. coli* B extract was used were completely

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eliminated when *E. coli* B/RE 130 extract was substituted. When the same experiment was performed with 10 μg Blu/ml, however, the inhibition of polyphenylalanine synthesis was again fully eliminated when *E. coli* B/RE 130 extract was used, but the degree of Blu-induced misreading seen with control (i.e. *E. coli* B) extract was reduced only about 60% when B/RE 130 extract was substituted. This suggested that the product of Sm inactivation, Sm adenylated at the 3′-hydroxyl group of the drug's *N*-methyl-L-glucosamine moiety (Yamada *et al.* 1968; Takasawa *et al.* 1968), caused neither misreading nor inhibition of polypeptide synthesis *in vitro*; however, Blu adenylated at the same site (Harwood & Smith, 1969), while incapable of inhibiting polypeptide synthesis under these conditions, appeared, nevertheless, to sustain a substantial level of misreading. Prior to this, Hori & Umezawa (1967) had obtained qualitatively similar results *in vitro* with Km-acetate, the product of an R-Km^R enzyme-catalysed reaction. Both of these findings implied the existence of naturally occurring aminoglycoside analogues which, at least *in vitro*, cause misreading but do not inhibit polypeptide synthesis.

There are, however, objections to such *in vitro* assays in that they do not accurately reflect conditions in the intact cell (e.g. Mg^{2+} concentration, type of mRNA, access of extracellular or periplasmic degradative enzymes to components of the *in vitro* system which would normally be protected from these enzymes due to their intracellular location, etc.). Furthermore, experiments in which the product of drug inactivation is purified and bioassayed fail to evaluate a crucial point; that is, the efficiency of the inactivated product in penetrating the indicator cells.

To obviate these objections, we have measured the capacity of Sm-adenylate and Blu-adenylate to cause phenotypic suppression *in vivo*.* This involves introducing an R factor, RE 130, known to mediate the adenylation of Sm and Blu (Harwood & Smith, 1969), into a conditionally Sm-dependent (CSD) *E. coli* strain. Since under certain conditions this host strain will grow only in the presence of aminoglycosides which can cause phenotypic suppression (Gorini & Kataja, 1964), one can determine whether Sm- or Blu-adenylate can cause phenotypic suppression by measuring growth of the CSD/RE 130 strain on low levels of Sm or Blu.

2. MATERIALS AND METHODS

(i) *Drugs*

Sm was used in the form of streptomycin sulphate (E. R. Squibb and Sons, New York). Bluensomycin, the gift of Dr George Whitfield of the Upjohn Co. (Kalamazoo, Mich.), was also used as the sulphate salt. Uniformly labelled [^{14}C]Sm (0·054 μc/mg of $CaCl_2$ complex of Sm) was obtained from Merck Sharp and Dohme Research Laboratories (Rahway, N.J.).

* Misreading, as it was originally defined *in vitro*, has not yet been directly demonstrated *in vivo*. Since the aminoglycosides which cause misreading *in vitro* are also those which permit phenotypic suppression *in vivo*, a close but as yet undefined relationship between these two processes probably exists (Weisblum & Davies, 1968). For this reason, the CSD assay system described actually measures *phenotypic suppression* by Sm-adenylate and Blu-adenylate rather than *misreading*; we elected to measure phenotypic suppression as that physiological phenomenon which best correlates with the *in vitro* phenomenon of misreading.

(ii) *Media*

Drug plates used to evaluate R factor resistances consisted of Levine-EMB-lactose agar (Baltimore Biological Lab., Baltimore, Md.) containing spectinomycin (Spc) at 50–100 μg/ml, chloramphenicol (Cm) at 25 μg/ml, tetracycline (Tc) at 25 μg/ml or Sm at 10–20 μg/ml.

'Medium A' (Davis & Mingioli, 1950) minus citrate was the basic constituent of (1) *minimal glucose medium* (medium A plus 0·2 % glucose), (2) *B buffer* (medium A minus $(NH_4)_2SO_4$) used as a diluent, and (3) *minimal glucose agar* (minimal glucose medium plus 1·5 % agar). We used trypticase soy broth (BBL) as a nutrient medium.

(iii) *Abbreviations*

Drug-AMP = drug-adenylate, $Sm^{S,R,D}$ = streptomycin-sensitive-resistant-dependent (except where otherwise noted, this refers to the host chromosomal locus), Su = sulphadiazine, Arg = arginine, Hg = mercuric chloride, and Nal = nalidixic acid. 'B/RE130' denotes host strain *E. coli* B carrying the R factor, RE130. Min = minimal glucose agar or minimal glucose liquid medium, where appropriate. Min Blu^{20}, Min Sm^{500}, etc. = minimal glucose agar containing Blu at 20 μg/ml. or Sm at 500 μg/ml, etc.

(iv) *Bacterial strains and recombinant selection*

The R factor, RE130, was originally discovered in a natural isolate of *E. coli* bearing transferable resistance to Sm, Su, Tc, Cm, Blu, Spc and Hg. This R factor has been demonstrated to confer Sm, Blu, and Spc resistance by virtue of an enzyme which adenylates these drugs (Harwood & Smith, 1969). Strains *E. coli* B 6–15Rc and 6–15S are the gift of Drs G. Jacoby and L. Gorini. They are *arg F40* (ornithine transcarbamylase deficient), *arg R15* (derepressed for the enzymes of Arg biosynthesis) (Jacoby & Gorini, 1967), and CSD such that in order to grow on minimal medium they require either Arg or an aminoglycoside (Sm or Blu) which will suppress the ornithine transcarbamylase-defective phenotype. Strain 6–15Rc (abbreviated 'Rc') is '*R*esistant' and 'competent'; that is, it is resistant to > 2000 μg/ml Sm (measured in minimal medium) by virtue of a chromosomal mutation affecting ribosome structure, and it is competent in allowing phenotypic suppression of the ornithine transcarbamylase defect by Sm or Blu. Strain 6–15S ('S') is a Sm^S derivative (by P1 transduction) of strain Rc.

E. coli AB1932–1 is a spontaneous mutant resistant to 100 μg/ml of Nal (Walton & Smith, 1969) and derived from AB1932 (K12, F^-, arg^- met^- xyl^- gal^- lac^- T_6^R) obtained from Dr E. A. Adelberg.

E. coli B (Sm^D) requires 200 μg/ml Sm or more in order to grow on nutrient medium and was selected in one step from wild-type *E. coli* B by plating 10^9–10^{10} cells on to EMB-Sm^{200} plates. After 3 days of incubation at 37 °C, colonies were purified several times on EMB-Sm^{200} plates, and B (Sm^D) was chosen on the basis of its ability to grow on EMB-Sm^{200} but not on EMB-Blu^{400}.

RE130 was transferred to *E. coli* B (Sm^D) recipient by conjugation (Watanabe,

1964) from an RE130-containing lac$^-$Sm^R multiply auxotrophic *E. coli* donor by selecting for recombinants which were prototrophic Cm^{R}Sm^R (500 μg/ml) and lac$^+$.

RE130 was transferred to the Rc strain by conjugation from an R$^+$Sm^S host by selecting for recombinants on EMB plates containing Tc 25 μg/ml and 500 μg/ml or more of Sm. In order to transfer RE130 to strain S, the original Sm^S natural *E. coli* isolate containing RE130 was mated with an intermediate recipient which was Sm^{R}lac$^-$, and lac$^-$ recombinants were selected on EMB plates containing Sm 500 μg/ml and Tc 25 μg/ml. Then the R factor was transferred to strain S by a mating from which recombinants were selected on minimal medium containing lactose 0·2% (sole carbon source) and Tc 25 μg/ml.

CSD/RE130 clones to be tested for RE130-mediated Sm resistance were transferred to strain AB1932-1 by conjugation and selected for recombinants on EMB plates containing Nal 50 μg/ml and either Cm 25 μg/ml or Tc 25 μg/ml.

(v) *Preparation of CSD cultures*

CSD strains, with or without RE130, were grown either in trypticase soy broth or Min + Arg until turbid, whereupon the cells were washed twice in the cold by pelleting and resuspending in B buffer before appropriate dilution (at least 100-fold) in B and plating.

(vi) *Nitrosoguanidine mutagenesis*

The mutagenesis protocol used to construct a Sm^{S}Spc^{S}Blu^S derivative of RE130 was previously described (Adelberg, Mandel & Chen, 1965) except that for this work citrate buffer at pH 6 was used. The mutagenized *E. coli* B/RE130 cells were replicated to appropriate drugs, and Sm^{S}Spc^S clones were mated with a Sm^S recipient, AB1932-1, in order to verify that these RE130 derivatives no longer determined resistance to Sm, Spc or Blu but still determined resistance to other drugs. One mutant R factor which fulfilled these criteria, RE130-17, was used in these studies.

(vii) [^{14}C]*Sm uptake*

Cells were grown in minimal glucose medium to a concentration of about 1–3 × 10^8 cells/ml whereupon the culture flask was chilled rapidly in an ice-water bath. When the culture temperature had dropped to 0 °C, [^{14}C]Sm was added to a final concentration of 100 μg/ml of culture (0-time). After immediate mixing, a 5 ml 0-time aliquot was removed, and the remainder of the culture was placed in a pre-warmed sidearm flask and incubated, shaking, at 37 °C. Five ml aliquots were removed at intervals, diluted quickly in 20 ml of B buffer at 0 °C, filtered on a 0·45 mμ membrane filter (Millipore Corp., Bedford, Mass.) of 47 mm diameter, and washed twice with 5 ml B buffer at 0 °C. The washed filters were taken directly to 10 ml of Bray's solution (Bray, 1960) and counted for at least 1500 counts per sample (standard deviation of the mean number of counts/min = 2%, equivalent here to no more than ± 1 count/min for any given value) in a Packard Tri-Carb Scintillation Spectrometer Model 3375. The background value used to correct all counts was measured as the number of counts/min adsorbed by a filter in the absence of cells. Viable cell counts were done at each time interval.

3. RESULTS

It has previously been shown that the adenylated form of Sm is inactive *in vitro* with respect to (*a*) inhibiting poly-U-directed polyphenylalanine synthesis and (*b*) promoting misreading as measured by isoleucine incorporation in the presence of poly-U (Yamada, Tipper & Davies, 1968; Harwood & Smith, 1969). In addition, Sm-AMP lacks bactericidal activity against Sm^S indicator bacteria in bioassay (Yamada, Tipper & Davies, 1968; Umezawa, Takasawa, Okanishi & Utahara, 1968; Harwood & Smith, 1969).

Table 1. *Protection conferred by the R factor, RE130, against lethal action of Sm or Blu**

	Aminoglycoside concentrations permitting growth	
E. coli strain	Sm (μg/ml)	Blu (μg/ml)
S	0–2	0–10
S/RE130	0–200	0–200†
Rc	0–2000	—
Rc/RE130	0–2000	—

* Growth was measured qualitatively after 5 days of incubation at 37 °C on Min agar plates containing 100 μg/ml Arg and either Sm or Blu at 0, 2, 5 ,10, 20, 40, 100, 200, 500, 1000 and 2000 μg/ml.

† Growth only followed as high as 200 μg/ml Blu.

In order to determine only the extent of protection against the lethal action of Sm or Blu conferred by RE130 on the two CSD host strains, Arg was incorporated into the medium so that growth of the CSD strain would not require drug-induced phenotypic suppression. Table 1 shows that on minimal plates containing Arg and varying concentrations of Sm or Blu, RE130 protected strain S against 200 μg/ml of Sm and > 200 μg/ml of Blu.

After the range of lethal drug concentrations over which the R factor (i.e. the adenylating enzyme) is effective had been established, the converse experiment was performed. Cells were plated on minimal glucose medium *minus* Arg and containing a graded series of *sublethal* Sm or Blu concentrations such that growth was dependent only upon drug-induced phenotypic suppression. CSD strain Rc with or without RE130 was chosen for this experiment rather than strain S because the arg^- defect of strain S is leaky, and therefore this strain grows slowly even in the absence of both Arg and Sm (or Blu). It was thought that under these conditions if Sm-AMP were *inactive* in suppressing the arg^- phenotype of the CSD host, the cells of strain Rc/RE130 would fail to grow unless the Sm concentration in the medium were increased above 200 μg/ml where, judging from Table 1, the adenylating capacity of the RE130 enzyme would be exceeded so that unadenylated Sm would be present in amounts sufficient to permit growth. If, on the other hand, the adenylated forms of Sm or Blu were *active* in causing phenotypic suppression, the Rc/RE130 cells would still grow on low Sm or Blu concentrations despite the fact

that the drug in these cells would be adenylated. From Table 2 one sees that without either Arg or drug, neither strain grows. In the presence of Arg, both strains grow. When various concentrations of Sm or Blu were substituted for Arg, essentially 100 % of the strain Rc cells which were plated grew. Similarly, at various Blu concentrations strain Rc/RE 130 grows well; however, of the Rc/RE 130 cells

Table 2. *Drug-dependent growth of* Escherichia coli *strains RC and RC/RE 130 on sublethal drug concentrations**

	Controls							
	(Arg) → 0	100						
Strain	(Drug) → 0	0						
Rc	0	145						
Rc/RE 130	0	210						
	Experimentals							
	(Arg) 0	0	0	0	0	0	0	0
		(Sm)				(Blu)		
	(Drug) → 2	5	10	20	2	5	10	20
Strain								
Rc	100	137	Not done	158	100	140	184	145
Rc/RE 130	0	5	10	18†	200	160	200	166

* Cultures of both strains were diluted and aliquots were plated on a set of control and experimental plates. The number of colonies per plate was recorded after 5 days of incubation at 37 °C on Min agar plates ± Arg ± Sm or Blu, all of whose concentrations are listed in μg/ml. Colony sizes on plates of a given drug level were not consistently different between strains Rc and Rc/RE 130.

† The percentage survival of strain Rc/RE 130 on Min Sm^{20} agar in this experiment was somewhat higher than in subsequent comparable experiments. It is possible that such relatively high-percentage survivals are obtained when the CSD cells, pregrown in Arg-containing medium, are not washed completely free of Arg. Residual Arg could permit several cell divisions to occur on the Min Sm plates so as to increase the effective number of plated cells and consequently increase the frequency, but not the rate of appearance, of R^- segregant clones in the plated cell population. This effective increase in the plated cell population would not, however, affect the viable count on the control Min + Arg plates because the Arg concentration is not limiting, and therefore segregation of RE 130 has no bearing on the number of clones which grow out on Min + Arg. Similarly the apparent correlation between percentage survival and Sm concentration in this experiment is not surprising if one postulates that the number of initial divisions which a freshly plated cell undergoes is proportional to the Sm concentration.

plated on to Min Sm^2, none grew. Only about 2 % of these cells grew on Min Sm^5 ('2 % survival'), 5 % on Min Sm^{10}, and 9 % on Min Sm^{20}. (In many subsequent experiments of this kind we have found consistently lower percentage survivals on Min Sm^{20}.)

The results from the series of Min Sm plates might be interpreted in two general ways. Either (*a*) Sm merely failed to support Rc/RE 130 cell growth under these conditions or (*b*) Sm actively inhibited the growth of Rc/RE 130. In order to distinguish between these two possibilities, Rc/RE 130 cells were plated in triplicate

on to Min (to monitor the arg$^+$ revertant frequency), Min + Arg (to determine the viable count) and Min Sm^{20} Blu^{20}. The percentage survivals on Min Sm^{20} Blu^{20} varied from 90 % to 100 %, and of 43 clones sampled from this population all proved to have retained the R factor and were still arg$^-$. This confirmed the first explanation, that the presence of Sm does not interfere with phenotypic suppression of Rc/RE 130's arg$^-$ defect by Blu.

The existence of this small fraction of the plated Rc/RE 130 cell population which could grow on Min Sm^{20} was of interest because it suggested that the few Rc/RE 130 clones which appeared were in some way genetically distinct from the large majority of the Rc/RE 130 population. Two possible explanations suggested themselves: (*a*) reversion of the CSD host to arg$^+$ and/or (*b*) loss of Sm-adenylating capacity. Therefore we examined 20 clones of this class for RE 130-mediated drug resistances and for growth on Min agar. None of these clones grew on Min agar and were, thus, still arg$^-$. Nineteen of the 20 had lost the R factor as judged by their sensitivity to all drugs tested. In these experiments R factor-mediated Sm resistance was evaluated after the R factor had been transferred from the clone in question into AB 1932-1 by conjugation. (The twentieth clone was cultured and plated on Min Sm^{20}; the population which survived this selection proved to include both R$^-$ and R$^+$ clones. Replating these R$^+$ clones on Min Sm^{20} gave the same result; this assured us that the original twentieth clone had not grown on Min Sm^{20} the first time by virtue of some mutation which we could not recognize and which might allow the mutant to remain arg$^-$ and R$^+$ but still grow on Min Sm^{20}. Potentially, however, further selective procedures of this kind could prove useful in enriching for such a class of mutants, some of which might make low levels of adenylating enzyme due to regulatory mutations.) In contrast, all 20 clones tested of 166 Rc/RE 130 clones which had grown up on Min Blu^{20} proved to be (1) arg$^-$ and (2) resistant to all the drugs tested. The controls for this experiment, clones of Rc/RE 130 from Min + Arg agar, were all still drug resistant and arg$^-$.

These results suggested that Sm and Blu are markedly different in their ability to cause phenotypic suppression with strain Rc/RE 130 despite the presence of the Sm-Blu-adenylating enzyme. Either of two alternatives might explain why Rc/RE 130 grows on Min Blu^{20}: (1) Blu-AMP can promote phenotypic suppression and/or (2) an amount of unadenylated Blu sufficient to suppress is present intracellularly. This might occur, for example, if enzyme-catalysed adenylation is incomplete or if the Blu-AMP complex is unstable. If either of these possibilities were true, Min Blu^{20} might *not* serve as a selective pressure favouring the outgrowth of R$^-$ segregants or Rc/RE 130 segregants which have lost the R factor's Sm-Blu-Spc locus.

In order to test this working model, several experiments were performed.

(*a*) If growth on Min Sm^{20} indeed selects those cells which have undergone a genetic change (loss of RE 130's Sm-Blu-Spc locus or loss of the entire R factor), such clones ought to grow at 100 % survival on Min Sm^{20} (and on Min Blu^{20}) when replated on these media. Conversely, if Rc/RE 130 is under no selective pressure to segregate the R when growing on Min Blu^{20}, clones from this medium ought to

have retained the RE130 factor and therefore ought *not* to grow when picked and replated on Min Sm^{20}. When ten Rc/RE130 control clones isolated from Min + Arg were plated on Min Sm^{20} none grew, but all ten grew on Min Blu^{20}. Of 15 Rc/RE130 experimental clones isolated originally on Min Sm^{20} and found to have lost the R factor, all 15 grew when plated subsequently on Min Sm^{20} as well as on Min Blu^{20}. Again, as expected, of 20 Rc/RE130 experimental clones isolated from Min Blu^{20}, none grew subsequently on Min Sm^{20}, but all 20 grew on Min Blu^{20}.

(*b*) The fact that R factor loss from Rc/RE130 was the most common event which allowed for growth of this strain on Min Sm^{20} agar suggested that retention of *some* RE130-associated function(s) was incompatible with growth under these conditions, but it did not prove that the pertinent R-factor function was the Sm-Blu-Spc adenylating enzyme locus. According to the model, the Rc host carrying a $Sm^{S}Spc^{S}Blu^{S}$ mutant of RE130 ought to grow normally on Min Sm^{20} agar. Accordingly, RE130-17, a $Sm^{S}Spc^{S}Blu^{S}$ point mutant of RE130 (D. H. Smith, J. A. Janjigian, N. Prescott & P. W. Anderson, in preparation), was crossed into strain Rc, and purified recombinants were plated onto Min, Min + Arg, and Min Sm^{20} agar in triplicate. Using the viable count on Min + Arg as the base line (100% survival = 450 colonies/plate), 0% grew on Min but 90–110% grew on Min Sm^{20}.

(*c*) At this stage u.v. mutagenesis was employed in order to obtain Rc/RE130 mutants which would survive the selection procedure on Min Sm^{20} agar by virtue of deletion of some *but not all* RE130 drug resistance determinants. It was hoped that this would provide a series of overlapping deletions which would cover the RE130 map, thereby permitting one to decide which of the known resistance determinants of this R factor interfere with the growth of strain Rc/RE130 on Min Sm^{20}. Examination of 190 clones which grew out on Min Sm^{20} agar following u.v. exposure revealed that all of the clones were either arg^{+} or contained a partial segregant R factor. The common characteristic of the latter category was that all these segregants were $Sm^{S}Spc^{S}Blu^{S}$ (discussed more fully in an accompanying communication: Harwood, Janjigian & Smith, 1969).

(*d*) Table 1 illustrates the fact that RE130 protects the Sm^{S}CSD host strain against the lethal effect of up to 200 μg/ml of Sm in Min + Arg agar. This may be interpreted to mean that, under such conditions, the Sm-adenylating enzyme in these cells is functioning 'at capacity' when the extracellular Sm concentration is 200 μg/ml. If this is so, raising the extracellular Sm concentration further might flood the enzyme, leaving the excess Sm unadenylated and thus able to cause phenotypic suppression and consequent growth of the CSD/R strain. Therefore, the percentage of Rc/RE130 cells which will grow out when plated on Min Sm^{500} agar, for example, ought to be substantially higher than that found normally on Min Sm^{20}. In two experiments of this kind, we observed that while the percentage survival of Rc/RE130 on Min Sm^{20} was somewhat variable, the percentage survival on Min Sm^{500} was at least 10–12 times higher than that growing on Min Sm^{20} for a given experiment. In addition, 9 out of 10 clones sampled from those that grew on Min Sm^{500} proved to be still arg^{-} and resistant to all drugs tested. Therefore,

plating Rc/RE130 on MinSm^{500} agar does not constitute a selective pressure which favours survival of those cells which have spontaneously lost the R factor or some fragment of it.

(*e*) If the presence of Sm-adenylating enzyme in a cell is incompatible with the cell's *conditional* Sm dependence, unconditionally Sm-dependent cells bearing RE130 also ought to grow poorly even on medium containing Sm. To test this prediction, RE130 was crossed into *E. coli* B (*Sm*^{D}), and the recombinants were checked for retention of (1) drug resistances and (2) Sm dependence. (The Sm concentration used to support the growth of the recipient in this cross (500 μg/ml) was low enough such that the mating conditions select not merely recombinants but recombinants which can grow on this Sm concentration despite the presence of adenylating enzyme.) Of 40 recombinant clones checked, *none* had lost any R-mediated drug resistance, but *all* were Sm^{R}-*independent*. This again confirmed the hypothesis and suggested that the reversion frequency from Sm-dependence to Sm-independence might be quite high with this Sm^{D} strain. Subsequently, the frequency of spontaneous Sm^{R}-independent revertants of B (*Sm*^{D}) was measured at approximately 7×10^{-3}. Since we have measured the spontaneous R^{-} segregant frequency of strain Rc/RE130 at $< 5 \times 10^{-4}$, the Sm$^{D} \rightarrow$ Sm^{R} reversion frequency is high enough to explain why Sm^{R}-independent revertants rather than R^{-} segregants predominate among those B (*Sm*^{D})/RE130 cells which are able to grow on Sm. (It should be emphasized that the mutation *frequency*, not rate, is 7×10^{-3} and probably reflects the fact that Sm^{R}-independent revertants grow considerably faster than the Sm^{D} parent.)

These five experimental tests of the model's predictions were confirmatory, but our conclusion that Sm-AMP does not promote phenotypic suppression *in vivo* was based on a major assumption: that Sm-AMP has access to the interior of the Rc/RE130 cell and presumably, therefore, to the cell's ribosomes. Since the adenylating enzyme appears to be located periplasmically (Yamada *et al.* 1968; Harwood & Smith, 1969), it is possible that the enzyme could confer Sm resistance by adenylating Sm molecules at either an extracellular or periplasmic site and that the Sm-AMP molecule cannot then permeate the cell membrane. To resolve this question, one would like to be able to measure the uptake of labelled Sm by strains Rc and Rc/RE130 at Sm concentrations far below the capacity of the adenylating enzyme such that essentially all of the uptake by strain Rc/RE130 would represent Sm-AMP having entered the cell. A substantially lower rate of uptake by strain Rc/RE130 as compared to strain Rc need not be interpreted as a lower permeability to Sm of strain Rc/RE130 for reasons recently invoked by Shaw & Unowski (1968) and Mise & Suzuki (1968). They argue that a lowered affinity for the ribosome of the enzymically altered drug can lead to an apparent decreased drug uptake as a secondary phenomenon. The relevance of this argument is furthered by the finding that Sm-AMP binds less tightly to the ribosome than Sm (Yamada *et al.* 1968). Therefore, even roughly comparable rates of labelled Sm uptake by both Rc and Rc/RE130 would strongly suggest that Sm-AMP does reach the intracellular space of the Rc/RE130 cell.

Performance of this experiment is complicated by several factors: (1) stable preparations of [^{3}H]dihydrostreptomycin have not been available to us at high enough purity (i.e. less than 0·1% of ^{3}H as ^3H_2O) to be confident that cell-associated counts of the relatively low magnitude obtained in such experiments truly reflect cell-associated Sm; (2) [^{14}C]Sm is presently available only at very low specific activity; and (3) the uptake of Sm by Sm^R strains of *E. coli* is lower than that of Sm^S strains (Anand, Davis & Armitage, 1960; Hurwitz & Rosano, 1962). For these reasons, we elected to compare the uptake of [^{14}C]Sm by CSD strains S and S/RE130 which are isogenic (except for the Sm locus) with strains Rc and Rc/RE130.

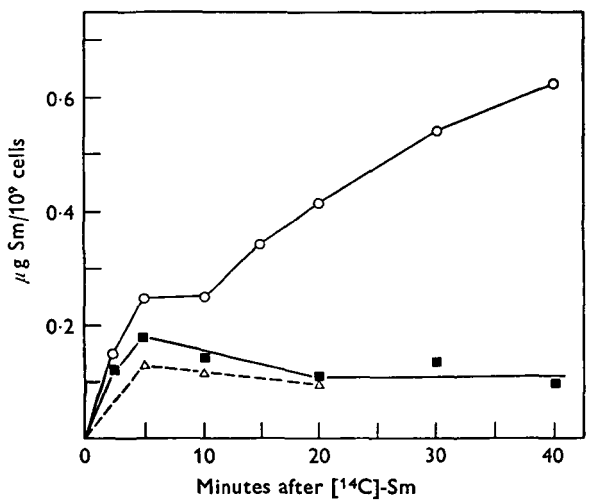

Fig. 1. Uptake of [^{14}C]Sm by *Escherichia coli* strains S (○—○), Rc (△ - - - △) and S/RE130 (■—■) growing in minimal glucose medium containing 100 μg Sm/ml.

The results illustrated in Fig. 1 demonstrate two points: (1) the biphasic kinetics of Sm uptake by strain S are similar to those of previous studies with Sm^S strains of *E. coli* (reviewed by Brock, 1966) and (2) strain Rc takes up less Sm than strain S as have the Sm^R strains used in the earlier studies described above. We also find that Sm uptake by strain S at 0-time is temperature-dependent. When the cells are exposed initially to Sm at 37 °C (not shown), a ten-fold higher 0-time uptake is observed. It appears, therefore, that the basal level of non-metabolic Sm uptake (e.g. adsorption to the cell envelope) consists of the level measured at 0-time in Fig. 1 (0·01–0·02 μg Sm/10^9 cells). The level of the primary plateau (0·25 μg Sm/10^9 cells, lasting generally until 10–15 min after [^{14}C]Sm addition in our experiments) must represent Sm which has actually penetrated the cell membrane since viable counts indicate that Sm has killed 90% of the strain S cells by 10 min. In addition, the data indicate that Sm is entering S/RE130 cells in amounts equal in magnitude to (or greater than) that which enters the Rc cells. The question remains: does Sm enter the S/RE130 cell as Sm or as Sm-AMP? Fig. 1 indicates that this entry is

mostly as Sm-AMP for the following reasons: (*a*) Since strain S is killed by 1 μg/ml Sm under these conditions, the primary plateau of strain S represents the amount of drug taken up when Sm is present at 100 times the lethal concentration (the secondary uptake of Sm, between 0·25 and 0·6 μg Sm/10^9 cells in Fig. 1 is probably due to membrane damage (Dubin, Hancock & Davies, 1963) and thus is not as useful an indication of the degree of uptake by intact strain S cells as is the primary plateau); (*b*) since killing occurs more rapidly the higher the extracellular Sm concentration, it is not unreasonable to suppose that considerably more Sm is entering strain S under these experimental conditions than the minimum number of drug molecules required to kill the cell; therefore, (*c*) because the ribosomes of strain S/RE 130 are Sm^S (Harwood & Smith, 1969) and because Fig. 1 shows that S/RE 130 takes up fully 50 % as much Sm as strain S yet is not killed by the drug (S/RE 130 viable counts continued to increase for at least 40 min. following addition of [^{14}C]Sm), Sm must get into these cells in substantial amounts but in a form which lacks bactericidal activity, i.e. Sm-AMP. By inference, Sm-AMP also enters Rc/RE 130 cells. Several experiments indicate that while the level of [^{14}C]Sm uptake by strain Rc/RE 130 is not large and thus difficult to measure precisely, it is quite comparable in magnitude to that of strain Rc.

The question as to whether or not the presence of unadenylated Blu in Rc/RE 130 cells is an adequate explanation for their growth on Min Blu has not been answered, but let us assume for the moment that unadenylated Blu is present. Examination of Table 1 reveals that the adenylating enzyme is able to deal with an extracellular Blu concentration of at least 200 μg/ml in conferring protection on strain S/RE 130 against Blu's lethal effect. Since strain S, when lacking RE 130, does not grow at extracellular Blu concentrations exceeding about 10 μg/ml, these data suggest that in S/RE 130, adenylation (viewed operationally) has reduced the *effective* extracellular Blu concentration by at least 95 %. If, then, a maximum of 5 % of the effective extracellular Blu concentration remains unadenylated by strain Rc/RE 130 and, as such, is solely responsible for the growth of strain Rc/RE 130 on Min Blu, one might expect that either the growth *rate* or the growth *yield* of strain Rc/RE 130 on Min Blu would be no more than 5 % of that of strain Rc on any given Blu concentration. (We are assuming that the efficiency of adenylation in strain Rc/RE 130 is the same as in strain S/RE 130. By 'growth yield' we mean the maximum number of cells comprising a given population after it has reached stationary phase.)

Inconsistent results were obtained when this experiment was performed in liquid medium, due in part to the outgrowth of arg^+ revertants. Growth on solid medium proved to be a better method for performing this experiment: colony area is an approximate but convenient measure of the number of cells per colony, and recognition of arg^+ revertants is facilitated. Since a visible colony (0·1 mm diameter) contains at least 10^7 cells and because the revertant frequency of strain Rc to arg prototrophy is about 10^{-7}, it was thought that in the course of the seven or more cell doublings during which the colony size increases from 0·1 to 1·0 mm or more, the arg^+ revertants would either make up a negligible (in this case, invisible)

fraction of the colony or, if their growth rate were high enough, be recognizable as papillae issuing from the edges of the arg⁻ CSD colonies.

Subsequently, we followed colony areas of strains Rc and Rc/RE130 on Min agar containing Blu at 2, 5, 10, 20, 40 and 100 μg/ml and observed that: (1) in accord with Gorini & Kataja (1964), who used Sm, above 5 μg/ml the growth *rate* of the Rc strain did *not* increase with increasing drug concentrations; rather (2) the growth *yield* of strain Rc proved to vary directly with the Blu concentrations as reported (for phenotypic suppression by Sm) by Molina, Calegari & Conte (1968). For example, the relative growth *yield* of strain Rc growing on 100 μg/ml of Blu was 9·0 (mean colony area in mm^2) and on 5 μg/ml, 0·2 (or 2·2% of the yield on 100 μg/ml). Thus, this measurement provided a reasonable index of the *effective* extracellular Blu concentration. Knowing this, we would have predicted (according to the theory that 5% of the Blu was remaining unadenylated in strain Rc/RE130) that at any given Blu concentration the growth yield of strain Rc/RE130 should be at most 5% of that of strain Rc. On the contrary, the *observed* percentages at all concentrations of Blu in the medium varied from 16 to 100% with an average of 55%. These growth yields of Rc/RE130 are, on the average, ten-fold too high to be explained merely by the hypothesis that a small fraction of the intracellular Blu remains unadenylated in strain Rc/RE130. Consequently, this model cannot provide an adequate explanation for the extensive growth of strain Rc/RE130 on MinBlu agar, and the possibility that Blu-AMP may cause phenotypic suppression *in vivo* remains open.

4. DISCUSSION

The genetic evidence presented suggests that Sm-AMP does not promote phenotypic suppression in an *in vivo* system. This, along with the *in vitro* data presented earlier (Yamada *et al.* 1968; Harwood & Smith, 1969), argues strongly that the 3′-OH group of the *N*-methyl-L-glucosamine moiety of Sm must be a functional group which is important for the biological activity (both for misreading and inhibition of protein synthesis) of the Sm molecule. While this work was in progress, two groups of investigators reported genetic data similar to our own, but in both cases the mechanism of Sm resistance conferred by the R factors used in their experiments had not been definitely established. Molina *et al.* (1968) employed a CSD mutant of *E. coli* B, and Hofemeister & Böhme (1968), using an R-Sm^R-infected Sm-dependent mutant of *Proteus mirabilis*, attributed the lethal interference found between Sm dependence and R-determined Sm resistance to intracellular inactivation of Sm.

The situation with Blu-AMP is somewhat less clear. First, the observation that growth of Rc/RE130 on MinBlu^{20} agar does not select for complete or partial RE130 segregation is consistent with either of two explanations: (1) Blu-AMP can cause phenotypic suppression or (2) sufficient unadenylated Blu exists intracellularly to cause phenotypic suppression and permit growth. Secondly, Blu adenylated by extracts of *E. coli* B/RE130 appears to be able to cause misreading of poly U

in vitro. This observation is not readily explained by extensive degradation of Blu-AMP to Blu or by incomplete adenylation of Blu since polyphenylalanine synthesis was not inhibited under these conditions. An alternative explanation for this finding which cannot at the moment be ruled out may be that Blu adenylation *in vitro* could be more than 99% complete, but minute amounts of unadenylated Blu would be responsible for the misreading observed. According to the concentration-activity curve for Sm-induced misreading of a poly U mRNA (Davies & Davis, 1968), very low concentrations of Sm can promote detectable levels of misreading without measureable inhibition of polypeptide synthesis. Analogous to this is the fact that *in vivo* 2 μg Sm/ml will support the growth of strain Rc but is not bactericidal for the isogenic strain S. Invoking this explanation would require the assumption that Blu misreads quantitatively like Sm in a poly U system. The third point which bears on this question is the existence of similar growth yields of strains Rc and Rc/RE130 on MinBlu agar. This finding suggests, but does not prove, that Blu-AMP can cause phenotypic suppression *in vivo*.

Regardless of which interpretation turns out to be correct, however, either of the two possible explanations is of interest. If Blu-AMP does allow phenotypic suppression *in vivo*, this fact would be consistent with the theory that misreading and the inhibition of protein synthesis are the results of two distinct *in vitro* actions of (certain aminoglycosides like) Blu because it would demonstrate that at least one functional group of this molecule which is indispensable for inhibition would not be necessary for misreading (phenotypic suppression). Secondly, it would allow one for the first time to approach the structural basis of the very slight differences in biological activity of Sm and Blu. Previously one of the few tests for distinguishing Sm and Blu on the basis of their biological activities has been the fact that not all Sm dependent strains are also Blu dependent (L. Gorini, personal communication).

Alternatively, if the existence of unadenylated intracellular Blu were to account for the growth of strain Rc/RE130 on MinBlu[20] agar, this observation would suggest another biological distinction between Blu and Sm. For example, the steady-state concentration of Sm-adenylate or Blu-adenylate in a cell can be expressed as a function of (1) the rate of formation of drug-adenylate and (2) the rate of breakdown of the drug-adenylate either spontaneously or via catalysis by a degradative enzyme such as phosphodiesterase (Yamada *et al.* 1968; Takasawa *et al.* 1968). A low level of unadenylated Blu (but not unadenylated Sm) in strain Rc/RE130 could be attributed, therefore, to differences in affinity of either (*a*) the adenylating enzyme for Sm *vs.* Blu or (*b*) some degradative enzyme for the adenylated forms of these two aminoglycosides. In both cases, differences in enzyme-substrate affinities would provide the basis for a biochemical differentiation of these two closely related drugs.

The *in vivo* assay for phenotypic suppression used in these experiments should be applicable to structure-activity studies with other aminoglycosides whose inactivation is R factor-mediated. For example, R factor-mediated Km and neomycin resistances have been reported to be enzymic in mechanism (H. Umezawa *et al.* 1967), and both of these drugs will support the growth of some CSD strains

(Gorini & Kataja, 1965). Therefore, it ought to be possible to determine whether the various products of R-mediated enzymic alteration of these aminoglycosides will promote phenotypic suppression in an *in vivo* system such as the one described. In addition, it will be of interest to test in this system Sm phosphate which is produced by another R factor-coded enzyme (J. Davies, personal communication). Such studies are currently in progress in this laboratory (Smith, Prescott & Janjigian, in preparation).

SUMMARY

An arg⁻ conditionally Sm-dependent *E. coli* B strain became unable to grow on minimal agar containing low levels of Sm when the strain acquired an R factor which mediates Sm and Blu adenylation. The same R-containing strain, however, grew normally when Blu was substituted for Sm in the agar. The data strongly suggest that Sm-adenylate does not suppress the arg⁻ phenotype in an *in vivo* system. The possibility that Blu-adenylate can cause phenotypic suppression without being bactericidal *in vivo* is discussed.

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