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Dr. Annette Baich

The effect of streptomycin on an early reaction of proline synthesis, the production of glutamic-γ-semialdehyde, in resting cell suspensions of antibiotic-sensitive, resistant, and dependent strains of Escherichia coli was examined, and the effect on glutamic-γ-semialdehyde production was found to correspond to the growth response of the organisms to streptomycin. Inhibition of glutamic-γ-semialdehyde production in sensitive strains was found not to be caused by streptomycin-induced changes in permeability to substrate or product of the reaction. The effects of pH, ionic strength, and the presence of purines, pyrimidines, and nucleotides on streptomycin action were examined, and possibilities for the mode and site of streptomycin action are discussed.

The Effect of Streptomycin on Proline Synthesis in *Escherichia coli*

by

David Bruce Couch

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Assistant Professor of Biochemistry and Biophysics

in charge of major

Redacted for Privacy

Acting Chairman, Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

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Typed by Gwendolyn Hansen for David Bruce Couch

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THE EFFECT OF STREPTOMYCIN ON PROLINE SYNTHESIS IN ESCHERICHIA COLI

INTRODUCTION

Metabolic Control

In order to make the most economical use of its environment, the cell controls the production of its products. Two methods available to the cell for the control of metabolic processes are (1) changing the rate at which an enzyme catalyzes a reaction and (2) changing the number of enzymes.

Feedback Inhibition

Umbarger (97) proposed the concept of regulation through interaction of an enzyme and the final product of the sequence in which the enzyme participates and termed the phenomenon end product, or feedback, inhibition. Suggestions concerning the nature of feedback inhibition from many investigators (9, 19, 41, 43, 59, 67, 97, 107) have served as the basis for many attempts to explain the kinetics of regulated enzymes, including those of Frieden (39), Monod, Wyman, and Changeux (68), Taketa and Pogell (93), and Koshland, Nemethy, and Filmer (60).

Nearly all models proposed to rationalize regulatory enzyme behavior involve a conformational change in the protein. The Monod,

Wyman, and Changeux model (68) assumes equilibrium between two conformations, one of which preferentially binds substrate and activator, and one which binds inhibitor, with ligand binding having no effect on conformation. Most other treatments have assumed the conformational change to be caused by binding effectors. The models differ also in the number of allowed conformations. The model of Monod, Wyman, and Changeux (68) deals primarily with two conformations, although the possibility of other forms is recognized; Koshland, Nemethy, and Filmer (60) propose for hemoglobin two conformations each for two subunits, with the number of possibilities for the whole molecule depending on the pattern of interaction of the subunits. All these studies did not lead to the adaptation of one model, since it was found that more than one model could account for the observed behavior, with appropriate adjustment of the parameters.

Repression

A regulatory system which is different from feedback inhibition has also been demonstrated; for many pathways in bacteria, enzymes of those pathways are formed only when they are needed, so that if the end product of such a pathway is supplied exogenously, the enzymes catalyzing its biosynthesis will be virtually absent (100). The synthesis of the enzymes is then said to be repressed.

Similarly, a number of enzymes required for the utilization of energy sources appear only when the appropriate energy source is present; such enzymes are termed inducible. A model for the control of protein synthesis has been presented by Jacob and Monod (54) and Monod, Changeux, and Jacob (66). McFadden and Howes (64) and Atkinson (7) suggest that feedback inhibition and repression are interrelated to provide the greatest degree of control, and in the model of Jacob and Monod the repressor, a protein (43, 77), is another example of an allosteric protein stimulated or inhibited by a small molecule, as in feedback inhibition.

Streptomycin (Sm) and Control

Sm has been shown to affect repression and feedback inhibition in organisms which require the antibiotic for growth; the effect on these regulatory mechanisms appears to be a consequence of other effects of the drug, especially on protein synthesis.

Several investigators (16, 21, 22, 28, 30, 73, 74, 75) found that the antibiotic appears to induce or derepress certain enzymes in Sm-dependent organisms; Polglase and Desai (29) examined the possibility that feedback inhibition is altered by mutation to Sm resistance and dependence and concluded that such mutation may cause a modification of allosteric interaction of the enzyme as a result of a change in protein structure, but the effect of Sm on

pre-formed enzyme is not clear.

Gibson and co-workers (42) have reported a system in which Sm has an effect on pre-formed enzymes. Indole synthesis in Sm-sensitive organisms was found to be inhibited by the antibiotic, while Sm-dependent strains showed stimulation of indole synthesis, provided that they were grown in suboptimal concentrations of the drug. Sm-resistant strains showed no inhibition of indole synthesis in the presence of the drug.

Streptomycin

Streptomycin (Sm), $C_{21}H_{39}N_7O_{12}$, a water soluble compound produced by Streptomyces griseus was discovered by Waksman, Bugie, and Schatz in 1944 (103). It is an optically active base with one free aldehyde group; the molecule has been shown (63) to be composed of the glycosidically linked saccharides streptidine, a diguanidinyl derivative of 1, 3-diaminohexose, N-methyl-L-glucosamine, and streptose, a 2-deoxyfuranoside. The glycosidic linkage between N-methyl-L-glucosamine and streptose was shown to be α and that between streptose and streptidine β (106). The structure of the antibiotic is given in Figure 1. Sm shows broad spectrum antibacterial activity and was of particular interest because of its effectiveness against Mycobacterium tuberculosis.

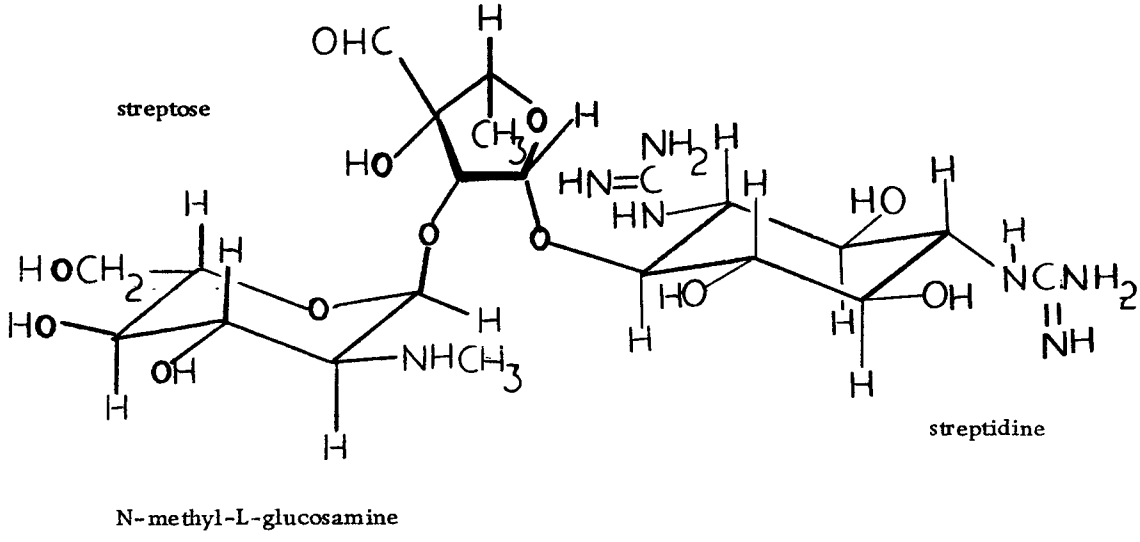

Figure 1. Structure of streptomycin.

Sm will inhibit the growth of E. coli when present in concentrations of 4×10^{-6} M (42).

The Effects of Sm

Although the precise nature of the mechanism of the antibacterial action of Sm is not known, the effects of the drug on certain aspects of cell metabolism have been described.

The Effect on Protein Synthesis. An early effect of Sm on growing, antibiotic-sensitive cells is the inhibition of protein synthesis (34, 105); that the ribosomes might be the site of action of the drug was suggested by Spotts and Stanier (86) and confirmed by other groups (36, 56, 85).

It was shown that Sm introduces ambiguity in cell-free amino

acid incorporating systems at the initial condon-anti-codon recognition step, but does not affect peptide bond formation (36, 70, 71, 85). Ambiguity was believed to be introduced by alterations of ribosome structure (25, 44), especially that of the 30S subunit (23, 24, 35, 36, 57, 85) and more specifically that of the 23S core particle of that subunit (87, 94), although there may be some involvement of the 50S subunit in the action of Sm and in determining the specificity of interaction of sRNA and mRNA (55). In cell-free systems using a specific polynucleotide and a specific amino acyl-sRNA the effect of ambiguity, the insertion of the incorrect amino acid, would appear to inhibit protein synthesis.

Sm-induced misreading in vivo was discovered when a class of mutants of E. coli were found which required Sm for growth, and in which a genetic defect could be overcome (suppressed) by Sm, showing the effect of the antibiotic on the translational process of protein synthesis (5, 25, 45, 47). Work on mutants of this type (26, 27, 46, 83, 99) led to the proposal that Sm suppresses genetic defects by producing ambiguity in base pairing, so that an incorrect message can lead to the production of a functional protein.

Gorini (44) suggested there is ambiguity with some, or all, codons, but under usual conditions the ribosomes, and competition of all charged sRNA's permit essentially one unambiguous reading of each. The alteration of the ribosome by Sm allows significant

ambiguity, even in the presence of all amino acids and charged sRNA's.

Other Effects. Other effects observed on antibiotic-sensitive cells in the presence of Sm include damage to the cell membrane (13, 33, 72, 82), change in permeability of the cell (3, 4, 33), impairment of respiration (13, 14, 16, 17, 98), and stimulation of RNA synthesis (20, 32, 37, 88, 89).

These effects may be additional aspects of the action of the drug, or they may be secondary to the effect on protein synthesis, since the production of faulty proteins due to the insertion of an incorrect amino acid might lead to alteration of the cellular component involved, for example, the membrane, and impair its function.

Sm Sensitivity, Resistance, and Dependence

Genetic studies have been made on Sm-sensitive, resistant, and dependent strains of *E. coli* (52); it was found that mutations to Sm dependence involved a single gene locus, and mutation to Sm resistance involved a closely linked site within the locus. Suppression mutations were observed to be responsible for reversions from dependence or resistance to sensitivity, and that locus appeared to be closely linked to the one governing resistance and dependence. Other workers (18, 45) suggest that Sm dependence can involve more

than one gene: one involved with sensitivity and resistance to Sm, believed to be one of the loci controlling 30S ribosome subunit structure, and a suppressor gene. It is suggested that mutations changing ribosome structure interfere with the action of genetic suppression, and the effect of the drug on the mutant ribosomes reestablishes suppression (40, 48).

Proline Synthesis and Control

In E. coli, the synthesis of proline from glutamic acid proceeds as indicated in Figure 2 (91, 102). Evidence for proline regulating its biosynthesis by feedback inhibition was obtained by Strecker (90), who demonstrated that proline inhibits the conversion of glutamic acid to pyrroline-5-carboxylic acid. Baich and Pierson (10) also found end product control of proline biosynthesis was exerted over the first step in the sequence, the formation of glutamic-γ-semi-aldenhyde from glutamic acid.

(1) L-glutamic acid → L-glutamic-γ-semialdehyde
(2) L-glutamic-γ-semialdehyde $\rightleftarrows$ Δ^1-pyrroline-5-carboxylic acid
(3) Δ^1-pyrroline-5-carboxylic acid → L-proline

Figure 2. Proline biosynthetic pathway.

Repression has also been shown to be involved in the control of proline synthesis (10, 96); in the presence of excess proline, the

synthesis of the proline pathway enzymes is reduced.

The object of these studies will be to determine the effect of Sm on proline biosynthesis in E. coli.

METHODS AND MATERIALS

Bacteria

The strains of E. coli used in these studies were W, wild type; W-2, wild type lacking the ability to synthesize glutamic-γ-semialdehyde (GSA); WP1-30, unable to control the proline pathway and lacking Δ^1-pyrroline-5-carboxylate reductase (PC reductase); 55-1, lacking PC reductase; 55-1SR, a mutant of 55-1 resistant to the bactericidal effects of Sm; and 55-1SD, a mutant of 55-1 which requires Sm for growth. All four strains which lack PC reductase excrete GSA into the medium, but only the control deficient WP1-30 does so in the presence of proline (Table 1).

Table 1. Strains of Bacteria Used.

Name	Description	Nutritional requirement
W	wild type	minimal medium (MM)
W-2	wild type lacking ability to synthesize GSA	MM + pro or GSA
WP1-30	W, deficient in control of proline synthesis, lacking PC reductase	MM + pro
55-1	W, lacking PC reductase	MM + pro
55-1SR	55-1, resistant to the bacterial action of Sm	MM + pro
55-1SD	55-1, dependent upon Sm for growth	MM + pro and Sm

Strains W, 55-1 and W-2 were obtained from Dr. D. F. Bacon of the Institute of Microbioloty, Rutgers, the State University. Strain 55-1SR was isolated from a culture of 55-1 which showed the ability to grow in Sm containing medium after exposure to ultraviolet irradiation, and 55-1SD was selected from a culture which showed the ability to grow in Sm containing medium by plating on Difco nutrient agar with and without added Sm. Strain WP1-30 was isolated in this laboratory (10).

Culture Conditions

Cultures of W, W-2, WP1-30, and 55-1 were maintained on Difco nutrient agar in the cold and transferred every three to four weeks to fresh medium. Cultures of 55-1SR and 55-1SD were maintained in the same way but with the addition of 1.3×10^{-3} M Sm to the agar.

The growth medium consisted of medium E(101) diluted 1 to 50 and autoclaved before use. Glucose was added at a concentration of 2.8×10^{-2} M, and the medium was supplemented with L-proline, 8.7×10^{-4} M, and Sm, 1.3×10^{-3} M, as required. Cells were grown without shaking at 37°C in a constant temperature water bath; growth was followed by measuring turbidity with a Klett-Summerson Photoelectric Colorimeter with a 660μ filter.

Fifty ml of medium in a 250 ml Erlenmeyer flask were inoculated with cells from a nutrient agar plate and allowed to grow

overnight; aliquots of these cultures were used to inoculate 250 ml of medium in a 500 ml flask. Growth was allowed to continue until early log phase was reached, corresponding to a turbidity of 50 Klett units, at which time the cells were harvested by centrifugation at 12,500 rpm for ten minutes in a Servall refrigerated centrifuge. The bacteria were washed twice with 0.1 M phosphate buffer, pH 7.0, and resuspended in 10 ml of the buffer. This suspension was kept refrigerated and used within 24 hours.

Glutamic-γ-semialdehyde Production

To follow GSA production and excretion by resting cells, a washed cell suspension was added to a solution 2.8×10^{-2} M in glucose and 0.02 M in phosphate buffer, pH 7.0, maintained at 37°C in a water bath. After incubation for 15 to 20 minutes, 9 ml aliquots of this suspension were added to test tubes containing L-glutamic acid, pH 7.0, with or without those materials whose effect on GSA production was to be studied. At times a series of glutamic acid concentrations was used, while at others only one concentration was used, 6.8×10^{-3} M. Two ml samples were withdrawn at regular intervals and placed in plastic centrifuge tubes containing 1.0 ml 3.6 M sodium acetate and 0.5 ml 4 mgm/ml o-aminobenzaldehyde to assay for GSA according to the method of Albrecht and Vogel (2). Two-tenths ml of 10% (v/v, aq.) trichloroacetic acid was also added

to prevent further GSA production. The centrifuge tubes were kept cold in an ice bath until all samples were collected.

The samples were centrifuged at 12,500 rpm for 10 minutes, and the absorbance of the supernatant at 440 mμ was determined in a Beckman DB spectraphotometer using cells with a light path of 1 cm.

Absorbance was related to concentration of GSA by comparing microbiological and colorimetric assays for measured amounts of s stock solution of GSA and a microbiological assay for proline (Figures 3, 4, 5). It was found that 1 μmole of GSA corresponds to 1.9 absorbance units.

The concentration of protein in each tube was determined by the method of Lowry, et al. (62); samples taken for protein assay at the onset and conclusion of these experiments confirmed that growth does not occur during the course of the experiment.

Growth Curves

Cells were grown overnight in minimal medium and harvested by centrifugation at 12,500 rpm for 10 minutes, washed twice with, and then resuspended in, 10 ml of phosphate buffer, pH 7.0. Aliquots of this suspension were used to inoculate 10 ml of medium in 250 ml Erlenmeyer flasks with Klett side arms; turbidity was

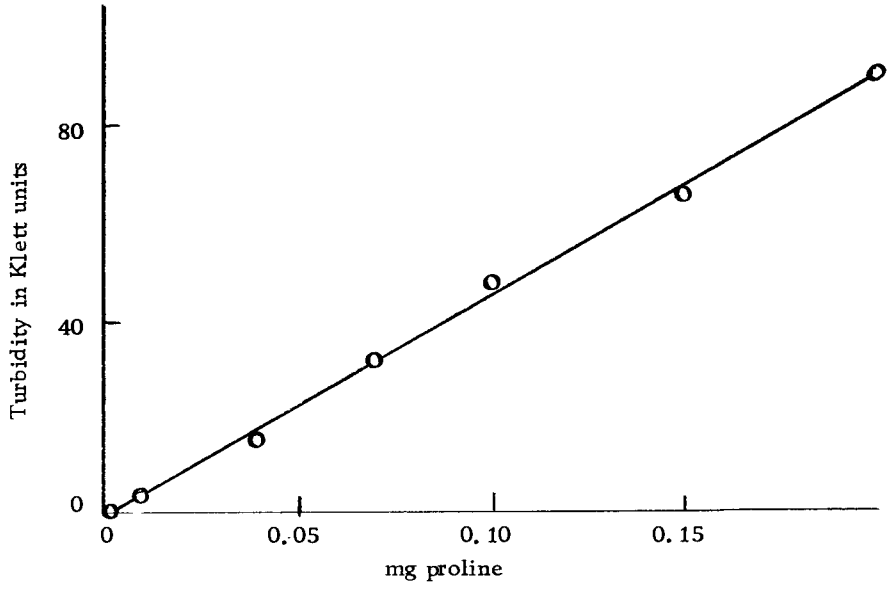

Figure 3. Growth of E. coli W-2 in increasing amounts of proline.

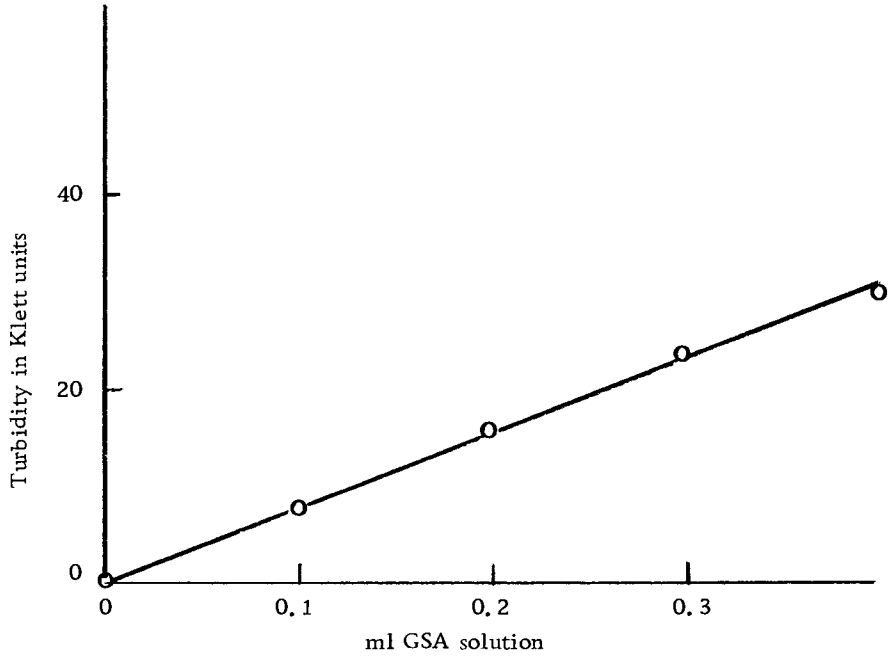

Figure 4. Growth of E. coli W-2 in increasing amounts of GSA.

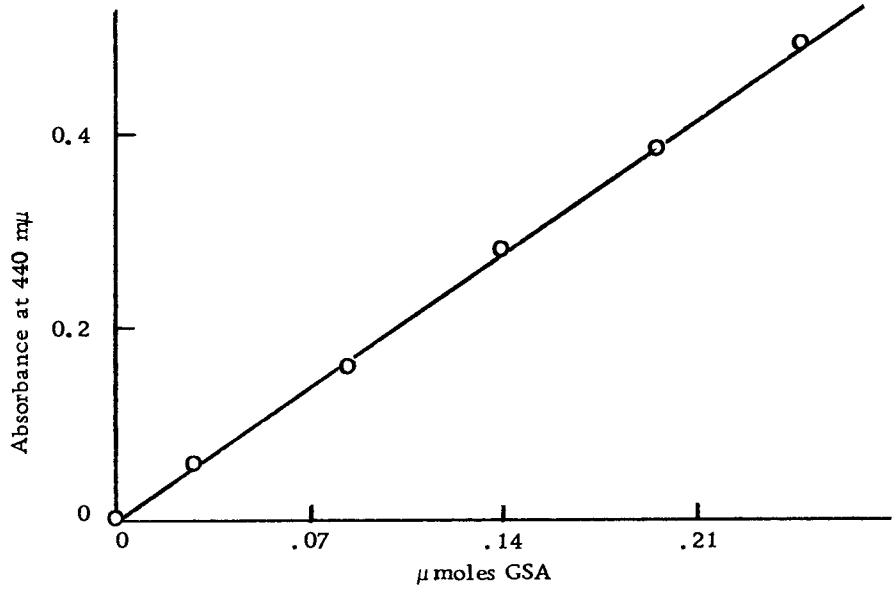

Figure 5. Amount GSA vs A_{440}.

estimated at measured intervals with a Klett-Summerson Photoelectric Colorimeter using a 660 mμ filter.

Permeability Studies

Permeability of cells to L-glutamic acid was studied in the presence and absence of Sm. Two mls of washed cell suspension were added to 50 ml of a solution 2.8×10^{-2} M in glucose and 0.02 M in phosphate buffer, pH 7.0, in an Erlenmeyer flask, and the flask was placed in a 37°C water bath. Aliquots of this suspension were added to test tubes containing uniformly labeled-C^{14}-L-glutamic acid, with or without Sm. Samples of 0.2 ml were taken at measured intervals and subjected to suction filtration through Millipore filters with pore diameter of 0.45 μ. The filters were washed with 0.1 M

phosphate buffer, pH 7.0, and allowed to dry. The dried filters were placed in planchettes and the radioactivity measured with a Tracerlab, Inc. planchette counter.

Materials

Sm sulfate was obtained from Sigma Chemical Corporation. Commercial Sm was used routinely after growth experiments with 55-1SD and Sm twice recrystallized from ethanol-water showed growth was dependent on Sm and not an impurity of the commercial preparation.

Streptidine was obtained by hydrolysis of Sm in 1 N H_2SO_4 at 37°C for 48 hours; the precipitated material collected after this treatment was recrystallized from water (38). The supernatant was chromatogramed on Whatman No. 1 paper using butanol-acetic acid-water (4:1:1 by volume) as the solvent. The three bands which were found using $AgNO_3$ indicator (95) were eluted from the paper with water and the solutions lyophillized. A solution of all three fractions will be referred to as 'streptobiosamine' ('SBA'), although the exact composition is unknown. Sm itself does not move from the spot of application under these conditions, so any unhydrolyzed Sm is separated, and thin layer chromatography on Silica Gel H layers using the same solvent and indicator showed no detectable streptidine in the mixture.

Uniformly labeled-C^{14}-L-glutamic acid was obtained from New England Nuclear Corporation.

Other materials whose effect on GSA production was studied were obtained from the following suppliers: chloramphenicol, Parke-Davis; tetracycline, The Upjohn Company; yeast nucleic acid, Cal Biochem; and uracil, guanine, adenine, and cytosine, Sigma Chemical Corporation.

Synthesis of Δ^3-pyrroline-2-carboxylic Acid

The synthesis of Δ^3-pyrroline-2-carboxylic acid (3, 4-dehydroproline -- 3, 4-DHP) was also undertaken, and, although not related to the thesis subject, will be briefly described.

A two-liter, three-neck flask was fitted with a mechanical stirrer and a soda lime drying tube and cooled in an acetone-dry ice bath. Ammonia gas was introduced through the remaining neck until 150 ml of liquid ammonia were collected.

Two grams (1.8×10^{-2} moles) pyrrole-2-carboxylic acid in 100 ml absolute alcohol were added; 2.3 gm (0.1 gm-atoms) sodium metal were then added in small pieces over 15 minutes with stirring. Stirring was continued until the color disappeared, 3.0 gm (5.5×10^{-2} moles) NH_4Cl was cautiously added, and stirring continued for an additional hour. The ammonia was then allowed to boil off.

A white precipitate which remained in the flask was collected

but was shown not to be 3,4-DHP. Thin layer chromatography of the supernatant on Silica Gel H layers using butanol-acetic acid-water (4:1:1 by volume) as solvent showed the presence of a material with the same Rf as for known 3,4-DHP, 0.19, as well as other components. The supernatant also showed the same biological activity as 3,4-DHP, that is, the ability to inhibit the growth of E. coli W, but not WP1, which was selected for resistance to the compound.

After acidifying a portion of the supernatant to pH 2-3, the addition of ethanol produced a precipitate; the remaining solution when evaporated to dryness produced a material after washing with hot ethanol which resembled 3,4-DHP in its biological properties and Rf value in various solvent systems and which had a melting point of 234-237°C compared to 236-237°C reported for 3,4-DHP (80). No attempt was made to collect this material, so no yield was calculated, but it is apparently not the major product.

This material decolorized bromine water, gave a pale yellow ninhydrin color, a faint purple isatin color, and did not react with o-aminobenzaldehyde.

3,4-DHP was previously prepared by Robertson and Witkop (80).

Pyrrole-2-carboxylic acid was obtained from the Aldrich Chemical Company, Inc. and was recrystallized twice from toluene before use.

RESULTS AND DISCUSSION

Growth

The Effect of Sm

The effect of increasing concentrations of Sm on the growth of E. coli 55-1 is shown in Figure 6; growth is completely inhibited by concentrations of Sm 1.3×10^{-5} M and greater. The growth of 55-1SR is indifferent to the antibiotic, as shown in Figure 7, and 55-1SD requires Sm for growth (Figure 8). For the last strain there is an increase in the amount of growth permitted with increasing concentrations of the antibiotic, and within a concentration range which permits growth to reach approximately the same final level ($0.7\text{-}5 \times 10^{-3}$ M), the increase in Sm concentration is reflected mainly by a decreased lag phase and a slightly increased growth rate, as shown in Figure 9.

The Effect of Hydrolysis Products of Sm

Hydrolysis products of Sm were tested for their effect on growth (Table 2). Streptidine (Sd) was observed to slow the growth rate of 55-1, and the degree of inhibition was the same whether the Sd was added at the onset of log phase growth or at the time of inoculation. Although Sd does reduce the growth rate, it is not bactericidal, even at relatively high concentrations (1.2×10^{-3} M).

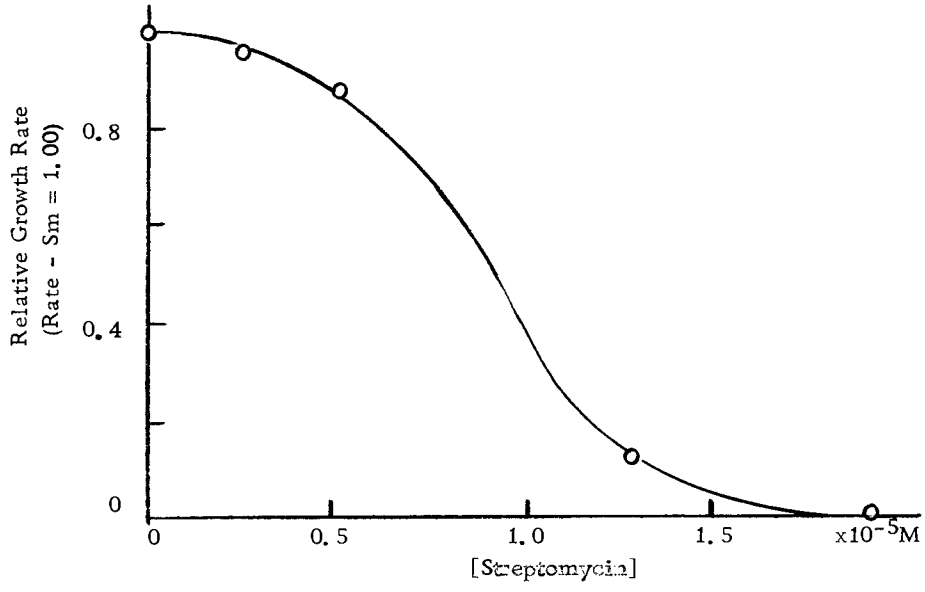

Figure 6. Inhibition of the growth of E. coli 55-1 by streptomycin.

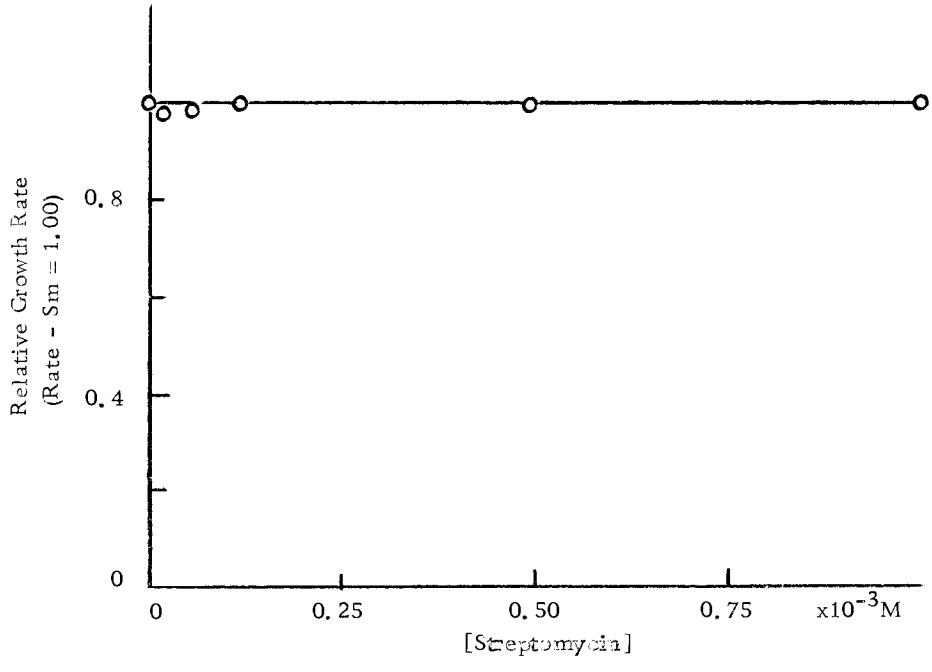

Figure 7. Effect of streptomycin on the growth rate of E. coli 55-1SR.

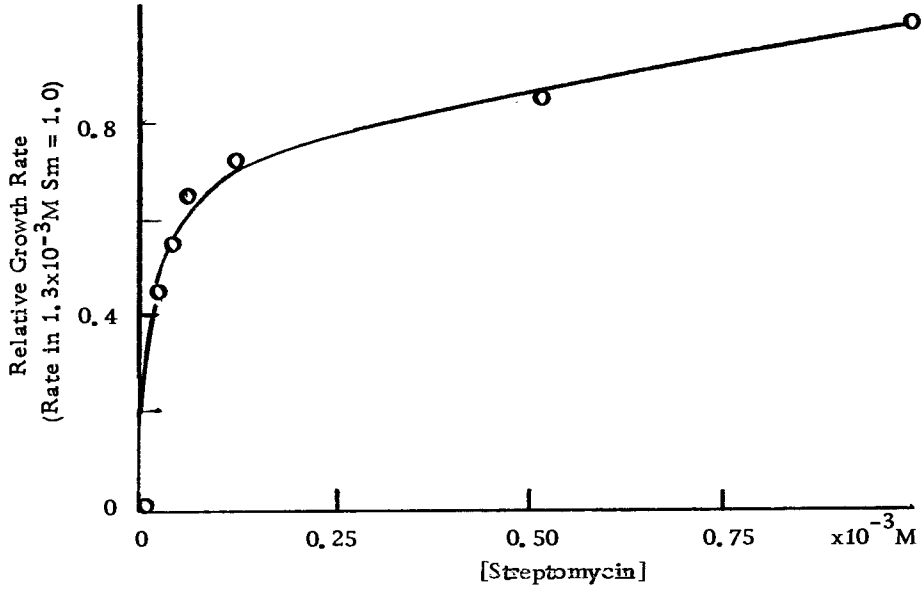

Figure 8. Effect of streptomycin on growth of E. coli 55-1SD.

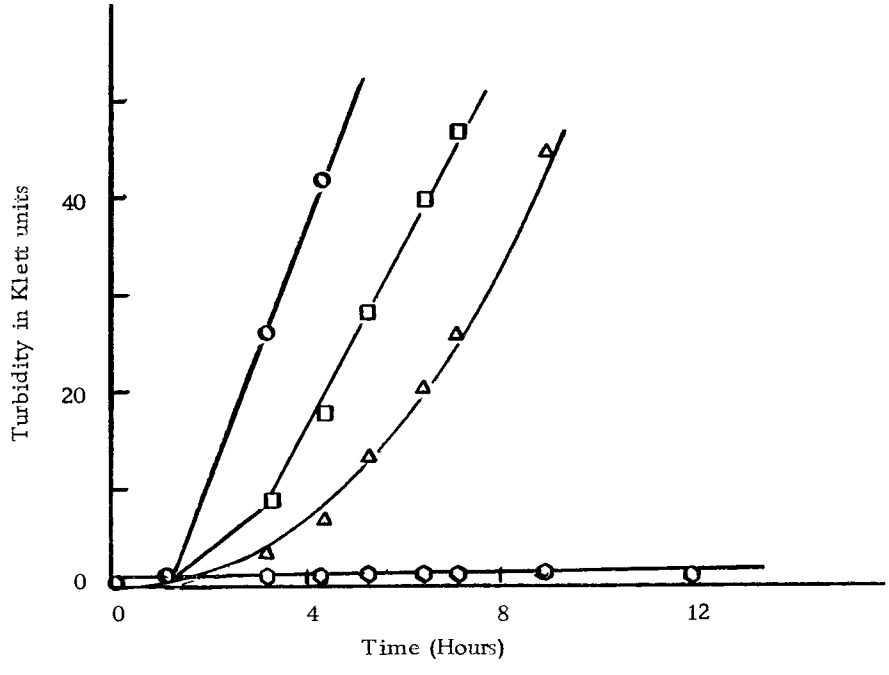

Figure 9. Growth of E. coli 55-1SD in increasing [Sm].

○—○ 5.2x10⁻⁴M Sm □—□ 1.3x10⁻⁴M Sm △—△ 0.65x10⁻⁴M Sm ○—○ no Sm

'Streptobiosamine' ('SBA'), the combination of the other hydrolysis products, had no effect on growth, and 'SBA' and Sd together showed the same decrease in growth rate as for Sd alone. Neither Sd nor 'SBA', alone or in combination, could fulfill the Sm requirement of 55-1SD, and recrystallized Sm allowed the same final level of growth to be achieved as did commercial Sm, although the growth rate was slightly lowered by successive recrystallizations from ethanol-water. The lowered rate may be a consequence of thermal decomposition of the antibiotic (79).

Table 2. Relative Growth Rates in Sm and Sm Products.

Strain	No additions	1.3×10^{-3} M streptidine	'SBA'	streptidine + 'SBA'	5.2×10^{-4} M Sm
W	1.0	0.63	1.0	0.68	0
55-1SD	0.06	0.04	0.05	0.08	0.52

The Effect of Adenine

Adenine, which was observed to affect GSA production, was also tested for an effect on growth on 55-1, but no change in the growth rate was observed in the presence of adenine, and adenine was not antagonistic to the bactericidal action of low concentrations of the antibiotic (Figure 10).

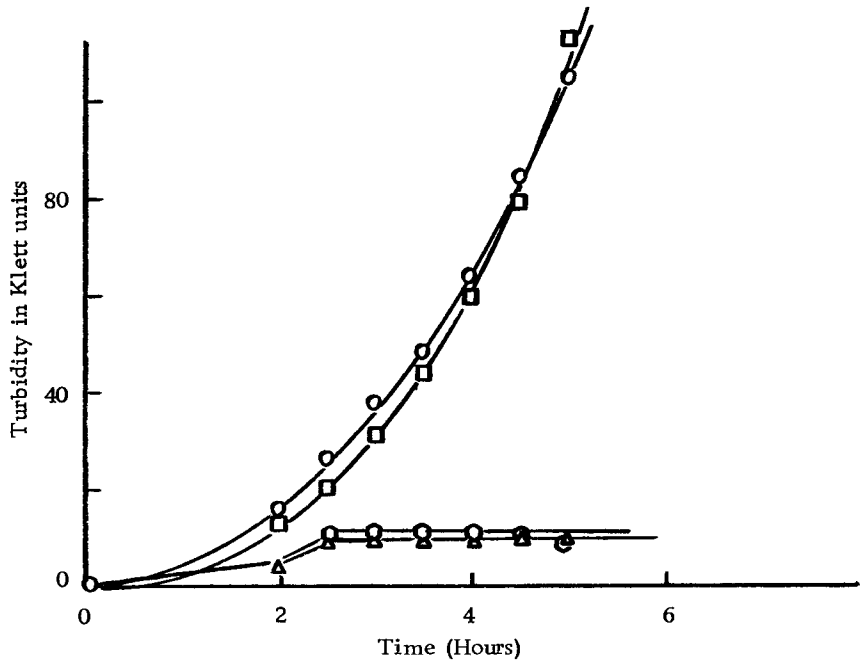

Figure 10. Growth of E. coli 55-1 in the presence of adenine and Sm.

No additions	7.6×10^{-5} M adenine	1.3×10^{-5} M Sm	adenine and Sm

Discussion

These results confirm reports that the entire Sm molecule is needed to support the growth of dependent organisms (65, 78) and indicate that the entire molecule is also needed to inhibit growth of sensitive strains. The dependent strains require Sm and not an impurity of commercial Sm preparations.

The dependent mutant described is not conditionally dependent, that is, dependence is not due to phenotypic repair of a genetic deficiency, namely the inoperative proline pathway.

GSA Production

The Effect of Sm

The Effect of Sm Concentration. The effects of Sm on GSA production in typical experiments with 55-1, WP1-30, 55-1SR, and 55-1SD are shown in Figures 11 and 12: GSA production is inhibited in 55-1; the relatively high concentrations of Sm shown for 55-1 will almost completely inhibit GSA production after 40 minutes, but the initial rate is lowered more by the higher concentration. GSA synthesis in WP1-30 is also inhibited by Sm, but not to the degree of GSA production in 55-1; the resistant strain does not exhibit a response to the drug, and in the dependent mutant GSA production is enhanced by the presence of Sm.

The effect of increasing concentrations of Sm on these organisms is shown in Figure 13; tabulation of the effect of Sm on the initial rate of GSA production appears in Table 3. For a series of concentrations which were tested on the same culture of 55-1 the results were always consistent, that is, the greater the concentration, the larger the degree of inhibition. The inhibition produced by the same amount of antibiotic on cultures grown up at different times was not always reproducible, however. For example, 6.5×10^{-5} M Sm might cause less inhibition than 4×10^{-5} M Sm on a different culture, but more than 4×10^{-5} M Sm tested on the same cell suspension.

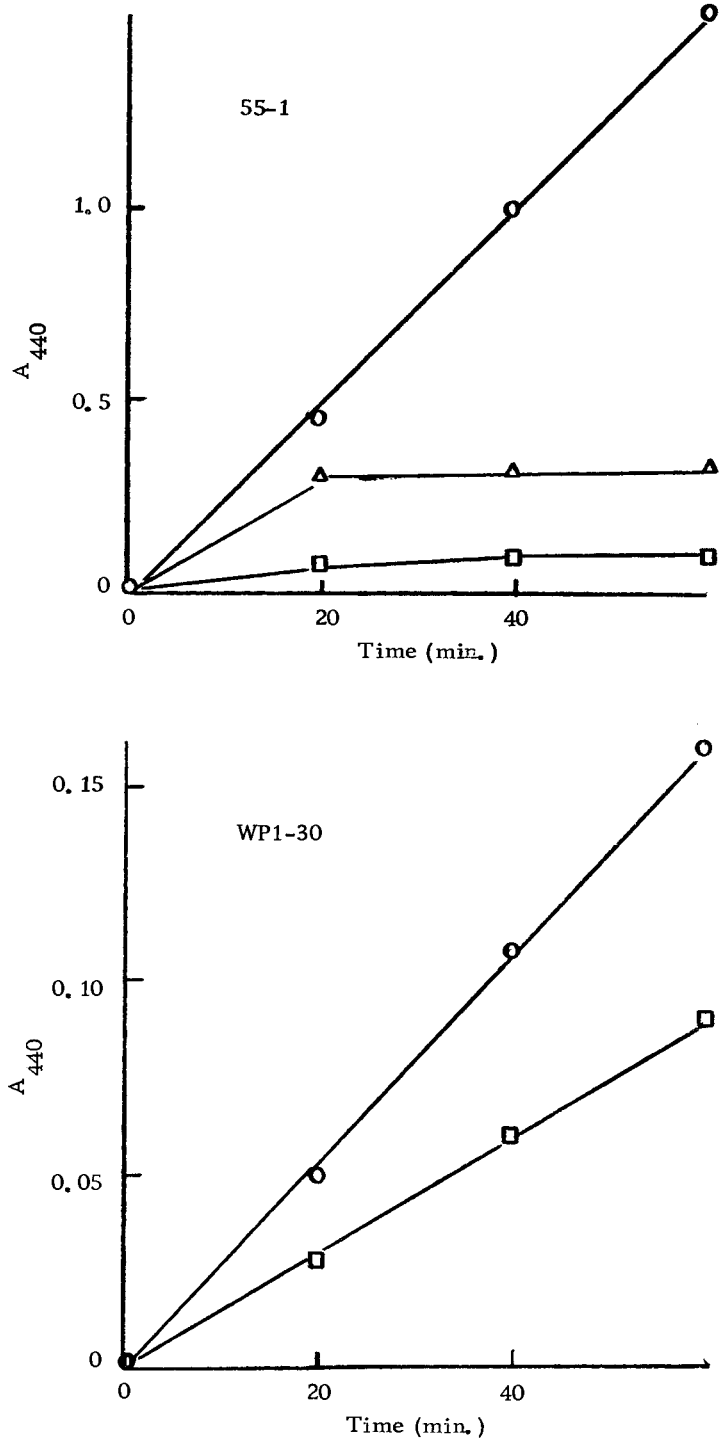

Figure 11. Effect of streptomycin on GSA production in E. coli 55-1 and WP1-30.

No Sm; 1.3×10^{-4} M Sm; 6.5×10^{-4} M Sm

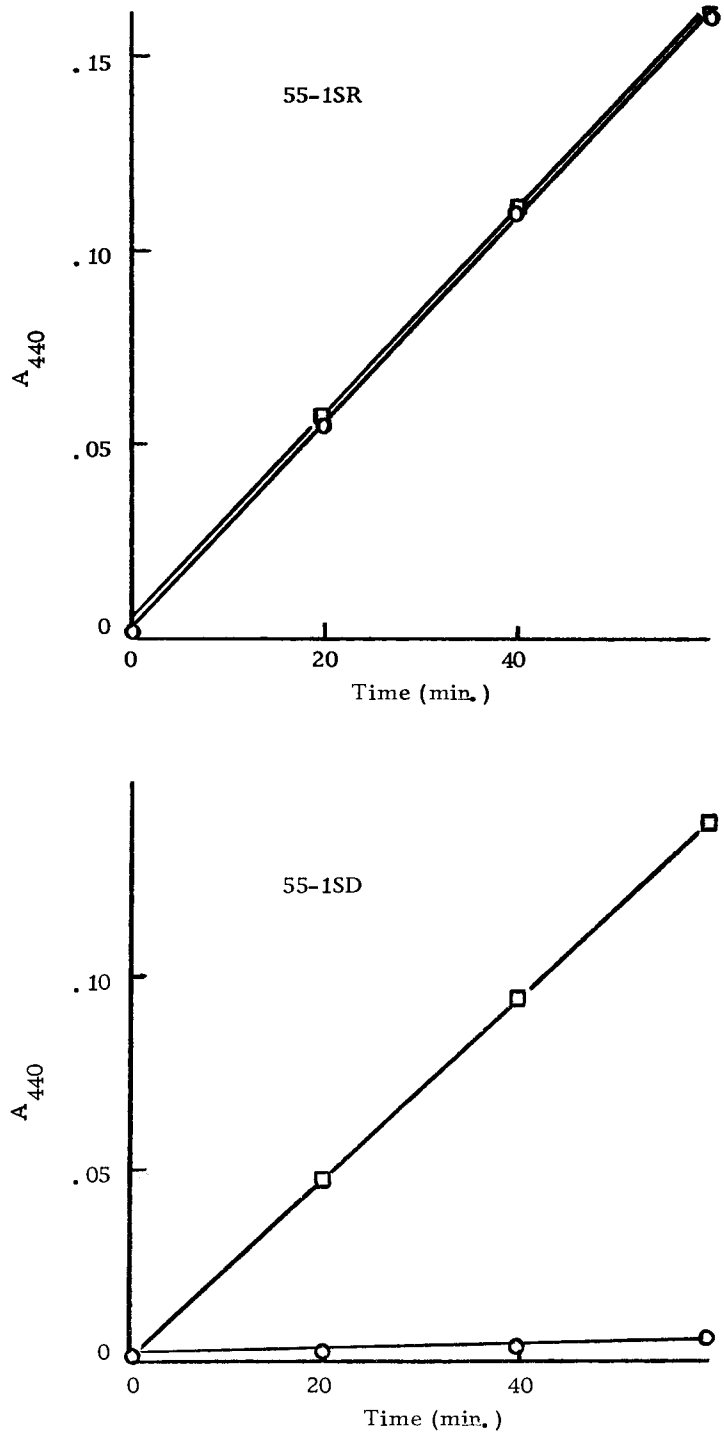

Figure 12. Effect of streptomycin on GSA production in E. coli 55-1SR and 55-1SD.

o——o no Sm; □——□ 6.5×10^{-4} M Sm

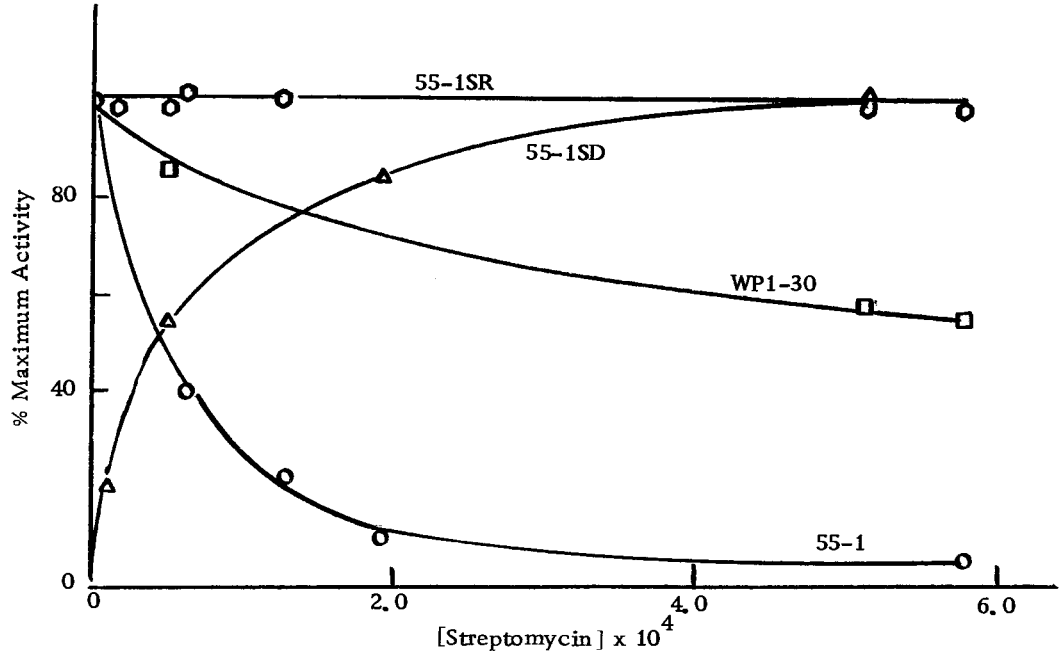

Figure 13. Effect of increasing [Sm] on GSA synthetase activity in E. coli 55-1, WP1-30, 55-1SR and 55-1-SD

The amount of antibiotic in the growth medium was found not to influence GSA production or its response to Sm in the resistant and dependent strains. 55-1SR showed the same indifference to Sm following growth in medium 1.3×10^{-3} M in Sm as it did after being grown without Sm, and growth of 55-1SD in a variety of Sm concentrations did not appreciably change the pattern of GSA excretion, although some concentrations were sub-optimal for growth. 55-1SD was observed to show large quantitative differences in response to the addition of Sm, even for cells grown at the same concentration of antibiotic. Although enhancement of GSA production was always observed, the degree of stimulation could vary several hundred percent

Table 3. Percent Change in the Initial Rate of GSA Production.

(Sm) x 10^3	55-1	WP1-30	55-1SR	55-1SD
0.005	- 5-10	- 20 - +9	0 - +3	+ 5-150
0.013	- 8-18		- 20 - +5	
0.020	- 37-47			
0.026	- 46-54	- 20 - 30		
0.033	- 50-56			
0.039	- 52 - 66			
0.045	- 59-67			
0.052	- 69-75	- 8 - 18	- 4 - +12	+ 16-336
0.058	- 66-77			
0.065	- 59-67		- 8 - +20	+ 20-425
0.072	- 64-73			
0.078	- 75-85			
0.091	- 75-85			
0.104	- 75-85			
0.13	- 69-88			
0.19	- 80-90			
0.26	- 81-90			
0.52	- 78-94	- 25 - 42	- 10 - +13	+ 5-550
0.65	- 90-95	- 30 - 57	0 - +5	
1.30	- 95-100		0 - +9	+ 10-425

The Effect of Hydrolysis Products of Sm. The effects of hydrolysis products of Sm on GSA production were tested on strains 55-1 and 55-1SD, and the results are given in Table 4. It can be seen that the Sd and 'SBA' fractions, alone or in combination, do not achieve the effect of Sm, indicating that to affect GSA production the entire Sm molecule is necessary.

Table 4. Relative Rates of GSA Production.

Addition	Strain	
	55-1	55-1SD
none	1.0	1.0
$6x10^{-5}$ M Sd	1.1	---
$3x10^{-4}$ M Sd	1.1	---
$1.5x10^{-3}$ M Sd	1.0	1.1
'SBA'	1.0	1.0
Sd + 'SBA'	1.0	1.0
$5.2x10^{-4}$ M Sm	0.1	1.5

The Effect of Substrate Concentration. The effect of Sm on GSA production was studied over a series of glutamic acid concentrations to permit a kinetic analysis of the system. Representative plots for 55-1 (Figure 14), WP1-30 (Figure 15), 55-1SR (Figure 16), and 55-1SD (Figure 17) are shown. While it is recognized that when an enzyme is within a cell the Michaelis-Menton kinetics no longer hold in their simple form and that the interpretation of the usual

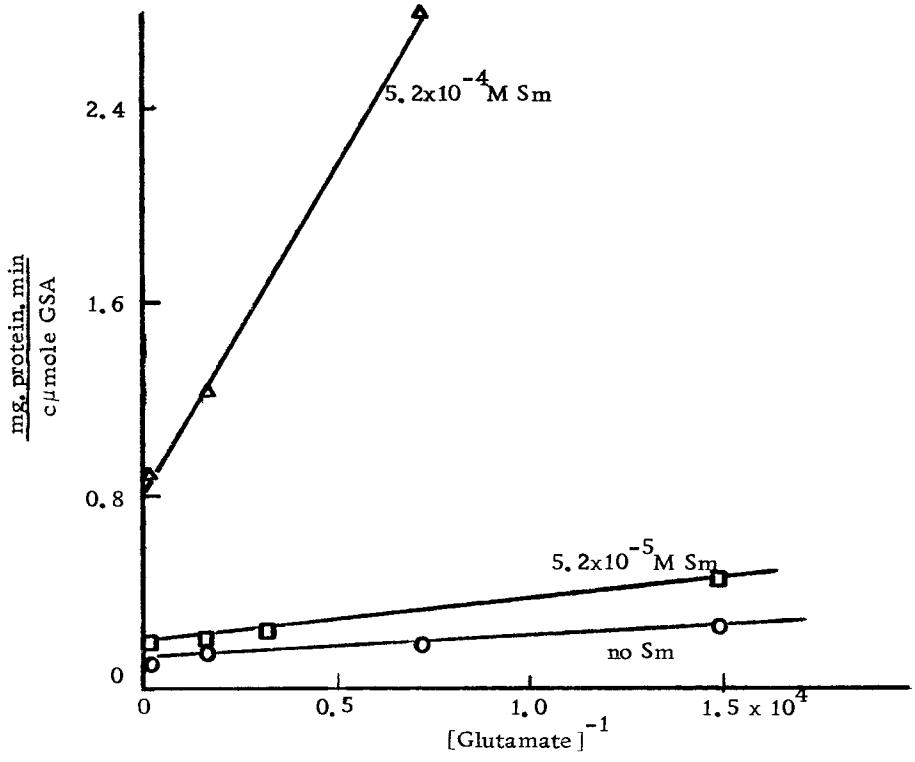

Figure 14. Lineweaver-Burk plots for E. coli 55-1.

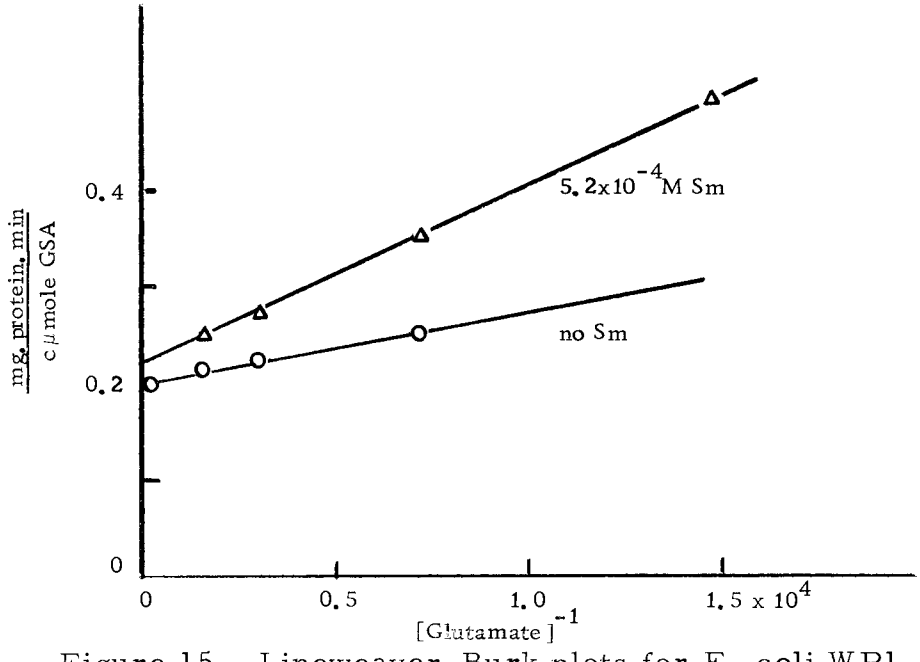

Figure 15. Lineweaver-Burk plots for E. coli WP1-30.

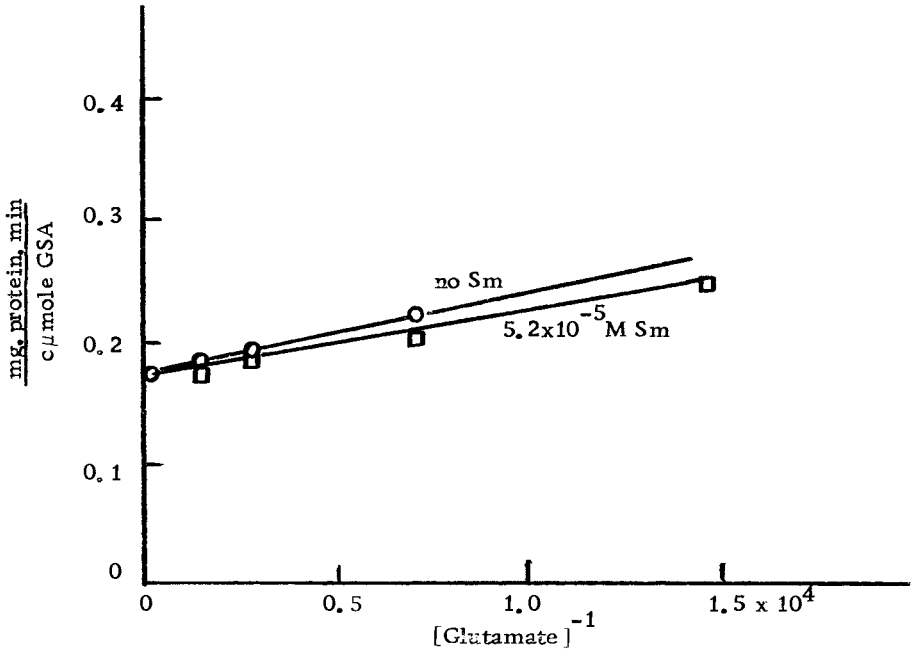

Figure 16. Lineweaver-Burk plots for E. coli 55-1SR.

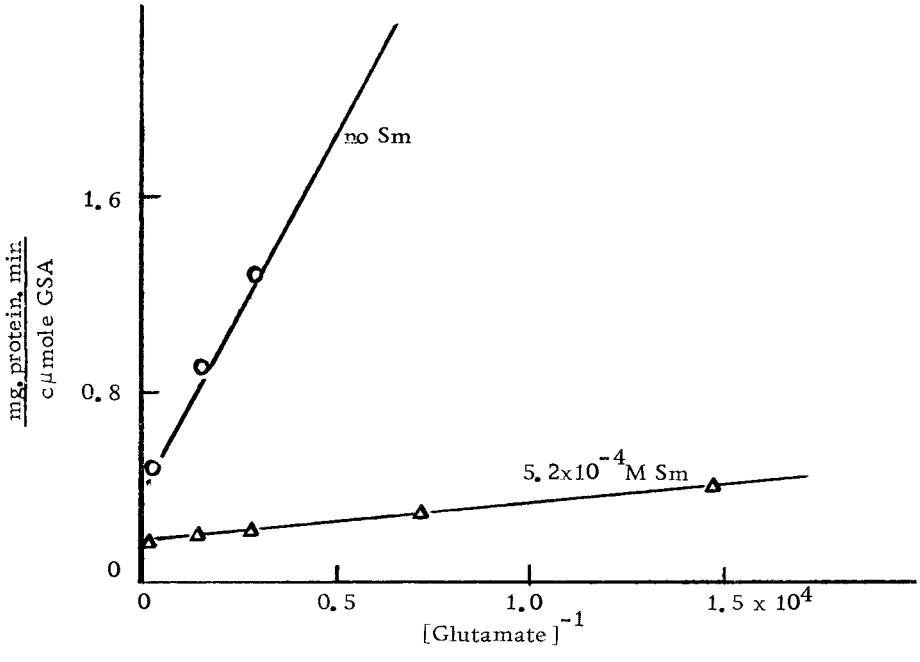

Figure 17. Lineweaver-Burk plots for E. coli 55-1SD.

reciprocal plots are not unambiguous, the average of all determinations of the Michaelis constant (Km) and maximum velocity (V max) for all strains appears in Table 5 to permit qualitative comparison of the effects of different concentrations of substrate and inhibitor and of different strains.

The addition of Sm causes an increase in Km and a decrease in Vmax in the GSA synthetase system of 55-1. GSA production in 55-1SR shows very little effect on either Km or Vmax, and the synthetase system in 55-1SD shows a decrease in Km and an increase in Vmax. WP1-30 behaves qualitatively like 55-1, although the changes are not as pronounced. The enzymes of 55-1 and the related strains 55-1SR and 55-1SD all show the same Km at conditions which give the lowest value of Km for each strain, but GSA appears to be synthesized more rapidly in 55-1. The type of inhibition conferred by Sm is neither wholly competitive nor wholly noncompetitive but is mixed.

The GSA synthetase system of WP1-30 was found to have a lower Km than that of 55-1, but the value in the table is an average of a fewer number of determinations than that for 55-1, which sometimes produced values as low as those for WP1-30, so the difference may not be significant.

In sensitive strains, Sm exerts an effect on the enzyme catalyzing GSA formation which influences the substrate site in such

Table 5. Km/Vmax (Vmax = $\frac{c\mu mole\ GSA}{min.\ mg.\ protein}$).

Strain	Addition				
	none	$5.2x10^{-6}$M Sm	$5.2x10^{-5}$M Sm	$5.2x10^{-4}$M Sm	$7.4x10^{-5}$M A
55-1	$5x10^{-5}$M 8.0	$8x10^{-5}$M 7.0	$1x10^{-4}$M 6.0	$3x10^{-4}$M 1.3	$1x10^{-7}$M 9.0
WP1-30	$3x10^{-5}$M 5.3	$6x10^{-5}$M 5.0	$6x10^{-5}$M 4.0	$8x10^{-5}$M 4.5	
55-1SR	$5x10^{-5}$M 7.0	$5x10^{-5}$M 7.0	$4x10^{-5}$M 7.0	$5x10^{-5}$M 7.0	
55-1SD	$1x10^{-3}$M 3.5	$2x10^{-4}$M 5.3	$9x10^{-5}$M 6.3	$5x10^{-5}$M 7.0	

The possibilities for the explanation of the action of Sm are (1) a direct action on the enzyme catalyzing GSA synthesis or (2) an indirect action, which can include effects on other enzymes and nonenzymic effects. As an example of the former type of indirect action, it has been reported (90) that the reduction of glutamic acid in washed cell suspensions of E. coli is strictly an aerobic process, and Sm has been shown to impair respiration in Sm sensitive but not in Sm dependent cells (13, 15, 16, 17, 49) or in cell extracts (14). Since within a cell, enzyme activity may depend on other enzymes or a structurally organized system, nonenzymic effects might alter conditions causing a change in the activity of the enzyme without a direct attack being made upon it.

Unfortunately it is usually difficult or impossible to distinguish between enzymic and nonenzymic action of inhibitors, and Webb (104) suggests no sort of kinetic analysis is useful in making the distinction.

Something of the nature of the action of Sm might be learned by considering the effect of other materials on GSA production and on Sm inhibition of GSA production.

The Effect of Salts

Magnesium Chloride. Reports (11, 31, 50, 58) that the presence of certain salts, including salts of magnesium, in the growth medium greatly influenced the bactericidal capabilities of Sm

raised the question of whether the action of Sm on GSA synthesis would be similarly affected. The possibility was tested, and the results appear in Table 6. The addition of magnesium chloride did not seem to change GSA production significantly; there was some increase in the rate of synthesis, but the change was within 10% of the rate in the absence of the salt. The presence of $MgCl_2$ likewise had a small effect on the inhibition produced by Sm; 5.2×10^{-5} M Sm gave the same degree of inhibition in all concentrations of $MgCl_2$ tested, and the change of inhibition produced by 1.3×10^{-5} M Sm was also within 10% of the inhibition in the absence of the salt.

The greater inhibition produced by 1.3×10^{-5} M Sm over that previously reported was due to preincubation of the cells with the antibiotic which was being tried at this time. Because preincubation resulted in complete (100%) inhibition in the initial rate for most concentrations of Sm used, the procedure was abandoned. Since the effect of $MgCl_2$ was not significant, the salt was not tested with other strains.

Other Salts. Table 7 shows results obtained with other salts; concentrations of KCl up to 10^{-4} M had no effect on GSA production. Up to 0.34M NaCl, which antagonizes the antibacterial action of Sm (11), had no effect when added to the excretion medium in the absence of Sm, although washing cells harvested from growth medium with

Table 6. Effect of Magnesium Chloride on GSA Production in E. coli 55-1.

Addition	Relative overall rate
none	1.0
4.2×10^{-7} M pro	0.5
1.3×10^{-5} M Sm	0.9
5.2×10^{-5} M Sm	0.1
0.005 M $MgCl_2$	1.1
+ 4.2×10^{-7} M pro	0.4
+ 1.3×10^{-5} M Sm	0.9
+ 5.2×10^{-5} M Sm	0.1
0.010 M $MgCl_2$	1.1
+ 4.2×10^{-7} M pro	0.5
+ 1.3×10^{-5} M Sm	0.9
+ 5.2×10^{-5} M Sm	0.1
0.020 M $MgCl_2$	1.1
+ 4.2×10^{-7} M pro	0.5
+ 1.3×10^{-5} M Sm	0.9
+ 5.2×10^{-5} M Sm	0.1
0.030 M $MgCl_2$	1.1
+ 4.2×10^{-7} M pro	0.5
+ 1.3×10^{-5} M Sm	0.95
+ 5.2×10^{-5} M Sm	0.1
0.045 M $MgCl_2$	1.1
+ 4.2×10^{-7} M pro	0.6
+ 1.3×10^{-5} M Sm	1.0
+ 5.2×10^{-5} M Sm	0.1

0.34M NaCl abolished the ability to produce GSA in cell suspensions of 55-1 and 55-1SD. Cell suspensions of 55-1 washed with 0.1M phosphate buffer, pH 7.0, following preincubation with Sm were not able to produce GSA when resuspended in the usual excretion medium. This observation, together with the fact that concentrations of Sm above $3x10^{-5}$ M usually gave complete inhibition after 60 minutes, suggests that at the usual conditions of pH and ionic strength Sm exerts irreversible inhibition, that is, enzymatic activity is not restored over the time interval of the experiment when the inhibited cells are placed in inhibitor free medium. This type of inhibition implies a high affinity for the inhibitor and its site.

Table 7. Effect of Salt Solutions on GSA Production in E. coli 55-1.

Addition	Ionic strength	% Change in overall rate of GSA production
$6.5x10^{-5}$ M Sm	$2x10^{-4}$	- 95
10^{-6} M KCl	10^{-6}	- 5
10^{-5} M KCl	10^{-5}	- 4
10^{-4} M KCl	10^{-4}	0
0.034 M NaCl	0.034	+ 7
$+6.5x10^{-5}$ M Sm		- 86
0.17M NaCl	0.17	+ 10
$+6.5x10^{-5}$ M Sm		- 66
0.34M NaCl	0.34	- 5
$+6.5x10^{-5}$ M Sm		- 20

NaCl solutions did have an effect on GSA production in the presence of Sm, however; it was found that NaCl reversed inhibition to some extent (Table 7). The reversal of Sm inhibition was not apparent initially but came about by the end of the experiments, so overall GSA production rather than the initial rate of production is compared in Table 7, making 6.5×10^{-5} M Sm appear more effective in inhibiting the reaction than previously noted. NaCl (0.34M) also lowered the degree to which Sm stimulated GSA synthesis in 55-1SD.

Discussion. Salt effects on cell suspensions are difficult to interpret, since one cannot be confident of a lack of specific ion effects, even when NaCl is used to change the ionic strength, and the concentration of the ions may be higher or lower in the area of the cell in which Sm acts than in the external medium, but it appears that the action of Sm is not brought about by a change in the ionic strength of the medium. The reversal of Sm inhibition by relatively high concentrations of NaCl might be caused by interference with the association of Sm to its site, since activity coefficients of ionic materials change in solutions of different ionic strength and could affect the association constant. The salt might interfere with Sm getting to its site; a similar suggestion was made for the ability of certain salts to antagonize the antibacterial action of Sm (72).

The Effect of Purines, Pyrimidines, and Nucleotides

In addition to inorganic salts, purine and pyrmidine bases were tested with 55-1 for their effect on GSA production (Table 8). Uracil, cytosine, adenine, and guanine all showed the ability to increase GSA production, but the largest stimulation was given by adenine, and a mixture of the four bases showed the same degree of stimulation as produced by adenine alone. ATP and AMP did not have an effect of GSA production.

Table 8. Effect of Purines, Pyrimidines, and Nucleotides on GSA Production in *E. coli* 55-1.

Addition	% Rate change
9×10^{-5} M uracil (U)	+ 4
9×10^{-5} M cytosine (C)	+ 15
6.6×10^{-5} M guanine (G)	+ 8
7.4×10^{-5} M adenine (A)	+ 40
U, C, G, A	+ 40
+4.4×10^{-5} M pro	- 100
9.5×10^{-5} M ATP	0
+1.3×10^{-4} M Sm	- 97
2×10^{-4} M AMP	0
+1.3×10^{-4} M Sm	- 95
0.5 mgm/ml yeast nucleic acid	+ 280
+4.4×10^{-5} M pro	- 100
+ 1.3×10^{-5} M Sm	+ 48

When 7.4×10^{-5} M adenine was present in the excretion medium, Sm was less effective an inhibitor of GSA synthesis, and increasing the concentration of Sm did not overcome the effect of the adenine (Figure 18).

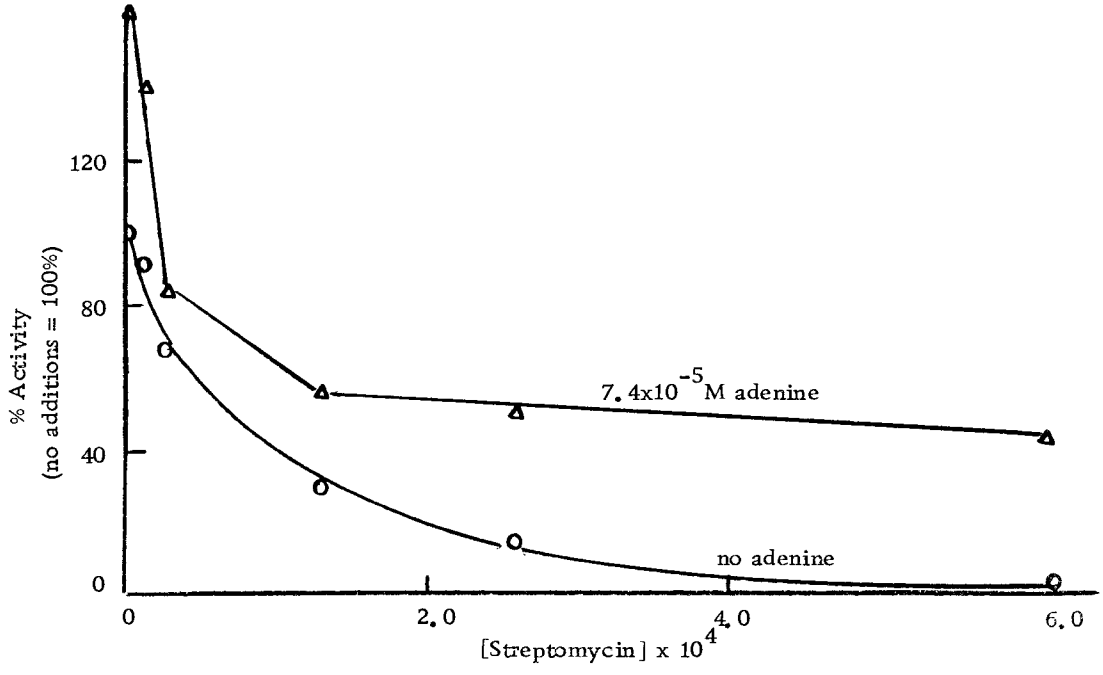

Figure 18. Effect of Sm concentration on adenine stimulation of GSA production in E. coli 55-1.

The Effect of Adenine Concentration. In the presence of 2.6×10^{-5} M Sm, increasing concentrations of adenine at first reversed inhibition, with 7.4×10^{-5} M adenine completely counteracting the effect of the antibiotic, but further increases in adenine concentration resulted in reinstatement of inhibition, and at

7.5×10^{-3} M adenine, the inhibition was like that with no adenine present (Figure 20). Increasing concentrations of adenine in the absence of Sm caused increasing GSA production up to 1.9×10^{-3} M then a decrease. However, even at the highest concentration of adenine used, 7.4×10^{-3} M, the GSA production was 10% higher than if no adenine was present, with 3.4×10^{-4} M glutamic acid (Figure 19).

The Effect of Substrate Concentration. Adenine was found to be much more effective in stimulating GSA production at low substrate concentration. Figure 19 compares the effect of increasing concentrations of adenine at 3.4×10^{-4} M and 6.8×10^{-3} M glutamic acid. Figure 21 shows Lineweaver-Burk plots for GSA production in 55-1 in the presence of adenine; the addition of adenine resulted in a lowered Km and a slightly increased Vmax (Table 5).

The Effect of Nucleic Acid. The observation that adenine stimulates GSA excretion suggested nucleic acid might also be effective, and it was discovered to have a profound stimulatory effect. Like adenine, yeast nucleic acid also lowered inhibition produced by Sm, but neither yeast nucleic acid nor adenine had any effect on inhibition by proline (Table 8).

Yeast nucleic acid also stimulated GSA production in WP1-30, but to a much lesser degree, and in 55-1SD, but did not overcome the slight amount of inhibition caused by 1.3×10^{-5} M Sm in WP1-30.

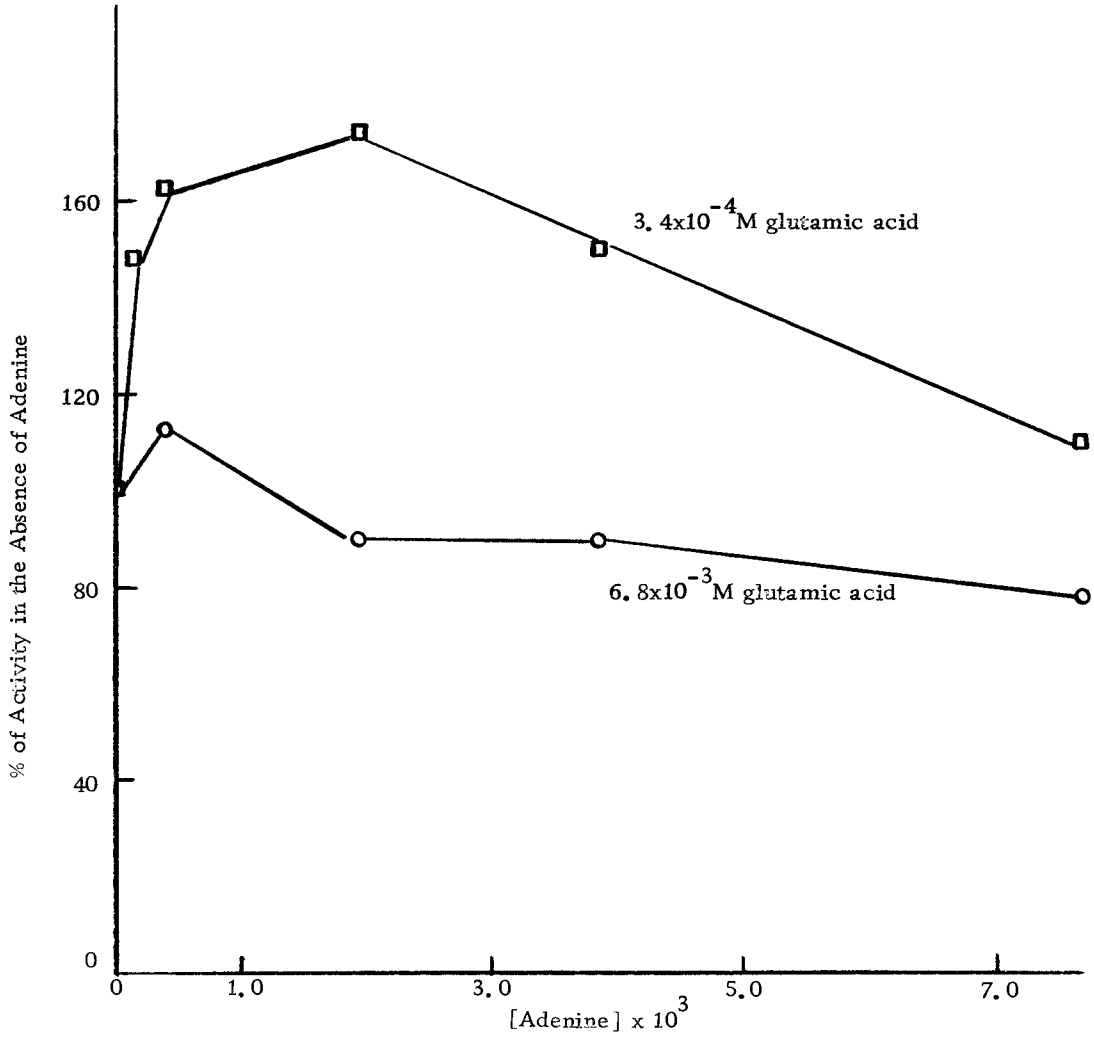

Figure 19. Effect of adenine concentration on GSA production in E. coli 55-1.

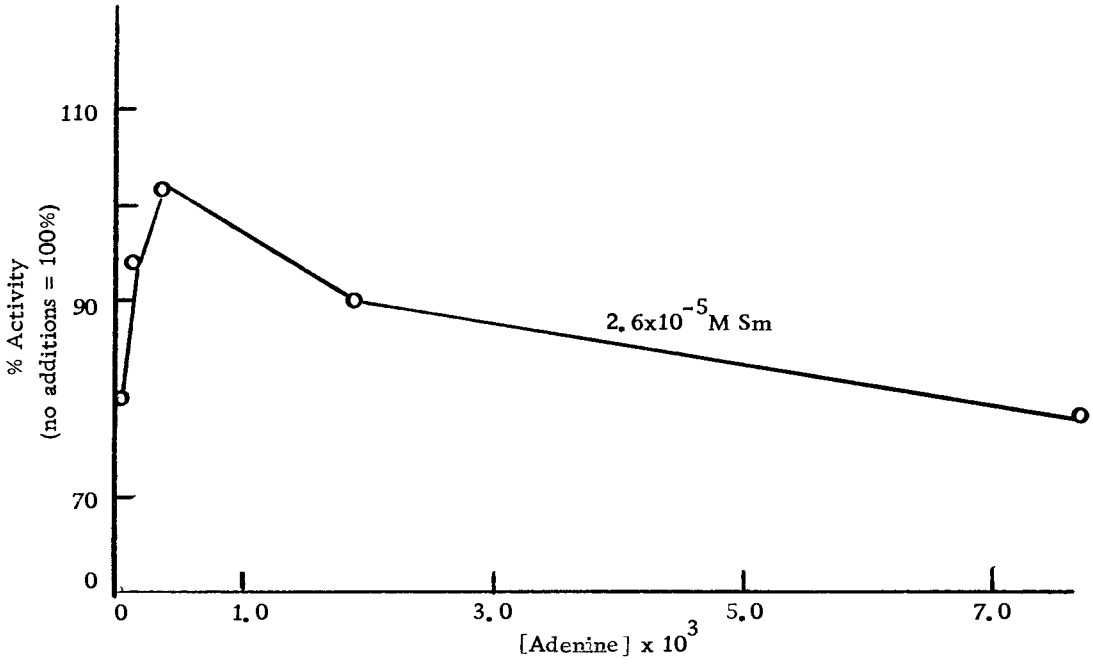

Figure 20. Effect of adenine concentration on streptomycin inhibition of GSA production in E. coli 55-1.

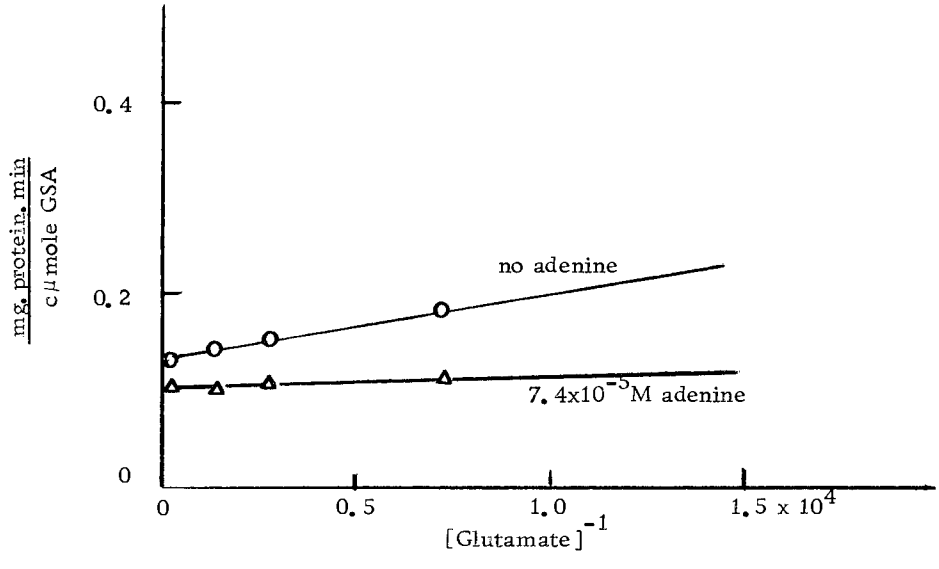

Figure 21. Lineweaver-Burk plots for E. coli 55-1.

Adenine did not appear to influence inhibition by Sm in this organism either. GSA production in 55-1SD was increased 25% by guanine, but the other bases did not show a significant effect (Table 8).

In addition to 55-1 cultures which produced GSA at a normal rate (70-80 mμmole/min/mgm protein), 55-1 cell suspensions were sometimes obtained which produced GSA only sparingly (0-15 mμmole/min/mgm protein); in the presence of 7.4×10^{-5} M adenine these cells showed enhancement of GSA production in excess of 250%, and yeast nucleic acid gave nearly five fold increases in GSA production. Some cultures produced GSA only in the presence of yeast nucleic acid or adenine.

The possibility that yeast nucleic acid and adenine could furnish substrate for the GSA forming reaction was tested, but these materials in excretion medium without glutamic acid did not allow GSA production by 55-1 cell suspensions.

The Mode of Action of Adenine and Nucleic Acid. The effect of adenine on GSA production in 55-1 is surprising because it is rather specific, adenine being much more effective than the other bases, including guanine, and also because adenine is presumably present in the cells already. It is likely that adenine, like Sm, can exert various actions on cellular metabolism. For example, adenine might act directly on the enzyme in the manner of a cofactor

or might act indirectly, such as by inhibiting a different, competing reaction. If adenine were to inhibit such a reaction, stimulation of GSA production might be brought about by increased availability of substrate; this interpretation accounts for the greater stimulation at low substrate concentrations, the inability to achieve the same degree of inhibition at high concentrations of Sm in the presence and in the absence of adenine, and the specificity for adenine. Adenine might also exert nonenzymic effects; the reduction in stimulation of GSA production at high adenine concentrations may be a consequence of secondary effects of the compound.

ATP did not show stimulation of GSA synthesis, but it may be that ATP has difficulty in entering the cell. Yeast nucleic acid would be expected to have an even greater difficulty, however, and yet this material has the same qualitative effect as adenine on GSA production. It may be that the means by which the two substances achieve the effect differs; for example, the nucleic acid may lower Sm inhibition by binding to the antibiotic, lowering the effective concentration, as proposed for the antagonism of nucleic acid to the bactericidal effect of Sm (6). A similar explanation for the lowering of Sm inhibition of GSA production by adenine does not explain the specificity for adenine or the failure of high Sm concentrations to reverse the adenine effect.

The Effect of Other Antibiotics

Other antibiotics known to have an effect on GSA synthesis were tested with 55-1SR and 55-1SD to see if mutation to Sm resistance or dependence conferred any resistance to these compounds, and the results are shown in Table 9. Tetracycline, chloramphenicol, and protamine sulfate, which was also found to inhibit the growth of 55-1, all inhibited GSA production in 55-1SR and 55-1SD, and Sm did not reverse the inhibition measurably. Protamine sulfate, a basic protein, would not be expected to act within the cell, an indication that the GSA forming system is near the outside of the cell or can be affected by something which is.

Table 9. Relative Rate of GSA Production in the Presence of Sm and Other Antibiotics.

Addition	Strain	
	55-1SR	55-1SD
none	1.0	1.0
6.5×10^{-5} M Sm	1.0	4.3
1.2×10^{-4} M tetracycline	0.0	0.0
50 μg/ml protamine sulfate	0.0	0.0
1.5×10^{-4} M chloramphenicol	0.1	0.0
Sm + tetracycline	0.0	0.0
Sm + protamine sulfate	0.1	0.0
Sm + chloramphenicol	0.0	0.0

The Effect of pH

The effect of pH on inhibition of GSA production by Sm and proline was examined (Figure 22); the maximum activity of the cells in GSA production was observed to occur at pH 7.0 and to fall off in acidic or basic suspensions. Also, percent inhibition by proline was at a maximum at pH 7.0 and decreased in acidic or basic suspensions to a greater degree than GSA synthesizing activity. Inhibition by Sm reached a maximum at pH 7.0, but only acidic conditions reversed inhibition. The activity of the GSA forming system in 55-1SR and 55-1SD were also pH dependent, and Sm showed a decreased ability to stimulate GSA production in acidic cell suspensions of 55-1SD.

Sm inhibition is observed to reach a maximum at approximately the same pH at which the molecule exists predominantly in the neutral form; since it is generally true that weak bases penetrate cells more rapidly in their uncharged forms, this observation is consistent with the idea that Sm cannot readily reach its site of action, presumably within the cell, in acidic conditions. The antibacterial action of Sm shows a similar pH dependence (103, 108). It may be that the active form of the antibiotic in inhibiting GSA production is charged; this interpretation requires only that the permeant form of Sm be uncharged.

It could also be that changes in the ionization of a species with which Sm interacts are responsible for the observed pH effect; if the

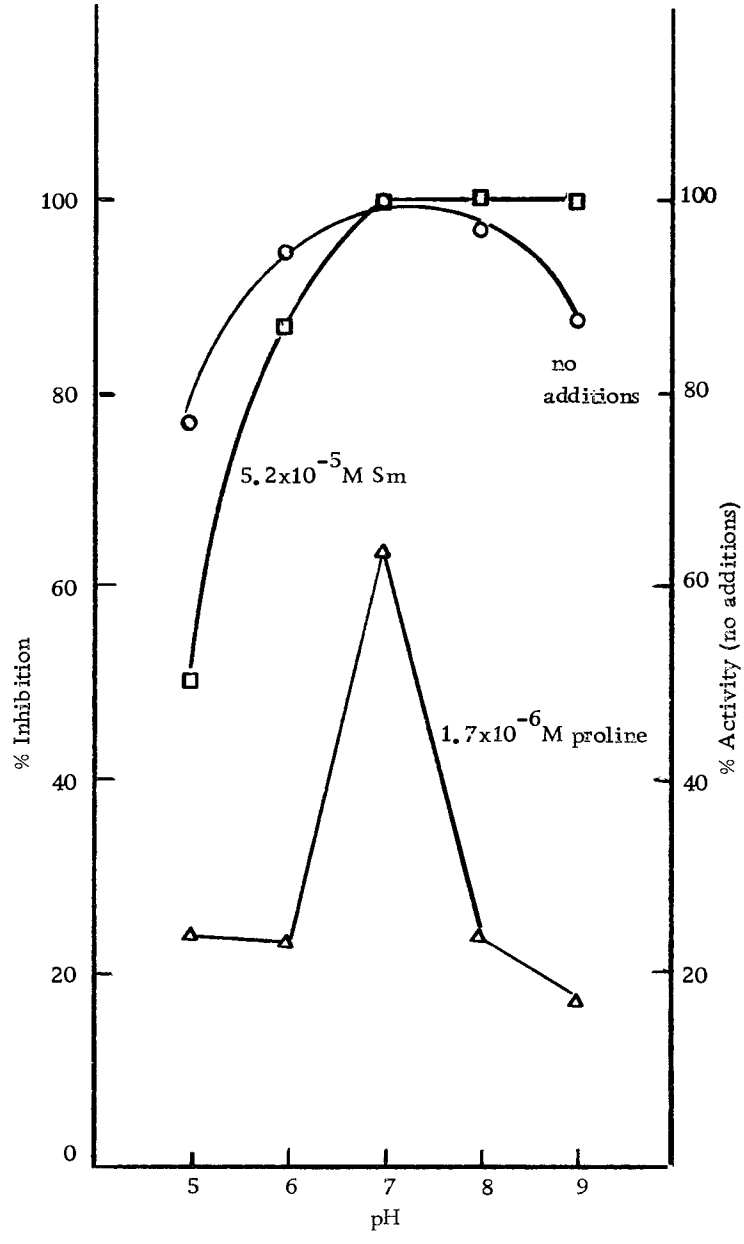

Figure 22. Effect of pH on production of GSA and its inhibition by proline and Sm in E. coli 55-1.

site of action of Sm is influenced by the pH of the medium, it may be that the site is quite near the surface of the cell.

Permeability

The GSA production as measured may reflect more than just the activity of the enzyme; for example, permeability of the cells to substrate and product may influence the measured rate. For this reason, the effect of Sm on uptake of L-glutamic acid was studied.

Table 10 compares the effect of 5.2×10^{-4} M Sm on uptake of L-glutamic acid and on GSA production; it can be seen that Sm inhibits the uptake of glutamic acid in all strains studied, and to approximately the same degree. It is evident, however, that the reduction in the rate of glutamic acid uptake is not sufficient to explain the effect on GSA synthesis. The data for 55-1SR show that the rate of substrate uptake, although reduced, is sufficient to allow GSA production at the maximal rate.

Table 11 shows the effect of increasing concentrations of Sm, as well as Sd and adenine, on uptake of glutamic acid using 55-1. The inhibition of uptake increases as concentration increases, but an equimolar concentration of Sd is not as inhibitory as Sm. Adenine, which was shown to enhance GSA production, is also inhibitory to uptake.

Table 10. Comparison of the Effect of Sm on GSA Production and on Glutamic Acid Uptake.

Strain	% Inhibition of GSA production by 5.2×10^{-4} M Sm	% Inhibition of glutamic acid uptake by 5.2×10^{-4} M Sm
55-1	90	55
WP1-30	42	60
55-1SR	2	57
55-1SD	- 500 (enhancement)	62

Table 11. Effect of Additions to the Excretion Medium on Uptake of Glutamic Acid in E. coli 55-1.

Addition	Average relative uptake in 1 min.
none	1.0
1.3×10^{-5} M Sm	0.9
2.6×10^{-5} M Sm	0.9
6.5×10^{-5} M Sm	0.7
1.3×10^{-4} M Sm	0.7
6.5×10^{-4} M Sm	0.4
	(0.4-WP1-30)
	(0.4-55-1SR)
	(0.4-55-1SD)
6.5×10^{-4} M Sd	0.8
7.4×10^{-5} M A	0.7
A + 1.3×10^{-5} M Sm	0.9

The possibility that Sm prevents the excretion of GSA by 55-1 was also tested, but assays of sonicates of 55-1 cells incubated in excretion medium plus 5.2×10^{-4}M Sm, which completely inhibited measured GSA production after 15 minutes, showed no GSA present within the cells.

Discussion

Reproducibility of the Results

During these studies significant variability in the GSA production of cell suspensions was noted, even for the same strain at the same substrate concentration and under conditions which should give optimal GSA synthesis. Attempts were made to keep the experimental conditions as nearly the same as possible in all experiments; cells were grown up to the same turbidity and the same concentrations of cells were used. The factor over which the least control was exerted was the amount of growth in the overnight cultures used to inoculate medium for growth up to 50 Klett units; no attempt was made to ensure these had incubated for the same length of time. Large inoculums of the overnight cultures were used, so a significant segment of the final cell population would be from the inoculum. Since it is not surprising that cells of different ages should show differences in metabolism, the differences in the overnight cultures

might account for some of the observed variability.

The large variation noted in the ability of Sm to enhance GSA production in 55-1SD may be a consequence of the lack of homogeniety of the cultures, since reversion to a resistant mutant frequently occurred with 55-1SD. Large enhancement of GSA production by Sm would then be considered typical of pure 55-1SD, and lower enhancement considered indicative of a significant population of the indifferent resistant mutant in the culture.

Alternatively, the larger stimulation might be considered atypical, since the effect is analogous to the effect of adenine on certain cell suspensions of 55-1 reported earlier: some cultures of 55-1 produced GSA slowly or not at all in the usual excretion medium, and adenine was found to increase GSA production several fold. However, the much greater frequency of the phenomenon with 55-1SD, together with the observation that cells which showed only slight stimulation of GSA synthesis in the presence of Sm, when tested for the ability to grow in medium without Sm, exhibited the characteristics of resistant bacteria, supports the mixed population idea. When the growth was found to be independent of Sm, the results of the excretion experiments were not, of course, reported as 55-1SD data, but results with cells which showed only slight enhancement but which were not tested for growth response to Sm are included.

Site of Sm Action

It can be seen that many similarities exist between Sm inhibition of growth and of GSA synthesis: GSA production in cells whose growth is inhibited by Sm is also inhibited; cells requiring Sm for growth show significant GSA production only in the presence of the drug; and GSA production in cells whose growth is unaffected by Sm shows a similar indifference to the antibiotic. Also, in both cases the entire Sm molecule seems to be necessary, and treatments or conditions which prevent or reverse Sm inhibition of GSA production, such as high salt concentration or acidic pH, generally also reverse inhibition of growth. These treatments are suggested to interfere with the uptake or binding of Sm, so the similarity does not necessarily indicate the site of action is the same. Further, antibiotics such as chloramphenicol and tetracycline and the basic protein protamine sulfate, which was also shown to inhibit growth, also inhibit GSA synthesis.

Differences in the two types of inhibition are also apparent: the concentration of antibiotic required to halt GSA synthesis entirely is higher than the concentration needed to kill the same organism; in WP1-30 a mutation which abolishes proline inhibition of the reaction also reduces the effectiveness of Sm inhibition compared to 55-1, but does not lessen growth inhibition; and adenine reverses inhibition of GSA production by low concentrations of Sm but does not

reverse growth inhibition.

In view of the similarities of the effects of Sm on GSA production and growth in antibiotic-sensitive, resistant, and sensitive strains, it might be well to consider the differences in these strains again. As has been emphasized (52, 86) Sm sensitivity, resistance, and dependence are multiple alleles at a single locus; this fact implies that the three phenotypes are due to alternative states of a single protein, suggested by Spotts and Stanier to be ribosomal. However, the interaction of Sm with the ribosomes has been shown (26, 27, 46, 61, 83, 99) to lead to ambiguity in the translational process of protein synthesis, so cells grown in the presence of Sm, as resistant and dependent strains were, may have other altered proteins compared to the sensitive strain. Also, the resistant and dependent strains could show variation in certain proteins, since the ribosomes differ and might influence miscoding.

Genetic analysis has not been carried out on strains 55-1, 55-1SR, and 55-1SD to confirm that the three phenotypes are due to mutation at a single locus, but if the assumption is made that they are, the conclusion can be made that Sm does not interact with the GSA synthetase system directly, since it is clear that the protein which differentiates the growth response to Sm is not the enzyme catalyzing GSA synthesis, and production of an altered enzyme due to Sm induced misreading does not account for the indifference of

GSA production to Sm in 55-1SR grown without Sm.

If the assumption is correct, then the effect of Sm is indirect, and the cell component which is different in the three strains should be involved, and according to Spotts and Stanier that component is ribosomal. Materials which are known to bind to nucleic acids, such as protamine sulfate, chloramphenicol, and tetracycline, have been shown to inhibit GSA formation in both sensitive and dependent strains; this observation is consistent with the report that ribosomes which are resistant to the effect of Sm in protein synthesis are still sensitive to other antibiotics (25).

It is difficult, however, to propose a function for the ribosomes in GSA synthesis. An enzyme has been isolated with a nucleic acid component (69), but the nucleic acid was removed by further purification without loss of enzyme activity; an interaction of protein and nucleic acid within the cell is nevertheless suggested, and Sm may disrupt such interactions by binding to the nucleic acid component, which might influence the activity of the enzyme.

Alternatively, the assumption that in these strains Sm sensitivity, resistance, and dependence are determined at the same locus may be incorrect. Sm resistance may differ from both sensitivity and dependence in that resistant cells lack the ability to accumulate the drug; resistant mutants of this type have been reported (51, 53, 81). Sm sensitivity and dependence could still be

due to differences in the ribosomes. In this case, a distinction between direct and indirect modes of action of Sm cannot be made, and this explanation requires that the Sm site be within the cell. The interpretation of the pH effect which suggests a requirement for the neutral form of the drug for permeance agrees with this conclusion.

There has been evidence presented (12, 76, 84) that many enzymes are localized at or near the cell membrane and are susceptible to influences which do extend within the cell, and the effect on GSA production of materials which are not supposed to enter the cell suggests that the GSA forming enzyme might be one of these. If the site of Sm is not intracellular, perhaps its effects might be mediated through an effect on the cell membrane. It has been reported that when Sm is added to a bacterial suspension there is an almost instantaneous binding to the cell, which has the features of an electrostatic interaction. This binding occurs in both growing and resting cells and is inhibited by some factors, such as the presence of certain salts, which also antagonize the antibacterial action of Sm (51, 72). However, this binding has been reported to occur almost equally in sensitive and resistant strains of E. coli (4).

SUMMARY

Summary

Strains of E. coli have been described in which Sm inhibits, enhances, or has no effect on GSA production in resting cells; growth of these strains is Sm-sensitive, dependent, and resistant respectively. Inhibition of GSA production is lessened in acidic medium, by the presence of certain salts, and by adenine and yeast nucleic acid, which at low concentration stimulate GSA production in the absence of Sm. That inhibition of measured GSA production could be due to an effect of Sm on permeability of the cells to substrate or product was ruled out. Other antibiotics were found to inhibit the formation of GSA in all strains.

The object of these studies was to determine the effect of Sm on proline synthesis in E. coli; effects have been described, but the mode of action of the drug was not ascertained.

Concluding Remarks

Two possibilities for the site of action of Sm have been discussed: (1) a direct action on the enzyme protein, and (2) an indirect action, including enzymic and nonenzymic effects. Genetic analysis might be useful in ruling out the direct mode of action, but the

answer to whether the effect of Sm is direct or indirect may, however, await the development of a cell-free GSA forming system.

If the effect is indirect does not mean that it is without interest or importance, but further study of the effect is a challenging problem. Distinguishing between the possibilities for indirect action discussed, including a Sm effect on a nucleic acid, an effect on respiration, and an effect on the membrane, might be difficult since the respiratory components of bacteria, as well as ribosomes, are believed to be associated with the cell membrane (1, 15, 92). Also, it is not a matter of determining if Sm interacts with these components, since these interactions are well known in other strains, but it must be determined if the interactions influence GSA production. It has also been emphasized that Sm can have more than one action, and it is conceivable more than one of these influences GSA production; some of the observed results seem to favor one possibility of the site of Sm action, whereas others are more readily interpreted in terms of another.

In conclusion, it can be seen that there are many difficulties in determining the site of Sm action using whole cells. It may be, however, that the effect of Sm requires whole cells or a structurally organized system, and, as Webb (104, p. 427) states, ". . . the full importance of inhibitor studies is manifested only when the inhibitors

are applied to living systems," so the use of whole cells is justified if the results are interpreted with caution.

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