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Silver Sulfadiazine: Effect on the Growth and Metabolism of Bacteria

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Even though the addition of silver sulfadiazine (AgSu) to purified deoxyribonucleic acid (DNA) results in the formation of AgSu-DNA complexes, no such complexes were detected in bacteria treated with AgSu. AgSu blocked macromolecular syntheses in treated bacteria, DNA synthesis being slightly more sensitive to this inhibitory action. The ribosomes, ribonucleic acid, and DNA isolated from treated cells were normal qualitatively. Bacteria deficient in DNA polymerase were not more sensitive than their parent strain to the lethal action of AgSu. Radioactive AgSu was localized mainly in the cytoplasmic membrane fraction of treated cells.

The proven efficacy of silver sulfadiazine (AgSu) in the treatment of *Pseudomonas* infections in burns (8-12, 14, 28, 31) has made a study of its mechanism of action desirable. It has been suggested (11), by analogy with the in vitro complex formation between silver nitrate and deoxyribonucleic acid (DNA; 6, 15, 30,[1] 32), that complex formation between AgSu and DNA was also the basis of its antibacterial action in vivo. Studies on the in vitro interaction of AgSu and DNA have shown (24) that an AgSu-DNA complex was indeed formed, but that it was very different from the $AgNO_3$-DNA complex. The properties of the AgSu-DNA complex have been characterized (24). The present study was undertaken to determine whether such complexes were also formed in vivo upon exposure of bacteria to AgSu and whether they could account for the biological activity of AgSu. It is reported herein that no such complexes could be detected and, moreover, that cellular DNA was not affected by AgSu. The findings suggest that AgSu acts on the external cell structures. A subsequent report (J. E. Coward, H. S. Carr, and H. S. Rosenkranz, *in preparation*) will provide visual evidence in support of this conclusion.

MATERIALS AND METHODS

Bacterial strains. The *P. aeruginosa* strain (no. 686) used in most of these studies was obtained from the Clinical Microbiology Laboratory of this institution. It had been recovered from a patient with burns. A thymine-requiring derivative of this strain was obtained. *Escherichia coli* W3110 thy$^-$ pol A_1^+ and its DNA polymerase-deficient derivative, *E. coli* p3478 pol A_1^-, were generously provided by John Cairns, Cold Spring Harbor Laboratory (7).

Growth of bacteria. At first, attempts were made to grow *P. aeruginosa* in media poor in chloride to prevent the transformation of AgSu into insoluble AgCl. However, when it was found (24) that AgSu did not ionize and that no insoluble AgCl was formed, bacteria were grown in medium HA (20). For determining the number of viable bacteria, serial dilutions of the cultures were spread onto Columbia-base agar plates (BBL); these were incubated for 16 hr, and colonies were then counted.

E. coli W3110 and p3478 were grown on medium HA supplemented with thymine (5 μg/ml). Because of the poor plating efficiency of *E. coli* p3478 on Columbia-base agar (20), the bacteria were spread on agar plates containing medium HA and thymine.

Metabolic techniques. Techniques for determining the incorporation of specific precursors of DNA, ribonucleic acid (RNA), and protein into acid-insoluble products have been described previously (16, 21-23).

Bacterial DNA was prelabeled by growing bacteria in the presence of ^{3}H-thymidine for several generations, harvesting the cells, and resuspending them in fresh medium. The bacteria were then incubated with aeration for 30 min to deplete the pool of radioactive precursor. A portion of the culture was then exposed to AgSu (5 μg/ml) for 1 hr.

Bacterial DNA was extracted from protoplasts (13) by the procedure of Bode and Kaiser (3), which was modified to include several extractions of the material at the interphase to increase the recovery of DNA.

Procedures for the physical-chemical characterization of macromolecules have been described previously (16, 21-24).

Bacterial RNA was isolated by a standard procedure (1) and analyzed on acrylamide gels (2.6%)

cross-linked by ethylene diacrylate as described by Bishop et al. (2). After electrophoresis, the gels were sectioned into slices 1 mm in thickness, which were placed on glass-fiber discs and dried. The discs were put into Omnifluor (4 g per liter of toluene) and radioactivity was determined.

Materials. Radioactive AgSu (^{110}AgSu, 40 μCi/mmole) was generously provided by Howard N. Harrison, Rochester General Hospital, Rochester, N.Y.

RESULTS

Growth of P. aeruginosa. Bacteria exposed to concentrations of AgSu exceeding 2.5 μg/ml were rapidly killed, whereas bacteria of the same strain required over 500 μg of sodium sulfadiazine for killing to be initiated (Fig. 1A). It should be noted that the lethal action of AgSu was not associated with an increase in the turbidity of the culture (Fig. 1B). This indicates that the bactericidal activity of AgSu was not accompanied by unbalanced growth (5), wherein DNA synthesis is blocked but RNA and protein production proceeds.

Effect of AgSu on the metabolism of P. aeruginosa. Studies on the incorporation of ^{3}H-thymidine, 5-^{3}H-uridine, and ^{3}H-leucine into DNA, RNA, and proteins, respectively, revealed (Fig. 2) that DNA synthesis was slightly more inhibited by AgSu than was the synthesis of RNA and proteins. Production of both RNA and DNA was almost completely blocked by AgSu after 15 min of treatment. Protein synthesis continued, but at a reduced rate, for the duration of the experiment. It should also be noted (Fig. 2B) that after a delay the amount of radioactive RNA decreased (i.e., it was degraded).

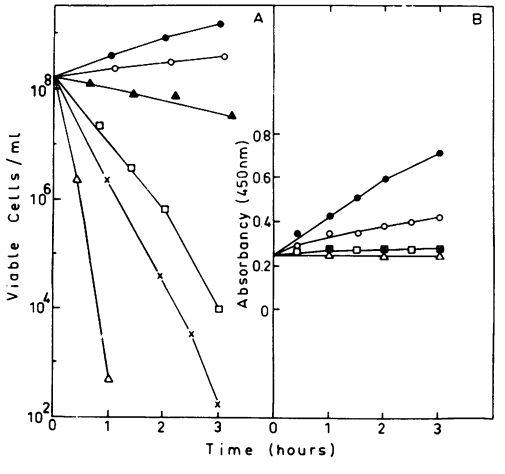

FIG. 1. *Effect of AgSu on the growth of P. aeruginosa.* (●) *Control;* (▲) *sodium sulfadiazine, 600 μg/ml;* (○, □, ×, *and* △) *2.5, 5, 7.5, and 10 μg of AgSu per ml, respectively.*

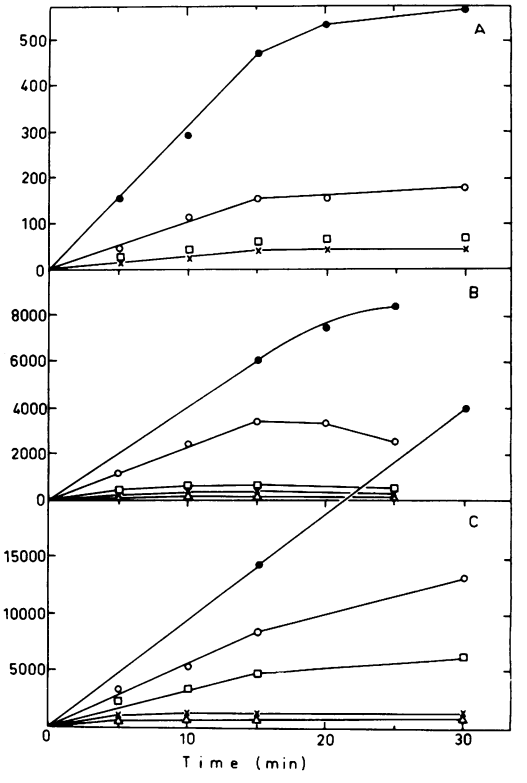

FIG. 2. *Effect of AgSu on macromolecular syntheses. Bacteria were brought to the exponential growth phase, at which time portions of the cultures were distributed into flasks containing the radioactive precursors and various amounts of AgSu. At intervals, samples were withdrawn and processed for determination of radioactivity incorporated into acid-insoluble form. (A) DNA (^{3}H-thymidine, 6.9 × 10^{-8} M, 11.3 Ci/mmole). (B) RNA (5-^{3}H-uridine, 2 × 10^{-4} M, 2 Ci/mmole). (C) Proteins (^{3}H-leucine, 5.4 × 10^{-5} M, 0.7 Ci/mmole). (●) Control; (⊙, □, ×, and △) 2.5, 5, 7.5, and 10 μg of AgSu per ml, respectively.*

Bacteria exposed to 3 μg of AgSu per ml for 20 min synthesized ribosomes that were normal structurally, as determined by analysis on sucrose gradients (*unpublished data*). Furthermore, analysis of ribosomal subunits (Fig. 3) failed to show any difference between AgSu-treated and normal ribosomes. This can be deduced from the superimposition of the radioactivity (AgSu-treated) and absorbance (normal cells) curves.

Analysis of cellular RNA on acrylamide gels revealed that in the presence of AgSu (3 μg/ml) normal species of RNA were made, although the

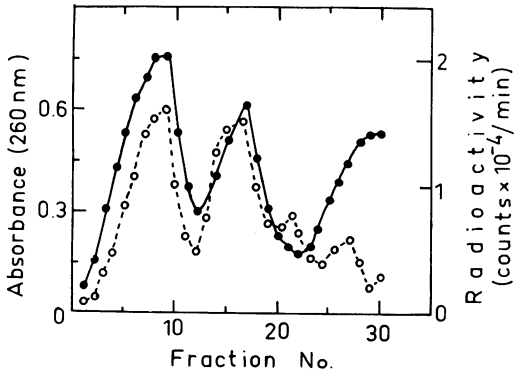

FIG. 3. *Effect of AgSu on ribosomal subunits. Bacteria (50 ml) in the exponential growth phase (2 × 10⁸/ml) received AgSu (3 μg/ml); 3 min later, the cultures received ³H-uridine (1 μCi/ml). At the end of 30 min of incubation, the culture was supplemented with 400 ml of untreated, unlabeled bacteria (also in exponential growth phase). The cells were processed for extraction of ribosomes (17), which were analyzed in linear sucrose gradients (5 to 20%) in 0.01 M tris-(hydroxymethyl)aminomethane buffer (pH 7.0) containing 10⁻⁴ M $MgCl_2$. Centrifugation was for 3 hr in the SW50 rotor of a Spinco model L-2 ultracentrifuge at 35,000 rev/min. The absorbance (260 nm) and radioactivity of each fraction were determined. The absorbance was mainly due to the excess of ribosomal material derived from normal cells. (●) Absorbance (260 nm); (⊙) radioactivity.*

amount of RNA made in treated cells was reduced (Fig. 4A and B). Analysis of the fate in the presence of AgSu of the RNA made prior to the addition of the drug revealed that, although normal species still existed (Fig. 4C and D), the amount of radioactivity present had decreased. This confirms the data of Fig. 2B, and it indicates also that the major species of RNA (except possibly 4 and 5*S* species) are degraded to the same extent.

Search for AgSu-DNA complex in AgSu-treated cells. The AgSu-DNA complex that is formed in vitro between AgSu and purified DNA is a very stable one which is not dissociated by high salt concentrations (24). Moreover, such complexes possess characteristic physical-chemical properties (24).

The physical-chemical characteristics of purified DNA derived from normal and AgSu-treated bacteria were compared by use of a culture of *P. aeruginosa* 686. The culture was brought to the exponential growth phase and divided into two parts. DNA was isolated from one half of the culture; the other half was exposed to AgSu (10 μg/ml) for 1.5 hr, whereupon its DNA was also isolated. For DNA from both the treated and the untreated cells, the T_m (midpoint of the thermal helix-coil transition profile) was 84 C, the density in cesium chloride was 1.727 g/cc, and the density in cesium sulfate was 1.432 g/cc. The sedimentation coefficients of the two DNA preparations differed slightly: 27.4*S* for the DNA from normal bacteria and 28.0*S* for the DNA from treated bacteria. Thus, the physical-chemical analyses of purified DNA derived from normal and AgSu-treated bacteria were indistinguishable by the criteria used. It is conceivable, however, that only a small portion of the cellular DNA was complexed with AgSu and that this escaped detection. To increase further the resolution, cellu-

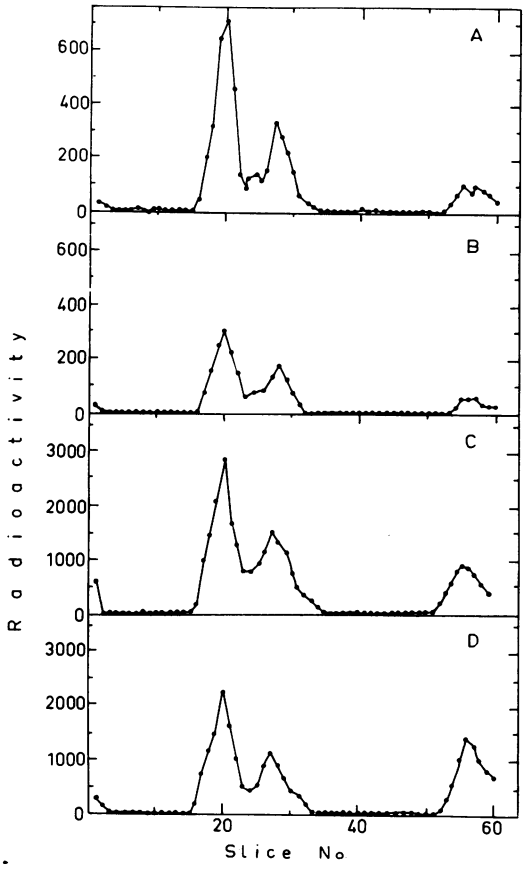

FIG. 4. *Effect of AgSu on cellular RNA. Bacteria in the exponential growth phase were exposed to AgSu (3 μg/ml) and 5-³H-uridine (5 μCi/ml) for 15 min, whereupon RNA was isolated and analyzed on acrylamide gels. (A) Control; (B) AgSu-treated. To study the fate of prelabeled RNA, bacteria were grown in the presence of 5-³H-uridine (0.5 μCi/ml) for several generations, after which they were washed and resuspended in fresh medium and exposed to AgSu (3 μg/ml) for 0.5 hr. (C) Control; (D) AgSu-treated.*

lar DNA was labeled with ^{3}H-thymidine prior to exposure to AgSu, and radioactive DNA was then subjected to fractionation procedures. (It was found, however, that ^{3}H-thymidine was not incorporated efficiently into *P. aeruginosa* DNA and that the presence of cold uridine to suppress a possible thymidine phosphorylase [4] did not increase the level of ^{3}H-thymidine incorporated. To overcome these difficulties a thymine-requiring strain of *P. aeruginosa* was used.)

It was thus found that by the criteria used (zonal centrifugation in sucrose [Fig. 5A] and density gradient centrifugation in cesium chloride [Fig. 5B] and in cesium sulfate [Fig. 5C]) that the DNA derived from AgSu-treated bacteria was identical to that from untreated bacteria. (After prolonged exposure to AgSu, some DNA degradation was observed [Fig. 5A].) The presence of material with properties identical to those of in vitro-formed AgSu-DNA complexes (i.e., increase in buoyant density in CsCl, decreased buoyant density in Cs_2SO_4, increased rate of sedimentation in gradient of sucrose) could not be detected by any of the procedures used.

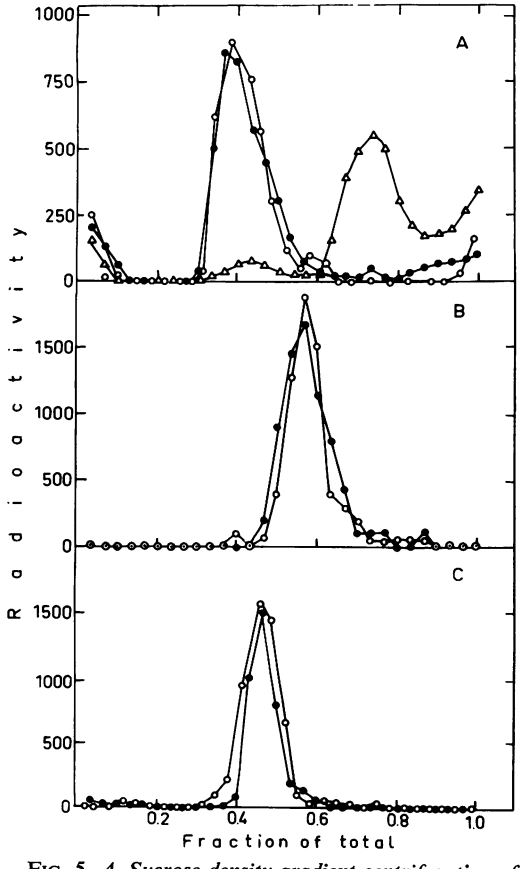

FIG. 5. *A, Sucrose density gradient centrifugation of DNA derived from normal and AgSu (5 μg/ml)-treated cells. (○) DNA from untreated cells; (● and △) DNA from cells exposed to AgSu for 1 and 3 hr, respectively. B, Cesium chloride density centrifugation of DNA from (○) normal and (●) AgSu-treated cells (5 μg/ml, 1 hr). C, Cesium sulfate density gradient centrifugation of DNA from (○) normal and (●) AgSu-treated cells (5 μg/ml, 1 hr).*

Effect of AgSu on DNA polymerase-deficient E. coli. It has been shown that bacterial mutants deficient in DNA polymerase (pol A$^-$) are more sensitive than the parent strain to agents that alter the cellular DNA (26). The agents shown to have such effects include radiation (7, 29), radiomimetic agents (7, 26), carcinogens (26), and compounds known to intercalate between DNA base pairs (18, 25, 27). Exposure of pol A$_1^-$ bacteria to AgSu did not reveal an increased lethal effect (Fig. 6), even though the effects of methyl methanesulfonate, an agent known to alkylate DNA, were reconfirmed. These results were confirmed by use of a bioassay on solid medium (Table 1).

Localization of radioactive AgSu. In a first series of experiments, growing cells were exposed to ^{110}AgSu for 1 hr, thoroughly washed, and lysed; the lysate was partitioned in a phenol-aqueous system. The radioactivity present in the various layers was determined. Most of the radioactivity was recovered (Table 2) at the interphase (i.e., in

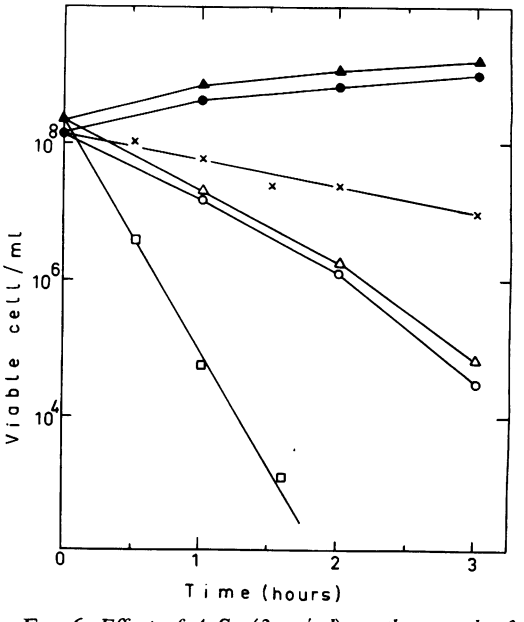

FIG. 6. *Effect of AgSu (3 μg/ml) on the growth of normal (E. coli W3110 thy$^-$ pol A$^+$) and DNA polymerase-deficient (E. coli p3478 pol A$_1^-$) bacteria. (●) E. coli pol A$^+$, untreated; (○) E. coli pol A$^+$ exposed to AgSu; (×) E. coli pol A$^+$ exposed to 0.01 M methyl methanesulfonate. (▲) E. coli pol A$_1^-$, untreated; (△) E. coli pol A$_1^-$ exposed to AgSu; (□) E. coli pol A$_1^-$ exposed to 0.01 M methyl methanesulfonate.*

TABLE 1. *Effects of various agents on the growth of a DNA polymerase-deficient strain*[a]

Agent	Amt	Size of zone of inhibition (mm)	
		Parent (*E. coli* W3110)	DNA polymerase-deficient (*E. coli* p3478)
Cycloserine	50 μg	62	62
Chloramphenicol	30 μg	30	30
Streptomycin	10 μg	26	26
Ampicillin	10 μg	29	28
Methyl methanesulfonate	10 μliter	44	60
Ethyl methanesulfonate	10 μliter	0	20
Nitrosomethylurea	0.5 μmole	42	79
Nitrosomethylurethane	0.5 μmole	20	87
Acridine orange	0.2 μmole	0	9
Proflavine	0.2 μmole	7	14
Ethidium bromide	0.2 μmole	0	20
Silver nitrate	10 μg	8	8
Silver sulfadiazine	10 μg	18	18
Sodium sulfadiazine	500 μg	3	3

[a] The procedure used was described in detail previously (26). Bacteria were grown in medium HA supplemented with thymine (5 μg/ml). Portions (0.1 ml) of the cultures were spread on the surface of agar plates of the same composition. When the surface of the agar had dried, sterile discs impregnated with various agents were deposited on the plates and these were incubated in the dark for 16 hr at 37 C, whereupon the diameter of the zones of inhibition was measured.

the proteinaceous layer). In a second series of experiments, cells were again grown in the presence of ^{110}AgSu, whereupon they were thoroughly washed and resuspended in 0.5 M sucrose. The cell wall was digested with lysozyme, and the suspension was centrifuged. Solubilized cell wall material was recovered in the supernatant fluid; the remainder of the cell (spheroplast) was in the pellet. The spheroplasts were lysed by dilution in tris(hydroxymethyl)aminomethane buffer. Upon centrifugation, RNA, DNA, and soluble proteins were recovered in the supernatant fluid whereas membranes were found in the pellet. The amount of ^{110}AgSu present in each fraction was determined, and it was thus found that most of the radioactivity was recovered in the membrane fraction (Table 3).

TABLE 2. *Distribution of radioactive silver sulfadiazine among cellular constituents*[a]

Phase	"Major" constituents	Percentage of radioactivity	
		Expt I	Expt II
Aqueous phase	DNA and RNA	2	1
Phenol layer	Polysaccharides	5	4
Interphase	Proteins	93	95

[a] Growing bacteria were exposed to ^{110}AgSu (25 μg/ml) for 1 hr. The cells were collected by centrifugation and washed twice with 0.01 M tris(hydroxymethyl)aminomethane buffer, *p*H 8.0. In experiment I, the cells were resuspended in the same buffer containing sodium ethylenediaminetetraacetic acid (2 mg/ml) supplemented with lysozyme, and then sodium dodecyl sulfate (SDS) was added to complete lysis. In experiment II, lysozyme was omitted and lysis was induced by SDS. The lysates received an equal volume of aqueous phenol, and after shaking the phases were separated. The radioactivity in each of the phases was determined and recoveries were calculated.

TABLE 3. *Localization of radioactive silver sulfadiazine in treated bacteria*[a]

Material	Percent radioactivity	
	Expt I[b]	Expt II
Cell wall	ca. 15[b]	26
Membrane	ca. 85	76
Soluble fraction (RNA, DNA, etc.)	0	<1

[a] Bacteria in the exponential growth phase were exposed to ^{110}AgSu (25 μg/ml) for 1 hr and were then washed with 0.5 M sucrose in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.0 (ST) and resuspended in ST. The suspension was supplemented with sodium ethylenediaminetetraacetic acid (200 μg/ml) and lysozyme (100 μg/ml) and incubated at 37 C for 1.5 hr. (Conversion to spheroplasts was ascertained microscopically.) The spheroplasts were collected by centrifugation; the supernatant fluid contained solubilized cell wall material. The spheroplasts (pellet) were lysed by resuspension in 0.01 M Tris buffer, *p*H 8.0 (free from sucrose), and disrupted membranes were collected by centrifugation. The supernatant fluid contained "soluble" RNA, DNA, and proteins. The radioactivity of each fraction was determined and recoveries were calculated.

[b] In experiment I, owing to technical reasons, the amount of radioactivity present in the cell wall fraction could be determined only by difference from the cumulative recoveries of the other two fractions.

DISCUSSION

Although AgSu binds to purified DNA (24), the present findings indicate that no such binding occurred when living bacteria were exposed to AgSu. As a matter of fact, the principal evidence suggests that DNA is not primarily involved in the action of AgSu. Thus, bacteria (pol A^-) more sensitive to agents which alter cellular DNA did not show enhanced sensitivities towards AgSu. DNA isolated from treated cells did not exhibit any of the properties associated with the AgSu-DNA complex, nor did radioactive AgSu bind to the DNA fraction. Unbalanced growth, which is characteristic of agents that preferentially block DNA synthesis, was not observed. Most of the present data indicate that AgSu was bound to the cell membrane fraction with some AgSu bound to cell wall material (Table 3). It is known that the cell membrane plays a crucial role in controlling DNA and RNA synthesis. It is not surprising, therefore, that an agent which affects the cell membrane should also cause a halt in the synthesis of DNA and RNA (and subsequently proteins). The exact chemical nature of the AgSu-induced modification of the external cell structure is currently under investigation. A subsequent report (Coward, Carr, and Rosenkranz, *in preparation*) will offer ultrastructural evidence in support of an effect of AgSu on the cell envelope.

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