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BINDING OF SILVER SULFADIAZINE TO THE CELLULAR COMPONENTS OF *PSEUDOMONAS AERUGINOSA**

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Abstract—The mechanism of action of silver sulfadiazine (AgSD) against *Pseudomonas aeruginosa* was investigated by use of the isotopes, ^{110}Ag and ^{35}S. AgSD was found to dissociate and only the silver moiety was bound to the cells; no cellular binding of sulfadiazine was detected. Silver was bound in considerable amounts, mainly in the fraction containing the cell proteins and carbohydrates. The DNA of log phase cells showed a degree of binding of silver which varied with time of incubation. The DNA-bound silver increased to a peak value during inhibition of growth and then declined to a low level. Growth did not occur until the Ag/DNA ratio had fallen below a certain level. The data suggest that inhibition of bacterial growth results from interference of DNA function by binding of silver ions along the helical chain.

THE EFFICACY of the silver salt of sulfadiazine (AgSD) as a topical agent to control bacterial infection, especially after burns, has been reported previously[1–6] and has been confirmed by others in the laboratory and in clinical practice.[7–10] A tentative hypothesis as to the mechanism of action, based on limited observation, has been advanced.[3] We will report here our further efforts, based on a more systematic attack on the problem.

Since *Pseudomonas aeruginosa* is the most common organism in burn wound infections, it has been used in our studies. The inhibition of its growth in culture medium by AgSD has been studied by use of the radioactive tracers ^{110m}Ag and ^{35}S.

MATERIALS AND METHODS

Radioactive silver

The isotope, silver-110m, was obtained from the International Chemical & Nuclear Corp. in the form of $AgNO_3$. This was reacted with sodium sulfadiazine (NaSD) to give $^{110m}AgSD$, which was obtained as a white powder with a specific activity of 1·14 mCi/mmole. In all comparison studies, the $^{110m}AgNO_3$ was diluted to the same activity.

Radioactive sulfadiazine

This was kindly supplied by Dr. Fred Williams of the Radiological Services Group of Iowa State University as $Na^{35}SD$. The $Ag^{35}SD$ prepared from it had a specific activity of 1·0 mCi/mmole.

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Silver sulfadiazine

Lot No. 5178 micronized powder was generously provided by Marion Laboratories.

Pseudomonas aeruginosa strains

The strain employed in most of our experiments has been used in our previous animal studies and is highly virulent to both rats and mice. It was originally obtained from Dr. Donald P. Dressler of Boston with the designation WHTG No. 2. As a confirmatory check, we also employed a Pseudomonas strain (No. 686) provided by Dr. Herbert Rosenkranz of this institution. The results obtained with these two strains were similar in all respects.

Incorporation of radioactive AgSD into pseudomonas cells

$^{110m}AgSD$. Cultures of pseudomonas were grown in nutrient broth and used in the early log phase, i.e. at an optical density near 0·2 (range 0·15–0·23), measured at 540 nm. Usually 50-ml or 100-ml aliquots of such cultures were added to 500-ml Erlenmeyer flasks containing the desired amount of AgSD or $AgNO_3$. The flasks were then incubated at 37° on a rotary shaker and removed at desired intervals. Cell growth was followed by optical density readings at 540 nm with a Bausch & Lomb Spectronic 20. All experiments were performed in duplicate. Bactericidal effect was determined by explants on agar plates, as described in a previous report.[6]

$Ag^{35}SD$. Early log phase cultures at an optical density near 0·25 were added in 30-ml aliquots into 250-ml Erlenmeyer flasks containing the required amounts of $Ag^{35}SD$, and incubated for 2 hr at 37°. Control flasks of $Ag^{35}SD$ in nutrient broth were set up simultaneously.

Separation of the cell mass

After incubation, the broth culture was centrifuged at 7000 rev/min for 10 min to separate the cells from the nutrient broth. The sediment was resuspended in 2 M sucrose and centrifuged at 5000 rev/min for 5 min to sediment precipitated silver salts, the bacteria remaining in the heavy sucrose supernatant. This supernatant was then made 1 M to sucrose by dilution, and centrifuged at 7000 rev/min for 10 min. The bacterial cells were collected in the sediment, while any dissolved silver salts were left in the supernatant. All centrifugations were carried out with a Sorvall type SS-1 instrument.

Extraction of cell components

The centrifuged cell mass was fractionated according to the method of Ogur and Rosen.[11] The DNA extraction, being of special interest, was also carried out on another aliquot of the same cell mass by the method of Marmur.[12] For equilibrum dialysis studies requiring the intact nucleic acids, RNA was extracted by the method of Adesnik and Levinthal,[13] while the Marmur[12] method was used for DNA. The cell residue was prepared by removing the lipids with ether–alcohol, and the nucleic acids by hot 10% NaCl extraction. The purity of the isolated DNA was checked by measure of the 260/280 nm ratio, repeating the chloroform–isoamyl alcohol extraction until the ratio exceeded 1·9. The DNA was estimated by the diphenylamine reaction.[14]

Measurement of radioactivity

The gamma emission of ^{110m}Ag was determined by a well-type scintillation detector with a 1 in dia (thallium-activated) sodium iodide crystal, connected to a Nuclear Chicago 720 series counter. The beta emission of ^{35}S was measured using Aquasol scintillator in a Nuclear Chicago 720 series liquid scintillation counter.

RESULTS

The growth curves of *P. aeruginosa* shown in Fig. 1 reveal the inhibitory effect of AgSD at various concentrations. In the initial 4 hr studied, increasing concentrations of AgSD cause a corresponding increase in inhibition, with complete inhibition being attained at a concentration of 0·05 μmole/ml. Concentrations up to 0·05 μmole/ml are bacteriostatic, while at 0·2 μmole/ml a bactericidal concentration is reached. Three

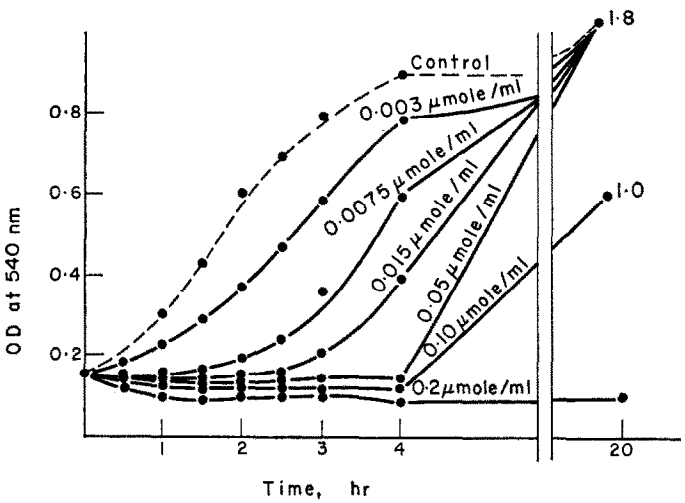

FIG. 1. Effect of silver sulfadiazine (AgSD) on the growth of *P. aeruginosa*. Early log phase cultures at an O.D. of 0·15 were added to tubes containing the concentrations of AgSD. These were incubated at 37° on a shaker and the O.D. was measured hourly and after 20 hr. Drug concentrations of 0·10 μmole/ml or below were bacteriostatic; the concentration of 0·2 μmole/ml was bactericidal.

concentrations were chosen for subsequent work: 0·0075, 0·015 and 0·05 μmole/ml, representing mild, moderate and complete inhibition respectively. The uptake of silver from AgSD by the pseudomonas cells during incubation, determined by the amount of isotopic ^{110m}Ag bound to cells, is shown in Fig. 2a with the corresponding effect on growth (as indicated by optical density) shown in Fig. 2b. The amount of silver absorbed reaches in 2 hr a constant level whose value depends on the drug concentration. Figure 3 depicts the results obtained after isolation and fractionation of the pseudomonas cells, followed by counting the radioactivity of the cell components. Practically all of the the silver was found in the DNA, RNA and cell residue; the total lipid fraction extracted by ether–alcohol had less than 0·5 per cent. The maximum activity was always found associated with the cell residue, consisting primarily of proteins and polysaccharides. This activity reached its maximum during 1–2 hr and

then remained almost stable. Even after growth resumed, there was no loss of radioactivity from this residue when examined at 20 hr.

It will be observed that the RNA fraction acquired about 3 per cent of the silver at the concentration causing complete inhibition; the silver present was almost negligible at the lower concentrations of AgSD. Of more interest was the activity in the DNA fractions, which attained a maximum within 2 hr and receded with further incubation. These peak values were 3, 4·8 and 12 per cent of the total silver absorbed under the conditions of mild, moderate and complete inhibition respectively. Further data relating the silver-binding of the DNA with the growth of the culture, are presented in Table 1. The peak values here were respectively 4·3, 7·6 and 13·6, expressed as the

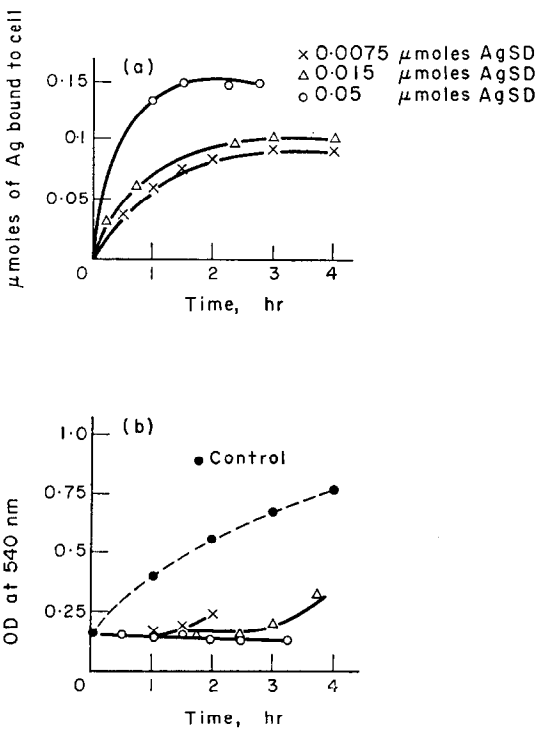

FIG. 2. Early log phase cultures of *P. aeruginosa* were incubated with each of three concentrations of ^{110m}AgSD. At hourly intervals 50-ml aliquots of each culture (containing approximately 10 mg dry wt) were removed, the cell mass was isolated by centrifugation, and freed from unbound silver by recentrifugation in sucrose (see Materials and Methods). The amount of radioactivity associated with the cell mass was taken as a measure of silver binding. The O.D. of each culture is shown in (b).

binding ratio of silver to DNA (μmoles/100 mg). Growth did not resume until the Ag/DNA ratio fell to around 2, with more time required for the higher ratios to fall to this value. After 20 hr, when the cells had completely overcome the bacteriostatic effect, the binding ratio was about 0·10.

Results at higher concentrations and with a different strain of pseudomonas are shown in Table 2. At an AgSD concentration of 0·1 μmole/ml, growth had resumed

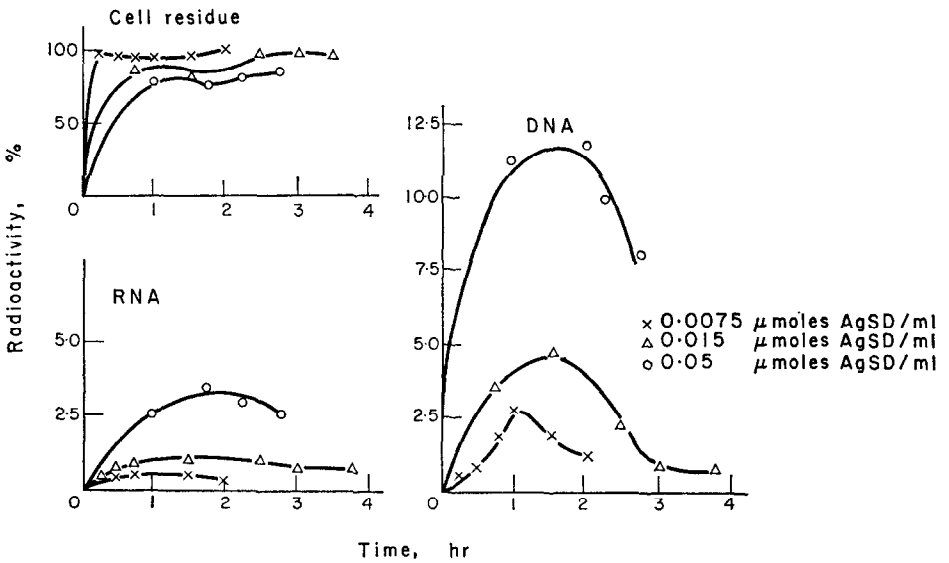

FIG. 3. Binding of silver to the cellular components. Early log-phase cultures of *P. aeruginosa* were incubated with each of three concentrations of ^{110m}AgSD. At hourly intervals 50-ml aliquots were removed from each culture, the cells were fractionated and the radioactivity was measured. The radioactivity is expressed as the percentage of the activity in the whole cells.

TABLE 1. BINDING OF SILVER TO DNA*

	Concn of AgSD used (μmoles/ml)									
	0 (Control)	0·0075			0·015			0·05		
			Binding ratios			Binding ratios			Binding ratios	
Time (min)	Growth (O.D.)	Growth (O.D.)	Ag/ DNA	Base pairs/ Ag	Growth (O.D.)	Ag/ DNA	Base pairs/ Ag	Growth (O.D.)	Ag/ DNA	Base pairs/ Ag
0	0·15	0·15			0·15			0·16		
15		0·15	0·63	254						
30	0·25	0·15	0·63	254						
45		0·15	2·64	61	0·15	4·6	35			
60	0·32	0·15	4·30	37				0·14	12	13
90	0·45	0·19	1·9	84	0·15	7·6	21			
105								0·12	13·6	12
120	0·54	0·25	0·85	190						
135								0·12	10·3	16
150	0·62				0·15	4·6	35			
165								0·12	6·1	26
180	0·75				0·21	2·0	80			
210										
225					0·38	0·79	200			
1200	1·9	1·8	0·11	1500	1·85	0·105	1500	1·8	0·09	1800

* Early log phase cultures at O.D. of 0·15 were grown in the presence of the indicated concentrations of ^{110m}AgSD. The DNA was isolated by Marmur's procedure[12] and the associated radioactivity measured. The DNA base pairs/Ag ratio was calculated on the basis that 100 mg DNA corresponds to 160 μmoles base pairs, assuming the phosphorous content of DNA to be 10 per cent and 2 phosphorous atoms per base pair. Ag/DNA = μmoles/100 mg) and base pairs/Ag = molar ratio.

TABLE 2. BINDING OF SILVER TO DNA AT HIGHER CONCENTRATIONS OF AgSD*

Concn of AgSD (μmoles/ml)	Time of incubation (min)					
	75			1200		
	Growth (O.D.)	Binding ratios Ag/DNA	Base pairs/Ag	Growth (O.D.)	Binding ratios Ag/DNA	Base pairs/Ag
0·1	0·17	20	8	0·6	0·9	180
0·25	0·15	36	5·0	0·15	15·6	11
0·50	0·15	54·5	3·0	0·15	18·6	9

* Early log phase cultures (O.D. = 0·18) were grown in presence of the indicated concentrations of ^{110m}AgSD (aqueous suspension); the DNA was isolated by Marmur's procedure[12] and the associated radioactivity measured. The DNA base pairs/Ag ratio was calculated on the basis that 100 mg DNA corresponds to 160 μmoles base pairs, assuming the phosphorus content of DNA to be 10 per cent and 2 phosphorus atoms per base pair. The Pseudomonas strain used was No. 686.

after 20 hr of incubation, the Ag/DNA ratio having fallen to a value of 0·9 from a value of 20 at 75 min. At a concentration of 0·25 μmole/ml, there was no resumption of growth at 20 hr, although the ratio had fallen to 15·6 from a 75-min value of 36; the results at a concentration of 0·5 μmole/ml were similar, but with somewhat higher binding ratios. (Aqueous suspensions of AgSD were used in the experiment of Table 2, as the amount of ammonia required to effect solution would increase the pH above acceptable limits. The optical density of these cultures was read against corresponding concentrations of AgSD in broth.)

The relative affinity of silver for the three cell fractions was measured by placing equal weights of each fraction into dialysis bags and dialyzing for 20 hr against an ammoniacal solution of ^{110m}AgSD. The DNA showed 10 times more affinity than the cell residue and 40 times more affinity than RNA (Table 3).

TABLE 3. RELATIVE AFFINITY OF AgSD TO DNA, RNA AND CELL RESIDUE PREPARED FROM *P. aeruginosa**

Macromolecule	(μmoles Ag/100 mg)
RNA	1·1
DNA	40·0
Cell residue	4·5

* Dialyzing bags were presoaked in a solution of radioactive AgSD (1·0 μmole/ml). Dialysis of 3 ml of the above macromolecules at a concentration of 20 μg/ml was carried out for 20 hr against 100 ml of 1·0 μmole/ml of ^{110m}AgSD in ammoniacal solution. The radioactivity inside and outside the bag was measured after the dialysis. The DNA content inside the bag after dialysis was estimated by the diphenylamine reaction.

The dissociation of AgSD and the role played by the sulfadiazine (SD) moiety in the process of growth inhibition were investigated in a series of experiments. In the first of these, the effect of sulfadiazine was tested by direct comparison of AgSD and $AgNO_3$ as inhibitors of bacterial growth. There was no apparent difference in their effect at the mild and complete inhibitory concentrations, either in growth or in the Ag/DNA binding ratio (Table 4). The minimum inhibitory concentration (MIC) for sulfadiazine was found to be 2·0 μmoles/ml, while it was only 0·005 μmole/ml for each of the two silver salts (Table 5); further, the addition of an equimolar concentration of *p*-aminobenzoic acid to SD and AgSD had no effect on the latter, while completely removing the inhibition by the sulfadiazine.

TABLE 4. COMPARISON OF GROWTH AND SILVER BINDING TO DNA BY AgSD AND $AgNO_3$*

	Concn (0·015 μmole/ml)				Concn (0·05 μmole/ml)			
	AgSD		$AgNO_3$		AgSD		$AgNO_3$	
Time (hr)	(O.D.)	(μmoles Ag/100 mg DNA)	(O.D.)	(μmoles Ag/100 mg DNA)	(O.D.)	(μmoles Ag/100 mg DNA)	(O.D.)	(μmoles Ag/100 mg DNA)
0	0·17		0·17		0·17		0·17	
1	0·17	3·3	0·17	2·0	0·17	15·0	0·17	14·0
2	0·17	9·0	0·17	7·0	0·17	13·8	0·17	12·0
4	0·19	2·1	0·2	1·9	0·17	9·7	0·17	9·0
20	2·0	0·02	2·0	0·07	1·5	0·07	1·5	0·1

* Early log phase cultures (O.D. = 0·17) were grown in the presence of the indicated concentrations of ^{110m}AgSD and $^{110m}AgNO_3$ (ammoniacal solution). The DNA was isolated by Marmur's procedure[12] and the associated radioactivity measured.

TABLE 5. COMPARATIVE ACTION *in vitro* OF SULFADIAZINE AND SILVER COMPOUNDS

Drug tested	MIC* (μmoles/ml)
Sulfadiazine	2·0
Silver sulfadiazine	0·005
Silver nitrate	0·005
Sulfadiazine + *p*-aminobenzoic acid†	No inhibition
Silver sulfadiazine + *p*-aminobenzoic acid†	No growth

* MIC = minimum inhibitory concentration. The inoculum was 0·1 ml of a 1/1000 dilution of a overnight broth culture in 5 ml broth containing the drugs. The endpoint for silver compounds was the lowest concentration of drug showing no turbidity after 24 hr of incubation and no growth on subculture; sulfadiazine was bacteriostatic only.

† *p*-Aminobenzoic acid was added in equimolar concentration to SD to AgSD.

Whether it is AgSD or silver alone which is being bound to the cells was investigated in another experiment. Here the uptake of sulfadiazine by log phase pseudomonas

cells was measured directly by use of aqueous AgSD labeled with ^{35}S. Three concentrations were used, and similar cultures containing ^{110m}AgSD were set up for a comparison. The results, shown in Table 6, indicate a negligible amount of sulfadiazine in the pseudomonas cells after a 2-hr incubation. The sulfadiazine uptake was only 0·0003 μmole/ml at each concentration, while that of the corresponding silver moiety was 0·0053 to 0·0203 μmole/ml.

TABLE 6. DISTRIBUTION OF SULFADIAZINE IN THE SUPERNATANT AND CELL MASS OF CULTURES INCUBATED WITH AQUEOUS Ag^{35}SD AND COMPARISON WITH ^{110m}AgSD*

		Sulfadiazine measured by ^{35}S (μmoles/ml)			Silver measured by ^{110m}Ag (μmoles/ml)
AgSD in culture (μmoles/ml)	Growth in 2 hr (O.D.)	Initial supernatant	Initial residue	Second supernatant (cell mass)†	Cell mass in sucrose†
0	0·90				
0·027	0·21	0·026	0·0008	0·0002	0·0053
0·062	0·20	0·060	0·0017	0·0003	0·0083
0·109	0·19	0·107	0·002	0·0003	0·0203

* Aliquots, 30 ml, of early log phase culture (O.D. = 0·25) were incubated with the indicated concentrations of Ag^{35}SD and ^{110m}AgSD separately for 2 hr. The cultures were fractionated by two centrifugations and the radioactivity was measured in each fraction.

† The initial residue was suspended in 2 M sucrose and recentrifuged, leaving the cell mass in the sucrose supernatant.

Mention will now be made of some crucial problems in laboratory technique.

Insolubility of AgSD. At room temperature, only 0·5 μmole AgSD dissolves in 100 ml water and 0·75 to 0·8 μmole in 100 ml nutrient broth. For obvious reasons, some method of solubilization was desired which would not affect the processes to be studied. Our procedure was to dissolve 100 μmoles AgSD in 0·5 to 0·75 ml of 28% ammonia and then dilute to the desired concentration. When added to broth in a final concentration of 0·1 μmole/ml, this caused an increase of pH from 6·5 to 7·0; however, addition of this amount of ammonia to control cultures had no effect on growth rate. There remained the question of whether ammonia would in itself influence silver uptake by cells.

An experiment was carried out to compare the effect of ammoniacal and aqueous AgSD on the binding of silver to pseudomonas cells incubated 90 min in broth containing four concentrations of ^{110m}AgSD. The results, in Fig. 4, show that the silver binding is slightly increased by the ammoniacal solutions. In a similar experiment the cells were fractionated and the binding ratio of silver to DNA was measured (Table 7). In both experiments, with concentrations above the aqueous solubility zone, the ammoniacal AgSD always produced more silver binding. The results with RNA and the cell residue were similar (not shown). At the maximum concentrations used, the cells grown in the ammoniacal culture had a DNA base pairs/Ag ratio of 2–4. The mixing of AgSD and DNA *in vitro* gave a saturation ratio of 2·8 (Table 8).

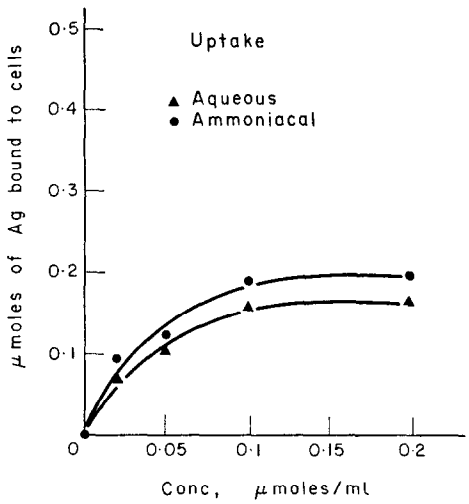

FIG. 4. Comparison of effect of aqueous and ammoniacal AgSD. Early log phase cultures, 50 ml, of *P. aeruginosa* (10 mg dry wt) were incubated at 37° with four concentrations of aqueous and ammoniacal ^{110m}AgSD. The cultures were shaken for 90 min and then removed for determination of radioactivity.

TABLE 7. BINDING OF SILVER TO DNA—COMPARISON OF AMMONIACAL AND AQUEOUS*

Concn of AgSD (μmoles/ml)	Ammoniacal AgSD (μmoles/100 mg)			Aqueous AgSD (μmoles/100 mg)		
		Binding ratios			Binding ratios	
	Growth (O.D.)	Ag/DNA	DNA base pairs/Ag	Growth (O.D.)	Ag/DNA	DNA base pairs/Ag
0·0075	0·20	3·5	46	0·20	3·2	50
0·02	0·18	6·2	26	0·19	3·9	41
0·05	0·17	13·8	12	0·18	8·2	20
0·1	0·17	36·0	4·5	0·18	12·6	13
0·19	0·16	70·0	2·3	0·17	27·0	6

* Cultures with an initial O.D. of 0·20 were grown for 19 min in the presence of the drug, the DNA was isolated, and the radioactivity associated was measured.

TABLE 8. BINDING OF ^{110m}Ag TO DNA BY EQUILIBRIUM DIALYSIS

AgSD in solution outside the bag* (μmoles/ml)	Binding ratios	
	(μmoles Ag/ 100 mg DNA)	(DNA base pairs/Ag)†
0·01	7	23
0·05	29	6
0·25	56	3
0·50	54	3

* The AgSD was in 100 ml ammoniacal solution. The dialysis bags contained 60 μg *E. coli* DNA in 3 ml water.

† The DNA base pairs/Ag ratio was calculated on the basis that 100 mg DNA corresponds to 160 μmoles base pairs, assuming the phosphorous content of DNA to be 10 per cent and 2 phosphorous atoms per base pair.

Since the inhibition by the ammonia-dissolved AgSD was only temporary, like that of aqueous AgSD, and since the only difference observed was the expected increase in silver uptake from the ammoniacal solution, we felt that no alteration in normal cell function resulted from use of ammonia.

Centrifugation in sucrose solution. When the cell mass is spun down after incubation, an uncertainty arises as to whether particulate AgSD (suspended rather than dissolved) may also precipitate in the case of aqueous solutions; also there is the possibility of silver precipitation by reaction with broth components, e.g. chlorides, in either the aqueous or ammoniacal solutions. To prevent this difficulty, the cell mass sedimented by an initial centrifugation was resuspended in 10 ml of 2 M aqueous sucrose and recentrifuged at 5000 rev/min for 5 min. The intention was to spin down any insoluble or precipitated silver compounds while the cells remained suspended in the dense sucrose solution. After careful decantation of the cell suspension and dilution with water to a sucrose concentration of 1 M, the bacteria were isolated by centrifugation at 7000 rev/min for 10 min. This use of sucrose raises questions as to whether (1) an effective separation is actually obtained and (2) whether sucrose may play a role in introducing silver into the cell by, for example, an effect on the cell membrane. This we attempted to answer by the experiment whose results are shown in Table 9. Aqueous AgSD solutions were used, as these gave a maximum test of the separation. Comparison of the sediments C and E in the three "Control" columns shows only a slight percentage of counts remaining in the 2 M sucrose (D), about half of which

TABLE 9. DISTRIBUTION OF ^{110m}Ag IN THE STAGES OF SEPARATION BY CENTRIFUGATION IN SUCROSE SOLUTION*

	Concn of AgSD (μmoles/ml)					
	0·02		0·05		0·10	
Fractions	Test†	Control	Test	Control	Test	Control
A	100	100	100	100	100	100
B	54	90	54	41·5	51·5	36·3
C	46	10	46	58·5	48·5	63·7
D	21·3	0·8	21·8	1·3	21·6	0·8
E	24·7	9·2	24·2	57·2	26·9	62·9
F	0·6	0·37	0·9	0·43	1·2	0·41
G	20·0	0·43	21·0	0·80	20·3	0·35

* Radioactivity is expressed as the per cent of activity initially in the culture (A). The fractionation procedure is represented in the diagram below:

Culture or Broth → Centrifuge at 7000 rev/min for 10 min (A) → Supt (B) / Sediment (C) (precipitated ^{110}Ag salts + bacteria) → Centrifuge in 2 M sucrose at 5000 rev/min for 5 min → Supt (D) (bacteria in 2 M sucrose) / Sediment (E) (precipitated ^{110}Ag salts) → Centrifuge in 1 M sucrose at 7000 rev/min for 10 min. → Supt (F) (dissolved ^{110m}Ag salts in 1 M sucrose / Sediment (G) (bacteria)

† Test: culture incubated with AgSD Control: broth innoculated with AgSD.

remains in supernatant (F) after centrifugation of the 1 M sucrose (representing dissolved silver salts). Thus the separation appears reasonably effective.

The three control values of the F supernatant average about 0·4 per cent. This represents the silver that would be available for introduction into the cell by hypothesized membrane damage, but it cannot account for the silver uptake observed for DNA. The DNA accounts for 10 per cent or more of the total cell count (at AgSD concentrations of 0·05 μmole/ml or above) and, since the cells absorb 20 per cent of the total isotope count, should account for 2 per cent or more of the counts added initially. It seems most unlikely that the intracellular isotope count observed could be an artifact of the sucrose centrifugation, since the amount of isotope available during this operation is insufficient.

Extraction of DNA. In the separation of DNA by the method of Ogur and Rosen,[11] performed by extraction at 70° with perchloric acid (PCA), the temperature was found to be critical. Even a slight elevation caused some release of silver from other cell macromolecules (proteins). If the temperature was carefully maintained at or slightly below 70° (65–70°), it was found that the cell residue, which contains more than 90 per cent of the cell radioactivity after the first PCA extraction (which removes all the DNA), lost less than 1 per cent of this activity by a second PCA extraction. To establish further the validity of this method in presence of silver, labeled pseudomonas cell residues were prepared *in vitro* by suspension of separated residues in broth and incubation with ^{110m}AgSD. Subjection to the 70° PCA extraction removed less than 1 per cent of the isotope. It was concluded, therefore, that the radioactivity found in the PCA extract represents silver bound to DNA, without any silver bound to other macromolecules and subsequently released by acid treatment.

Feeling that a confirmation by another method was desirable, we performed the conventional phenol extraction, followed by precipitation with alcohol. It was then observed that phenol picked up a large quantity (70 per cent) of the silver bound to DNA (Table 10). This loss of silver into buffer-saturated phenol was reduced to a 40 per cent loss by use of water-saturated phenol, but we abandoned further efforts with this procedure in favor of Marmur's method,[12] modifying it to exclude all the buffers. Over 95 per cent of the silver-bound DNA was extracted by this method.

TABLE 10. LOSS OF RADIOACTIVITY DURING EXTRACTION OF DNA

	Extraction of DNA*		Extraction of DNA from bacterial cells†	
Method of extraction	^{110m}Ag (cpm/mg DNA)	(% of original)	^{110m}Ag (cpm/mg DNA)	(% of original)
Tris–EDTA-saturated phenol	7050	30		
H_2O-saturated phenol	14,100	60	6520	40
Marmur[12]	22,300	95	16,300	95
Ogur and Rosen[11]			17,600	102

* DNA isolated from Pseudomonas was incubated with ammoniacal ^{110m}AgSD. The ^{110m}Ag-DNA precipitated with alcohol contained 23,500 cpm/mg and was taken at 100 per cent.

† *P. aeruginosa* were grown in the presence of ^{110m}AgSD and the DNA was isolated by these extraction procedures. The value obtained by Marmur's method[12] was used as a standard for comparison.

In our modification the cells were broken by sodium dodecyl sulfate in aqueous solution and, after addition of sodium perchlorate to a final concentration of 1 M, were extracted with chloroform–isoamyl alcohol. The loss of ^{110m}Ag after perchlorate treatment was less than 1 per cent, and the DNA values obtained were in good agreement with those of the Ogur and Rosen[11] procedure (Table 10).

Inasmuch as the lipid and RNA fractions contained negligible amounts of radioactivity, these were not processed further.

DISCUSSION

The silver ion appears to be of central importance in the antibacterial effect of AgSD, at least on the pseudomonas cell and presumably on others, for the following reasons: (1) AgSD dissociates in the culture medium and only silver is bound to the cells—no binding of sulfadiazine occurs; (2) the antibacterial effect of AgSD *in vitro* against various organisms is practically the same as that of $AgNO_3$; and (3) while the MIC of AgSD is near or identical to that of $AgNO_3$ for most organisms tested, the MIC of sulfadiazine is considerably (200 ×) higher.

The absorption of silver by the cellular proteins and polysaccharides (called the cell residue in our analytical scheme) is apparently of minimal concern in the mechanism of inhibition, inasmuch as growth proceeds in spite of it. This bound silver remains practically constant during and after the inhibitory phase; and 20 hr later, after cell proliferation, the level is either unchanged or shows a slight increase. Evidence that the binding of silver to DNA is intimately connected to the inhibitory mechanism includes the following: (1) silver binds to DNA with an affinity that is 10 times that of the cell residue materials and 40 times that of RNA; (2) in the inhibitory period following addition of AgSD to a log phase culture, there is a rise in silver bound to DNA which proceeds to a peak value, then recedes—and growth does not begin until the bound silver has fallen to a certain level; (3) the similar effect of $AgNO_3$ on inhibition of growth also shows a strikingly similar rise and fall of DNA-bound silver in the period of inhibition; (4) after cell proliferation at 20 hr, the DNA-bound silver has fallen to a very small value, in contrast to the cell residue-bound silver; (5) at those higher concentrations of AgSD in which the DNA-bound silver has not fallen to a small value at 20 hr, no growth has occurred. This decline in DNA-bound silver cannot be due to a dilution effect, since the optical density is not increasing during the period of decline.

The possibility cannot yet be excluded that some other mechanism is involved, acting in conjunction with, or primary to, the binding of silver to DNA. For instance, there could be injury to the cell membrane followed by repair. Investigation of this possibility by cell leakage experiments is planned. However, the assumption of another mechanism as the primary cause of cell inhibition leaves unexplained the fall of silver-bound DNA which always precedes a resumption of growth. The percentage of the silver binding to the cell protein fraction remained constant or slightly increased during inhibition and growth. This suggests a lack of relation to the inhibition process.

Previously we have proposed a binding of silver *in vivo* to bacterial DNA[3] analogous to the binding *in vitro* investigated by Jensen and Davidson.[15] This seemed plausible in view of the absorption of isotopic silver by the cells and the breakdown of AgSD *in vitro* by both cells and DNA, with release of sulfadiazine.[3] The present studies support this view. If the Ag/DNA ratio is calculated to form a base pair/Ag ratio, this

latter figure varies from a value of about 3, obtained after binding *in vitro*, to a value of 1500 after cell proliferation. A decrease to about 80 base pairs per Ag atom permits growth to begin again. The lowest value obtained *in vivo* (corresponding to the peak Ag/DNA ratio) is very near the value obtained *in vitro*, which presumably represents the maximum for a particular concentration. (Compare Tables 1 and 7 with Table 8.) A plausible interpretation would be that inhibition results when numerous base pairs along the DNA helix are bound by silver; and when only a few base pairs are thus bound growth can occur.

The rise of DNA-bound silver to a peak value *in vivo*, followed by a decline to a low value agrees with the findings by Jensen and Davidson[15] that the silver ion complexing reaction is chemically and biologically reversible. That *P. aeruginosa* cells bind large amounts of labeled ions from either AgSD or $AgNO_3$ has been observed also by Lowbury *et al.*[7] and Ricketts *et al.*[16] After washing labeled cells with cold 0·1 N $AgNO_3$, they observed a sharp fall in radioactivity and concluded that silver from AgSD and $AgNO_3$ is bound reversibly. However, in growing cells, the possibility that silver-containing DNA segments are selectively cleaved by the repair mechanism of the cells is not ruled out.

It has previously been proposed by Waring[17] that some antimicrobial drugs may function by attachment to DNA, such as mitomycin and actinomycin. More recently the anti-tumor action of certain platinum compounds has been ascribed to binding of platinum by tumor cell DNA.[18,19]

The silver salts (or complexes) of many other biological compounds, e.g. sulfonamides, antibiotics, metabolites, anti-metabolites, have been prepared in this laboratory; in general they showed strong antibacterial action *in vitro* but proved ineffectual *in vivo*.[1,20] This indicates that the sulfadiazine moiety does play a valuable role. The specific nature of this role is not clear. One possibility is to localize the action of the drug to microbial cells. Another may relate to the extreme insolubility of silver sulfadiazine and the known ionization of the sulfadiazine.[21] Although most of the silver is unable to react with the chloride ions, etc., of body fluids, enough silver ions are available to produce a strong antibacterial effect. The correspondingly low level of sulfadiazine ions is innocuous.

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