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EFFECT OF INHIBITORS ON LYTIC ENZYME SYNTHESIS BY *BACILLUS SUBTILIS* R

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SUMMARY

8-aza-guanine has been shown to be a potent inhibitor of lytic enzyme synthesis in *B. subtilis* R. Its action is detectable within 5 min of addition to the system and is complete before incorporation of the analogue into the RNA fraction of the cells is appreciable. 8-aza-guanine was also found to be a potent inhibitor of general protein synthesis in the cell whether measured by the incorporation of L-[1-^{14}C]-leucine, L-[1-^{14}C]phenylalanine, DL-[1-^{14}C]alanine or [^{14}C]glycine. Cell wall synthesis, as measured by the incorporation of DL-[^{14}C]alanine into the egg-white lysozyme sensitive portion of heat-killed *B. subtilis* R, was not effected significantly by concentrations of the analogue causing inhibition of protein synthesis.

A method is suggested whereby the amino acid composition of small quantities of cell wall may be determined.

INTRODUCTION

It has been shown previously[1,2] that, during exponential growth in simple synthetic media, cultures of *Bacillus subtilis* R synthesise an extracellular lytic enzyme similar to egg-white lysozyme. As the extracellular lytic activity at any instant is a constant proportion of the total enzyme activity in the culture, the appearance of lytic activity in the growth medium may be used as a measure of enzyme synthesis. In the past a

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number of antibiotics and metabolite analogues have proved useful in the study of protein and nucleic acid synthesis in micro-organisms. It is well established that 8-aza-guanine causes a change from exponential to linear growth rate when added to cultures of susceptible bacteria[3,4] and that the analogue is incorporated into the ribose nucleic acid (RNA) of the cell in the place of guanine. CREASER[5] has shown that inducible β-galactosidase synthesis in *Staphylococcus aureus* can be inhibited by 8-aza-guanine and that the inhibition can be reversed by guanine, xanthine and hypoxanthine. Subsequently, SMITH AND MATTHEWS[3] and MANDEL AND MARKHAM[4] have shown that the aza-guanine is incorporated predominantly into the terminal positions of the RNA chains; furthermore the total amount of analogue incorporated is not directly related to the degree of inhibition of growth[6]. It is suggested that the primary inhibitory action of aza-guanine is upon some guanine-containing cofactor involved in protein synthesis. GALE AND FOLKES[7] have reported that protein synthesis by washed suspensions of *Staphylococcus aureus* can be inhibited by growth inhibitory concentrations of chloramphenicol. The synthesis of certain enzymes is equally sensitive[8] and it seems that this antibiotic acts as a powerful inhibitor of protein synthesis within the cell. Aureomycin seems, superficially, to be similar to chloramphenicol in its action[7]. Certain phenanthridines possess trypanocidal activity and NEWTON[9] has shown that one of these, ethidium bromide, is capable of inhibiting DNA synthesis in a protozoan, *Strigomonas oncopelti*. GALE AND FOLKES[10] have shown that this same compound inhibits the incorporation of radioactive glycine into preparations of disrupted *Staphylococcus aureus*. WOOLLEY[11,12] has synthesised several "aggregate analogues" of dimethylbenzimidazole and *p*-amino-benzoic acid which will inhibit the growth of a number of Gram-positive and Gram-negative bacteria. One of these compounds, DCDNS, is a powerful inhibitor of the incorporation of glycine in GALE's staphylococcal preparation[10]. A number of other analogues of dimethylbenzimidazole are active inhibitors of influenza virus multiplication[13]. Some also inhibit the incorporation of radioactive alanine into the protein of isolated thymus nuclei[14] and glycine into preparations of disrupted *Staphylococcus aureus*[10]. This paper describes the effect on lytic enzyme synthesis by *Bacillus subtilis* R of a number of antibiotics and metabolite analogues known to interfere with protein and nucleic acid metabolism.

METHODS AND MATERIALS

The organism, *Bacillus subtilis* R, the media, growth conditions, methods of estimating bacterial cell density and lytic enzyme synthesis used in these studies, together with the preparation of the "Total extractable" and "Extracellular" enzyme fractions have been described previously[1].

Cell fractionation. Cultures were normally fractionated with perchloric acid using a modification of the method reported by OGUR AND ROSEN[15]. In certain cases residual traces of perchloric acid interfered with subsequent steps and for these purposes cells were fractionated with trichloracetic acid (TCA).

Perchloric acid fractionation

2.0 ml of culture was mixed with an equal volume of cold 0.4 *N* $HClO_4$ and stored at 4° for 1 h. The resulting precipitate was removed on the centrifuge,

washed once with cold 0.2 *N* $HClO_4$ and then treated as follows to prepare the required fraction.

Total nucleic acid fraction. The precipitate obtained after washing with 0.2 *N* $HClO_4$ was treated for 20 min with two portions of 0.5 *N* $HClO_4$ at 80°. The extracts prepared were decanted after centrifuging, and when combined contained more than 95% of RNA of the cell together with about 90% of the DNA.

RNA fraction. RNA was extracted by suspending the precipitate obtained after washing with 0.2 *N* $HClO_4$ in 2.0 ml of *N* $HClO_4$ and storing at 4° overnight. This extract contained more than 90% of the total cell RNA (determined by repeated extraction of similar material with 5% TCA at 80°).

DNA fraction. DNA was estimated directly after extraction of RNA either on the residual cell material suspended in 1.0 ml distilled water or on an extract prepared with 0.5 *N* $HClO_4$ as described for the total nucleic acid extract (see above). As the RNA has already been removed by *N* $HClO_4$ treatment, the extract contains the cell DNA with only traces of RNA as contaminant.

"Protein + cell wall" fraction. The material remaining after the extraction of RNA and DNA contained the majority of the protein and cell wall material of the organism. Over 90% of the protein may be dissolved from the fraction by treatment overnight with *N* NH_4OH at room temperature.

Removal of perchloric acid from fractions. Large quantities of residual perchloric acid interfere with subsequent estimation of radioactivity and, therefore, for this purpose fractions were treated with KOH in the cold followed by removal of the precipitated $KClO_4$ in a centrifuge.

TCA fractionation

2.0 ml of the culture to be fractionated was mixed with an equal volume of cold 10% (w/v) TCA and allowed to stand for 3 h at 4°. The precipitate was removed on the centrifuge and washed once with cold 5% TCA.

Total nucleic acid fractionation. The material prepared in this way was extracted successively for 20 min with two portions of 5% (w/v) TCA at 80°. This treatment extracts all the RNA from the preparation together with more than 90% of the DNA.

"Protein + cell wall" fraction. The residue after removal of the nucleic acid contained most of the protein and cell wall material of the organism and is similar to the material remaining after extraction of nucleic acid by the perchloric acid method.

Removal of TCA. TCA was removed where required by extraction three times with equal volumes of ether; the ether layer was discarded.

Estimation of RNA and total nucleic acid. RNA and total nucleic acid were estimated in the relevant fractions by their specific absorption at 260 mμ in the Beckman Spectrophotometer, Model DU. A 1.0 mg/ml solution of pure RNA was assumed to have a specific extinction $(\log I_0/I)_{260} = 28$[16]. TCA was removed before estimation; perchloric acid does not interfere with estimations at 260 mμ.

Estimation of DNA. DNA was estimated as deoxyribose by the method of BURTON[17].

Hydrolysis of nucleic acid fractions. 0.1 *N* HCl, as described by MCQUILLEN AND ROBERTS[18] released free purine bases and pyrimidine nucleotides. *N* KOH as described by SMITH AND MATTHEWS[3], liberated purine and pyrimidine nucleotides. The KOH was removed by repeated precipitation with $HClO_4$ followed by chilling at 0° and

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removal of the precipitated $KClO_4$ on the centrifuge. This method does not, however, free the preparation from salt completely.

Chromatography. Purine bases and pyrimidine nucleotides prepared by 0.1 *N*-HCl hydrolysis were separated by chromatography in isopropanol–HCl[19]. Purine bases and pyrimidine nucleotides prepared by hydrolysis in *N* KOH were separated in *n*-butanol–ammonia[20]. Whatman No. 3 paper was used throughout.

Location of material on chromatograms: u.v.-absorbing material was located on chromatograms by the u.v. contact print method of MARKHAM AND SMITH[20]. Derivatives of aza-guanine were located by the fluorescence photography method as described by MATTHEWS[21].

Radioactive materials. Glycine (generally labelled), L-[1-^{14}C]leucine, L-[1-^{14}C]-phenylalanine, DL-[1-^{14}C]alanine and [8-^{14}C]adenine were supplied by the Radiochemical Centre, Amersham, Berkshire, England. The amino acids were used at a specific activity of 0.06 μC/μmole unless otherwise stated. [8-^{14}C]Adenine was used at a specific activity of 0.5 μC/μmole. 5.0 mμC of the glycine gave 587 counts/min in the equipment used.

Radioactive determinations were carried out as described by GALE AND FOLKES[7].

Inhibitors. The following compounds are referred to subsequently by the numbers succeeding them:

2,3-dichloro-5-(*p*-nitrobenzenesulphonamido)-benzene (I).
2,3-dimethyl-5-(*p*-nitrobenzenesulphonamido)-benzene (II).
p-nitrobenzenesulphonamide (III).
5,6-dimethyl-benzimidazole (IV).
5,6-dimethyl-1-β-D-ribofuranosyl-benzimidazole (V).
5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (VI).
4,5,6-trichloro-benzimidazole (VII).
4,5,6-trichloro-1-β-D-ribofuranosyl-benzimidazole (VIII).
4,5,6-trichloro-1-α-D-ribofuranosyl-benzimidazole (IX).

I am indebted to Dr. B. A. NEWTON for a gift of ethidium bromide (2,7 diamino-9-phenyl-10-ethyl-phenanthridinium bromide); to Dr. E. F. GALE, for gifts of DCDNS (2,3-dichloro-5-(*p*-nitrobenzene-sulphonamido)-6-nitro-benzene), 6-amino-4-hydroxy-benzimidazole and 6-amino-4-hydroxy-benzotriazole and Compounds IV to IX; and to Dr. J. D. SMITH, Molteno Institute for Parasitology, University of Cambridge, Cambridge, England, for a generous gift of 8-aza-guanine (AZA). Compounds I and II were synthesised by the method of WOOLLEY[11] by condensing *p*-nitrobenzene-sulphonylchloride (Kodak) with 3,4-dichloro-aniline and 3,4-dimethylaniline (both Lights) respectively. Crystalline egg-white lysozyme (Armour) was used throughout these experiments.

EXPERIMENTAL.

8-*Aza-guanine. Effect on lytic enzyme synthesis*

The addition of 20 μg/ml aza-guanine to cultures of *Bacillus subtilis* R growing exponentially in CGG medium[1] resulted in cessation of lytic enzyme synthesis and a change from exponential to linear growth. Fig. 1 shows that the synthesis of lytic enzyme in the culture ceased within 10 min of addition of the analogue although enzyme continued to accumulate in the growth medium until 90% was extracellular.

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The inhibitory effect of aza-guanine could be completely prevented by the addition of 2.0 μg/ml guanine or guanosine to the growth medium. The protection obtained in this way (Fig. 2) was complete only if the guanine was added within 5 min of the analogue; greater delays resulted in less complete protection.

GALE AND FOLKES[10] have reported that 6-amino-4-hydroxy-benzotriazole and

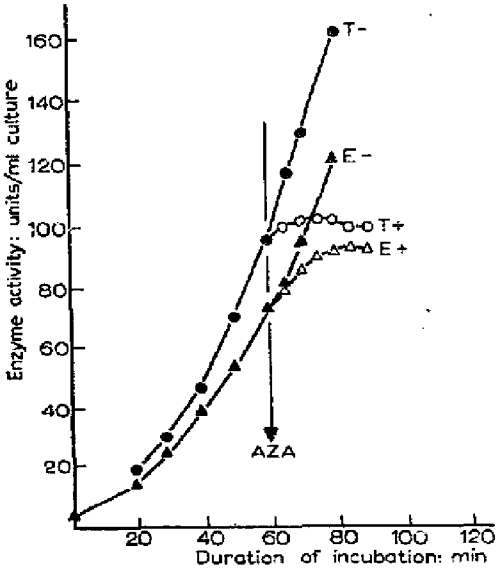

Fig. 1.

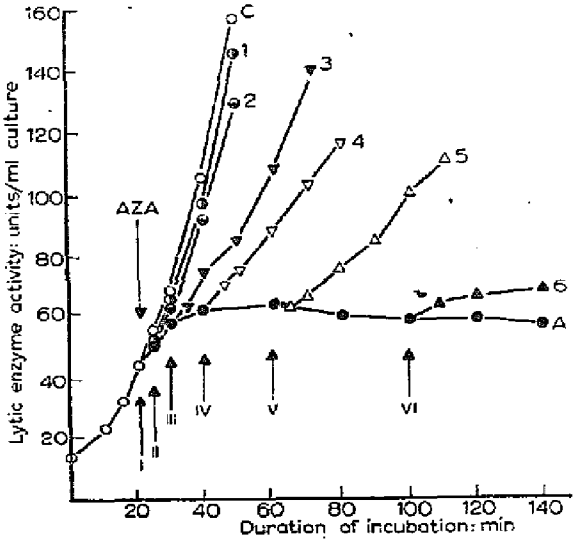

Fig. 2.

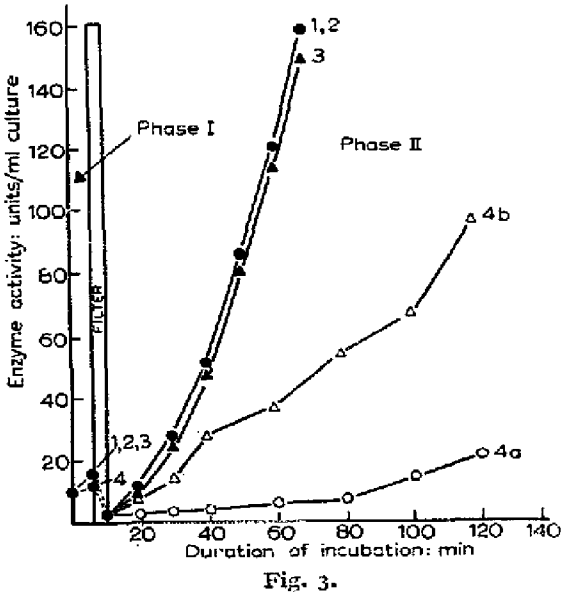

Fig. 3.

Fig. 1. Effect of aza-guanine on the appearance of "Total extractable" and "Extracellular" lytic activity. Growth medium: CGG. Aza-guanine (AZA) 20 μg/ml added at the point indicated. T, total extractable activity; E, extracellular activity: —, in absence; +, in presence of azaguanine.

Fig. 2. Effect of guanine in reversing inhibition (due to aza-guanine) of appearance of extracellular lytic activity. Growth medium: CGG. Aza-guanine (AZA) (20 μg/ml) added as indicated. Guanine (2 μg/ml) added at points i–vi. C = Control without aza-guanine or guanine; A = with aza-guanine but without guanine; 1–6 with aza-guanine, guanine added at points i–vi.

Fig. 3. Effect of brief treatment with aza-guanine on ability of cultures to synthesise lytic enzyme subsequently, in absence of the analogue. Growth media: No. 1; Phase I and II, CGG medium. No. 2; Phase I and II, CGG medium + guanine. No. 3; Phase I, CGG medium + guanine + aza-guanine, Phase II CGG medium. No. 4a, b; Phase I, CGG + aza-guanine; No. 4a; Phase II, CGG medium; No. 4b; Phase II, CGG medium + guanine. Aza-guanine: 20 μg/ml; guanine 2.0 μg/ml final concentration.

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6-amino-4-hydroxy-benzimidazole, both of which are structural analogues of guanine and aza-guanine, stimulate the incorporation of radioactive glycine into preparations of disrupted *Staphylococcus aureus*. Neither compound (at concentrations up to 100 μg/ml) was able to reverse the inhibition due to 20 μg/ml aza-guanine. To determine how quickly aza-guanine affects the synthesis of lytic enzyme, a culture of *B. subtilis* R growing exponentially in CGG medium was centrifuged and resuspended to a density of 170 μg/ml in CGG medium containing 20 μg/ml aza-guanine. After 3 min incubation in this medium, the culture was passed rapidly through a membrane filter to remove the aza-guanine, washed once on the filter with 5.0 ml CGG medium and resuspended to the initial suspension density in fresh CGG medium. A second culture, incubated throughout in the absence of aza-guanine, acted as a control. Incubation for 3 min in the presence of aza-guanine (Fig. 3) completely inhibited lytic enzyme synthesis for about 90 min; subsequently the cells regained their synthetic ability. Addition of 2.0 μg/ml guanine to the aza-guanine in the preliminary incubation medium completely prevented the inhibitory effect of the analogue. Furthermore, if organisms incubated in the presence of aza-guanine for 3 min were washed and resuspended in CGG medium containing 2.0 μg/ml guanine, lytic enzyme synthesis commenced immediately at about half the control rate and a normal rate of synthesis was achieved after about 100 min incubation. Treatment of cultures with aza-guanine for periods shorter than 3 min led to less complete inhibition of enzyme formation. After 1.0 min in the presence of the analogue the synthetic ability of cultures was variable, ranging from 60–90% of the control values in different experiments. It seems, therefore, that aza-guanine causes complete inhibition of lytic enzyme synthesis within 3 min of addition to a growing culture and that the inhibitory effect persists for a considerable time after the removal of the analogue.

Effect of aza-guanine on total protein and nucleic acid synthesis

Aza-guanine is known to inhibit the synthesis of several enzymes, notably the inducible β-galactosidase of *Staphylococcus aureus*[5,23] and the inducible penicillinase of *Bacillus cereus*[22]. MANDEL[24] and SMITH AND MATTHEWS[3] have shown that aza-guanine is incorporated into the RNA of the cell under conditions in which exponential growth is limited but that the *total* amount of analogue incorporated cannot be responsible for the change to linear growth as incorporation of the analogue continues for several hours after the effect on growth is complete. As aza-guanine affects lytic enzyme synthesis within 3 min of addition to the system, experiments were carried out to decide whether the analogue specifically inhibited enzyme synthesis or whether other synthetic processes in the cell were affected simultaneously.

Inhibitory concentrations of aza-guanine had little immediate effect on the synthesis of total nucleic acid, RNA or DNA, whether measured by the optical density of material extracted by the methods described above (Fig. 4) or by growing cells in CGG medium + [8-^{14}C]adenine and following the incorporation of radioactivity into the RNA and DNA fractions (Fig. 5).

In all cases synthesis of total nucleic acid (Fig. 4a and 5a) and RNA and DNA (Fig. 4b and 5b) continued at nearly the control rates for about 45 min before beginning to fall off.

SMITH AND MATTHEWS[3] have reported that aza-guanine is incorporated into RNA in the place of guanine when *Bacillus cereus* is incubated in growth inhibitory

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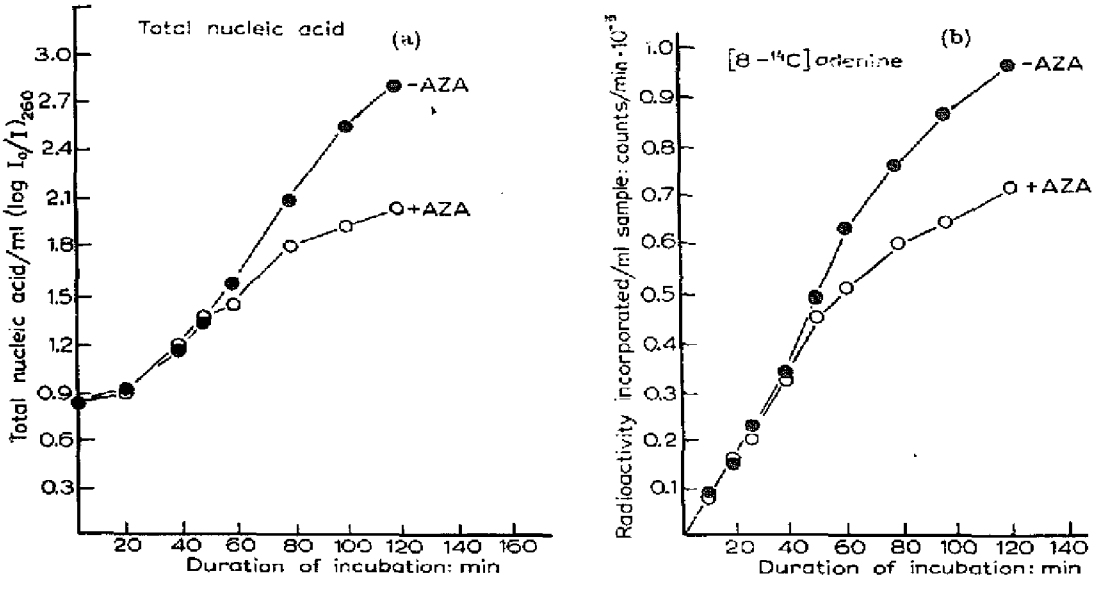

Fig. 4. Effect of aza-guanine on total nucleic acid synthesis measured (Fig. 4a) by extinction at 260 mμ and (Fig. 4b) by incorporation of radioactivity from [8-^{14}C]adenine. Fig. 4a. Medium: CGG medium + aza-guanine (AZA) 20 μg/ml final concentration.

Fig. 4b. Medium: CGG + [8-^{14}C]adenine (200 μM, final concentration) ± aza-guanine (AZA), 20 μg/ml final concentration.

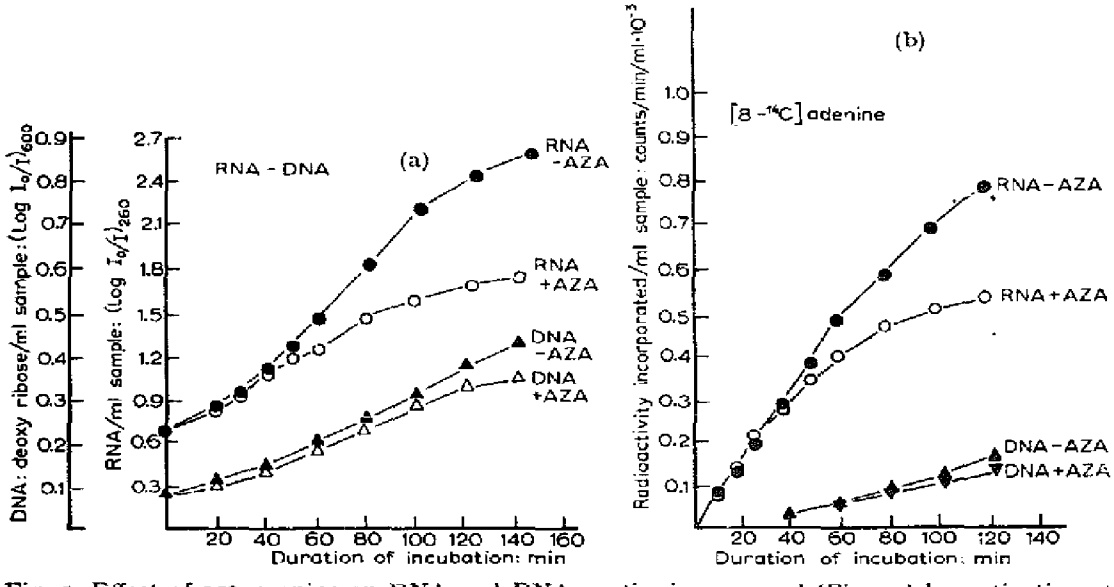

Fig. 5. Effect of aza-guanine on RNA and DNA synthesis measured (Fig. 5a) by extinction at 260 mμ (RNA) and extinction at 600 mμ due to estimation of deoxyribose (DNA), and (Fig. 5b) by incorporation of radioactivity from [8-^{14}C]adenine into RNA and DNA fractions (see text).

Fig. 5a. Medium: CGG medium ± aza-guanine (AZA) at 20 μg/ml final concentration.

Fig. 5b. Medium: CGG ± 200 μM [8-^{14}C]adenine ± aza-guanine (AZA) 20 μg/ml, final concentration.

concentrations of the analogue. Aza-guanine might be expected, therefore, to interfere with the incorporation of guanine into the RNA of *B. subtilis* R grown in the presence of the analogue. Attempts to study this process by following the incorporation of [8-^{14}C]guanine into the RNA of the cell were unsuccessful because the specific activity of the guanine available was too low to allow sufficient radioactivity to be added to the system without adding simultaneously enough guanine partially to reverse the inhibitory effect of the analogue. To avoid this difficulty, the effect of aza-guanine was studied on the incorporation of [^{14}C]glycine into the adenine and guanine of the nucleic acid fractions.

Glycine incorporation in normal cells

ROBERTS *et al.* have shown that *Escherichia coli*, grown in a glucose–ammonia-salts medium containing [^{14}C]glycine, incorporates radioactivity into both nucleic acid and the protein of the cell; similar results have been obtained by MCQUILLEN[26] with *Bacillus megaterium*. Addition of [^{14}C]glycine to a culture of *Bacillus subtilis* R grown exponentially in CGG medium resulted in a similar pattern of incorporation, 38% of the radioactivity being incorporated into the nucleic acid and 56% into the "protein + cell wall" fraction of the cells (see Table I). The distribution of radioactivity was also determined in the RNA and the DNA components (prepared by the perchloric fractionation procedure) and in the total nucleic acid (prepared by both the perchloric and TCA methods). Distribution of radioactivity within the RNA and DNA fractions was followed by hydrolysing the fractions in 0.1 *N* HCl to release free purine bases and pyrimidine nucleotides. These were separated by chromatography of the hydrolysates in isopropanol–HCl; guanine and 2.0 mg pure yeast nucleic acid, all hydrolysed under the same conditions, were used as markers. After development, the position of the purines on the chromatograms was determined by u.v. transmission photography[20], and the area containing the material was cut out and eluted in distilled water. Samples of this material were used for determination of radioactivity and confirmation of the identity of the purine bases under acid and alkaline conditions. Table II shows the distribution of radioactivity incorporated into the nucleic acid of organisms incubated under these conditions. Approximately the same proportion of radioactivity appeared in the total nucleic acid fraction of the cells whether it was prepared by the TCA or perchloric acid fractionation pro-

TABLE I

DISTRIBUTION OF RADIOACTIVITY FROM [^{14}C]GLYCINE INTO THE VARIOUS FRACTIONS OF *Bacillus subtilis* R

Cells fractionated for this purpose by the method of MCQUILLEN AND ROBERTS[18]

Results expressed as: $\frac{\text{(counts/min in extract)}}{\text{(total counts/min incorporated)}} \times 100$.

Fraction[a]	*Expt. 1* %	*Expt. 2* %	*Extraction procedure*
Cells before fractionation	100	100	
Free internal "pool"	4.3	3.8	5% TCA, 4°, 3 h
Lipid- and alcohol-soluble protein	5.2	5.4	75% aq. EtOH, 75°, 30 min, twice
Total nucleic acid	38.4	39.2	5% TCA, 95°, 20 min, three times
"Protein + cell wall"	48.5	49.4	Residue
% Recovery	96.4	97.8	

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TABLE II

DISTRIBUTION OF RADIOACTIVITY FROM [^{14}C]GLYCINE IN VARIOUS PARTS OF THE NUCLEIC ACID FRACTION PREPARED FROM CELLS BY DIFFERENT METHODS

	Perchloric acid fractionation: % total radioactivity incorporated	*T.A.A. fractionation: % total activity incorporated*
Total in cells before fractionation	100	100
Total nucleic acid	38.1	36.9
RNA	36.4	—
DNA	0.1	—
Total nucleic acid		
Total purines	33.6	34.0
adenine	17.9	18.4
guanine	16.0	15.6
[^{14}C]adenine/[^{14}C]guanine	1.12	1.17
RNA		
Total purines	27.7	—
adenine	14.4	—
guanine	13.3	—
[^{14}C]adenine/[^{14}C]guanine	1.08	—

cedures. Of the radioactivity in the total nucleic acid fraction, about 80% was in the RNA and 15% in the DNA. More than 90% of the radioactivity in the total nucleic acid and RNA fractions was in the purine bases, the ratio of radioactivity in the adenine to that in the guanine ([^{14}C]adenine/[^{14}C]guanine) being about 1.1 in all the fractions measured. In subsequent experiments this ratio fluctuated between 1.05 to 1.43, but was always approximately constant within one experiment. The time course of incorporation of [^{14}C]glycine into *B. subtilis* R was followed in cells grown exponentially in CGG medium containing 400 μM glycine (0.06 μC/μmole). Samples removed at intervals were fractionated as described in the previous experiment to measure the distribution of radioactivity in the various cell fractions. Table III

TABLE III

TIME COURSE OF INCORPORATION OF RADIOACTIVITY FROM [^{14}C]GLYCINE INTO VARIOUS CELL FRACTIONS OF *B. subtilis* R

Initial culture density: 240 μg bacterial dry weight/ml. Sample size: 1.0 ml

Time	*Protein + cell wall fraction counts/min/sample*	*RNA fraction counts/min/sample*	[^{14}C]adenine/[^{14}C]guanine
5	51	36	—
15	158	117	—
30	342	257	1.21
45	491	355	1.17
60	663	492	1.18
90	1021	798	1.10
120	1271	992	1.23
180	1581	1175	1.15

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shows that a similar pattern of incorporation is obtained in all the samples, [^{14}C]-adenine/[^{14}C]guanine ratio being approximately constant throughout the experiment at about 1.17.

Effect of aza-guanine on glycine incorporation

To study the effect of aza-guanine on the pattern of incorporation of [^{14}C]glycine the organisms were grown in CGG medium containing [^{14}C]glycine as described above. After 20 min growth in this medium, aza-guanine was added to a final concentration of 20 μg/ml and samples were removed at intervals from the culture for analysis. A second culture incubated throughout in the absence of aza-guanine and sampled under identical conditions, acted as control. Table IV shows the effect of aza-guanine on the distribution of radioactivity in the various fractions of the cells. The incorporation of glycine into the "protein + cell wall" fraction ceased within 5 min of addition of the analogue. As the cell wall of *B. subtilis* R contains no glycine or serine and as glycine is not converted appreciably to any amino acid other than serine under these conditions[27], these results imply an inhibition by aza-guanine of glycine incorporation into the protein fraction of the cell. Aza-guanine had no detectable effect on the incorporation of [^{14}C]glycine into the DNA fraction of the cells, and little effect, for the first 45 min, on incorporation into the RNA. The radioactivity appearing in the guanine, however, was depressed causing a progressive increase in the [^{14}C]adenine ratio and aza-guanine appeared in the RNA hydrolysate. The change in [^{14}C]adenine/[^{14}C]guanine could result either from a lowering of the amount of guanine incorporated into the RNA or a differential effect of aza-guanine on the conversion of glycine to adenine and guanine within the cell. To decide between these two possibilities, the specific radioactivity (μC/μmole) of the adenine and guanine in the RNA fractions prepared from a similar experiment was determined and the

TABLE IV

EFFECT OF AZA-GUANINE ON THE DISTRIBUTION OF RADIOACTIVITY FROM [^{14}C]GLYCINE INTO VARIOUS CELL FRACTIONS OF *Bacillus subtilis* R

+ aza: aza-guanine added (20 μg/ml final concentration). — aza: equivalent volume of water added. Sample volume: 1.0 ml. Initial suspension density: 170 μg bacterial cell dry weight/ml.

Time	Protein + cell wall counts/min/sample		RNA				DNA	
			Total incorporation counts/min/sample		[^{14}C]adenine/[^{14}C]guanine		counts/min/sample	
	— aza	— aza	— aza	— aza				
5	41	—	31	29	—	—	—	—
10	88	83	66	71	—	—	—	—
15	134	138	106	103	—	—	—	—
20	173	187	131	140	1.12	1.10	—	—
	— aza	+ aza	— aza	+ aza	— aza	+ aza	— aza	+ aza
25	220	203	173	168	1.10	1.13	—	—
30	253	207	194	184	1.08	1.17	37	40
45	407	196	301	285	1.13	1.38	69	71
60	548	213	420	397	1.07	1.49	74	88
90	812	204	603	515	—	1.63	99	104
120	1170	188	943	687	1.12	2.02	136	129
180	1371	190	1024	735	1.19	2.23	167	145

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TABLE V

EFFECT OF AZA-GUANINE ON THE SPECIFIC RADIOACTIVITY (μC/μmole) OF ADENINE AND GUANINE OF RNA SYNTHESISED FROM [^{14}C] GLYCINE BY *Bacillus subtilis* R

— aza: No aza-guanine added. + aza: aza-guanine added (20 μ/ml final concentration).

Time	RNA: Total radioactivity incorporated counts min sample		Specific radioactivity of adenine and guanine μC μM: adenine		guanine	
	— aza	+ aza	— aza	+ aza	— aza	+ aza
5	27	24				
20	128	120				
	— aza	+ aza	— aza	+ aza	— aza	+ aza
30	184	171	0.02	0.02	0.02	0.02
60	390	377	0.03	0.03	0.03	0.03
90	579	495	0.041	0.037	0.037	0.03
120	890	647	0.051	0.047	0.041	0.04

results are shown in Table V. The aza-guanine had little effect on the specific radioactivity of either purine and, therefore, had no differential effect on the conversion of glycine to adenine and guanine in the cell. It has been shown above that the formation of RNA under these conditions was only slightly affected by aza-guanine during the first 45 min of incubation. As the appearance of radioactivity from glycine followed a similar course, the progressive change in the [^{14}C]adenine/[^{14}C]guanine ratio must be due to a depression in the incorporation of guanine into the RNA of the cell.

Incorporation of aza-guanine into RNA of B. subtilis *R*

Several workers[3,4,23] have reported that aza-guanine can be incorporated in the place of guanine into the RNA fraction of a variety of organisms. The effects on RNA metabolism described above, together with the appearance of aza-guanine in the RNA hydrolysates, shows a similar process is likely in *B. subtilis* R. However, the amount of aza-guanine in the RNA hydrolysates was found, on analysis, to be much more than would be required to replace the guanine missing from the RNA (measured by the depression of incorporation of radioactivity from [^{14}C]glycine). It has been pointed out that the level of aza-guanine in RNA hydrolysates is not a valid measurement of the amount incorporated[24]. Aza-guanine is extremely insoluble in acid solutions and any free-aza-guanine precipitated in the RNA fraction or adsorbed on the surface would appear as the free base on hydrolysis in 0.1 *N* HCl. In an attempt to avoid this difficulty, RNA formed in the presence of aza-guanine was hydrolysed in *N* KOH to liberate the purine and pyrimidine nucleotides. It was impossible, however, to free these hydrolysates from salt and the subsequent separation achieved by chromatography in *n*-BuOH–ammonia was too poor to allow sufficiently accurate estimation of the aza-guanylic acid found in the hydrolysates.

Effect of aza-guanine on the incorporation of DL-[^{14}C]*alanine,* L-[^{14}C]*phenylalanine and* L-[^{14}C]*leucine*

To decide whether the inhibition by aza-guanine of glycine incorporation into

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the "protein + cell wall" fraction reflected a general inhibition of amino-acid incorporation, separate cultures were grown exponentially in CGG medium containing radioactive DL-alanine, L-phenylalanine and L-leucine respectively. After 20 min incubation, aza-guanine was added to the cultures and the distribution of radioactivity followed in samples removed at intervals for analysis. A second series of cultures, incubated in the absence of added tracer, acted as controls. Fig. 6 shows the results of this experiment. Aza-guanine completely blocked the incorporation of phenylalanine and leucine within 5 min of addition of the analogue; the incorporation of alanine was only partially inhibited under similar conditions. As the cell wall of *B. subtilis* R contains alanine but no phenylalanine or leucine[27], it is possible that the aza-guanine-insensitive incorporation of alanine occurs into the cell wall.

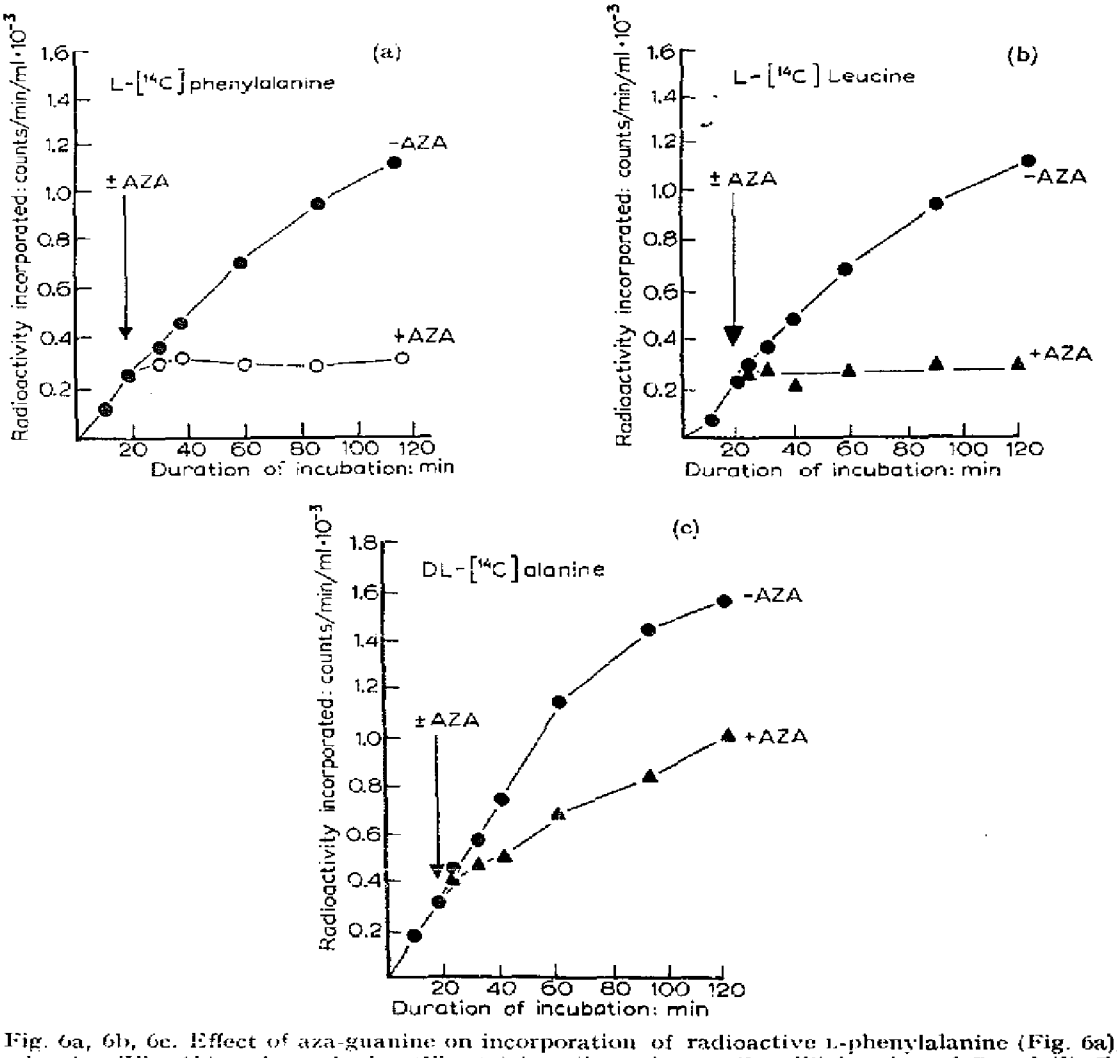

Fig. 6a, 6b, 6c. Effect of aza-guanine on incorporation of radioactive L-phenylalanine (Fig. 6a), L-leucine (Fig. 6b) and DL-alanine (Fig. 6c) into "protein + cell wall" fraction of *B. subtilis* R. Growth medium: CGG medium + 400 μM amino acid. Aza-guanine (AZA) 20 μg/ml final concentration, added where indicated.

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Effect of aza-guanine on cell wall formation

It has not been possible to prepare cell wall from *Bacillus subtilis* R by classical methods[28,29] as rapid autolysis of the wall occurs as soon as the cell is broken. However, it has been shown that heat-killed cells of *Bacillus subtilis* R are sensitive to the action of egg-white lysozyme[2] and SALTON[30] has shown that this enzyme is able partially to digest cell wall preparations from several strains of *Bacillus subtilis*. The material liberated from heat-killed *B. subtilis* R by egg-white lysozyme contained the amino acids alanine, diaminopimelic acid and glutamic acid with traces of aspartic acid and valine[27]. The main components of this wall agree well with those found by CUMMINS AND HARRIS[31] in *B. subtilis*.

To decide whether DL-[^{14}C]alanine was incorporated into the cell wall in the presence of sufficient aza-guanine to stop the incorporation of glycine, phenylalanine and leucine into protein, a culture was grown in the presence of radioactive DL-alanine as described in the previous experiment. After 20 min in this medium, aza-guanine was added and sets of three samples, taken at intervals, were fractionated as follows. Sample 1 was precipitated with 5% TCA at 4° and fractionated in the normal way to give the "protein + cell wall" fraction. Samples 2 and 3 were heated on a water bath at 100° for 30 min, centrifuged and washed twice with distilled water. The cell pads were resuspended in 5.0 ml 0.06 *M* Na_2HPO_4/KH_2PO_4 buffer, pH 6.5, and portions removed for radioactivity determinations. Egg-white lysozyme 200 μg/ml (final concentration) was added to sample 2; an equivalent volume of water to sample 3. Chloroform (0.2 ml) was added to each sample and they were shaken at 37° in stoppered tubes for 24 h. At the end of this period, the digests were centrifuged, the pads resuspended in 5.0 ml water and samples from this material and the supernatant fraction removed for radioactivity determinations. Fig. 7 shows that all but about 10% of the alanine incorporated in the presence of aza-guanine was released from the preparation by digestion with egg-white lysozyme. The level of radioactivity appearing in the "protein + cell wall" fraction after boiling the cells for 30 min followed by incubation at 37° overnight was approximately the same as that in the "protein + cell wall' fraction prepared by TCA fractionation. More than 90% of the radioactivity released in the heat-killed cell preparation could be recovered in the soluble digestion products.

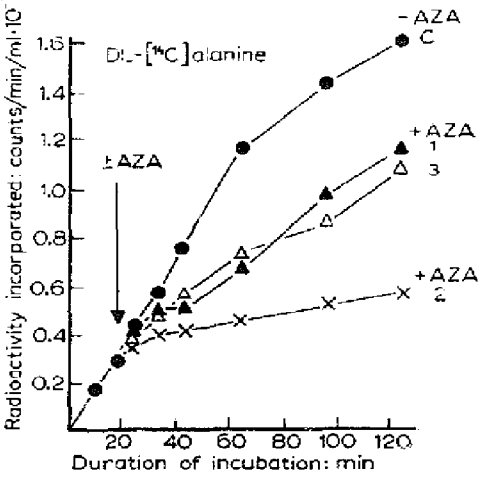

Fig. 7. Effect of egg-white lysozyme on the radioactivity incorporated into the "protein + cell wall" fraction of *B. subtilis* R grown in the presence of aza-guanine. Growth medium, CGG medium: Aza-guanine (AZA) 20 μg/ml added at point indicated. C, culture incubated in absence of aza-guanine and fractionated by TCA method (see METHODS AND MATERIALS); 1–3, samples 1–3 (see text).

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Effect of other inhibitors on synthesis of lytic enzyme

A number of compounds known to interfere with protein or nucleic acid metabolism were tested for their effect on lytic enzyme synthesis. Table VI shows that the compounds known to be potent inhibitors of protein synthesis[8] (such as chloramphenicol, aureomycin, neomycin, terramycin) all inhibited the synthesis of lytic enzyme at or near growth inhibitory levels. Ethidium bromide inhibited lytic enzyme synthesis immediately at concentrations as low as $5 \cdot 10^{-5}$ *M*. DCDNS, and the related compounds I and II, were also inhibitors of lytic enzyme synthesis, but DCDNS inhibits the accumulation of proline within *S. aureus* in a manner similar to 2:4 dinitrophenol (HANCOCK, personal communication) and may, therefore, act as an uncoupling agent. Compounds IV–IX (see METHODS AND MATERIALS) had only feeble inhibitory action on lytic enzyme synthesis (Table VI).

TABLE VI

ACTION OF SOME INHIBITORS ON SYNTHESIS OF LYTIC ENZYME BY *Bacillus subtilis* R

Cultures of *Bacillus subtilis* R grown exponentially in CGG medium were tested for their ability to form lytic enzyme with or without inhibitor added at the concentrations as below.

	Concentration		Inhibition %
	μg/ml	mM	
Chloramphenicol	10	—	69
	30	—	95
	100	—	100
Aureomycin	10	—	52
	30	—	100
Neomycin	10	—	68
Terramycin	10	—	75
8-hydroxy-quinoline	—	0.01	83
	—	0.03	95
Ethidium bromide	—	0.001	52
	—	0.005	100
DCDNS	—	0.1	100
	—	0.05	31
Cpd I	—	0.5	74
	—	1.0	87
Cpd II	—	0.5	27
	—	1.0	63
Cpd III	—	1.0	0
Cpd IV	—	1.0	10
Cpd V	—	0.2	7
Cpd VI	—	0.2	12
Cpd VII	—	0.2	10
Cpd VIII	—	0.2	17
Cpd IX	—	0.2	23

DISCUSSION

The results reported above agree closely with those reported by SMITH AND MATTHEWS[3] and MANDEL[4,24] on the mode of action of 8-aza-guanine. Although the analogue completely inhibited lytic enzyme formation within 10 min of addition to the system, incorporation of the analogue into the RNA fraction of the cells continued for a period of up to 3 h. Little change in the total amount of RNA synthesised

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occurred during the first 45 min and thes eresults support the conclusion of CREASER[32] that aza-guanine has little immediate effect on the incorporation of [^{14}C]uracil into the RNA of *Staphylococcus aureus* incubated in the presence of sufficient aza-guanine to inhibit inducible β-galactosidase synthesis. The results are not compatible, however, with the suggestion[32] that aza-guanine inhibits the synthesis of inducible enzymes but has markedly less effect on constitutive enzyme formation. In the strain used in these experiments, growth inhibitory concentrations of aza-guanine completely inhibited lytic enzyme synthesis together with the incorporation of L-phenylalanine, L-leucine and glycine into the protein fraction of the cells. No differential effect of aza-guanine at lower concentrations could be detected on these processes; similar findings have been reported recently by CHANTRENNE AND DEVREUX[33]. It seems likely, therefore, that the primary effect of aza-guanine is to inhibit protein synthesis and the amount of analogue incorporated into the RNA is not directly related to this. It is possible that the inhibitory effect of the analogue is exerted on some guanine-containing co-factor; the GTP necessary for the system reported by HOAGLAND *et al.*[34] being an obvious candidate.

Synthesis of the bacterial cell wall continued in the presence of concentrations of aza-guanine inhibitory towards protein synthesis. These results are similar to those reported recently by ROGERS AND MANDELSTAM[35] and HANCOCK AND PARK[36] who showed that cell wall synthesis in *Staphylococcus aureus* continues in the presence of concentrations of chloramphenicol sufficient to inhibit the incorporation of amino acids into protein. This suggests that the synthesis of cell wall and protein are not closely related processes in the bacterial cell.

Several workers[2,30] have reported that the cell wall of *Bacillus subtilis* is only partially digested to soluble products by egg-white lysozyme. Recently, ARMSTRONG *et al.*[37] have shown that about half the cell wall of *Bacillus subtilis* consists of polymer containing ribitol, glucose, alanine and phosphate and have called this substance "teichoic acid". About 10% of the alanine incorporated into *Bacillus subtilis* R in the presence of aza-guanine was not released on digestion with egg-white lysozyme. It is possible that this represents the ester-bound alanine of teichoic acid which is not sensitive to lysozyme digestion.

The method used above to locate alanine in the cell wall of *Bacillus subtilis* R may be of more general use in determining the composition of cell walls from those species of bacteria from which pure cell wall cannot be prepared by classical techniques because of autolysis on disruption of the cell. The method is most applicable to organisms sensitive to egg-white lysozyme. However, enzymes capable of digesting bacterial cell walls and having a different activity spectrum from egg-white lysozyme have been detected and might be used to remove cell wall material from organisms not sensitive to lysozyme. Furthermore, as chloramphenicol, at growth-inhibitory concentrations, will inhibit protein but not cell wall synthesis, it could be used in the place of aza-guanine.

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