Studies on the Mode of Action of the Streptogramin Antibiotics

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SUMMARY

Streptogramin is a complex antibiotic containing two components, streptogramin A and streptogramin B. The individual components are bacteriostatic whereas the mixture is bactericidal. At growth-limiting concentrations streptogramin A and the streptogramin complex inhibit *Staphylococcus aureus* by blocking protein synthesis but not nucleic acid and cell wall synthesis. Protein, nucleic acid and cell wall synthesis were all reduced in the presence of streptogramin B.

Streptogramin A, but not streptogramin B, inhibits the binding of radioactive chloramphenicol to bacterial ribosomes. However, streptogramin B enhances this effect of streptogramin A in a cell-free system containing both bacterial soluble fraction and ribosomes. The results suggest that streptogramin A inhibits protein synthesis by bacteria at the ribosome level, and that this effect is enhanced by streptogramin B.

INTRODUCTION

The antibiotic streptogramin was first obtained from culture filtrates of a species of *Streptomyces*, now classified as *Streptomyces graminofaciens* (Charney *et al.* 1953). Other antibiotics closely related to streptogramin have been described, for example, virginycin or staphylomycin (De Somer & Van Dijk, 1955), E 129 or ostreogrycin (Garrod & Waterworth, 1956; Ball *et al.* 1958), synergistin or PA 114 (Celmer & Sobin, 1955/1956), mikamycin (Arai *et al.* 1956), pristinamycin or pyostacin (Benazet *et al.* 1962) and vernamycin (Bodanszky & Ondetti, 1963). All these antibiotics are mixtures of two or more different active compounds, and there are numerous reports on the isolation and properties of the individual components from PA 114, staphylomycin, ostreogrycin, mikamycin and vernamycin. All these complex antibiotics consist of at least two components belonging to two major groups, A and B. The antibiotics in group A show a marked synergism with those in group B in their activity against Gram-positive bacteria and consequently the complex antibiotics of the streptogramin family have a markedly higher activity than the individual components. The classification of the antibiotics of the streptogramin family has been reviewed by Lester Smith (1963).

The antibiotics in group A are mainly active against Gram-positive cocci and the antibiotics in group B against Gram-positive bacilli. Although there is a strong synergism between the antibiotics of groups A and B in their antibacterial effect against Gram-positive bacteria in general, there is no such effect on Gram-negative organisms; consequently, the antibiotic complexes are effective mainly against Gram-positive bacteria.

This work describes investigations of the mode of action of streptogramin and its components in an attempt to clarify the mechanism of the remarkable synergism shown by mixtures of the individual antibiotics. Preliminary reports of this work have been published (Vazquez, 1962, 1963*a*, 1964*b*).

METHODS

Organisms and growth conditions. The organisms used in this work were *Staphylococcus aureus* strain Duncan, *Bacillus megaterium* strain KM (NCIB 9521) and *Escherichia coli*, strain B. *Staphylococcus aureus* was grown overnight at 37° in medium A of Gale (1947). Next morning the culture was diluted 10 times with fresh medium and incubation continued. For studies of synthesis of cellular constituents or isotope incorporation, *S. aureus* was resuspended in the defined medium of Gale & Folkes (1953*a*) containing glucose, amino acids, purines, pyrimidines and the buffered salt solution of Gale (1947). *Bacillus megaterium* and *E. coli* were grown overnight aerobically in CG medium containing 0.03 % (w/v) glucose (McQuillen & Roberts 1954) at 30° and 37° respectively. In the morning, excess glucose was added and incubation continued. Growth of bacteria was followed by measuring the optical density as previously described (Vazquez, 1965*a*).

Viable counts. Viable counts were made in triplicate on medium A + 2 % (w/v) agar using the method described by Miles & Misra (1938) after serial dilution of the bacteria in saline.

Synthesis of cellular constituents

(*a*) *Net synthesis.* Suspensions of *Staphylococcus aureus* at an initial concentration equivalent 1 mg. dry wt./ml. were incubated statically at 37° in the defined medium. Samples of 3 ml. were taken at intervals and cooled rapidly. Bacteria were harvested by centrifugation and washed with the buffered salt solution. The bacterial pellet was extracted twice with 2 ml. of 0.2 N-perchloric acid at 4° for 60 min. and the supernatant fluids from these extractions combined. This constituted the pool fraction. The nucleic acids were extracted from the pellet with 3 ml. 0.5 N-perchloric acid at 70° for 20 min. (three extractions were sufficient). The nucleic acids were estimated by measuring the absorption at 260 m μ in a Unicam SP 500 spectrophotometer. A solution of 100 μ g. nucleic acid/ml. was assumed to have an absorption of 2.8 (Gale & Folkes, 1953*a*). The DNA in the 0.5 N-perchloric extracts was estimated by the diphenylamine method of Burton (1956).

The residual pellet was taken up in N-NaOH and protein in this solution was estimated by the method of Lowry *et al.* (1951).

(*b*) *Isotope incorporation.* Suspensions of *Staphylococcus aureus* at an initial cell concentration equivalent 1 mg. dry wt./ml. were incubated at 37° statically in the defined medium to which a ¹⁴C-labelled compound had been added. Samples of 1 ml. were taken at intervals and cooled rapidly. The bacteria were harvested by centrifugation and washed with 5 ml. of the buffered salt solution. The bacterial pellet was extracted with 3 ml. 5 % (w/v) trichloroacetic acid for 1 hr at 4°; nucleic acids were extracted from the pellet with 3 ml. of 5 % (w/v) trichloroacetic acid at 90° for 15 min. The insoluble material remaining after this extraction was washed once with 2 ml. 0.01 M-NH₄HCO₃, resuspended in 2 ml. of this solution, and digested with 2 mg. trypsin to separate the protein and the cell-wall fractions (Hancock & Park, 1958).

Samples of every fraction were transferred to aluminium planchets and radioactivity determined as previously described (Vazquez, 1965*b*).

Uptake of chloramphenicol. The effect of the streptogramin antibiotics on the uptake of ^{14}C -labelled chloramphenicol (methylene ^{14}C ; $9.90\ \mu\text{C./}\mu\text{mol}$; [^{14}C]CAP) by *Staphylococcus aureus* suspended in medium A was studied as described by Vazquez (1965*b*) for *Bacillus megaterium*. The non-radioactive antibiotics were mixed in the tubes with [^{14}C]CAP prior to the addition of bacterial suspensions.

Preparation of bacterial cell sap and ribosomes. Suspensions of *Staphylococcus aureus* (100 mg. dry wt./ml.) in the buffered salt solution were disrupted in a Mullard ultrasonic disintegrator (model SL 82) for 10 min. Broken cell walls and membranes were separated by centrifugation at $40,000g$ for 20 min. and discarded. The supernatant cell sap was diluted with buffered salt solution when required. The buffered salt solution contained $0.002\ \text{M-Mg}^{2+}$ and under these conditions the ribosomes were split into 30S and 50S ribosomal subunits. Ribosomes from *Bacillus megaterium* and *Escherichia coli* were obtained as previously described (Vazquez, 1965*a*). These ribosomes were resuspended in $0.01\ \text{M-tris} + \text{HCl}$ buffer, pH 7.4, containing $0.2\ \text{M-K}^+$.

[^{14}C]CAP binding to ribosomes. The effect of the streptogramin antibiotics on [^{14}C]CAP binding to *Staphylococcus aureus* ribosomes was studied without separating the ribosomes from the bacterial 'soluble fraction' using bacterial cell sap. Suspensions of *Bacillus megaterium* ribosomes in $0.01\ \text{M-tris-HCl}$ buffer, pH 7.4, containing $0.2\ \text{M-K}^+$ were also used for binding experiments. The experiments were carried out as described by Vazquez (1965*b*). The non-radioactive antibiotics were mixed in the tubes with [^{14}C]CAP before adding of ribosome suspensions. *B. megaterium* and *S. aureus* ribosomes were harvested by centrifugation at $150,000g$ for 100 min. and 150 min., respectively.

Antibiotics. Streptogramin, PA 114-A-1 and PA 114-B-1 were kindly supplied by Dr H. B. Woodruff. The two major components of streptogramin (streptogramin A and streptogramin B) were separated and purified following the methods described by Vanderhaeghe, Van Dijk, Parmentier & De Somer (1957) for the separation of the components of staphylomycin. Streptogramin A was further purified by the method described by Sarin (1962) for the purification of ostreogrycin A. The ultraviolet and infrared spectra of the streptogramins A and B were determined and compared with those of PA 114-A-1 and PA 114-B-1. There were no significant differences between streptogramin A and PA 114-A-1, or streptogramin B and PA 114-B-1. The chemical structures of the individual components of the streptogramin complex have not been published, but Dr Woodruff and Dr Lester Smith (personal communications) have suggested that streptogramins A and B are identical with PA 114-A-1 (synonym, ostreogrycin A) and PA 114-B-1 (synonym, ostreogrycin B), respectively.

PA 114-A-1 and PA 114-B-1 were used in this work, and will be referred to as component A and component B of streptogramin. In many experiments the individual components isolated from streptogramin were also used to confirm results obtained.

The sources of other antibiotics used in this work are given by Vazquez (1965*b*).

Isotopically labelled compounds were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Effect on the viability of Staphylococcus aureus. The limiting growth-inhibitory concentrations for *Staphylococcus aureus* of streptogramin, component A and component B were respectively, 0.6, 6, and 10 $\mu\text{g./ml.}$ Streptogramin was bactericidal whereas the individual components A and B were bacteriostatic, even when tested at three times the minimum growth-inhibitory concentrations. The effect of mixtures of component A with other antibiotics on the viability of *S. aureus* was studied with the results shown in Table 1. Bactericidal effects were found when component A was mixed with either viridogrisein (Garcia-Mendoza, 1965) or component B and to a lesser extent when mixed with aureomycin or terramycin. On the other hand, component A prevented the lethal effect of streptomycin but not that of echinomycin.

Table 1. *The effect of component A with other antibiotics on the viability of Staphylococcus aureus*

Antibiotics	Colony count		
	0 min.	75 min.	150 min.
None		8.0×10^7	1.5×10^8
Component A ('A')		3.3×10^7	3.5×10^7
'A' + Component B		1.2×10^6	$< 10^5$
Viridogrisein		3.5×10^7	4.5×10^7
'A' + Viridogrisein		1.0×10^6	$< 10^5$
Aureomycin		2.5×10^7	2.1×10^7
'A' + Aureomycin		9.2×10^6	3.0×10^6
Terramycin	3.0×10^7	3.0×10^7	2.8×10^7
'A' + Terramycin		1.2×10^7	8.0×10^6
Chloramphenicol		5.4×10^7	9.0×10^7
'A' + Chloramphenicol		3.4×10^7	3.5×10^7
Streptomycin		8.0×10^6	2.5×10^6
'A' + Streptomycin		3.0×10^7	3.1×10^7
Echinomycin		7.5×10^6	2.5×10^6
'A' + Echinomycin		7.2×10^6	3.0×10^6

Staphylococcus aureus (3.0×10^7 bacteria/ml.) was incubated at 37° for 150 min. in medium A. Antibiotics were added simultaneously (final concentrations 2×10^{-6} M), at the beginning of the incubation period. The colony count was determined by the method of Miles & Misra (1938).

Chloramphenicol, aureomycin, terramycin, erythromycin and carbomycin prevent the bactericidal action of penicillin and streptomycin on many bacteria (Jawetz, Gunnison, Bruff & Coleman, 1952; Coleman, Gunnison & Jawetz, 1953; Anand & Davis, 1960; Hurwitz & Rosano, 1962). A similar protective effect of chloramphenicol was found when it was added with streptogramin to an exponentially growing culture of *Staphylococcus aureus*. The protective effect of chloramphenicol occurred to the same extent when it was added to the bacterial culture before, or at the same time as, streptogramin, and to some extent when added after streptogramin (Table 2).

Penicillin and streptomycin are bactericidal only when the bacteria are growing (Kirby & Burnell, 1954; Prestidge & Pardee, 1957; Anand & Davis, 1960), and it was thought that chloramphenicol might protect by preventing growth. However,

this may not be the complete explanation in the case of streptogramin, as chloramphenicol at 3 $\mu\text{g./ml.}$ inhibited the growth of *Staphylococcus aureus* by only 60% but still prevented the bactericidal effect of streptogramin (Fig. 1).

Erythromycin, puromycin, aureomycin and terramycin are bacteriostatic and inhibit growth of *Staphylococcus aureus* at concentrations of 1, 50, 1 and 1 $\mu\text{g./ml.}$ respectively. At growth inhibitory concentrations erythromycin also prevented the bactericidal effect of streptogramin, and puromycin had no such effect, while aureomycin and terramycin increased its bactericidal effect. When growth of *S. aureus* was prevented by using a defined medium with glutamic acid omitted, the bactericidal effect of streptogramin was not significantly decreased. These findings suggested that streptogramin can kill *S. aureus*, even when growth is lessened, and that the effect of chloramphenicol and erythromycin in preventing killing by streptogramin cannot be explained simply by their effect in inhibiting bacterial growth.

Table 2. *The effect of time of addition of chloramphenicol on the bactericidal action of streptogramin*

Time of addition of antibiotics (min.)					Colony count after 90 min. incubation	% Decrease in colony count
0	1	3	5	10		
—	—	—	—	—	6.3×10^6	—
CAP	—	—	—	—	2.7×10^6	—
S	—	—	—	—	8.1×10^4	96
CAP + S	—	—	—	—	2.5×10^6	—
S	CAP	—	—	—	2.2×10^6	12
S	—	CAP	—	—	1.5×10^6	40
S	—	—	CAP	—	9.0×10^5	64
S	—	—	—	CAP	6.0×10^5	76
CAP	S	—	—	—	2.6×10^6	—
CAP	—	S	—	—	2.2×10^6	12
CAP	—	—	S	—	2.2×10^6	12
CAP	—	—	—	S	2.4×10^6	6

Staphylococcus aureus (2.5×10^6 bacteria/ml.) was incubated at 37° in medium A for 90 min. Chloramphenicol at 10 $\mu\text{g./ml.}$ (CAP) and streptogramin at 0.6 $\mu\text{g./ml.}$ (S) were added at the times shown in the Table. The colony count was determined by the method of Miles & Misra (1938).

Respiration and fermentation. Streptogramin and the individual components A and B at concentrations of up to 60 $\mu\text{g./ml.}$, had no effect on endogenous respiration, oxidation of ethanol or glucose, or anaerobic fermentation of glucose when added to washed suspensions of *Staphylococcus aureus* in the Warburg vessels. The studies were carried out using the manometric techniques described by Umbreit, Burris & Stauffer (1949).

Synthesis of cellular constituents. The effect of component A on net synthesis and on the incorporation of radioactive isotopes into the different cell fractions is shown in Tables 3 and 4. These results indicate that the antibiotic inhibits bacterial growth by blocking protein synthesis. The inhibitory effect of the antibiotic on the incorporation of [U- ^{14}C]glycine into the protein fraction was detected within 30 sec. of addition of the antibiotic (Fig. 2). Similar results were obtained with L-[U- ^{14}C]-lysine. The increased rate of glutamic acid accumulation in the pool in the presence

of component A was probably due to the amino acid not being incorporated in the protein fraction, and has been observed in the presence of other inhibitors of protein synthesis like chloramphenicol (Hancock, 1960). The increased nucleic acid content in the presence of component A was due to an increase of RNA accumulation; the synthesis of DNA was unaffected. An increase in the rate of accumulation of RNA has also been observed in bacteria treated with chloramphenicol (see Gale, 1963).

Fig. 1.

Fig. 2.

Fig. 1. The effect of chloramphenicol on the bactericidal action of streptogramin. Suspensions of *Staphylococcus aureus* (7×10^5 bacteria/ml.) were incubated in medium A at 37° for 90 min. and viable bacteria were counted at the beginning and at the end of the incubation period by the method of Miles & Misra (1938). ●—●—, No streptogramin added; —▲—▲—, 0.6 µg. streptogramin/ml.

Fig. 2. The effect of component A on the incorporation of [U- 14 C] glycine into the protein fraction of *Staphylococcus aureus*. Suspensions of 1 mg. *S. aureus*/ml. were incubated at 37° for 15 min. in the defined medium without adenine or guanine and supplemented with [U- 14 C]glycine (36 µg./ml.; 0.4 µc./ml.). Samples were taken at intervals and fractionated by the method of Hancock & Park (1958). —●—●—, Control; ▲—▲—, 6 µg. component A/ml.; —■—■—, 20 µg. component A/ml.

Similar experiments with component B suggest that it has a complex effect on *Staphylococcus aureus* (Tables 3 and 4). Protein, nucleic acid, and cell-wall synthesis were all reduced. However, the incorporation of glycine into the protein fraction was affected by component B within 30 sec., whereas the effect on nucleic acid and cell wall fractions was not established until after 1–2 min. Similar results were obtained for the incorporation of L-[U- 14 C]lysine.

These results are very similar to those reported by Garcia-Mendoza (1965) for the effect of viridogrisein on *Staphylococcus aureus*. Viridogrisein is included in group B of the streptogramin family of antibiotics and is more active than the other members of this group in inhibiting bacterial growth and protein synthesis. The effects of equal concentrations of viridogrisein and component B on nucleic acid and cell-wall synthesis were found to be very similar.

Effect of the streptogramin complex. The effect of the streptogramin complex at limiting growth-inhibitory concentrations on the net synthesis and incorporation of radioactive isotopes in the different cell fractions is shown in Tables 3 and 4, and resembles those obtained with component A (at $10 \times$ concentration). The main

Table 3. *The effect of the streptogramin antibiotics on synthesis of cellular constituents*

Antibiotic	$\mu\text{g./ml.}$	Net synthesis of control		Protein estimated in the fraction insoluble in hot perchloric acid
		260 m μ absorbing material		
		Pool	NA* fraction	
Component A	6.0	100	173	32
	20.0	100	158	16
Component B	10.0	87	61	56
	30.0	80	57	42
Streptogramin	0.6	100	174	38
	2.0	100	163	15

NA*: total nucleic acid. Suspensions of 1 mg. *Staphylococcus aureus*/ml. were incubated at 37° for 15 min. in the defined medium. Samples were taken at the beginning and at the end of the incubation period, fractionated by perchloric acid treatment, and protein estimated in the hot perchloric acid-insoluble fraction.

Table 4. *The effect of the streptogramin antibiotics on synthesis of cellular constituents*

Incorporation of radioactive compounds						
Antibiotic	$\mu\text{g./ml.}$	Radioactive compound	% control incorporation			
			Pool	NA*	Protein	Wall
Component A	6.0	[U- ^{14}C]Glycine	100	100	26	100
	20.0		100	100	12	100
	6.0	L-[U- ^{14}C]Glutamic acid	120	100	27	100
	20.0		128	100	11	100
	6.0	[8- ^{14}C]Adenine	126	204	—	—
	20.0		121	189	—	—
Component B	10.0	[U- ^{14}C]Glycine	77	59	54	57
	30.0		73	49	43	46
	10.0	L-[U- ^{14}C]Glutamic acid	83	57	52	58
	30.0		76	46	40	48
	10.0	[8- ^{14}C]Adenine	74	61	—	—
	30.0		69	58	—	—
Streptogramin	0.6	[U- ^{14}C]Glycine	100	100	34	100
	2.0		100	100	11	100
	0.6	L-[U- ^{14}C]Glutamic acid	118	100	35	100
	2.0		127	100	10	100
	0.6	[8- ^{14}C]Adenine	130	206	—	—
	2.0		120	176	—	—

*NA: total nucleic acid. [U- ^{14}C]Glycine added $0.4 \mu\text{C./ml.}$; $36 \mu\text{g./ml.}$ L-[U- ^{14}C]Glutamic acid added $0.1 \mu\text{C./ml.}$; $100 \mu\text{g./ml.}$ [8- ^{14}C]Adenine added $0.05 \mu\text{C./ml.}$; $10 \mu\text{g./ml.}$

Suspensions of 1 mg. *Staphylococcus aureus*/ml. were incubated at 37° for 15 min. in the defined medium supplemented with the required radioactive compound. Samples were taken at the end of the incubation period, fractionated by the method of Hancock & Park (1958), and the radioactivity in the fractions determined.

difference is that component A had an immediate effect on protein synthesis, whereas a lag of about 2 min. was observed before the synergistic mixture exerts its effect.

Gale & Folkes (1958a) showed that the action of chloramphenicol on bacterial protein is reversible; synthesis begins again immediately the antibiotic is removed. A comparison was made of the inhibitory effects of chloramphenicol and streptogramin on net protein synthesis as measured by increase in the perchloric acid-insoluble fraction and their reversal in *Staphylococcus aureus*. The inhibitory effect of streptogramin was not readily reversible, and removal of the antibiotic was followed by only a slight increase in the rate of protein synthesis.

The effect of a mixture of chloramphenicol and streptogramin on L-[U-¹⁴C]-glutamic acid incorporation into the protein fraction was studied, and showed that chloramphenicol at a concentration which prevented the lethal effect of streptogramin did not also block its inhibitory action on protein synthesis (Table 5).

Results similar to those found with streptogramin were obtained when an equivalent mixture of component A (80 %) and B (20 %) was used. (Fig. 3).

Table 5. *Incorporation of ¹⁴C-labelled glutamic acid into protein. The effect of streptogramin with chloramphenicol*

	Bacteria (counts/ min./mg.)
Control	1135
+ CAP (3.5 µg./ml.)	485
+ Streptogramin (0.9 µg./ml.)	161
+ CAP (3.5 µg./ml.) + Streptogramin (0.9 µg./ml.)	150

Suspensions of 1 mg. *Staphylococcus aureus*/ml. were incubated at 37° for 30 min. in the defined medium without adenine nor guanine in the presence of ¹⁴C-labelled glutamic acid 0.1 µC./ml.; 100 µg./ml. Samples were taken and fractionated by the method of Hancock & Park (1958).

Effect of the streptogramin antibiotics on the binding of chloramphenicol. Studies of the uptake of antibiotics by bacteria and their binding to different structures or components within the cell have been very helpful in elucidating their initial site of action.

As radioactive components A and B were not available, the uptake and binding of these antibiotics could not be studied directly. However, the results described above showed that component A had an overall effect resembling that of chloramphenicol, and it was thought that component A and chloramphenicol might compete for the same binding site(s) in sensitive bacteria. The binding of ¹⁴C-labelled chloramphenicol [¹⁴C]CAP has been used to test this hypothesis. Component A significantly reduced the uptake of [¹⁴C]CAP by *Staphylococcus aureus* and the effect was somewhat enhanced by component B (Table 6). Component B also reduced [¹⁴C]CAP uptake, but to a lesser extent than component A. When cells are exposed to [¹⁴C]CAP and then broken and fractionated in the centrifuge, all the radioactivity is associated with the ribosomal and soluble fraction (Vazquez, 1968b).

The effect on the binding of chloramphenicol by ribosome preparations was next studied. In a cell-free system consisting of a mixture of ribosome subunits (30 S and 50 S) and soluble fraction in the buffered salt solution, the ribosome bound

Fig. 3. Net protein synthesis in *Staphylococcus aureus* after streptogramin or chloramphenicol treatment. Suspensions of 1 mg. *Staphylococcus aureus*/ml. were incubated at 37° for 90 min. in the defined medium without antibiotic (—●—●—), with 30 µg. chloramphenicol/ml. (—▲—▲—), or with 3 µg. streptogramin/ml. (—■—■—). At time 30 min. (at arrow) portions of the bacteria were spun down, washed twice and resuspended in fresh medium free from antibiotic. Broken lines show increase in total protein synthesis after resuspending *S. aureus* in fresh medium.

Table 6. *The effect of the streptogramin antibiotics on [¹⁴C]chloramphenicol uptake by Staphylococcus aureus*

Antibiotic concentration		µmoles [¹⁴ C]CAP taken up, mg. bacteria
Component A	Component B	
—	—	56
1 × 10 ⁻⁶ M	—	48
3 × 10 ⁻⁶ M	—	33
1 × 10 ⁻⁵ M*	—	21
3 × 10 ⁻⁵ M	—	14
1 × 10 ⁻⁴ M	—	9
—	1 × 10 ⁻⁶ M	52
—	3 × 10 ⁻⁶ M	42
—	1 × 10 ⁻⁵ M*	34
—	3 × 10 ⁻⁵ M	27
—	1 × 10 ⁻⁴ M	23
(1 × 10 ⁻⁶ M + 1 × 10 ⁻⁷ M)*		32
(1 × 10 ⁻⁵ M + 1 × 10 ⁻⁶ M)		12

* Minimum growth-inhibitory concentration of the antibiotic(s).

Suspensions of *Staphylococcus aureus* (0.25 mg./ml.) in medium A were incubated at 37° in the presence of (3 × 10⁻⁶ M) [¹⁴C]chloramphenicol with the required antibiotic. After 15 min. the bacteria were harvested by centrifugation and the surface of the bacterial pellet rinsed with buffered salt solution. Finally bacteria were resuspended in water, and the radioactivity determined. Corrections were made for [¹⁴C]chloramphenicol present in the intercellular fluid as previously described (Vazquez, 1965b).

[¹⁴C]CAP. This binding was inhibited by component A but not by component B. However, the addition of components A and B to ribosomes slightly reduced [¹⁴C]CAP binding still further. Nevertheless, the effect of component B was too small to explain the strong synergistic effect of the two streptogramin components on intact cells. Addition of components A and B before or after [¹⁴C]CAP did not significantly change the results. In further experiments suspensions of *Staphylococcus aureus* were incubated with streptogramin or mixtures of components A

Table 7. *Binding of [¹⁴C]chloramphenicol to bacterial ribosomes. Intact bacteria pretreated with the streptogramin antibiotics*

Organism	Conditions of incubation			Antibiotic concentration		[¹⁴ C]CAP binding (% of control)
	Medium	Temperature	Time (min.)	Component A	Component B	
<i>S. aureus</i>	A	37°	20	1 × 10 ⁻⁶ M	—	97
				5 × 10 ⁻⁶ M	—	78
				1 × 10 ⁻⁵ M	—	54
				3 × 10 ⁻⁵ M	—	45
				—	5 × 10 ⁻⁷ M	100
				1 × 10 ⁻⁶ M	1 × 10 ⁻⁷ M	24
				5 × 10 ⁻⁶ M	5 × 10 ⁻⁷ M	4
	Buffer*	4°	10	1 × 10 ⁻⁵ M	—	50
	A	4°	10	1 × 10 ⁻⁵ M	—	56
	A	37°	10	1 × 10 ⁻⁵ M	—	58
	Buffer*	4°	10	1 × 10 ⁻⁶ M	1 × 10 ⁻⁷ M	88
	A	4°	10	1 × 10 ⁻⁶ M	1 × 10 ⁻⁷ M	92
	A	37°	10	1 × 10 ⁻⁶ M	1 × 10 ⁻⁷ M	38
<i>E. coli</i>	Peptone†	37°	20	1 × 10 ⁻⁵ M	—	58
				—	2 × 10 ⁻⁶ M	100
				1 × 10 ⁻⁵ M	2 × 10 ⁻⁶ M	55

* The buffered salt solution of Gale (1947).

† Medium containing 5 mg. Difco Bacto peptone/ml.

Suspensions of bacteria (3 mg./ml.) were incubated under the specified conditions. At the end of the incubation period, cells were harvested by centrifugation and the surface of the bacterial pellet rinsed with the buffered salt solution. The bacteria were resuspended in the buffered salt solution, prior to ultrasonic breakage and addition of (3 × 10⁻⁶ M) [¹⁴C]chloramphenicol to the cell-sap containing the bacterial soluble fraction and ribosome subunits. After 5 min. at 4° the ribosome subunits were harvested by centrifugation at 150,000 g. for 150 min. and the radioactivity in the pellet estimated. Ribosomes and soluble fraction from 60 mg. bacteria were added per tube (2 ml. tubes).

and B, before breakage of the cocci and the addition of [¹⁴C]CAP to the cell sap; the amount of chloramphenicol bound to ribosomes was then markedly decreased (Table 7), and component B enhanced the effect of component A on [¹⁴C]CAP binding. Component A alone was also effective when cocci were incubated at 4° during the pretreatment with this antibiotic but the additional inhibition due to component B only occurred under conditions where growth would normally occur in the absence of the antibiotics (Table 7). Components A and B had no synergistic inhibitory effect on the growth of *Escherichia coli* and, in this organism, pre-treatment of intact cells with A+B did not enhance the inhibitory effect of component A in the binding of [¹⁴C]CAP to ribosomes.

Since chloramphenicol protects *Staphylococcus aureus* against the bactericidal

effect of streptogramin, chloramphenicol might have inhibited the association of streptogramin with ribosomes. This was tested by treating *S. aureus* with a mixture of streptogramin and chloramphenicol before breakage of the cells and treatment of the cell-cap with [^{14}C]CAP (Table 8), but this did not annul the inhibitory effect of

Table 8. *Binding of [^{14}C]chloramphenicol to Staphylococcus aureus ribosomes after pre-treatment of the bacteria with the streptogramin antibiotics*

Antibiotics added during the initial incubation	[^{14}C]chloramphenicol binding (% of control)
Chloramphenicol (3.3 $\mu\text{g.}/\text{ml.}$)	64
Streptogramin (0.6 $\mu\text{g.}/\text{ml.}$)	36
Chloramphenicol and (3.3 $\mu\text{g.}/\text{ml.}$) + streptogramin (0.6 $\mu\text{g.}/\text{ml.}$)	21

Suspensions of *Staphylococcus aureus* (3 mg. bacteria/ml.) in medium A were incubated at 37° for 15 min. in the presence of the required antibiotic(s). At the end of the incubation period, cells were harvested by centrifugation and the experiment continued as described in Table 7.

Table 9. *The effect of the streptogramin antibiotics on the binding of [^{14}C]chloramphenicol to Bacillus megaterium ribosomes*

Soluble fraction		M conc. of antibiotic		[^{14}C]CAP binding (% of control)
Amount	Treatment	Component A	Component B	
—	—	6×10^{-8}	—	90
—	—	6×10^{-7}	—	38
—	—	6×10^{-6}	—	19
—	—	—	6×10^{-6}	100
—	—	6×10^{-8}	6×10^{-6}	92
From 50 mg. bacteria	—	6×10^{-8}	6×10^{-6}	68
From 100 mg. bacteria	—	6×10^{-8}	6×10^{-6}	56
From 50 mg. bacteria	Incubated 15 min. at 30° with the antibiotics	6×10^{-8}	6×10^{-6}	69
From 50 mg. bacteria	Boiled 10 min. and coagulated protein removed	6×10^{-8}	6×10^{-6}	71
From 50 mg. bacteria	Dialysed against buffer 24 hr. at 4°	6×10^{-8}	6×10^{-6}	92

The different experimental conditions set out above produced no alteration in the separate effects of component A (6×10^{-8} M) or component B (6×10^{-6} M) on the binding of ^{14}C -chloramphenicol.

Ribosome suspensions in 0.01 M-tris + HCl buffer, pH 7.4, containing 0.01 M-Mg $^{2+}$ and 0.2 M-K $^{+}$ were incubated for 5 min. at 4° with a mixture of (3×10^{-6} M) [^{14}C]chloramphenicol and the required antibiotic(s) and the additions as shown in the table. The ribosomes were harvested by centrifugation at 150,000 g for 100 min. and the radioactivity in the pellet estimated. 2 mg. of ribosomes were added per tube (2 ml. tubes).

streptogramin on the binding of [^{14}C]CAP by ribosomes. Similar results to those found with streptogramin were obtained with the equivalent mixture of components A and B.

Bacillus megaterium is more easily broken than *Staphylococcus aureus*, and further studies on the interaction of the streptogramin components have been carried out mainly with ribosomes from *B. megaterium*. The binding of [^{14}C]CAP to bacterial

ribosomes is not dependent on the presence of the soluble fraction; maximum binding to 70 S ribosomes occurs in 0.01 M-tris+HCl buffer, pH 7.4, containing 0.01 M-Mg²⁺ and 0.2 M-K⁺ or NH₄⁺ (Vazquez, 1964*b*). The previous finding that component B enhanced the inhibitory effect of component A on the binding of [¹⁴C]CAP to ribosomes was not confirmed with ribosomes from *B. megaterium*, *E. coli* or *S. aureus* when these were suspended in 0.01 M-tris-HCl buffer containing either 0.01 M-Mg²⁺ and 0.2 M-K⁺ (70 S ribosomes) or 0.001 M-Mg²⁺ and 0.06 M-K⁺ (30 S and 50 S ribosome subunits). It was then found that enhancement of inhibition required the presence of the soluble fraction and that its extent depended upon the amount of soluble fraction added (Table 9).

Treatment of components A and B with soluble fraction before the addition of ribosomes did not increase the inhibitory effect on [¹⁴C]CAP binding and the effect of the soluble fraction is not likely to be due to enzymic modification of either component A or B (Table 9). Removal of protein by boiling and centrifugation did not alter the effect of the supernatant fluid. After dialysis against buffer the non-dialyzable part of the soluble fraction was inactive (Table 9); ashed soluble fraction taken up as chloride or nitrate was inactive.

These results were obtained with ribosomes from *Bacillus megaterium* and have been confirmed with *Escherichia coli* ribosomes. Since enhancement can be demonstrated in cell-free systems from *E. coli*, but not with whole organisms, component B may be unable to penetrate the intact cells of Gram-negative bacteria.

DISCUSSION

These results show that the overall effects of streptogramin components A and chloramphenicol are very similar. The inhibitory effect of component A on [¹⁴C]CAP binding suggests that it acts at the ribosome level. This suggestion is supported by the inhibitory effect of the antibiotic on amino acid incorporation (from aminoacyl-s-RNA into protein) in cell-free systems (Vazquez, 1965*c*). Inhibition of amino acid incorporation by other antibiotics of group A has also been observed in bacterial cell-free systems by other workers (Yamaguchi & Tanaka, 1964; Laskin & May Chan, 1964). However, chloramphenicol has a greater effect on the incorporation of proline and lysine than on the incorporation of phenylalanine, whereas component A inhibits the incorporation of the three amino acids to a similar extent (Vazquez, 1965*c*), which suggests that component A does not attach to ribosomes at precisely the same site as chloramphenicol. If so, the inhibitory effect of component A on [¹⁴C]CAP binding to ribosomes might be due to an allosteric effect.

The site of action of streptogramin component B cannot be deduced from the present results. It has an immediate inhibitory effect on protein synthesis, and a delayed effect on cell wall and nucleic acid synthesis. The inhibitory effect of component B on protein synthesis has been confirmed in cell-free systems (Vazquez, 1965*c*).

The overall effects of streptogramin, or of equivalent mixtures of component A and component B, are similar to those of component A. There are, however, three important differences: (a) the minimum growth-inhibitory concentration of the mixture for *Staphylococcus aureus* is 10% of that of component B alone; (b) component A has an immediate effect on protein synthesis, whereas a lag period of about two minutes is observed before the synergistic mixture exerts its effect; (c) com-

ponent A is bacteriostatic, whereas the mixture of components A and B is bactericidal. No synergism between component A and component B has been found for inhibition of incorporation of proline, lysine and phenylalanine in cell-free systems directed respectively by polycytidylic, polyadenylic and polyuridylic acids (Vazquez, unpublished data). The above results suggest that the synergism of components A and B can be explained by an enhancement by component B of the binding of component A on ribosomes. A dialysable, low molecular weight, heat stable constituent of the soluble fraction, whose nature is at present unknown, is required for this enhancement.

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