An Investigation into the Mode of Action of Actinonin

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SUMMARY

The mode of action of actinonin, a pseudo-peptide antibiotic has been investigated. When added to cultures of *Bacillus subtilis* 7198 and 3610 actinonin inhibits growth. Evidence is presented which shows that this inhibition of growth reflects a bacteriostatic rather than bactericidal effect and that the site of action of the antibiotic is associated with RNA synthesis.

INTRODUCTION

The antibiotic actinonin first obtained from the culture filtrates of a *Streptomyces* species classified as *Streptomyces* sp. CUTTER C/2 NCIB 8845 (Gordon, Kelly & Miller, 1962) has been shown to have the constitutional formula of the pseudo-peptide shown in Fig. 1 (Ollis, East, Gordon & Sutherland, 1964). As a natural product this structure is of interest since it is the first known naturally occurring derivative of L-prolinol and is also the first known naturally occurring hydroxamic acid of the type R—CO—NHOH. Actinonin shows some structural relationship to other polypeptide antibiotics but it

Fig. 1. The constitution of actinonin.

contains no D- α -amino acid residue. This absence of D- α -amino acid residues in association with antibiotic activity should be considered in relation to the idea put forward by Arnstein (1957) that amongst the polypeptide antibiotics the minimum requirement for biological activity is the presence of one amino acid residue with the D-configuration. Actinonin has been shown to be active against, in the main, Gram-positive bacteria, with some evidence of activity against Gram-negative bacteria, the acid-fast bacteria and a number, of phage strains (Gordon *et al.* 1962). This wide and in some instances unusual biological activity coupled with its chemical stability and low toxicity makes actinonin an interesting antibiotic. This work describes investigations directed towards an understanding of the mode of action of actinonin.

METHODS

Organisms and growth conditions. The organisms used in this investigation were *Bacillus subtilis* (NCIB 7198) and *B. subtilis* (NCIB 3610).

The bacteria were grown overnight at 30° with constant shaking in a defined medium of Davis mineral medium (Davis & Mingioli, 1960) supplemented with L-asparagine and L-glutamic acid (10 µg./ml.). Bacteria from this suspension were used to inoculate fresh medium and the growth of the bacteria was followed by measuring the extinction of the suspension in a Unicam SP 600 at 610 mµ. Any additions to the system were made at the end of the lag period of bacterial growth unless otherwise stated. Actinonin was always added as an aqueous solution and was stable under the experimental conditions.

Viable counts were made in triplicate on the defined medium plus agar (1.5%, w/v) using the surface-spreading method after serial dilution of the bacteria in saline to ensure that 100 to 200 bacteria were distributed on each plate.

Synthesis of cellular constituents. (1) *Net synthesis of cellular RNA, DNA and protein.* Suspensions of *Bacillus subtilis* 7198 were incubated at 30° with shaking in the defined medium in the presence and absence of actinonin. Samples (3 ml.) were taken at intervals and cooled rapidly. The bacteria were harvested by centrifugation and washed with a buffered salt solution. The bacterial pellet was extracted three times with 0.2 N-perchloric acid at 4° for 60 min. and the supernatant fluids from the extractions combined. This constituted the bacterial pool fraction. The nucleic acids were extracted from the pellet with 0.5 N-perchloric acid (3 ml.) at 70° for 15 min. Three extractions were required.

The RNA was estimated by the orcinol method (Schneider, 1957) and the DNA by the diphenylamine method of Burton (1956). The residual pellet was taken up in N-NaOH and the protein present estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). All estimations were performed in triplicate and gave an accuracy of ± 5%.

(2) *The production of bacteriophage infection centres.* *Bacillus subtilis* 3610 growing exponentially in nutrient broth was harvested when a titre of 2×10^8 bacteria/ml. was obtained and then infected with phage øE at a multiplicity of three. The mixture was shaken and incubated for 15 min. at 30° to allow the phage to become absorbed to the bacteria. The infected suspension was then centrifuged (5000g) and the excess free phage particles removed with the supernatant fluid. The bacteria were resuspended in nutrient broth and diluted 4×10^6 -fold with fresh growth medium. The latent period of the phage infection in this system is 70 to 80 min. Actinonin at concentrations sub-inhibitory to bacterial growth was added or removed at various times throughout the latent period. Samples were taken at the end of the latent period and assayed for total infective centres (plaque formers) by the procedure described by Adams (1959).

RESULTS

The effect of actinonin upon the viability of Bacillus subtilis 7198. The limiting growth inhibitory concentration for *B. subtilis* 7198 under these conditions was seen to be 2 mg./ml. At this concentration the effect was bacteriostatic rather than bactericidal. The results are shown in Table 1.

Resistance of *Bacillus subtilis* to repeated additions of actinonin. Bacteria grown in the presence of actinonin for 24 hr or more were resistant to further treatment with actinonin (Fig. 2) but had a slower growth rate than the untreated control bacteria. No information has been obtained from which the possible mechanism of this resistance could be ascertained.

Respiration and fermentation. Actinonin (at 100, 1000 and 3000 $\mu\text{g./ml.}$) had no effect on endogenous respiration, oxidation of glucose or anaerobic fermentation of glucose when added to washed suspensions of *Bacillus subtilis* 7198 in Warburg vessels. These studies were made by the standard manometric techniques (Umbreit, Burris & Stauffer, 1964).

Permeability and integrity of the cell membrane. Bacteria previously grown in a rich medium were suspended in buffer in the presence and absence of actinonin

Fig. 2

Fig. 3

Fig. 2. The resistance of 'actinonin pretreated' *Bacillus subtilis* 7198 to further treatment with actinonin. A suspension of *B. subtilis* 7198 was grown in defined medium containing actinonin (100 $\mu\text{g./ml.}$) for 24 hr. This culture became the 'pretreated' inoculum. Another suspension of *B. subtilis* 7198 was grown in defined medium alone for 24 hr—this culture became the control inoculum. Both types of inoculum were then used to inoculate fresh medium alone (○—○, control inoculum; and △—△, pretreated inoculum). After 3 hr incubation (at arrow) actinonin (100 $\mu\text{g./ml.}$ final conc.) was added to one of the control inoculum cultures (●—●) and to one of the pretreated cultures (▲—▲). Growth was followed as described in the Methods section. When actinonin (100 $\mu\text{g./ml.}$) was added to the pretreated inoculum (▲—▲) growth of this sample and the control pretreated inoculum was identical (△—△).

Fig. 3. The effect of adding Mg^{2+} ions to a suspension of *Bacillus subtilis* 7198 growing in the presence of actinonin. A suspension of *B. subtilis* 7198 was incubated at 30° in the defined medium containing actinonin (100 $\mu\text{g./ml.}$) and 100 $\mu\text{g. Mg}^{2+}$ ions/ml. (△—△); 200 $\mu\text{g. Mg}^{2+}$ /ml. (▲—▲); 500 $\mu\text{g. Mg}^{2+}$ ions/ml. (□—□) or 1000 $\mu\text{g. Mg}^{2+}$ ions/ml. (●—●). Control suspensions of *B. subtilis* were incubated in the defined medium alone (○—○); defined medium with 1000 Mg^{2+} ions/ml. (●—●) and defined medium containing actinonin (100 $\mu\text{g./ml.}$) only (●—●). The growth was followed as described in the Methods section.

(100 and 1000 $\mu\text{g./ml.}$). The rate of release of amino acids, purines and pyrimidines from the bacteria into the medium was measured (Gale & Taylor, 1947). There was no evidence of increased permeability of the bacteria to these molecules in the presence of the antibiotic.

Chelation and antimetabolite studies. The addition of the metal ions Mg^{2+} , Mn^{2+} , Fe^{2+} , K^{+} and Ca^{2+} (0 to 1000 $\mu\text{g./ml.}$), the amino acids L-valine and L-isoleucine, D-alanine or L-aspartic acid (0 to 500 $\mu\text{g./ml.}$) to bacteria growing in the presence of actinonin failed to annul the growth inhibitory effect of the antibiotic. Figure 3 shows

Fig. 4. The constitution of *O*-benzylactinonin.

Table 1. *Viability of Bacillus subtilis 7198 grown in the presence of increasing concentrations of actinonin*

B. subtilis (6×10^8 bacteria/5 ml.) was incubated at 30° for up to 4 hr in the defined medium. Actinonin in sterile distilled water was added in the concentrations shown at the beginning of the incubation period. Samples (0.1 ml.) were taken at the times indicated below and plated out in triplicate on to defined media as described in the Methods section and incubated for 24 hr at 30° . The colonies formed were then counted. Accuracy obtained was $\pm 5\%$.

Actinonin added (mg./ml.)	Time (hr)			
	0	1	2	3
0		7.3×10^6	1.2×10^7	3.7×10^7
0.1	6×10^6	6.2×10^6	8.0×10^6	2.2×10^7
1.0		5.9×10^6	6.6×10^6	1.8×10^7
2.0		6.1×10^6	6.4×10^6	6.2×10^6
3.0		6.1×10^6	6.0×10^6	5.7×10^6

Table 2. *Growth inhibitory effect of actinonin and O-benzylactinonin on a culture of Bacillus subtilis 7198 growing in the test system*

Organisms were grown in defined medium in Monod flasks in presence of compound under test. The concentration of compounds varied from 100 to 2000 $\mu\text{g./ml.}$ Growth was measured at 610 $m\mu$. Inhibition was calculated as the percentage of the growth measured in the absence of any added compound.

Compound added	Concn. ($\mu\text{g./ml.}$)	% growth inhibition after 3 hr incubation in presence of test compound
Actinonin	100	44
	1000	50
<i>O</i> -Benzylactinonin	500	5
	1000	14
	2000	38

the results obtained using Mg^{2+} ions. Identical relationships were seen when the other additions were made to the system. Furthermore, the addition of a synthetic analogue of actinonin, *O*-benzylactinonin (kindly supplied by Mr J. P. Devlin) consistently and reproducibly produced an inhibition of bacterial growth. Results shown in Table 2. This analogue has the free hydroxamic acid residue blocked by the benzyl group (see Fig. 4).

Synthesis of cellular constituents. An investigation during the first 75 min. of growth after the addition of the antibiotic shows that the total synthesis of RNA and protein are inhibited (Table 3), but that DNA synthesis is not significantly affected. However, the initial site of action of the antibiotic appears to be associated with RNA synthesis since a significant reduction of total RNA is evident after 15 min. incubation, whereas a reduction in total protein is only evident after 30 min. incubation.

Table 3. *The effect of actinonin upon the growth and total synthesis of protein, RNA and DNA of Bacillus subtilis*

Six Monod flasks containing defined medium were inoculated with *B. subtilis* 7198 and incubated with shaking until the exponential growth phase was established (O.D. 0.405 at 610 m μ which gave a dry wt of 6.6 mg./3 ml. sample). To one set of three flasks actinonin was added in aqueous solution to give a final concentration of 1 mg./ml., to the other remaining three flasks an equal volume of sterile distilled water was added. The flasks were then returned to the water bath 37° and incubated with shaking. At intervals of 15 min. growth was measured (see Methods section) and samples (3 ml.) taken and the total protein, RNA and DNA estimated (see Methods section).

Time (min.)	Growth		Total protein (mg.)		Total RNA (mg.)		Total DNA (mg.)	
	– Actinonin	+ Actinonin	– Actinonin	+ Actinonin	– Actinonin	+ Actinonin	– Actinonin	+ Actinonin
0	0.41	0.41	3.30	3.30	1.05	1.05	0.15	0.15
15	0.52	0.52	3.84	3.80	1.32	1.20	0.15	0.14
30	0.64	0.63	4.50	4.29	1.56	1.22	0.15	0.15
45	0.77	0.71	5.43	5.01	1.87	1.31	0.17	0.16
60	0.92	0.75	6.45	5.60	2.28	1.37	0.29	0.28
75	1.10	0.89	7.29	5.63	2.43	1.42	0.31	0.29

Table 4. *The percentage inhibition of formation of infective centres caused by addition of actinonin to the Bacillus subtilis 3610/phage ϕ E system*

Phage particles were allowed to adsorb and infect the bacteria: (A) After increasing time intervals actinonin was added to the system and the incubation continued (see Methods section). (B) Immediately after phage infection actinonin was added and removed by dilution at time intervals throughout the latent period (see Methods section). In both cases at the end of the latent period the bacterial suspensions were analysed for infective centre formation.

A. Time when actinonin added (min.)	% inhibition of infective centre production	B. Time when actinonin removed (min.)	% inhibition of infective centre production
0	98	0	—
10	98	10	57
20	97	20	67
30	98	30	57
40	98	40	57
50	—	50	57
60	95	60	79
70	—	70	74

Effect of actinonin upon the production of infective phage centres in Bacillus subtilis 3610 phage ϕ E system. The addition of actinonin, only for the adsorptive period for the phage, showed that the antibiotic did not inhibit phage adsorption. Further studies during which actinonin was added: (a) at intervals throughout the adsorption period, and (b) for increasing time into the latent period before being removed from the system, demonstrated that actinonin affected some process which occurred during the first 10 min. of the latent period. Moreover, if the actinonin was not added until after an increasing time interval during the latent period and then left in the system for the rest of the latent period, the antibiotic affected some process which occurs late in the latent period. These results are shown in Table 4. When other antibiotics whose site of action have been reported were added to the system in such a manner and the results compared with those obtained for actinonin (Table 5), actinonin was shown to resemble actinomycin-D rather than chloramphenicol or 5-aminoacridine.

Table 5. (A) *Percentage inhibition of formation of infective centres caused by the addition of growth inhibitory substances at time intervals throughout the latent period after infection. Bacillus subtilis 3610/phage ϕ E system*

Bacteria were infected with phage particles and after increasing time intervals growth inhibitory substances were added to the system and the incubation continued (see Methods section). At the end of the latent period the cell suspensions were analysed for infective centre formation.

Time when substance added (min.)	Growth inhibiting substance added			
	Actinomycin-D (1 μ g./ml.)	5'-Aminoacridine (20 μ g./ml.)	Chloramphenicol (100 μ g./ml.)	Actinonin (100 μ g./ml.)
0	99	100	97	98
10	99	92	91	98
20	97	95	80	97
30	95	98	46	98
40	96	87	50	98
50	97	74	—	—
60	95	46	43	95
70	50	34	54	71

(B) *Percentage inhibition of formation of infective centres caused by addition of growth inhibitory substances during adsorption of the phage and then removed at time intervals throughout the latent period. Bacillus subtilis 3610/phage ϕ E system*

Bacteria were infected with phage particles and immediately the growth inhibitory substances were added (see Methods section). At time intervals throughout the latent period the inhibiting substances were removed from the infected cells by dilution and incubation continued. At the end of the latent period the cells were analysed for infective centre formation.

Time when substance removed (min.)	Growth inhibiting substance			
	Actinomycin-D (1 μ g./ml.)	5'-Aminoacridine (20 μ g./ml.)	Chloramphenicol (100 μ g./ml.)	Actinonin (100 μ g./ml.)
10	81	0	0	57
20	94	0	0	67
30	85	76	52	57
40	100	76	44	57
50	87	83	100	57
60	100	91	100	79
70	94	80	96	74

DISCUSSION

When the structure of actinonin with its free hydroxamic acid residue is considered the failure to reverse the inhibitory action of actinonin by the addition of metal ions to the system is both interesting and surprising. This is further emphasized by the reports that the growth inhibitory action of aspergillic acid, a cyclic hydroxamic acid, has been reversed by the addition of ferrous ions (Goth, 1945). Furthermore, as a hydroxamic acid derivative there is some structural similarity between actinonin, D-cycloserine and Hadacidin (Fig. 5). Thus from these structural considerations it appeared possible that the biological activity of actinonin could involve an anti-metabolite/essential metabolite relationship. However, the addition of the respective essential metabolites D-alanine and L-aspartic acid failed to annul the growth inhibitory effect of actinonin. Hogg, Biswas & Broquist (1965) reported that cyclic

Fig. 5. The constitution of actinonin (A), D-cycloserine (B) and Hadacidin (C).

hydroxamic acids inhibited the growth of *Escherichia coli* by interfering with the biosynthesis of L-valine and L-isoleucine. They showed that the toxicity of cyclic hydroxamates could be counteracted by the addition of mixtures of L-valine and L-isoleucine or by L-valine and L-isoleucine separately. Again the addition of L-valine and L-isoleucine, together or individually, did not reverse the inhibitory effect of actinonin.

This inability to reverse the growth inhibitory action of actinonin, particularly when essential metal ions were added, suggested that the hydroxamic acid residue is not part of the active site of the actinonin molecule. This idea was supported when an analogue of actinonin, *O*-benzylactinonin, in which the hydroxamic acid residue had been chemically blocked proved to be biologically active. The growth inhibitory action of this compound proved to be very small but was reproducible. When further analogues become available the position of the hydroxamic acid residue with respect to the active site of the molecule will be investigated further.

The results presented here are compatible with the idea that the over-all grown inhibitory effect of actinonin is associated with RNA synthesis. This is supported by the inhibitory effect of the antibiotic on the total amount of RNA synthesized within the first 75 min. of incubation in the presence of actinonin. Within this time total RNA, and protein synthesis together with growth is seen to be inhibited but the initial inhibitory effect is associated with RNA synthesis. Moreover, the pattern of infective centre formation obtained when actinonin is added or removed from the phage ϕ E/*Bacillus subtilis* 3610 system during the latent period demonstrates two possible sites of antibiotic action with respect to time within the latent period. This fact is also compatible with the site of action being associated with RNA rather than DNA or protein synthesis. The activity of actinonin also resembles the pattern obtained when actinomycin-D, a known inhibitor of DNA directed RNA synthesis, is used in the system instead of actinonin and does not resemble the patterns obtained in the system when the protein synthesis inhibitor, chloramphenicol or 5-aminoacridine, which is known to inhibit viral DNA synthesis, is used. Thus these results indicate that actinonin inhibits bacterial growth by interfering with total cellular RNA synthesis. However, the actual site of action of actinonin cannot be deduced from these results.

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