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Thiostrepton, an Inhibitor of 50S Ribosome Subunit Function

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Thiostrepton inhibits ^{14}C-leucine incorporation by intact cells of *Bacillus megaterium* as well as ^{14}C-phenylalanine incorporation by a poly U-directed extract of *Escherichia coli*. Extracts of *E. coli* which are pretreated by incubation with thiostrepton cannot be reactivated by dialysis to more than 5% of their former activity. The 50S ribosome subunit appears to be the site of thiostrepton action, since protein-synthesizing activity can be restored to dialyzed pretreated extracts by supplementation with 50S ribosome subunits but not with 30S ribosome subunits. This technique also provides a simple sensitive method for detection of the biological activity of very small amounts of 50S ribosome subunits.

Thiostrepton (synonym for bryamycin) is a peptide antibiotic which primarily inhibits gram-positive organisms. Recent results of chemical and X-ray crystallographic studies on thiostrepton have been reported, respectively, by Anderson et al. (1) and by Bodanszky et al. (1*a*). Many of the known chemical, physical, and structural properties of this antibiotic have been summarized by Umezawa et al. (8).

Neubart and Marmur (*personal communication*) have identified bryamycin as an inhibitor of protein synthesis, and Dubnau et al. (5) have reported that a genetic determinant of resistance to these antibiotics in *Bacillus subtilis* is closely linked, by transformation analysis, to genetic markers which determine ribosomal ribonucleic acids (RNA) as well as resistance to several inhibitors of ribosome function. In the present communication, we present evidence that thiostrepton is an inhibitor of ribosome function and that its site of action is on the 50S ribosome subunit.

We first tested the effect of thiostrepton on ^{14}C-thymidine, -uracil, and -leucine incorporation by intact cells of *B. megaterium* (strain B-5, obtained from H. Halvorson). This organism was used because intact cells of *E. coli* are relatively more resistant to the action of thiostrepton. Thiostrepton inhibited leucine incorporation within less than 5 min after addition, whereas the rate of thymidine and uracil incorporation remained unchanged for about 15 min after addition of the antibiotic (Fig. 1). Further, poly U-directed ^{14}C-phenylalanine incorporation in cell-free extracts of *E. coli* (strain C-3000) was inhibited more than 95% at 1.7×10^{-5} M thiostrepton (Table 1A). These data imply that thiostrepton acts by inhibiting protein synthesis. Because thiostrepton has very low solubility in water, stock solutions (2.5 mg/ml) were prepared by using dimethyl sulfoxide (DMSO) as solvent. The final concentration of DMSO utilized in cell-free protein synthesis was 2%, and a control was performed in which 2% DMSO alone was used. Stimulation of ^{14}C-phenylalanine incorporation in response to DMSO as well as a net stimulation attributable to the antibiotic at 1.7×10^{-7} M was seen. The basis of these findings is not understood; however, they do not alter our conclusions.

S-30 fractions of *E. coli* prepared by the method of Matthaei and Nirenberg (6), which have been pretreated with thiostrepton and then dialyzed, lose about 95% of their ability to incorporate ^{14}C-phenylalanine (Table 1B). When purified ribosomes and supernatant fractions were recombined with thiostrepton-pretreated ribosomes and supernatant fractions in four possible complementary combinations, activity was seen only when untreated ribosomes were used (Table 1C). This clearly indicates that the ribosome contains the sensitive site of thiostrepton action.

Rather than pretreat purified 30S and 50S subunits, we attempted to identify the subunit specificity of thiostrepton action by determining the ability of active, purified 30S and 50S subunits to restore activity to a thiostrepton-inactivated crude extract. We note that activity could be restored only by supplementing the extract with 50S subunits, but not with 30S subunits (Table 1D). Thus, thiostrepton appears to inhibit specifically the 50S subunit, and, from Table 1E, we can infer that the subunits are relatively clean and active. The apparent stimulation

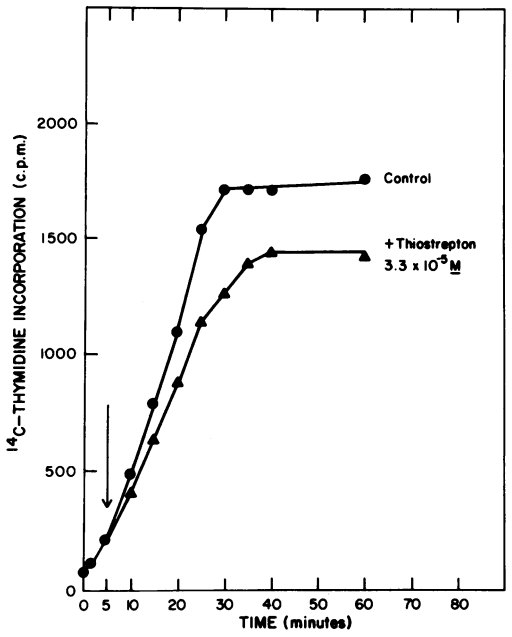

FIG. 1. *Effects of thiostrepton on incorporation of ^{14}C-thymidine by intact cells of B. megaterium. Time of addition of thiostrepton is indicated by the arrow. Experimental conditions were otherwise identical to those described previously* (7).

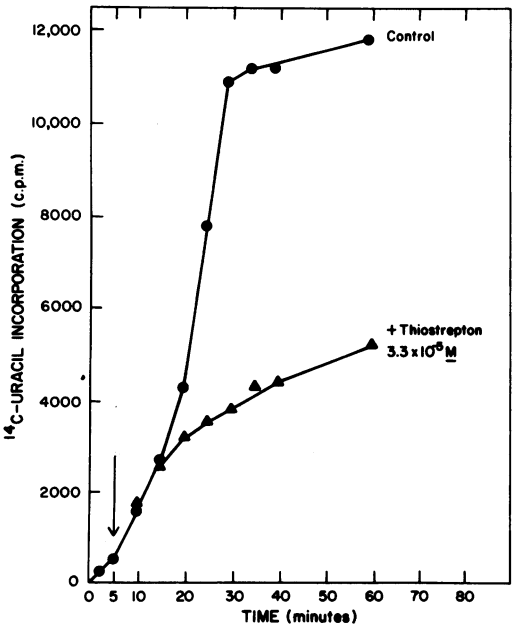

FIG. 2. *Effects of thiostrepton on incorporation of ^{14}C-uracil by intact cells of B. megaterium. Time of addition of thiostrepton is indicated by the arrow. Experimental conditions were otherwise identical to those described previously* (7).

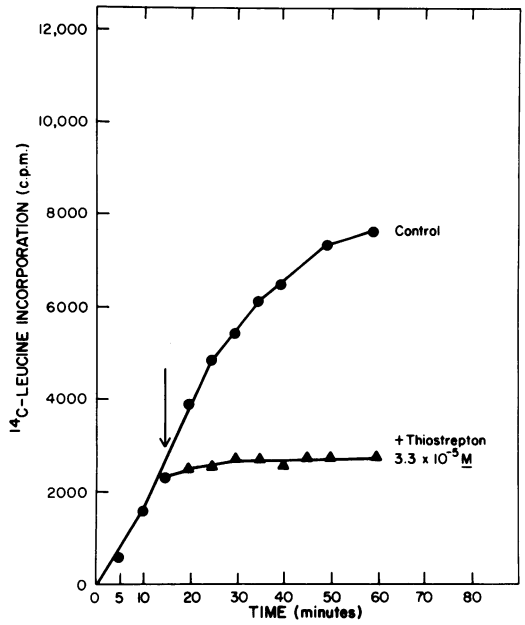

FIG. 3. *Effects of thiostrepton on incorporation of ^{14}C-leucine by intact cells of B. megaterium. Time of addition of thiostrepton is indicated by the arrow. Experimental conditions were otherwise identical to those described previously.*

of protein synthesis seen in the presence of antibiotic or in reconstituted systems, or both, has been observed previously by other investigators as well as in our own studies (3, 4). The relation of such apparent stimulations to the mechanism of action still remains unclear. In addition, the requirements for thiostrepton binding to ribosomes will have to await its availability in a labeled form.

In more recent studies (*data not shown*), we have observed that resistance to thiostrepton cannot be induced by erythromycin in strains of *Staphylococcus aureus* which show dissociated resistance to erythromycin (9), nor does thiostrepton inhibit ^{14}C-chloramphenicol binding to *B. stearothermophilus* ribosomes. This latter observation is exceptional in view of our recent studies on the ribosomal binding of ^{14}C-chloramphenicol (2), in which we noted that known inhibitors of 50*S* ribosome subunit function are also effective inhibitors of chloramphenicol binding to ribosomes.

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TABLE 1. *Effect of thiostrepton (THS) on cell-free protein synthesis*[a]

Experimental condition	^{14}C-phenylalanine incorporation[b]		
	+poly U	−poly U	net
A. Effect of THS in a crude (S-30) system			
Complete	31,756	2,322	
+THS (1.7×10^{-7} M)	54,946		
+THS (1.7×10^{-6} M)	26,649		
+THS (1.7×10^{-5} M)	1,998		
+ 2% DMSO	48,036		
B. Irreversibility of inhibition in a THS-treated extract			
Complete (untreated S-30)	22,975	1,003	21,972
Complete (THS-treated S-30)	1,461	425	1,036
C. Reconstitution of protein synthesis from THS-treated and untreated ribosomes and supernatant, respectively			
Treated, untreated	179	119	60
Untreated, treated	5,705	221	5,484
Untreated, untreated	1,705	234	1,471
Treated, treated	244	193	51
D. Reconstitution of protein synthesis from THS-treated extract and purified ribosomal subunits			
Complete (untreated S-30)	22,975	1,003	21,972
Complete (THS-treated S-30)	1,461	425	1,036
+30*S* subunits (0.25 OD_{260} units)	1,440	434	1,006
+30*S* subunits (0.50 OD_{260} units)	2,339	432	1,907
+50*S* subunits (0.60 OD_{260} units)	4,541	577	3,964
+50*S* subunits (1.20 OD_{260} units)	14,434	751	13,683
E. Control for part D			
S-100	681	475	206
S-100 + 30*S*	736	510	226
S-100 + 50*S*	858	606	252
S-100 + 30*S* + 50*S*	15,955	710	15,245

[a] Compositions of buffers and incubation mixtures for protein synthesis are the same as those which we used previously (3, 4), except where modified as indicated. Thiostrepton-treated ribosomes or extracts were incubated with thiostrepton (1.4×10^{-5} M) at 37 C for 15 min. The reaction mixtures (200 μliters) were chilled and dialyzed at 4 C for 3 hr with three changes (1 liter each) of standard buffer. For phenylalanine incorporation, a total volume of 100 μliters was used in parts A and B, and 50 μliters was used in parts C and D. The reaction mixtures contained, in addition to the usual components cited above: part B, 10 μliters of ribosomes (200 OD units/ml) and 10 μliters of S-100; part C, 30*S* subunits (50 OD units/ml), 5 and 10 μliters, respectively, and 50*S* subunits (200 OD units/ml), 5 and 10 μliters, respectively; part D, 5 μliters of S-100, 5 μliters of 30*S* subunits, and 5 μliters of 50*S* subunits. All OD measurements given are at 260 nm. Stock solutions of thiostrepton were prepared by dissolving 2.5 mg in 1 ml of dimethyl sulfoxide.

[b] Values expressed as counts per min.

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