THE MODE OF ACTION OF THIOSTREPTON *IN VIVO*

by ERIC CUNDLIFFE,
Department of Pharmacology,
Cambridge University Medical School,
Hills Road, Cambridge, England.

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In cell-free systems derived from *E. coli* thiostrepton was shown to interact with the 50s ribosomal subunit (1) and to prevent the formation of a (G factor-guanine nucleotide-ribosome) complex believed to be involved in the translocation process (2,3). Since the drug failed to inhibit both the puromycin reaction and the non-enzymic binding of amino acyl-tRNA to the ribosome messenger RNA complex *in vitro* this led to the classification of thiostrepton as an inhibitor of translocation (4). Here I report that the primary action of thiostrepton *in vivo* is to inhibit the functional binding of amino acyl-tRNA to the ribosomal A site. This conclusion was reached as a result of studying the effects of thiostrepton on the puromycin reaction in bacterial protoplasts.

METHODS

These were closely similar to those described previously (5) and are given here only in outline. Protoplasts of *Bacillus megaterium KM* were prepared from cells previously steady-state labelled with ^{32}P-phosphate to label ribosomal RNA and were incubated in protoplast medium (5) supplemented with 0.03 (^{W}/v) peptone. Nascent peptides were labelled by incubating protoplasts for 30 sec. at 37^{o} with ^{3}H-leucine (17.6 C/mM; used at a final activity of 40 μc/ml) whereupon incorporation was terminated by the addition of an antibiotic. Subsequently puromycin was added and the progress of the puromycin reaction was followed by analysing lysates on sucrose density-gradients. As previously described these lysates were treated before analysis with ribonuclease to degrade polyribosomes to 70s

material and thereby localize ribosomes in the gradients. The sucrose density-gradients used here differed from those used previously in containing 5mM Mg^{++} (rather than 10mM). Under these conditions of analysis free 70s ribosomes are unstable and there is a qualitative (though not quantitative) conversion of polyribosomes to ribosomal subunits as the puromycin reaction proceeds.

COMPUTATION OF RESULTS.

In control experiments when polyribosomes were extensively degraded to ribosomal subunits following a 30 sec. labelling of protoplasts with ^{3}H-leucine, it was observed that the released subunits were almost completely devoid of ^{3}H-radioactivity. Accordingly the amount of nascent peptide material on ribosomes in any given sucrose gradient was estimated as (^{3}H in polyribosomes/ total ^{32}P in ribosomes) i.e. as (^{3}H at 70s/^{32}P at 70s + 50s + 30s) to compensate for any differences in the numbers of ribosomes applied to different gradients.

RESULTS AND DISCUSSION

To assay release of ^{3}H-leucine-labelled peptides from ribosomes by puromycin *in vivo*, it is necessary to stop protein synthesis promptly and essentially completely at a known time in order to prevent continued incorporation of ^{3}H-leucine into the proteins of mature ribosomes. Therefore it is necessary to use rather high concentrations of antibiotics in studies such as these. Table 1 shows that thiostrepton fulfils this requirement: the specific radioactivity (^{3}H in polyribosomes/^{32}P in ribosomes) rapidly attained a constant value when thiostrepton was added to terminate 30 sec. of labelling of protoplasts with ^{3}H-leucine. Table 1 also shows that polyribosomes were stable indefinitely in the presence of high concentrations of thiostrepton as is the case with many other ribosome-inhibitors. The subsequent addition of low concentrations of puromycin was followed by rapid and quantitative release of nascent peptides from ribosomes and extensive breakdown of polyribosomes (Table 2A and Fig. 1). The significance of the residual 70s material in Fig. 1B is not clear since traces

TABLE 1

EFFECTS OF THIOSTREPTON ON INCORPORATION OF ^{3}H-LEUCINE INTO POLYRIBOSOMES AND ON THE CELLULAR CONTENT OF POLYRIBOSOMES

	Time after addition of thiostrepton 1 Min.	3 Min.
Specific Radioactivity* (^{3}H in polysomes/^{32}P in ribosomes)	100	98
Ribosomes involved in polyribosomes** (% of total)	82	79

^{32}P-labelled protoplasts were incubated with ^{3}H-leucine for 30 sec. at 37° then thiostrepton (300 µg/ml) was added and incubation was continued. Samples were taken and analysed as in Fig.1.

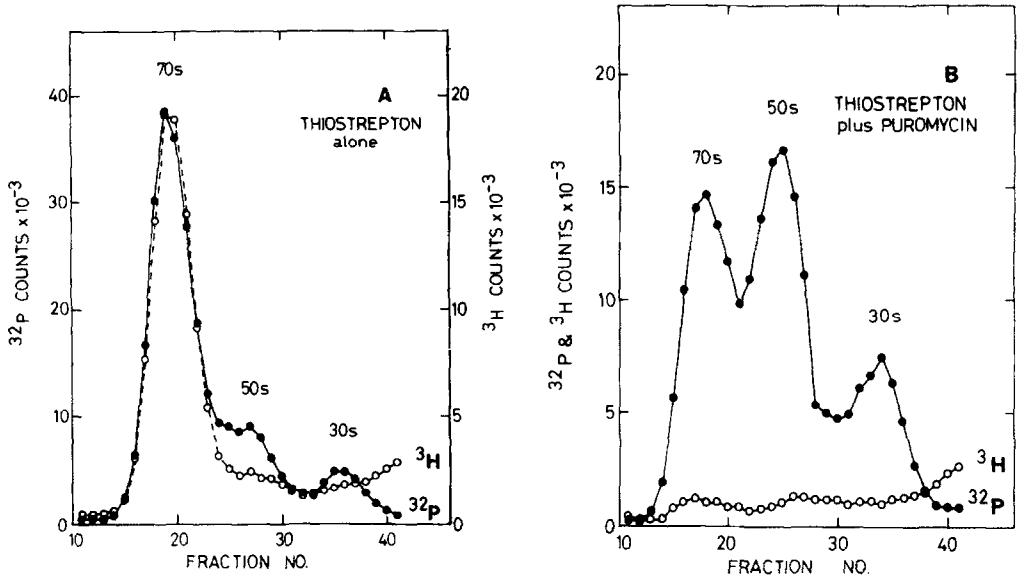
* Values of this ratio were normalised for ease of comparison.

** Computed as (^{32}P at 70s/^{32}P at 70s + 50s + 30s)

These experiments were clearly carried out at the limit of solubility of thiostrepton since incubations containing the drug exhibited a marked turbidity.

(1% ^{w}/v) of dimethyl sulphoxide, the solvent used for thiostrepton, prevented dissociation of ribosomes in control experiments.

We have previously argued (5) that inhibitors of translocation should inhibit the puromycin reaction *in vivo* by confining nascent peptides to the A sites of functional ribosomes. Conversely, in systems where nascent peptides are confined to the P sites of ribosomes and are free to react with puromycin, inhibitors of the translocation process should be without effect. This latter situation can be created *in vivo* by incubating cells or protoplasts with chlortetracycline, an antibiotic which prevents the enzymic binding of amino acyl-tRNA to the ribosomal A site (6, 7, 8) and thereby restricts nascent peptides to the P sites of ribosomes without impairing their ability to react with puromycin (5; also Table 2B). The characteristics of the inhibition of the puromycin reaction by erythromycin (see Table 2A and B) led us to suggest that this antibiotic inhibits the translocation process *in vivo* (5). By comparison (Table 2) it can be seen



LEGEND TO FIGURE 1.

THIOSTREPTON AND THE PUROMYCIN REACTION IN VIVO

Protoplasts, steady-state labelled with ^{32}P-phosphate were incubated for 30 sec. with ^{3}H-leucine before thiostrepton (300 µg/ml) final concentration) was added. After 2 min. with thiostrepton, puromycin (10 µg/ml final concentration) was added and incubation was continued at 37°. Samples were taken into DNase and Triton X100 as previously described (5) and lysates were analysed on sucrose density-gradients. Fractions from sucrose gradients were treated with trichloroacetic acid, filtered on glass fibre discs and their radioactivity estimated as described previously.

(A) Protoplasts treated with thiostrepton for 2 min.

(B) Protoplasts treated with thiostrepton and then with puromycin for 2 min.

●——● ^{32}P-radioactivity (acid precipitable)

O - - - O ^{3}H -radioactivity (acid precipitable)

that thiostrepton does not inhibit the puromycin reaction under any of these conditions and resembles chlortetracycline rather than erythromycin in its action. This can be seen most clearly in Table 2B where thiostrepton mimics chlortetracycline in preventing the inhibitory action of erythromycin. This result is also important since it indicates that the failure of thiostrepton to inhibit the puromycin reaction was not due to a slow residual rate of functioning of ribosomes in the presence of the drug.

TABLE 2

EFFECTS OF ANTIBIOTICS ON THE PUROMYCIN REACTION IN VIVO SINGLY AND IN COMBINATION

ADDITIONS	NASCENT PEPTIDES OF RIBOSOMES (% of those present when puromycin added) Time after addition of puromycin 1 Min.	3 Min.	5 Min.
(A)			
THS_{300}	12	6	5
CTC_{150}	10	6	5
ERY_{300}	104	103	95
(B)			
CTC_{200} then ERY_{300}	20	-	8
CTC_{200} then THS_{300}	6	-	-
THS_{300} then ERY_{300}	12	10	9

Experiments carried out as in legend to Fig. 1. In experiments involving more than one antibiotic in addition to puromycin (Section B) the antibiotics were added at intervals of 2 min. Puromycin was used at a final concentration of 10 µg/ml.

Abbreviations: THS = thiostrepton; CTC = chlortetracycline; ERY = erythromycin. Subscripts e.g. CTC_{150} give drug concentrations in "µg/ml".

This might have allowed nascent peptides to enter a puromycin-sensitive state albeit at a reduced rate. However if this had been so, nascent peptides should also have been able to enter an erythromycin-sensitive state and to be thus rendered resistant to puromycin. Accordingly I conclude that thiostrepton inhibits protein synthesis in vivo so as to leave nascent peptides in the ribosomal P sites, free to react with puromycin. The obvious corollary of this conclusion is that thiostrepton achieves this by preventing the functional binding of amino acyl-tRNA into the ribosomal A site. Since thiostrepton inhibits translocation - assays in vitro but apparently does not inhibit translocation in vivo it would appear that the drug can only bind to the ribosome in the post-translocation state prior to the binding of amino acyl-tRNA i.e. when the ribosomal A site is vacant.

A similar conclusion has recently been reached by Modolell and co-

workers (9) who have observed that thiostrepton inhibits the enzymic binding of amino acyl-tRNA to ribosomes *in vitro* at concentrations similar to those at which it prevents interaction between G factor, GTP and ribosomes. They further showed that, unlike chlortetracyline, thiostrepton inhibits the GTPase reaction normally associated with the enzymic binding reaction. A plausible conclusion (see also ref. 9) from all the available data is that 50s ribosomes possess a single "GTPase" site located in or near the 50s moiety of the A site and that thiostrepton binds at or near this GTPase site. Consequently *in vitro* the drug can inhibit either (i) the G factor - GTPase reaction, involved in translocation or (ii) the T_u factor - GTPase reaction, involved in binding amino acyl-tRNA to the A site. However the present results show that *in vivo* only an inhibition of the amino acyl-tRNA binding reaction was observed. This difference presumably reflects the different circumstances under which the drug is presented to the ribosome in various experiments. On the one hand (*in vitro*) thiostrepton may be added to washed ribosomes devoid of factors and other components necessary for activity, alternatively (*in vivo*) the drug is added to functional ribosomes bathed in such factors and components.

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