IN VIVO AND IN VITRO BINDING OF DIHYDROSTREPTOMYCIN TO *ESCHERICHIA COLI* RIBOSOMES

Elaine PRIZANT and Margot KOGUT
Department of Biochemistry, University of London King's College, Strand, London WC2R 2LS, U.K.

Received 23 November 1974

1. Introduction

Since Spotts and Stanier first suggested that streptomycin exerts its antibiotic by inhibiting protein synthesis in sensitive bacteria [1], the antibiotic has been shown to act on such ribosomes in vivo and in vitro [2–4]. In vitro studies have shown that streptomycin combines with the 30S ribosomal subunit [5–10].

We have now studied the distribution of dihydrostreptomycin taken up by ribosomes in vivo at different stages of growth inhibition. This is important because one cannot necessarily apply conclusions drawn from in vitro effects to in vivo mechanisms.

This report present results which show for the first time that the combination in vivo of dihydrostreptomycin with 30S ribosomal subunits is proportional to inhibition of growth and protein synthesis [11,12].

We have also studied the addition of dihydrostreptomycin to ribosomal preparations in vitro to find out which binding sites are accessible, and the relation of such in vitro binding to in vivo effects. We found no competition between antibiotic added to intact cells during growth and antibiotic added to cell-free extracts; in other words, dihydrostreptomycin could bind to 30S ribosomes in vitro after in vivo treatment had produced complete inhibition of growth.

2. Materials and methods

2.1. *Growth of culture*

Escherichia coli MRE 600 was grown aerobically in mineral salts medium with 0.4% (w/v) glucose and 0.2% (w/v) casamino acids at 32°C [13,14]. Cultures were labelled with [^{14}C]uracil (8.63×10^{-2} μCi/mg, 15 μg/ml of culture) to a constant specific activity. For this we allowed 2 generations of growth [15]. Growth was measured as absorbance at 500 nm and plotted as Log_2 [16,13].

2.2. *In vivo dihydrostreptomycin treatment*

[^{3}H]dihydrostreptomycin (75 μCi/mg, 15 μg/ml culture) was added to logarithmically growing cultures. This high specific activity is necessary because streptomycin is effective at only 1 or a few molecules per ribosome (2,9,10,17).

At various times after addition of dihydrostreptomycin the degree of growth inhibition was determined in the manner previously described [13,18]: portions of cultures were rapidly filtered, washed 3 times with the mineral salts medium containing double the PO_4^{3-} concentration and 10 mM Mg^{2+}, and resuspended in antibiotic-free medium. Further incubation then allowed determination of the inhibited growth rates.

Determinations of intracellular dihydrostreptomycin were carried out on the remaining portions of cultures. These were harvested by rapidly cooling to below 4°C, centrifuging and washing in medium enriched with PO_4^{3-} and Mg^{2+} as above. Since it had been calculated from studies on streptomycin uptake [18] that the minimum concentration to produce complete inhibition of growth is of the order of 0.1 μg/mg dry weight cells, we argued that a level of labelled antibiotic below this figure, remaining with the cell pellet, would indicate insignificant adsorption to the cell surface. It was found that 3 washes of the harvested cultures under these conditions were sufficient to reduce the dihydrostreptomycin concentrations to below this figure.

Cells were broken by alumina grinding and extracted with 10 mM Tris–HCl buffer pH 7.3 containing 10 mM Mg^{2+} and 60 mM KCl. Alumina and unbroken cells were removed by centrifuging at 20 000 *g* for 15 min.

Parts of the crude extracts were centrifuged at 139 000 *g* for 5 1/4 hr in a 10 X 10 aluminium angle-head rotor on an MSE 50 centrifuge in order to pellet all the ribosomes (M. Cannon – personal communication). The ^{3}H and ^{14}C counts and absorbance at 260 nm were measured for the ribosomal pellet and supernatant fractions.

Another part of the crude extract was subjected to centrifugation through a 15–30% (w/v) linear sucrose gradient in the same Tris MG KCl buffer for 6 1/4 hr at 94 000 *g* in the 3 X 23 aluminium swing-out rotor on an MSE-65 centrifuge at 5°C. The gradients were fractionated and collected as 25 drop fractions in parallel sets of tubes; the middle 3 drops of each fraction were used for absorbance measurements at 260 nm. After plotting the profiles of the absorbance at 260 nm, the 22 drop tubes containing polysomes, 70S monomers, 50S and 30S subunits and the top fractions of the gradients were pooled separately. The ^{3}H and ^{14}C counts in each of these pooled fractions were then measured. For counting, 0.2 ml samples were taken, and 0.8 ml of water added. They were then counted in 10 mls of a scintillation fluid containing 4 g PPO, 0.1 g dimethyl POPOP, 500 ml Triton X-100 and 1 litre Toluene.

For controls, untreated exponentially growing cultures (labelled with [^{14}C]uracil) were harvested and after alumina grinding [^{3}H]dihydrostreptomycin (approx. 0.1 μg/mg dry weight harvested cells) was added during extraction with buffer. These extracts were then further treated as above.

2.3. *In vitro treatment after complete inhibition in vivo*

In order to investigate any interaction between streptomycin added in vitro and in vivo, exponentially

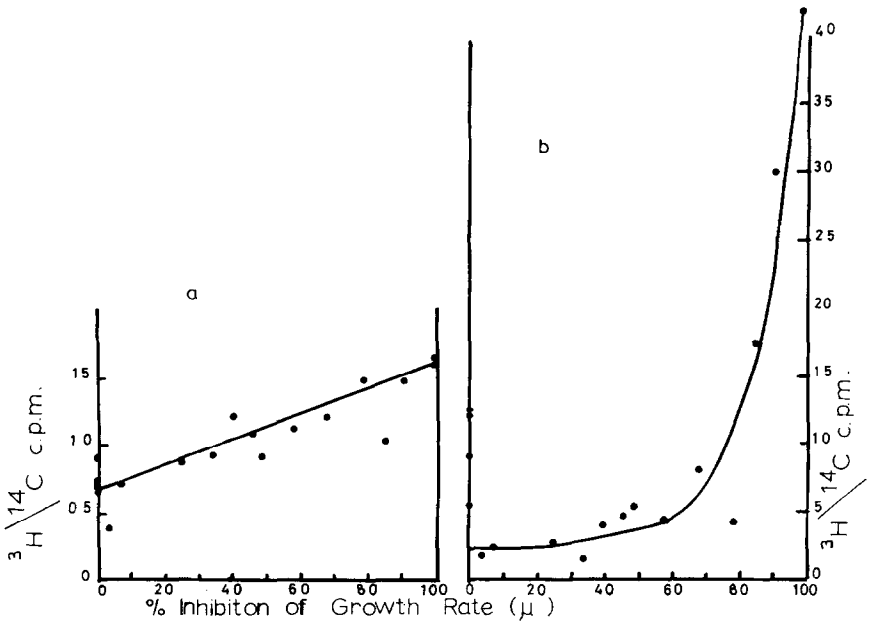

Fig.1. Distribution of intracellular [^{3}H]dihydrostreptomycin between ribosomes and supernatant fractions as a function of growth inhibition. Logarithmically growing [^{14}C]uracil-labelled cultures, treated with [^{3}H]dihydrostreptomycin (15 μg/ml) for various periods, were divided into two portions. One of these, after removal of extracellular antibiotic, as described in Materials and methods, was further incubated to determine the extent of growth inhibition at the termination of treatment. The other portion. harvested, freed from extra-cellular and adsorbed dihydrostreptomycin, was used for preparation of cell-free extract, as described, and one portion of this centrifuged to divide into ribosomal pellet and supernatant fraction. For each culture the intracellular dihydrostreptomycin in the 2 fractions, estimated as $^{3}H/^{14}C$ c.p.m., is plotted against the extent of growth inhibition. a) Ribosomal pellet; b) supernatant fraction.

growing cultures were treated with [^{3}H]dihydrostreptomycin, as above, until growth had completely ceased. They were then harvested, ground with alumina, and varying concentrations of [^{3}H]dihydrostreptomycin (in the range of 0.1–2.0 μg per mg dry weight of cells harvested) were added to the crude extracts. These were then centrifuged through 15–30% (w/v) sucrose gradients as before and the 25 drop fractions collected and pooled for counting and OD_{260} measurements as above.

For this series also, controls of untreated exponentially growing cultures (labelled with [^{14}C]uracil) were harvested, and the crude extracts treated with [^{3}H] dihydrostreptomycin in the same way as for the in vivo treated cultures.

[^{3}H]dihydrostreptomycin (3 Ci/mmol) and [^{4}C] uracil (60 mCi/mmol) were obtained from the Radiochemical Centre, Amersham. Unlabelled dihydrostreptomycin sulphate was a standard preparation generously supplied by Messrs. Glaxo Ltd. and has a specific activity of 780 I.U. per mg base. All other reagents were reagent grade.

3. Results

Fig.1. shows the distribution of [^{3}H]dihydrostreptomycin between total ribosomal pellet and the supernatant after centrifugation at 139 000 *g*. In the pellet the ratio of ^{3}H (that is dihydrostreptomycin) to ^{14}C (that is, RNA) increases linearly with increasing inhibition of growth rate, as shown by the calculated regression line. The supernatant graph shows a fairly constant ratio of ^{3}H/^{14}C counts until growth inhibition has proceeded to about 60 percent, and then a rapidly increasing rise at the later stages of inhibition, similar to the increases in total intracellular streptomycin previously observed [18].

Fig.2 shows the distribution of [^{3}H]dihydrostreptomycin between different ribosome fractions obtained from the sucrose gradients. The picture for the 30S ribosomal subunits parallels the picture for the total ribosomal pellet. This confirms that dihydrostreptomycin, during its in vivo action combines with the 30S subunits [14,19].

The graph for the 50S subunits rises during the early stages of growth inhibition, but then plateaus at approx. 40% inhibition. Similarly, the graph representing the [^{3}H]dihydrostreptomycin on polysomes and 70S monomers also shows that there is no straight line relationship between the ^{3}H/^{14}C ratio and percent inhibition of growth rate.

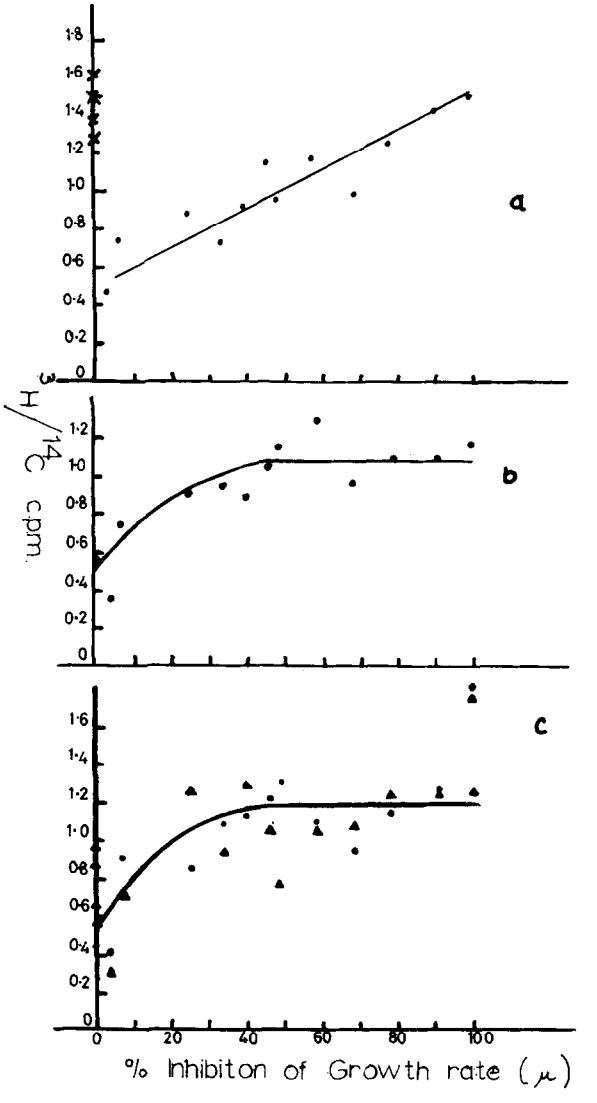

Fig.2. Distribution of intracellular [^{3}H]dihydrostreptomycin between various ribosomal components, as a function of growth inhibition. Portions of the crude cell-free extracts, obtained as in fig.1, were sedimented through a 15–30% (w/v) linear sucrose gradient, in Tris/mg/KCl buffer, as described in Materials and methods, and the intracellular antibiotic associated with the various ribosomal components, estimated as ^{3}H/^{14}C c.p.m. plotted against growth inhibition of cultures from which they were obtained. a) 30S ribosomal subunits; b) 50S ribosomal subunits; c) 70S ribosomes (○); and polysomes (△).

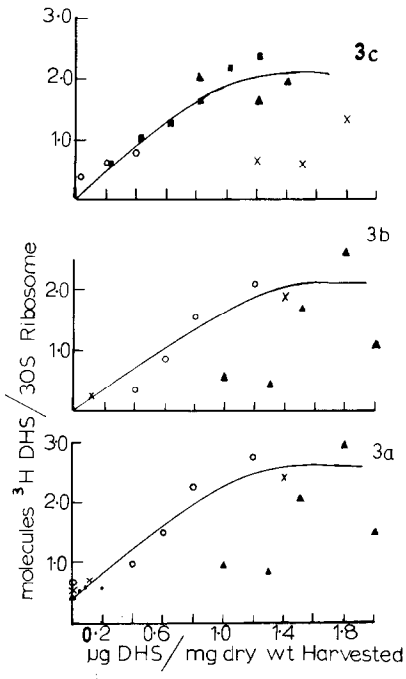

Fig.3. Binding of [^{3}H]dihydrostreptomycin to 30S ribosomal subunits in vitro and in vivo. Crude extracts, obtained from cultures without dihydrostreptomycin treatment, or after treatment with [^{3}H]dihydrostreptomycin to complete growth inhibition, were further treated with various concentrations of [^{3}H]dihydrostreptomycin after removal of alumina and unbroken cells. 30S ribosomal subunits were then isolated by sedimentation through 15–30% (w/v) linear sucrose gradients as described, and the tritium counts and mg of ribosomes determined. From the specific activity of the [^{3}H]dihydrostreptomycin used, the molecules of DHS per 30S subunit were determined and plotted against µg dihydrostreptomycin added in vitro per mg of dry weight of culture harvested for each preparation. a) In vitro binding of [^{3}H]dihydrostreptomycin to 30S ribosomes in extracts obtained from cultures treated with [^{3}H]dihydrostreptomycin to complete growth inhibition. b) Calculated in vitro binding of [^{3}H]dihydrostreptomycin to in vivo-treated 30S ribosomes, by substracting the values after in vivo treatment only from those in 3a. c) In vitro binding of [^{3}H]dihydrostreptomycin to 30S ribosomes in extracts from untreated control cultures.

On the graph for the 30S subunits the 3 points from the control cultures, i.e. at 0 inhibition, are very high. In fact, this in vitro binding seems to be as high as in vivo binding at 100% inhibition. This would suggest that in vitro binding differs from in vivo binding which is related to the antibiotic effect. The question at this stage was: 'does the antibiotic, in fact, bind to the same sites on the 30S ribosomal subunits when added in vitro as it does during in vivo treatment?'

In order to investigate this we added [^{3}H]dihydrostreptomycin to crude extracts from cultures harvested after in vivo treatment with dihydrostreptomycin had produced 100% inhibition of growth. We compared the in vitro binding under these conditions with in vitro binding in similar extracts from control cultures. If competition for in vivo binding sites and those accessible to the antibiotic in vitro did occur, then the binding to 30S ribosomes present in cell-free extracts from in vivo treated cultures should be less than such in vitro binding to ribosomes from controls.

Fig.3 shows the ratios of molecules [^{3}H]DHS bound per 30S subunit, plotted against the concentrations of [^{3}H]dihydrostreptomycin added to cell-free extracts (on the basis of mg dry weight of cells from which the extracts were prepared). The graph in 3a represents in vitro binding after in vivo inhibition; 3b is the calculated in vitro binding, obtained by subtracting the in vivo binding (the points at 0 on the abscissa of graph 3a) from the results in fig.3a. Fig.3c represents in vitro binding alone, i.e. to the controls (see Materials and methods).

Comparing graphs 3b and 3c, it is quite clear that there is no competition for the sites accessible under these conditions in vitro and those occupied during in vivo treatment. Furthermore, this in vitro binding increases with increasing concentrations of DHS added to the crude extracts, to reach a plateau of approx. 2 mol DHS per 30S ribosome. The number of molecules [^{3}H]DHS bound by 30S subunits during treatment in vivo, i.e. at the stage when growth had just ceased, was estimated in a number of experiments and found to be in the range of 0.45–0.8 molecules/30S ribosome.

4. Discussion

The results presented above fall into 2 sections. In the first we have studied the distribution of intracellular dihydrostreptomycin as a function of growth inhibition.

We have shown (fig.1) that, whilst the antibiotic associated with the ribosomal pellet increases linearly with increasing inhibition of growth, there is no such proportionality between the [^{3}H]dihydrostreptomycin in the supernatant fraction and growth inhibition. This

would seem to confirm the idea that not all intracellular streptomycin is inhibitory at all stages [20] and some of the antibiotic in the latter fraction may correspond to the intracellular 'pool streptomycin' suggested on the basis of earlier work by Kogut et al. [18,19].

On comparing fig.2 with fig.1, it can be seen that the 30S subunits show the same picture as the total ribosomal pellet, that is the ribosome-bound streptomycin increases linearly with the extent of growth inhibition. Such a relationship is not seen with any other ribosomal component. This would seem to constitute the first real evidence for the generally accepted hypothesis that combination of dihydrostreptomycin (and streptomycin) with the 30S ribosomal subunits in vivo is directly involved in inhibition of growth and protein synthesis, i.e. its antibiotic action. Furthermore, the high levels of the 3 control points on the 30S graph of fig.2 (in vitro addition of dihydrostreptomycin to cell-free extracts) suggested that the accessibility of binding sites on the 30S subunits is different under in vivo and our in vitro conditions. We suggest therefore that the in vivo binding which is correlated with growth inhibition may be to specific inhibitory sites.

The above observations led us to further investigations, namely comparing in vitro binding of dihydrostreptomycin to 30S ribosomes in crude extracts after in vivo inhibition, with such in vitro binding alone. The results show that the addition and binding of the antibiotic to 30S ribosomes in cell-free extracts is not prevented by previous in vivo treatment and binding to the 'inhibitory' sites. It would also appear, from our results, that such separate in vitro binding sites on 30S ribosomes are limited, i.e. approx. 2 per subunit.

It is of interest, in this connection, that other workers [9,10], studying in vitro binding of [^{3}H]dihydrostreptomycin to isolated and purified 30S subunits (by equilibrium dialysis), have found approx. 1 molecule of DHS = 1 binding site per subunit. In our experiments, the values for in vivo binding at complete growth inhibition were somewhat below one molecule DHS per 30S subunit. Thus, it would appear that not all 30S subunits in the cell need to have combined with the antibiotic for growth to cease; perhaps not all 30S subunits are accessible to intracellular streptomycin [14,18,19]. Alternatively, the possibility that some of the 30S subunits may have lost their in vivo bound DHS during the isolation procedure (i.e. sucrose gradient centrifugation) cannot be ruled out. However, the in vitro binding of DHS to 30S ribosomes present in crude extracts, which we observed, could not be due merely to the presence of such unaffected 30S subunits in inhibited cultures, since two molecules DHS, hence 2 binding sites were found per 30S ribosome.

The apparent conflict between our results and the observations on in vitro binding to isolated 30S ribosomes may be due to the different preparations and techniques used. Thus, additional binding sites or factors, present on 30S ribosomes in crude cell-free extracts, may be lost during isolation and purification, and/or conformational changes of the 30S subunits during the above procedures could alter the accessibility of binding sites to the antibiotic. Whether the one site per isolated 30S subunit determined by Chang and Flaks [9] and Schreiner and Nierhaus [10], is the same as our 'in vivo binding site' could be established by measuring the in vitro binding (or sites) by such isolated 30S ribosomes obtained from cultures which had been inhibited by streptomycin in vivo. Conformational changes of ribosomal structure, caused by streptomycin treatment in vitro have been suggested by Sherman and Simpson [21].

Acknowledgement

On of us (E.P) is in receipt of a Wellcome Foundation Studentship.

References

[1] Spotts, C. R. and Stanier, R. Y. (1961) Nature 192, 633–637.
[2] Flaks, J. G., Cox, E. C. and White, J. R. (1962) Biochem. Biophys. Res. Commun. 7, 385–389.
[3] Luzzatto, L., Apirion, D. and Schlessinger, D. (1968) Proc. Natl. Acad. Sci. U.S. 60, 873–880.
[4] Kogut, M. (1969) J. Gen. Microbiol. 57, 25.
[5] Cox. E. C., White, J. R. and Flaks, J. G. (1964) Proc. Natl. Acad. Sci. U.S. 54, 703–709.
[6] Davies, J. E. (1964) Proc. Natl. Acad. Sci. U.S. 51, 659–664.
[7] Chang, F. N. and Flaks, J. G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 1321–1328.
[8] Chang, F. N. and Flaks, J. G. (1972) Antimicrobial Agents & Chemoth. 2, 294–307.
[9] Chang, F. N. and Flaks, J. G. (1972) Antimicrobial Agents & Chemoth. 2, 308–319.

[10] Schreiner, G. and Nierhaus, K. H. (1973) J. Mol. Biol. 81, 71–82.
[11] Prizant, E. and Kogut, M. (1973) J. Gen. Microbiol. 75, (part 2) xi.
[12] Hipkiss, A. R. and Kogut, M. (1973) Transact. Biochem. Soc. 1, 594–596.
[13] Kogut, M., Lightbown, J. W. and Isaacson, P. (1965) J. Gen. Microbiol. 39, 155–164.
[14] Kogut, M. and Harris, M. (1969) Eur. J. Biochem. 9, 42–49.
[15] Kogut, M., Maltby, E. N. and Prizant, E. (1970) Biochem. J. 119, 121–123.
[16] Finney, D. J., Hazlewood, T. and Smith, M. J. (1955) J. Gen. Microbiol. 12, 222.
[17] Kaji, H. and Tanaka, Y. (1968) J. Mol. Biol. 32, 221–230.
[18] Kogut, M., Lightbown, J. W. and Isaacson, P. (1966) J. Gen. Microbiol. 42, 333–344.
[19] Kogut, M., Lightbown, J. W. and Isaacson, P. (1965) J. Gen. Microbiol. 39, 165–183.
[20] Hancock, R. (1962) J. Gen. Microbiol. 28, 503–516.
[21] Sherman, M. I. and Simpson, M. U. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1388–1395.