EFFECTS OF DIHYDROSTREPTOMYCIN TREATMENT *IN VIVO* ON THE RIBOSOME CYCLE IN *ESCHERICHIA COLI*

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

Received 20 October 1970
Revised version received 10 November 1970

1. Introduction

The gradual decline in polyribosomes and accumulation of 70 S ribosomes observed in cell lysates from cultures treated with streptomycin *in vivo* [1–4] has been interpreted by Luzzatto et al. [1, 2] as accumulation of special "streptomycin-monosomes" or "stuck initiation complexes". Although this interpretation seemed consistent with a number of earlier observations and models of streptomycin action [5–7] it is based upon indirect evidence which is inconclusive and in some cases contradictory [4]. A more direct examination of this hypothesis was suggested by reports [8, 9] that ribosomes which appear as 70 S particles in 10 mM Mg^{2+} can be classified into "run-off" ribosomes on the one hand, and polysome-derived on the other, by their sedimentation behaviour in 2 mM Mg^{2+}. Using this approach we have compared dihydrostreptomycin-treated cultures with log-phase controls and cultures harvested during amino acid deprivation. We found that log-phase controls contain 70 S ribosomes which behave largely as "stuck" polysomal ribosomes, whilst amino acid deprivation causes rapid accumulation of "run-off" ribosomes. Treatment with low concentrations of dihydrostreptomycin which produces gradual inhibition of growth rates [10, 11] leads to the gradual accumulation of "run-off" ribosomes as growth inhibition proceeds.

2. Materials and methods

Escherichia coli C600 was grown aerobically in mineral salts medium with 0.2% (w/v) glucose and 0.2% (w/v) casaminoacids at 32° [12, 7]. Cultures were labelled with ^{14}C-uracil (0.13 μCi/ml, 15 μg/ml) for at least two generations. Growth was measured as absorbance at 500 nm and plotted as $\log_2$ [13]. Harvesting and lysis in 10 mM Mg^{2+} was as described by Godson and Sinsheimer [14]. Lysates were layered onto 4.0 ml of 15–30% (w/v) linear sucrose gradients above a 0.5 ml "cushion" of 50% sucrose, all in 10 mM tris-HCl buffer, pH 7.3, containing 10 mM Mg^{2+} and 6 mM KCl. They were centrifuged immediately at 5° in the SW rotor of the Beckman Spinco L2 centrifuge for 70 min at 50,000 rpm. The gradients were fractionated and collected as 6-drop fractions in parallel sets of tubes; the middle 2-drops of each fraction were used for absorbance measurements at 260 nm. After plotting the profiles from the A_{260} readings, the 70 S peaks (3 or 4 tubes) of the 4-drop fractions were pooled and diluted two-fold in the Brij-lysing mixture to reduce the sucrose concentration to below 12%. After addition of 0.1 μg/ml RNase to the pooled fractions, and incubation for 5 min on ice, 0.5 ml portions were layered onto similar sucrose gradients in 10 mM tris-HCl buffer, pH 7.3 containing 2 mM Mg^{2+} and 6 mM KCl [8], and centrifuged immediately for 2 hr at 48,000 rpm in the SW 50 rotor. These gradients were collected as before in 6-drop fractions and the fractions precipitated and counted (in a Nuclear-Chicago end-window, gas-flow counter) as described [4]. Dihydrostreptomycin was obtained as a gift from Glaxo Laboratories and contained 780 IU/mg base. ^{14}C-Uracil was obtained from the Radiochemical Centre, Amersham. Brij 58 was a gift from Honeywell-Atlas Ltd. (Carshalton, Surrey). All other reagents were reagent grade.

3. Results

Lysates from untreated cultures, harvested during exponential growth and centrifuged as above in 10 mM Mg^{2+} yield ribosome patterns which show considerable proportions of polysomes and variable amounts of 70 S particles. Cultures which have been deprived of nitrogen source by filtration, washing and resuspension in fresh medium lacking casaminoacids, cease to grow within 15–20 min. Samples harvested during this period show rapid disappearance of polysomes and accumulation of 70 S particles [3]. However, Ron et al. [8] showed that lysates prepared in 10 mM Mg^{2+} from cultures subjected to "run-off" conditions had different sedimentation properties in 2 mM Mg^{2+} than did similar lysates prepared from exponentially growing cultures, after brief treatment with RNase to disrupt polysomes. We have confirmed this distinction, although with our centrifugation programme the "run-off" ribosomes do not appear as a single, displaced peak, but are dissociated into 50 S and 30 S subunits. Fig. 1 illustrates an experiment in which a portion of a logphase

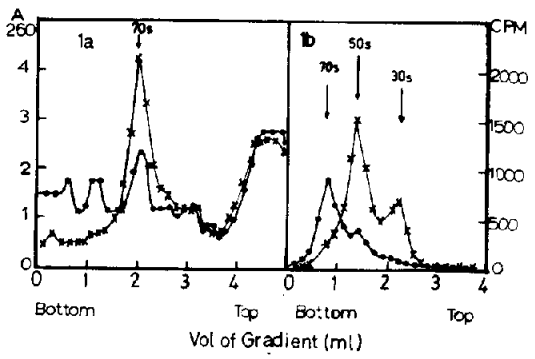

Fig. 1. Ribosome sedimentation patterns from log-phase control and nitrogen-deprived cultures of *Escherichia coli* C 600.
a) Sucrose gradient profiles of lysates L1 and L2, prepared as described and centrifuged for 70 min at 50,000 rpm in 10 mM Mg^{2+}.
b) Sucrose gradient profiles of the 70 S peak material harvested from the gradients in 10 mM Mg^{2+} and recentrifuged for 2 hr at 48,000 rpm in 2 mM Mg^{2+}. ●—● L1 = control; x—x L2 = nitrogen-deprived culture.

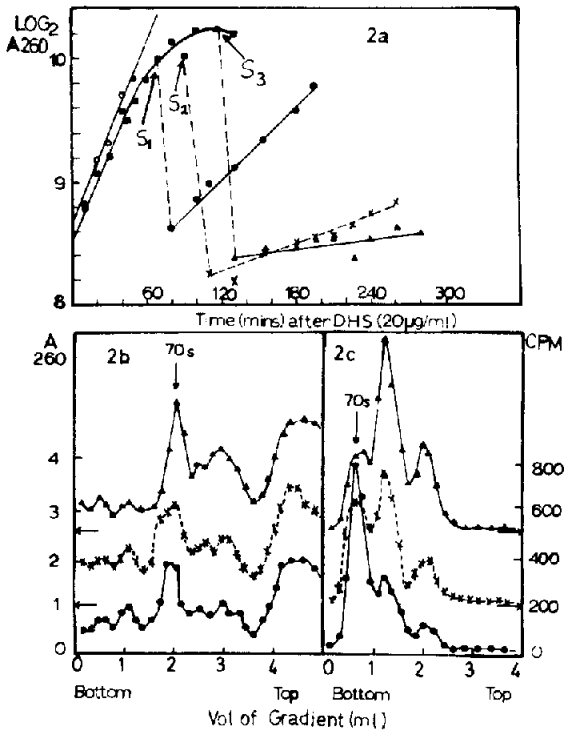

Fig. 2. Ribosome sedimentation patterns from cultures of *Escherichia coli* C 600 treated with dihydrostreptomycin.
a) Growth, as $\log_2 A_{500}$ of control and culture after addition of 20 µg/ml dihydrostreptomycin (DHS). Arrows show times at which three samples from the DHS-treated culture were harvested for preparation of lysates and for measurement of residual growth rates after filtration and resuspension in antibiotic-free medium. o—o growth of control; ■—■ growth after addition of DHS; ●—● S1 = growth of sample resuspended after 70 min growth in the presence of DHS; x- - -x S2 = growth of sample resuspended after 90 min growth in the presence of DHS; ▲—▲ S3 = growth of sample resuspended after 115 min growth in the presence of DHS.
b) Sucrose gradient profiles of lysates S1, S2 and S3, prepared as described and centrifuged for 70 min at 50,000 rpm in 10 mM Mg^{2+}.
c) Sucrose gradient profiles of the 70 S peak materials harvested from the gradients in 10 mM Mg^{2+}, and recentrifuged for 2 hr at 48,000 rpm in 2 mM Mg^{2+}. ●—● S1; x- - -x S2; ▲—▲ S3.
To give greater clarity the base lines for the three gradients in 2b and 2c are separated as indicated by the arrows: the scales are the same.

control was deprived of amino acids as described and lysates from both analysed on sucrose density gradients. In 1a the gradient profiles show the relative proportions of polysomal and 70 S material at 10 mM Mg^{2+}. Upon harvesting the 3 tubes from each of the 70 S peaks in 10 mM Mg^{2+} and recentrifuging in 2 mM Mg^{2+} for 2 hr the control sedimented almost entirely as 70 S, whereas the material from the amino acid-starved cultures dissociated completely into 50 S and 30 S subunits (fig. 1b). Fig. 2 illustrates an experiment in which dihydrostreptomycin (20 μg/ml) was added to an exponentially growing culture and 3 samples removed at the indicated times (2a) and assayed for ribosomal sedimentation patterns and for growth rates after removal of extracellular antibiotic [10, 7]. The gradient profiles in 10 mM Mg^{2+} (2b) show that the proportion of 70 S to polysomal material increased in the successive samples. Recentrifugation in 2 mM Mg^{2+} of the three 70 S peak tubes from each of these gradients shows 3 different patterns (2c): the earliest sample, harvested when growth rate was about 60% inhibited, is similar to the log-phase control in fig. 1. The last sample, harvested when growth was almost completely inhibited, resembles the sample from the amino acid-starved culture, showing predominantly 50 S and 30 S peaks, although there is also a small peak at 70 S. The intermediate sample, harvested when growth was approximately 80% inhibited, shows intermediate behaviour, i.e. equivalent amounts of 70 S and 50 S material.

4. Discussion

If dihydrostreptomycin, by combination with free 30 S ribosomal subunits [5–7, 15, 16] led to the formation of "stuck initiation complexes" [1] one would expect these to accumulate at the expense of polysomes in parallel with the antibiotic effect. However, loss of polysomes and accumulation of 70 S ribosomes is a concomitant of many conditions which slow or inhibit growth, including conditions imposed during harvesting [3, 4, 8, 14]. The crucial question then is: "What is the nature of the 70 S material obtained in 10 mM magnesium?" The "special streptomycin-monosomes" proposed by Luzzatto et al. [1, 2], having attached mRNA, and perhaps a dipeptidyl-tRNA, should behave like monosomes found in extracts from log-phase cultures prepared by alumina-grinding, or derived from polysomes by brief treatment with RNase [8]. Such monosomes have been characterized here (control, fig. 1) and by Ron et al. [8] as sedimenting at 70 S in 2 mM Mg^{2+}. In contrast, "run-off" 70 S ribosomes, free of mRNA and peptide chains dissociate completely into 50 S and 30 S subunits in 2 mM Mg^{2+} under our conditions of preparation and centrifugation (fig. 1). Applying this differentiation to a culture whose growth is gradually inhibited by accumulation of intracellular dihydrostreptomycin [11] and its interaction with 30 S ribosomes, we find at the earlier stages of growth inhibition mainly monosomes sedimenting as 70 S in 2 mM Mg^{2+}. The progress of growth inhibition, however, is paralleled by increasing accumulation of "run-off" 70 S ribosomes. Clearly this conflicts with the above prediction from the model of Luzzatto et al. [1, 2] and suggests that the interaction of dihydrostreptomycin with ribosomes *in vivo* must allow some translational movement, subsequent to initiation. This is also consistent with our earlier conclusions [3, 4] and with the interpretation of recent *in vitro* studies [17].

Acknowledgements

This work was supported by a grant from the S.R.C. to M.K. and greatly helped by the willing and conscientious assistance of Miss E.Weller.

References

[1] L.Luzzatto, D.Apirion and D.Schlessinger, Proc. Natl. Acad. Sci. U.S. 60 (1968) 873.
[2] L.Luzzatto, D.Apirion and D.Schlessinger, J. Mol. Biol. 42 (1969) 315.
[3] M.Kogut, J. Gen. Microbiol. 57 (1969) 25.
[4] M.Kogut, E.M.Maltby and E.Prizant, Biochem. J. 119 (1970) 121.
[5] D.T.Dubin, J. Mol. Biol. 8 (1964) 749.
[6] E.C.Cox, J.R.White and J.G.Flaks, Proc. Natl. Acad. Sci. U.S. 54 (1964) 703.
[7] M.Kogut and M.Harris, European J. Biochem. 9 (1969) 42.
[8] E.Ron, R.Kohler and B.D.Davis, J. Mol. Biol. 36 (1968) 83.
[9] R.Kohler, E.Ron and B.D.Davis, J. Mol. Biol. 36 (1968) 71.

[10] M.Kogut, J.W.Lightbrown and P.Isaacson, J. Gen. Microbiol. 39 (1965) 165.
[11] M.Kogut, J.W.Lightbrown and P.Isaacson, J.Gen. Microbiol. 42 (1966) 333.
[12] M.Kogut, J.W.Lightbrown and P.Isaacson, J. Gen. Microbiol. 39 (1965) 155.
[13] D.J.Finney, T.Hazlewood and M.J.Smith, J. Gen. Microbiol. 12 (1955) 222.
[14] N.Godson and R.L.Sinsheimer, Biochim. Biophys. Acta 149 (1967) 476.
[15] R.W.Wolfgang and N.L.Lawrence, J. Bacteriol. 95 (1968) 1295.
[16] A.Kaji, Biochim. Biophys. Acta 134 (1967) 134.
[17] J.Modolell and B.D.Davis, Nature 224 (1969) 345.