THE EFFECT OF CYCLOHEXIMIDE ON POLYRIBOSOMES FROM HAMSTER CELLS

Clifford P. Stanners

Department of Medical Biophysics, University of Toronto,
(and The Ontario Cancer Institute)
500 Sherbourne Street, Toronto 5, Canada.

Received August 5, 1966

The antibiotic cycloheximide strongly inhibits protein synthesis in cell free systems (Bennett et al., 1965), in cultured cells (Ennis and Lubin, 1964) and in whole animals (Trakatellis et al., 1965). In general, the polysome-ribosome profiles obtained by velocity centrifugation analysis of cellular extracts show little or no change after drug treatment, possibly because the drug simply stops the attachment and movement of ribosomes along the messenger RNA (mRNA) in the polysomes (Wettstein et al., 1964, Colombo et al., 1965, and Trakatellis et al., 1965). In these studies, highly inhibitory drug concentrations were employed. This report describes the effect of less inhibitory doses of cycloheximide on the polysomes of hamster embryo cells grown in vitro. Under such conditions the free ribosomes in the cells disappear completely and become attached to mRNA in polysomes. The results are consistent with the hypothesis that low concentrations of the drug cause the ribosomes to move more slowly along the mRNA, but have less effect on ribosome attachment.

MATERIALS AND METHODS

Syrian hamster embryo cells were grown in monolayer culture on glass as described elsewhere (Reader and Stanners, 1966). The cells were used in the 3rd to 8th subculture after explantation from the animal. Cultures were treated with growth medium containing cycloheximide ("Actidione", Upjohn Co.) at various concentrations while in the exponential phase of growth (the doubling time was about 11 hours). To obtain preparations of ribosomes and

polysomes, the cells were removed from the glass with a 1% solution of Bacto trypsin (Difco) in phosphate buffered saline (PBS) (Dulbecco and Vogt, 1954) at 0°C. The cell suspension was washed 3 times with cold PBS, then finally suspended in 1.4 ml extraction buffer (0.1 M NaCl, 0.02 M $MgCl_2$, 0.01 M Tris pH 7.8). Brij-58 (Atlas Chemical Industries, Brantford, Ontario) was added to 0.5% and the cell membranes disrupted by subjecting the suspension to 10 strokes of a tight fitting Dounce glass homogenizer. The nuclei were removed by light centrifugation. The extracts were usually not treated with deoxycholate, as this was found to have no effect on the polysome spectrum. Ribosomes and polysomes were detected by zonal centrifugation through 27 ml of 15 to 30% w/w sucrose gradients followed by continuous measurement of the optical density (OD) at 260 mμ by a Gilford recording spectrophotometer.

RESULTS

In a preliminary experiment, a high dose of cycloheximide (100 μg/ml) was found to have no effect on the cellular polysomes or free ribosomes. If high doses stop ribosome movement, it was reasoned that lower doses might slow down the movement, resulting perhaps in a lower ratio of free to polysome-bound ribosomes. To determine the effect of low drug doses on protein synthesis in these cells, a series of replicate monolayer cultures growing exponentially in complete growth medium were treated with various concentrations of cycloheximide from 0.1 μg/ml to 100 μg/ml. The rate of protein synthesis was measured by the incorporation of radioactivity into alkali-stable acid-precipitable cellular material as a function of time after the addition of C^{14}-valine to the cultures. The incorporation at all drug doses was strictly linear with time over a 6-hour period. Fig. 1 shows the rate of protein synthesis, as per cent of the control rate, as a function of inhibitor concentration.

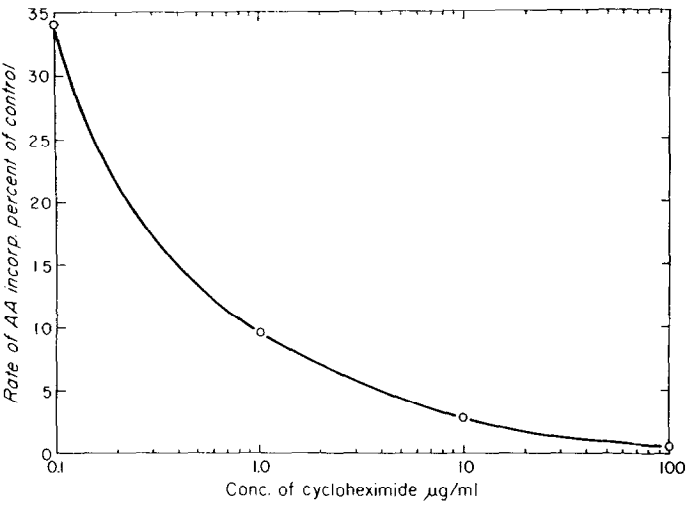

Fig. 1. The effect of various doses of cycloheximide on protein synthesis.

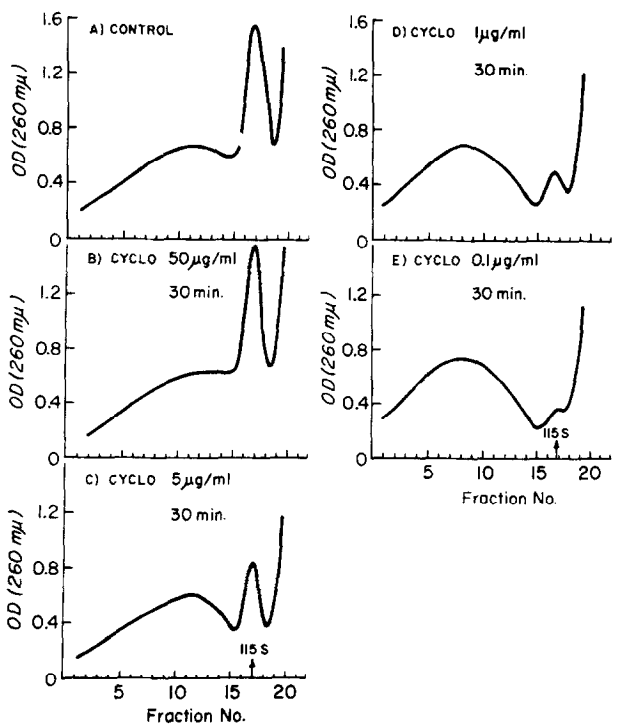

Fig. 2. The effect of various doses of cycloheximide on cytoplasmic polysomes and ribosomes in cells harvested 30 min. after addition of the drug. The direction of centrifugation was from right to left.

Fig. 2 shows the effect of various concentrations of cycloheximide on the cytoplasmic polysomes and ribosomes in cells harvested 30 min. after addition of the drug. The profiles show two peaks: a broad heavy peak and a lighter narrow one. The heavy region has been shown to consist of polysomes by the following criteria. A broad peak of acid-precipitable radioactivity is found in this region after brief exposure of the cells to radioactive amino acids; the OD and radioactivity shift quantitatively after mild RNase treatment to single ribosomes sedimenting at about 80S (Reader and Stanners, 1966). The lighter peak is comprised of ribosome dimers (approx. 115S) which are inactive in protein synthesis and which are believed to form from the free single ribosomes of the cell in the high salt, high Mg^{++} buffer used in the extraction procedure.*

The profiles of Fig. 2 show that high concentrations of cycloheximide have no effect on the polysome profile, in agreement with the results of others (Wettstein et al., 1964, Colombo et al., 1965, and Trakatellis et al., 1965). Lower concentrations, however, show a progressive shift of the free inactive ribosomes into polysomes. At 0.1 µg/ml there are virtually no free ribosomes in the extracts.

It seemed possible that the concentration-dependent shift of free ribosomes into polysomes seen in Fig. 2 at a fixed time was due to a decreasing rate of shift as the drug concentration was increased. To test this hypothesis the following experiment was carried out. Ribosomes were first attached to polysomes by incubating the cells for 20 min. in cycloheximide at 0.1 µg/ml. They were then detached by a 5-min. incubation in puromycin at 100 µg/ml (Villa-Trevino et al., 1964). The preliminary attachment step was designed to ensure proximity of all of the ribosomes to mRNA before detachment with puromycin (Joklik and Becker, 1965). The

* This extraction buffer results in a much improved yield of larger polysomes over that obtained with conventional extraction buffers (Reader and Stanners, 1966).

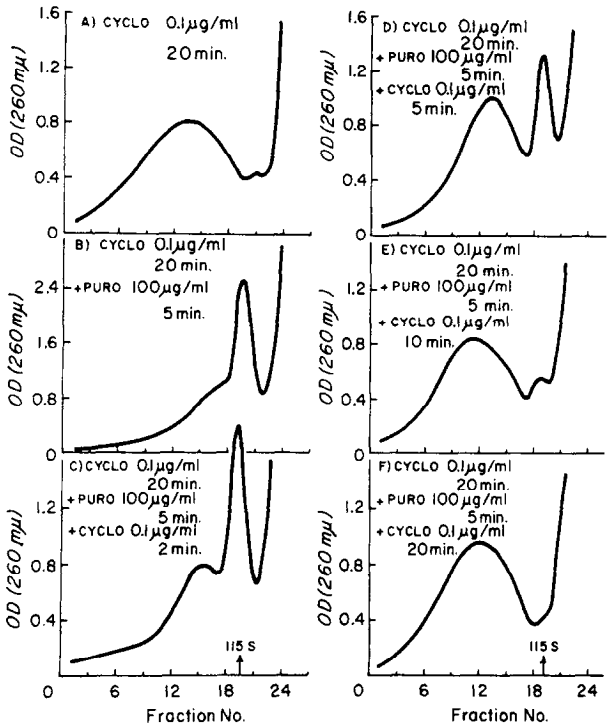

Fig. 3. The regeneration of polysomes from free ribosomes in the presence of cycloheximide at 0.1 μg/ml. See text for details. The direction of centrifugation was from right to left.

rate of disappearance of free ribosomes was then measured by washing the cultures and incubating them with medium containing cycloheximide at 0.1, 1.0, or 10 μg/ml. The ribosome-polysome profiles for cycloheximide at 0.1 μg/ml are shown in Fig. 3. The profiles for the other concentrations of cycloheximide are not shown. As a measure of the rate of disappearance of free ribosomes, the fraction of the total OD in the peak of free ribosomes was estimated for each time and expressed as a percentage of the fraction just after the 5-min. puromycin incubation. The "surviving percentage" of free ribosomes was then plotted against time of incubation in cycloheximide-containing medium. The results are shown in Fig. 4. The time required for 50% of the free ribosomes to attach to polysomes was 3.5 min., 10 min. and 47 min. in medium containing cycloheximide at 0.1,

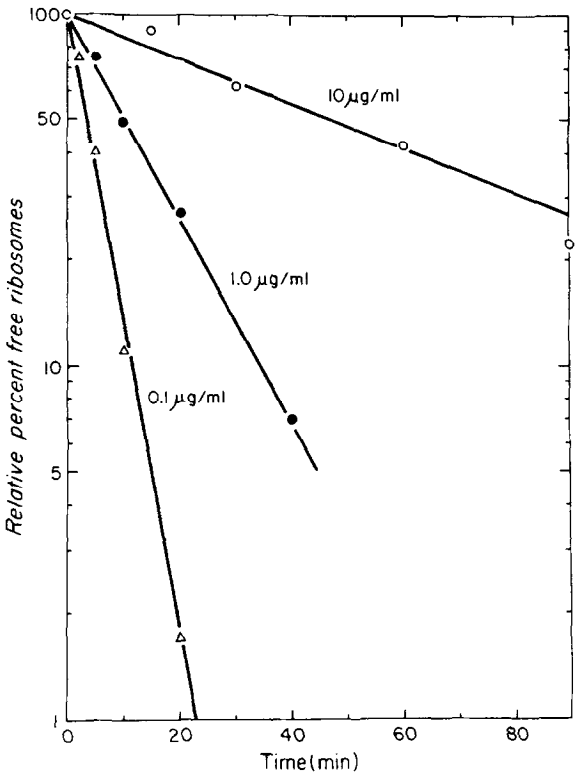

Fig. 4. The "surviving percentage" of free ribosomes at various times after addition of cycloheximide at 0.1 μg/ml : Δ, 1.0 μg/ml : ●, and 10 μg/ml : 0. See text for details.

1.0, and 10 μg/ml respectively. These rates are in approximately the same ratios as the rates of protein synthesis for these concentrations shown in Fig. 1. Similar results were obtained with cells not given the cycloheximide-puromycin pretreatment.

DISCUSSION

The experiments described demonstrate that in the presence of low concentrations of cycloheximide nearly all of the free ribosomes in the cell become attached to polysomes. Non-specific attachment cannot be excluded but is considered unlikely. Assuming that the newly attached ribosomes function properly, though perhaps more slowly, in the translation mechanism, the results can be interpreted using the generally accepted model of the polysome as presented by Warner et al. (1963). The rates of three ribosomal processes must be considered: attachment, movement along the mRNA and

detachment. The shift of free ribosomes into polysomes can be most easily explained by a decreased rate of movement giving a reduced rate of detachment, coupled with a normal (or less reduced) rate of attachment. The rate of disappearance of free ribosomes, however, was observed to decrease with increasing concentration of the drug. It should be pointed out that this rate is not the attachment rate of free ribosomes but the difference between the attachment and detachment rates. This difference must be positive and its magnitude must decrease with increasing drug concentration to account for the observed results. A possible explanation consistent with the slow motion hypothesis is that the probability of successful attachment of a free ribosome in the presence of cycloheximide depends both on physical proximity with the end of a mRNA molecule and on the rate of translation. The attachment rate could thus be reduced in proportion to the degree of translation inhibition but to a lesser extent than the detachment rate. Other self-consistent models are, of course, possible.

ACKNOWLEDGEMENTS

I wish to thank Mr. G. McKenzie and Mr. W. Mehring for capable technical assistance. This work was supported by grant MA-1877 from the Medical Research Council of Canada, and by the National Cancer Institute of Canada.

REFERENCES

Bennett, L.L., Ward, V.L., and Brockman, R.W., Biochim. Biophys. Acta 103, 478 (1965).
Colombo, B., Felicetti, L., and Baglioni, C., Biochem. Biophys. Res. Commun. 18, 389 (1965).
Dulbecco, R., and Vogt, M., J. Exp. Med. 99, 167 (1954).
Ennis, H.L., and Lubin, M., Science 146, 1474 (1964).
Joklik, W.K., and Becker, Y., J. Mol. Biol. 13, 496 (1965).
Reader, R.W., and Stanners, C.P., In preparation (1966).
Trakatellis, A.C., Montjar, M., and Axelrod, A.E., Biochem. 4, 2065 (1965).
Villa-Trevino, S., Farber, E., Staehelin, T., Wettstein, F.O., and Noll, H., J. Biol. Chem. 239, 3826 (1964).
Warner, J.R., Knopf, P.M., and Rich, A., Proc. Natl. Acad. Sci. 49, 122 (1963).
Wettstein, F.O., Noll, H., and Penman, S., Biochim. Biophys. Acta 87, 525 (1964).