Differential Effects of Cycloheximide on Protein and RNA Synthesis as a Function of Dose

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Received February 12,1973

SUMMARY. The in vivo dose response of rat liver protein and DNA synthesis to cycloheximide have been determined. Protein synthesis was quite sensitive to relatively low doses of cycloheximide being inhibited by more than 90% with 1.5 mg/kg. Maximal inhibition of 98% was achieved with 5 mg/kg. There was no inhibition of RNA synthesis with this dose of cycloheximide. Larger doses of cycloheximide did lead to quite marked inhibition of RNA synthesis without any change in the already maximally inhibited rate of protein synthesis. This differential effect of cycloheximide on protein and RNA synthesis as a function of dose indicates that the inhibition of RNA synthesis caused by the antibiotic is not a consequence of the inhibition of protein synthesis but related otherwise to the effects of large doses of cycloheximide.

The dependence of eukaryotic, nuclear RNA synthesis on active cytoplasmic protein synthesis has been a problem of continuing interest over the last few years (1-11). Most of these studies have utilized rapidly growing cells in tissue culture, but there have been several studies of the effect of cycloheximide, an inhibitor of protein synthesis, on the rate of rat liver RNA synthesis (9,10,11). The in vivo administration of cycloheximide was shown to be associated with a time dependent inhibition of ribosomal RNA synthesis in rat liver nuclei (10,11). This observation was interpreted as implicating a role of short-lived protein(s) in the normal synthesis of ribosomal RNA (10,11).

A significant feature of these reports is the very large doses of cycloheximide employed [20 mg/kg (10) and 30 mg/kg (11)]. A dose of 1.5 mg/kg will inhibit protein synthesis by greater than 90% in vivo in rat liver cells (12). There may, of course, be strain differences in the sensitivity to cycloheximide, and there may be variations in the potency of different

preparations of the antibiotic. These considerations aside, the 10 to 20 fold difference between the dose of cycloheximide reported to produce virtually complete inhibition of protein synthesis (12) and that used in the above reports (10,11) suggested to us the possibility that the inhibition of ribosomal RNA synthesis caused by cycloheximide may not be the consequence of the inhibition of protein synthesis but rather related otherwise to very large doses of cycloheximide. In this paper we present data indicating that this indeed may be the case.

We have compared the dose response of rat liver protein and RNA synthesis to *in vivo* administered cycloheximide. We were interested to determine if we could dissociate the inhibition of protein synthesis from the inhibition of RNA synthesis with a dose of cycloheximide that would maximally inhibit the former without affecting the latter.

Fasted rats were injected with doses of cycloheximide from 0.5 to 20 mg/kg body weight. Three hours later the *in vivo* rate of protein synthesis was measured by the incorporation of ^{14}C-leucine into protein during a 20 minute pulse. The results of this experiment are shown in Figure 1. Cycloheximide throughout the range of doses employed had no effect on the uptake of ^{14}C-leucine into the total acid-soluble pool of the liver cells (data not shown). Figure 1, however, shows a dose-dependent inhibition of protein synthesis. This inhibition reaches 94% with a dose of 1.5 mg/kg and is constant at 98% with doses of 5, 10 and 20 mg/kg body weight.

The effect of the same dose range of cycloheximide on the *in vivo* rate of RNA synthesis is quite different. Fasted animals were again injected with cycloheximide (0.5 to 20 mg/kg). Since the earlier studies with cycloheximide (10,11) demonstrated a time dependent inhibition of RNA synthesis with a half-time for the decay of RNA polymerase activity of 1/2 to 1-1/2 hours, we waited for three hours after the administration of cycloheximide to measure the *in vivo* rate of RNA synthesis. At this time, the animals were injected with ^{3}H-orotic acid. Thirty minutes later the extent of the

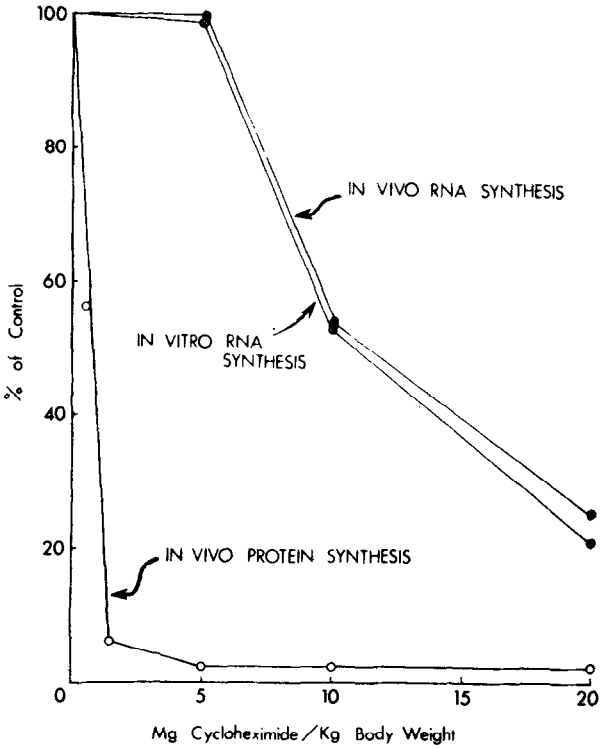

Figure 1. The effect of cycloheximide on rat liver protein and RNA synthesis. (a) Measurement of *in vivo* protein synthesis. Female Wistar rats obtained from Carworth Farms and weighing between 140 and 160 grams were fasted over night. Cycloheximide (Upjohn - Lot 6836-LMH-53) was injected intraperitoneally as a 1 mg/ml solution at the indicated doses. Three hours later the animals were given an intraperitoneal injection of 10 μCi L-leucine-^{14}C (U) (New England Nuclear - 300 mc/mm). Twenty minutes later the animals were sacrificed, the livers excised and homogenized in 2 volumes 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4, 0.025 M KCl and 0.005 M $MgCl_2$. 0.5 ml of the homogenate was precipitated with 5 ml 10% TCA and centrifuged at 500 x *g* for 10 minutes. An aliquot of the supernatant was counted as the acid soluble fraction, and the precipitate resuspended in 5 ml 10% TCA, heated at 90° for 15 minutes and centrifuged. The pellet was washed successively in 10% TCA, 95% ethanol containing 10% potassium acetate, absolute ethanol, ethanol-ether (3:1) at 60° for 5 minutes, ethanol-ether at room temperature and twice in ether. 5 mg of the dried proteins were solubilized in NCS-solubilizer (Amersham) and counted. (b) Measurement of *in vivo* RNA synthesis. The animals were injected intraperitoneally with 25 μCi ^{3}H-orotic acid (New England Nuclear - 12 Ci/mm). Thirty minutes later the animals were sacrificed, the livers excised and homogenized in 2 volumes 0.34 M sucrose, 0.015 M Mg acetate and 0.25 mM spermine. The homogenate was diluted with 2 volumes 2.1 M sucrose and 30 ml aliquots layered over 10 ml 2.1 M sucrose. The nuclei were sedimented by a 1 hour centrifugation at 25,000 rpm in the SW-27 rotor of the Spinco L2-65B ultracentrifuge. The nuclei were resuspended in 0.34 M sucrose and an aliquot precipitated with 5 ml cold 10% TCA. The precipitate was collected on a glass fiber filter, washed with 25 ml cold 10% TCA, solubilized in NCS-solubilizer and counted. DNA was determined by the diphenylamine assay. (c) Measurement of *in vitro* RNA synthesis with whole nuclei. Nuclei were prepared as above and resuspended in 0.05 M Tris-HCl, pH 7.9 containing 0.005 M $MgCl_2$; 0.01 mM EDTA;

incorporation of the isotope into nuclear RNA was determined as described in the legend to Figure 1. Cycloheximide administered in doses of 0.5, 1.5 and 5 mg/kg had no effect on the *in vivo* rate of nuclear RNA synthesis (Figure 1). With larger doses, however, there was increasing inhibition of RNA synthesis, 45% with 10 mg/kg and 75% with 20 mg/kg. There was no effect of cycloheximide on the uptake of orotic acid by the liver (data not shown).

An *in vitro* assay of RNA synthesis with nuclei isolated from animals treated with the same doses of cycloheximide confirmed that there is inhibition of RNA synthesis only with large doses of cycloheximide. There was no inhibition of *in vitro* RNA synthesis with *in vivo* doses less than 10 mg/kg (Figure 1). The extent of the inhibition of RNA synthesis with nuclei from animals receiving 10 and 20 mg/kg of cycloheximide was virtually the same as the *in vivo* inhibition of RNA synthesis produced by the same doses.

The results of the above experiments clearly show dose-dependent differential effects of cycloheximide on protein and RNA synthesis. *In vivo* protein synthesis in the rat is quite sensitive to relatively low doses of cycloheximide with the rate falling off to less than 10% of the control rate with 1.5 mg/kg. The inhibition is 98% with 5 mg/kg and does not change with larger doses. The residual protein synthesis measured with doses of 5 mg/kg and higher may reflect mitochondrial protein synthesis. This would imply that there is virtually no residual microsomal protein synthesis. Nuclear RNA synthesis, on the other hand, is relatively resistant to the doses of cycloheximide that virtually completely inhibit liver protein synthesis. The rate of RNA synthesis does decrease with larger doses of cycloheximide

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0.05 mM dithiothreitol and 25% glycerol. Aliquots were incubated for 10 minutes at 37° in a final volume of 0.25 ml containing 1.6 mM $MnCl_2$; 8 mM KCl; 6 mM NaF; 2 mM β-mercaptoethanol; 0.04 M phosphoenolpyruvate; 1.6 µg pyruvate kinase/ml; 0.6 mM ATP, GTP and CTP, and 40 µM UTP (1.0 µCi/µmole-^{3}H-UTP, New England Nuclear) and 0.08 M $(NH_4)_2SO_4$. The final $MgCl_2$ concentration was 0.005 M. The reaction was stopped by the addition of 5 ml cold 10% TCA. The precipitate was collected on a glass fiber filter, washed, solubilized and counted. All of the results are the mean of three individual animals and are accurate to a S.D. of ± 10% of the mean. Results are expressed as % of the control.

without any change in the already maximally inhibited rate or protein synthesis. These results suggest very clearly that the inhibition of RNA synthesis produced by cycloheximide is not a consequence of the drug's effect on protein synthesis.

Two possibilities can be considered for the mechanism of inhibition of RNA synthesis by large doses of cycloheximide. There may be a very low rate of metabolism of the drug with inhibition of nuclear RNA polymerase activity by the metabolite(s). Increasing concentrations of cycloheximide would lead to greater inhibition due to greater metabolism. Equally possible is some as yet unknown toxic effect of these large doses of cycloheximide manifested indirectly in the inhibition of nuclear RNA synthesis. Our experiments do not allow a distinction to be made between these alternative explanations. Our studies do imply that interpretation of experiments based on the use of very large doses of cycloheximide involve considerations in addition to the inhibition of protein synthesis. Further, the inhibition of protein synthesis by 98% for three hours without any significant effect on RNA synthesis is inconsistent with the postulated existence of very short-lived protein(s) controlling the normal rate of ribosomal RNA synthesis.

Acknowledgment: This work was supported by NIH grant CA 12073. Mr. Farmar was supported by NIH grant CA 12227.

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