CYCLOHEXIMIDE, AN INHIBITOR OF PEPTIDE CHAIN TERMINATION OR RELEASE IN LIVER IN VIVO AND IN VITRO[1]

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SUMMARY: It has been found that cycloheximide at low dosage has a selective inhibitory effect on peptide chain termination or release in the liver in vivo and in vitro. In vivo, cycloheximide in a dosage that inhibits total liver synthesis by over 90% has little or no effect on labeling of nascent protein on polysomes by radioactive leucine but a marked effect on the labeling of sap protein. In vitro, cycloheximide in a dosage range of 0.018 to 0.89 mM shows a progressive inhibitory effect on the labeling of soluble protein and no effect on the labeling of nascent protein on polysomes. Only at a much higher concentration range (above 1.88 mM) is there an increasing effect on incorporation of radioactive amino acids into nascent protein.

It has been found that polysomes, functionally active in vitro, are induced from ribosomal precursors by cycloheximide in the liver of animals pretreated with ethionine or puromycin, despite an almost complete inhibition of protein synthesis in vivo (1). This finding appeared to be incompatible with a primary or major effect of cycloheximide either on peptide chain initiation (2) or on translocation (2-5) but rather suggested that this inhibitor of protein synthesis was acting at or near the site of peptide chain termination or release (6). This suggestion was previously made by Godchaux et al. (7) in studies on intact reticulocytes in vitro.

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The studies that followed from this, which are the subject of this communication, have clearly shown that in the liver, cycloheximide in a lower dosage range has indeed an inhibitory effect at or near the site of peptide chain termination both *in vivo* and *in vitro* and that one or more additional effects became evident at much higher doses.

EXPERIMENTAL PROCEDURE AND RESULTS

IN VIVO. Early in the *in vivo* studies, it became evident that a meaningful result would be most easily obtained if the experiment were limited to a single polysome-ribosome cycle. Although this is not accurately known for any liver protein, the little data available suggested that some liver

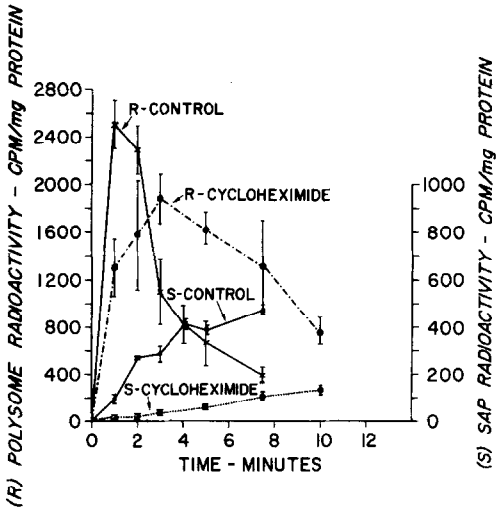

Figure 1. Specific radioactivity (cpm per mg protein) of polysomal (R) and soluble supernatant (S) protein of liver at various time intervals following the injection of 5 μCi U-^{14}C-L-leucine (10 to 30 mCi/mmole, Amersham/Searle) with 0.9% NaCl (saline) or cycloheximide (0.08, 0.15 or 0.30 mg/kg) into the portal vein of anesthetized animals (6 mg Nembutal/100 g/body weight). The polysomes were obtained as a pellet from the postmitochondrial supernatant (PMS) after adding sodium deoxycholate (DOC) (1% final concentration) and centrifuging through a discontinuous sucrose gradient (0.5 M and 2.0 M sucrose) as previously described (14). The soluble supernatant was obtained by centrifuging the PMS without added DOC for 4 hrs. at 40,000 rpm in a 40 rotor in a Spinco L-2 centrifuge. The R and S proteins were precipitated with 10% trichloracetic acid (TCA) containing 20 mg/ml celite. The precipitates in the TCA were extracted at 90° C for 30 minutes, filtered and washed with 5% TCA, propanol-ether and ether. The protein was dissolved in soluene (Packard solubilizer) and counted in a Packard liquid scintillation spectrometer. Protein was determined by the biuret method.

proteins are synthesized in a period of 2 to 3 minutes (8). In order to control the timing as accurately as possible, rats (male or female white Wistar, Carworth Farms) weighing from 170 to 220 g were initially anesthetized with Nembutal (6 mg per 100 g body weight) and then were injected into the portal vein after laparotomy. The compounds administered were cycloheximide, uniformly-labeled leucine-^{14}C, saline (0.9% NaCl) or puromycin.

The injection of radioactive leucine intraportally at zero time produces a pulse labeling of polysomes, as seen in Figure 1. The polysomes show a peak labeling between zero and 1 minute. This remains more or less at the same level for an additional 1 minute after which there is a rapid decline in the specific activity of the ribosomal proteins. By 4 to 5 minutes, the polysomes have lost 75% or more of their labeled protein. Essentially all of the radioactivity in the polysomal pellet not removed by treatment with hot trichloracetic acid is nascent protein, since the bulk of the label is lost within a few minutes and virtually no intrinsic ribosomal protein would be labeled in the liver in such short time intervals. Also, approximately 93% of the labeled trichloracetic acid precipitable material on the polysomes was rapidly removed by treatment with puromycin (Table 1).

The specific activity of the supernatant proteins shows a fairly rapid increase during the first 5 to 7.5 minutes (Figure 1). Since the pool size of unlabeled protein is much higher in the sap than in the polysomes, the specific activity of the sap proteins remains much lower than that of the ribosomal proteins at the peak of labeling of nascent protein with a single pulse.

The administration of cycloheximide simultaneously with the radioactive leucine results in considerable distortion of the labeling patterns (Figure 1). The nascent protein labels a little more slowly than in the controls but reaches a value close to that of the controls. In the experiment illustrated in Figure 1, the maximum value for the polysomal protein is 75% that in the control shown. However, in other experiments, the value has varied from 75% to 120%. The loss of label from the polysomes is considerably slower than in the controls. This

TABLE 1

EFFECTS OF CYCLOHEXIMIDE AND PUROMYCIN IN VIVO UPON PROTEIN RADIOACTIVITY IN LIVER POLYSOMES AND SUPERNATANT PRELABELED FOR 1 MINUTE WITH U-^{14}C-LEUCINE

Experiment No.	Group[b]	Specific Activity[a] of Protein (cpm/mg) at							
		1 minute		4 minutes		10 minutes		20 minutes	
		R	S	R	S	R	S	R	S
1	Control	3110 (2257-3963)	51 (19-83)	1409 (1284-1535)	213 (179-248)	600 (569-632)	575 (446-704)		
	Cycloheximide (0.15 mg/Kg)			3248 (2736-3760)	94 (58-130)	1538 (1363-1713)	201 (196-206)		
2	Control	4252 (4024-4481)	43 (38-48)	1825 (1595-2056)	274 (273-276)			447 (326-569)	442 (397-487)
	Cycloheximide (0.3 mg/Kg)			4773 (4618-4928)	147 (115-179)			1358 (1164-1552)	169 (127-212)
	Puromycin (25 mg/Kg)			280 (78-481)	247 (240-254)				

[a] Each value is the mean of the values from two animals. The two values are given in parentheses. R = polysomal nascent protein, S = soluble supernatant protein. The protein was prepared and counted as described in the legend to Figure 1.

[b] The saline (control), cycloheximide and puromycin were given by injection, into the portal vein, at exactly one minute after the injection of radioactive leucine.

varies with the dose of cycloheximide, the larger the dose (up to 1.5 mg/Kg), the slower the loss of the label. Concomitantly, the increase in labeling of sap protein is very much slower than in the controls (Figure 1). For example, the sap proteins in the period from 1 to 5 minutes show 85 to 93% decrease in specific activity with the dose of cycloheximide used (0.08 mg/Kg) as compared to control values. The discrepancy between the dynamics of labeling of polysomes and sap is shown graphically in Figure 2. The sap/polysome radioactivity increases so much more quickly in the control than in the cycloheximide-treated.

If the cycloheximide is administered 1 minute after the radioactive leucine, the polysomes retain their radioactivity for a much longer period of time than in controls while the sap proteins are now only slowly labeled, even over periods of 10 to 20 minutes (Table 1). Not infrequently, the level of labeling of the polysomes may actually increase somewhat after the administration of the cycloheximide. In contrast, with puromycin, there is a very rapid loss of nascent labeled material from the polysomes (Table 1). The sap shows the same increase in trichloracetic acid precipitable material as in the controls, thus suggesting that some of the labeled material released by puromycin from the polysomes consists of peptide chains sufficiently large to be precipitable with this acid.

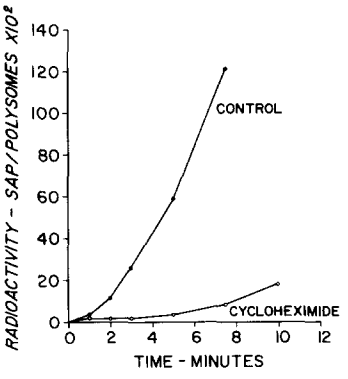

Figure 2. Ratio of the specific radioactivity of sap to polysomal nascent protein from control (saline treated) and cycloheximide treated rats as a function of time after the intraportal injection of U-^{14}C-L-leucine with saline or cycloheximide (0.3 mg/kg).

IN VITRO. As seen in Figure 3, cycloheximide in the range of 5 to 250 µg/ml shows progressive inhibition of labeling of sap protein without any effect on labeling of ribosomal protein. Above this range, a second effect becomes evident - the progressive decrease in radioactivity of ribosomal as well as of sap protein. This second concentration range is that in which cycloheximide was active in suppressing protein synthesis in cell free preparations of reticulocytes (4) or of liver (2,3,5). The differences between the labeling of sap and of polysomal nascent protein remained the same whether the ribosomes and soluble fractions were separated after incubation by centrifuging at 105,000 g for 2 hours or for 24 hours. Virtually no labeling of protein was found when sap or polyribosomes were incubated separately.

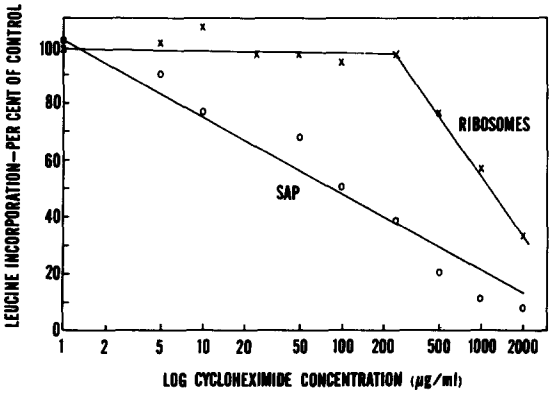

Figure 3. Effect of cycloheximide on the labeling of liver polysomal nascent protein (R) and supernatant protein in vitro. The incubation medium contained the following in a final volume of 1 ml: 1 µmole ATP, 0.4 µmole GTP, 10 µmole phosphoenolpyruvate, 50 µg pyruvate kinase, 5 µmole $MgCl_2$, 25 µmole KCl, 50 µmole Tris buffer (pH 7.4), 50 µmole NH_4Cl, 5 µmole mercaptoethanol, 0.1 µCi U-^{14}C-L-leucine 0.1 ml cell sap and 0.1 ml cycloheximide. Incubation was at 37° C for 10 minutes The tubes were rapidly chilled and the ribosomes and cell sap were separated by centrifuging in a 40 rotor at 105,000 g for 2 or for 24 hrs. The proteins from the ribosomal pellet and the supernatant were prepared and counted as described in the legend for Figure 1.

DISCUSSION

It is evident from this study that cycloheximide has at least two inhibitory effects on protein synthesis - a potent effect on chain termination or release at low concentration and an effect on amino acid incorporation into nascent protein

at higher concentration. The latter is likely due to inhibition of translocase (2-5) The *in vivo* effect at dosages producing over 90% inhibition of overall protein synthesis is the one on chain termination or release. This action of cycloheximide is probably not unique to liver since similar effects have been reported for reticulocytes *in vivo* (7) and *in vitro* (9). Different effects in protein synthesis of different concentrations of cycloheximide have been reported (2,7,10).

An attractive hypothesis that could explain the effects of cycloheximide on protein synthesis is an action of the inhibitor on the binding of GTP. Consistent with this are the following: a) GTP is required for initiation, translocation and peptide release (11). Cycloheximide at different concentration ranges has been reported to inhibit at each of these loci (2-5,7,10); b) GTP prevents the inhibitory effect of cycloheximide on translocase (5); and c) cycloheximide *in vitro* arrests the progress of mitosis in some cells (12), a process involving microtubular proteins which avidly bind GTP (13).

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