proliferation alone. Since drugs were added 48 hr after planting, the present experiments exclude drug effects during the initial 48-hr period. To further define the loci of inhibition with respect to induction, proliferation and maturation of immunocompetent cells, we are currently examining antibiotic effects at various temporal intervals after cell culture.

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The effect of cycloheximide on ribonucleic acid and protein synthesis in rat liver*

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NUMEROUS carcinogens[1] and antitumor agents[2–4] produce alterations in the function and ultrastructure of the nucleolus, the site of synthesis and maturation of the ribosomal precursors.[5,6] Separation of nucleolar granules and fibrils into two or more distinct zones is designated as nucleolar segregation or nucleolar "cap" formation.

Cycloheximide produces nucleolar ultrastructural lesions in hepatic and pancreatic parenchymal cells of rats[7] and inhibits protein synthesis at the polysomal level,[8,9] but exerts no direct effect on

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RNA synthesis.[10,11] Nevertheless, the inhibition of protein synthesis by cycloheximide does have important secondary effects on RNA synthesis, stability and transport.[12–16]

MATERIALS AND METHODS

Male F344 rats fasted for 24 hr and weighing between 80 and 150 g were used in all experiments. Cycloheximide (Actidione), dissolved in normal saline, was given by intraperitoneal injection (5 mg/kg body wt.) at 2, 12 and 24 hr before sacrifice. Thirty min prior to sacrifice, uridine-^{3}H (2·3 c/mM, Nuclear-Chicago) or leucine-^{14}C (170 mc/mM, Nuclear-Chicago) was injected intraperitoneally in doses of 100 μc/100 g body wt. and 5 μc/100 g body wt. respectively.

The methods employed for determination of RNA synthesis have been described in detail elsewhere.[17,18] Nuclei and nucleoli were isolated by using the methods of Widnell and Tata[19] and Muramatsu *et al.*[20] respectively. RNA synthesis *in vitro*, incorporation of UTP-^{3}H (2·7 c/mM, New England Nuclear Corp.), was measured in nuclear fractions of liver from normal and cycloheximide-treated rats. The nuclear fractions were assayed in duplicate after 30 min of incubation in the presence of 0·32 M ammonium sulfate.[21] For comparison, nuclei from livers of untreated rats were also incubated in the presence of actinomycin D (10 μg) or cycloheximide (10 μg) and RNA synthesis *in vitro* was assayed.

For extraction of nucleic acids and proteins, aliquots of the nuclear, nucleolar and whole cell homogenates were suspended in a final concentration of 10% trichloracetic acid (TCA), the mixture allowed to stand 10 min and the precipitate centrifuged. The pellet was successively washed, once with 10% TCA and twice with 5% TCA, and the lipids were extracted with ethanol and an ethanol–chloroform mixture. The precipitate was dried and then hydrolyzed in 5% TCA at 90° for 15 min. Nucleic acids were estimated by the method of Schneider.[22] The TCA-insoluble residue was solubilized in 0·3 N KOH and the protein content was estimated by the method of Lowry.[23] The radioactivity of the TCA-soluble and insoluble fractions was measured in a Packard Tri-Carb liquid scintillation spectrometer by using a mixture of NCS (Nuclear-Chicago), BBOT (Packard Instrument Company, Inc.)[24] and toluene.

RESULTS

Protein synthesis. In rats with cycloheximide, maximum inhibition of protein synthesis in the nuclear fraction and whole cell homogenate was observed at 2 hr (Fig. 1). Incorporation of leucine-^{14}C into protein did not return to normal by 24 hr. No significant differences in protein synthesis were observed in nuclear fractions compared to whole cell homogenates.

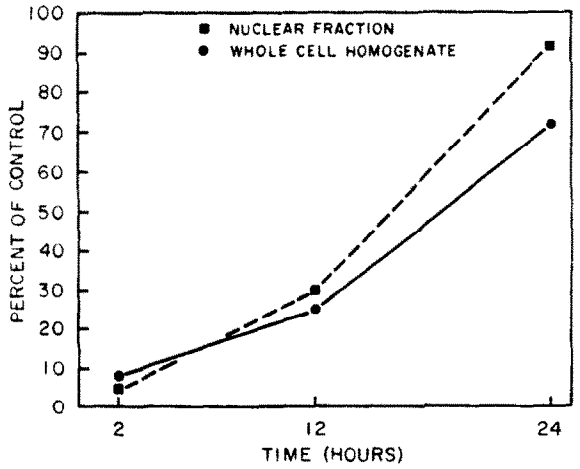

FIG. 1. Thirty min incorporation *in vivo* of leucine-^{14}C (5 μc/100 g) after cycloheximide administration (5 mg/kg).

RNA synthesis in vivo *and* in vitro. The results of incorporation *in vivo* of uridine-^{3}H into nucleolar RNA after a single dose of cycloheximide are shown in Fig. 2. Nucleolar RNA synthesis appeared most sensitive to cycloheximide at 24 hr, when the nucleolus showed conspicuous ultrastructural aberrations consisting of disarrangement of the normal reticular pattern.

As shown in Table 1, the addition of cycloheximide (10 μg) to the reaction mixture did not inhibit RNA synthesis *in vitro* in normal rat liver nuclei, whereas the addition of actinomycin D (10 μg) resulted in a marked decrease in activity at 30 min of incubation. On the other hand, the RNA synthetic activity in liver nuclei isolated from cycloheximide-treated rats was reduced to 60 per cent of control levels.

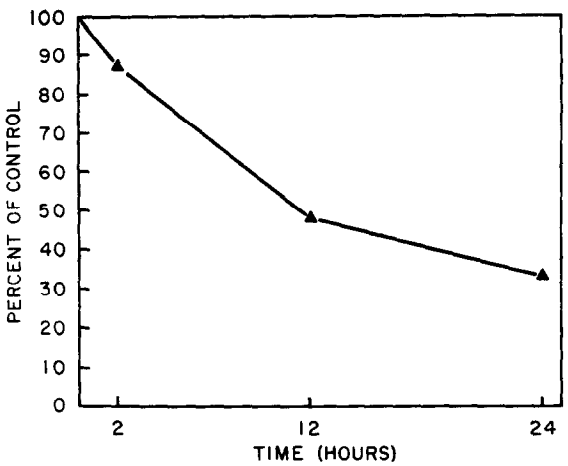

FIG. 2. Thirty min incorporation *in vivo* of uridine-^{3}H (100 μc/100 g) in nucleolar RNA after cycloheximide administration (5 mg/kg).

TABLE 1. EFFECT OF CYCLOHEXIMIDE ON RNA SYNTHESIS *IN VITRO* IN RAT LIVER NUCLEI*

	Treatment *in vitro* with cycloheximide (10 μg) or actinomycin D (10 μg) (%)	24 hr Pretreatment *in vivo* with cycloheximide (5 mg/kg) (%)
Control	100	100
Cycloheximide	125	60
Actinomycin D	31	

* Reaction mixture contained: 100 μmoles Tris, pH 8·0; 10μmoles cysteine; 3 μmoles $MgCl_2$; 0·5 μmole each of adenosine, cytosine and guanosine triphosphates; 0·04 μmole of tritiated uridine triphosphate; 160 μmoles $(NH_4)_2SO_4$; and nuclei in a final volume of 0·5 ml. Each experiment was carried out in duplicate.

DISCUSSION

Cycloheximide has been utilized to study the RNA-protein inter-relationship, since it has no known direct effect on RNA synthesis.[10,11,25] The absence of inhibiting effect of cycloheximide *in vitro* on RNA synthesis, (Table 1) compared to actinomycin D, for example, supports the lack of direct effect of cycloheximide. Liver nuclei isolated from cycloheximide-treated rats, however, have reduced RNA synthetic activity at 24 hr (Table 1), demonstrating the interdependence of protein and RNA synthesis.

Cycloheximide causes rapid inhibition of protein synthesis (Fig. 1), which is followed by a reciprocal decrease in RNA synthesis[12,16,26] (Fig. 2) and decreased stability of rapidly labeled RNA.[13,26] The normal synthesis of the 18S ribosomal RNA does not occur in the presence of cycloheximide,[10,14]

and Ennis[12] hypothesized that the newly synthesized 18S ribosomal RNA is unstable, " . . . perhaps due to a requirement for the continued synthesis of a specific protein for stability." Cycloheximide appears preferentially to inhibit nucleolar RNA synthesis at 24 hr,* the same time at which it produces ultrastructural changes.[7] Nucleolar RNA and protein synthesis are also preferentially inhibited by puromycin and actinomycin D.[5,27]

Nucleolar segregation cannot as yet be attributed solely to reduced RNA synthesis,[3] but appears to be a nonspecific ultrastructural pattern, clearly associated with inhibition of RNA synthesis, and possibly with other biochemical alterations in nucleolar function.

Note added in proof—Inhibition of nucleolar RNA synthesis by cycloheximide has also been reported recently by HIGASHI *et. al. Biochim. Biophys. Acta.* **166,** 338 (1968).

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* Nuclear RNA synthesis was depressed to 65 per cent of control (unpublished data), whereas nucleolar RNA nthesis was depressed to 34 per cent of control (Fig. 2).