a viral inhibitor with killed *E. coli* and endotoxin has been demonstrated, its identity is not yet definite. Most of its properties suggest that it is an interferon, but this cannot be categorically stated since a precise definition of interferon is lacking (*9*). The characteristics of the inhibitor here described which differentiate it from interferon are its heat and acid lability. While heat-labile interferon from mouse cells has been described (*11*), circulating interferon of rabbits induced by viruses rather than by endotoxin was stable at 56°C for 1 hour (*1*). Possibly there may be more than one type of interferon-like inhibitors in one animal species. The phenomenon described here may explain the sparing effect of endotoxins on certain viral infections (*2, 12*). It suggests further proof of the relation between bacteria and viral inhibitors, and particularly between interferon, endotoxin, and fever.

Note added in proof: W. R. Stinebring and J. S. Youngner have obtained in mice an inhibitor in high titer which was more definitely characterized as interferon (*13*).

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Cycloheximide: Aspects of Inhibition of Protein Synthesis in Mammalian Cells

Abstract. *Cycloheximide and acetoxycycloheximide specifically inhibit protein synthesis in L-cells growing in suspension culture. In extracts of rat liver, the drugs inhibit transfer of amino acid from soluble RNA to polypeptide. Unlike puromycin, these drugs do not accelerate release of nascent polypeptide chains. The drugs have no effect on protein synthesis in extracts of* Escherichia coli.

Cycloheximide (*1*), an antibiotic produced by *Streptomyces griseus*, inhibits the growth of many yeasts and fungi and inhibits protein synthesis in intact rats, but has little effect on growth of bacteria (*2*). Opinion is divided on the question of whether the primary effect of low doses of cycloheximide is on DNA synthesis or on protein synthesis (*3–5*). Our results reported here indicate that in L-cells the effect of this antibiotic is greater on protein synthesis.

Two laboratories have found that cycloheximide inhibits transfer of amino acids from aminoacyl sRNA (*6*) to polypeptide. Siegel and Sisler have recently reported that cycloheximide decreases transfer of amino acids to polypeptide in extracts of *Saccharomyces partorianus* by about two-thirds (*7*). Our results with extracts of rat liver (*8*) are given in detail below. The present report also describes experiments on the release of nascent polypeptide chains, on the reversibility of inhibition by cycloheximide and acetoxycycloheximide (*9*), and on the sensitivity of systems stimulated by natural and synthetic messenger RNA.

For measurements on intact cells, L-cells were grown in suspension culture, and various concentrations of drug and either C^{14}-leucine or C^{14}-uridine were added. After 1 hour of incubation the cells were collected, and the radioactivity in the fraction precipitated by cold TCA was determined. Cycloheximide and acetoxycycloheximide markedly inhibited incorporation of C^{14}-leucine, but, even at concentrations 100-fold greater than that required for maximum inhibition of protein synthesis, incorporation of C^{14}-uridine into the TCA precipitable fraction was not inhibited (Fig. 1). No inhibition of C^{14}-uridine incorporation was apparent until 90 to 120 minutes after addition of drug.

To measure the rate of DNA synthesis, portions of the cell suspension were removed at intervals during a 90-minute period of incubation in the presence of C^{14}-uridine, and 10*N* NaOH was added to produce a final concentration of 1*N*. The mixture was then kept at 37°C for 2 hours, by which time RNA had been degraded (*10*). The mixture was neutralized with concentrated HCl, an equal volume of 10 percent TCA was added, and the precipitate was collected and counted. At a concentration of cycloheximide (10 μg/ml) that inhibited protein synthesis by 95 percent, DNA synthesis was inhibited by only about 50 percent (three experiments). This result is similar to the findings reported for HeLa cells by Young *et al.* (*4*). On the other hand, Bennett *et al.* have reported that, in cell lines derived from human epidermoid carcinoma and from mouse adenocarcinoma, DNA and protein synthesis are nearly equally depressed by cycloheximide (*5*).

When cells were incubated in the presence of either drug for 3 hours and then washed free of drug, protein synthesis resumed promptly at nearly nor-

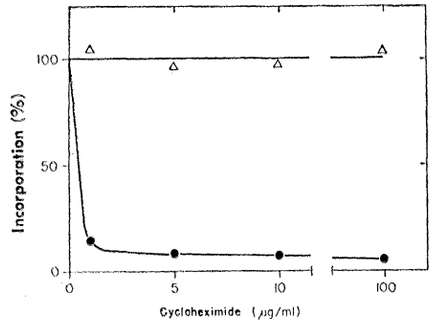

Fig. 1. Effect of cycloheximide on the incorporation of C^{14}-leucine and C^{14}-uridine into growing L-cells. A growing suspension (3×10^5 cells/ml) with a division time of about 24 hours was inoculated into Eagle's MEM medium (*16*) containing either C^{14}-leucine (0.1 μc/μmole, 0.3 μmole/ml) or C^{14}-uridine (1.5 μc/μmole, 0.24 μmole/ml). The indicated quantity of cycloheximide was added to 10-ml portions of each of the suspensions, and the cells were incubated at 37°C with shaking. At the end of 60 minutes, 1-ml samples (in duplicate) were filtered through Millipore filters (3-μ pore) under slight suction (2 cm H_2O). The cells were washed with 10 ml of Earle's salt solution, and then washed with 10 ml of 5 percent TCA containing 1 mg of nonradioactive leucine and uridine per milliliter. The filters were mounted on planchets and counted with a Nuclear-Chicago end-window counter. C^{14}-uridine, (△——△); C^{14}-leucine, (●——●).

mal rate. Inhibition by these drugs appears, therefore, to be reversible.

In a cell-free system derived from rat liver (*11*), both antibiotics inhibited the stimulation by polyU of polyphenylalanine synthesis (Fig. 2). Acetoxycycloheximide was about 10 times more potent than cycloheximide. When the drugs were added to the reaction mixture as late as 15 minutes after the start of incorporation of phenylalanine, further synthesis of polypeptide was sharply inhibited. Marked inhibition of polypeptide synthesis was also found in the presence of either natural messenger RNA or polyAC. When ribosomes (prepared by deoxycholate treatment of microsomes) and enzymes from the supernatant were substituted for the S-30 fraction (*12*) the results were similar. In contrast, protein synthesis in cell-free extracts of *Escherichia coli*, in the presence of either natural messenger RNA or synthetic polyU, was not inhibited by either drug.

In rat liver extracts, concentrations of the drugs as high as 200 μg/ml did not inhibit synthesis of C^{14}-phenylalanyl sRNA from C^{14}-phenylalanine. However, the transfer of radioactivity from C^{14}-phenylalanyl sRNA to polypeptide, in the presence of polyU, was markedly inhibited by both drugs. (Figure 3 shows inhibition of transfer by acetoxycycloheximide.)

The effect of cycloheximide and acetoxycycloheximide on the release of nascent polypeptide chains from microsomes was measured. Microsomes were labeled with C^{14}-lysine by 10 minutes of incubation in a complete system. The labeled microsomes were then isolated by centrifugation at 100,000*g* for 90 minutes, and they were again incubated in a complete system but without added label. Samples were taken at intervals, and the microsomes were again isolated by centrifugation. The residual radioactivity attached to these microsomes was measured. The results (Fig. 4, one of four similar experiments) show that the rate of release of labeled polypeptide in the presence of acetoxycycloheximide was not significantly different from the rate of release in its absence. As expected, puromycin (100 μg/ml) caused extensive release of labeled polypeptide from microsomes (*13*).

The reversibility of inhibition was also tested in the cell-free system. Microsomes were incubated in a complete system containing acetoxycycloheximide (25 μg/ml) for 30 minutes. After this time, they were harvested by centrifugation, and then were washed in, and dialyzed against the homogenizing medium (*11*). They were then incubated in a complete system with polyU, and the incorporation of C^{14}-phenylalanine was measured. In a parallel control experiment, microsomes from a reaction mixture that did not contain drug were similarly isolated, washed, dialyzed, and tested for the capacity to synthesize polyphenylalanine. The results showed no significant difference between microsomes that had been incubated with acetoxycycloheximide and washed and the controls that had not. Inhibition in the cell-free system, as with intact cells, was reversible.

Cycloheximide and acetoxycycloheximide resemble chloramphenicol (*14*) and chlortetracycline (*15*) in their apparent mode of action, except that cycloheximide and acetoxycycloheximide strongly inhibit mammalian and yeast

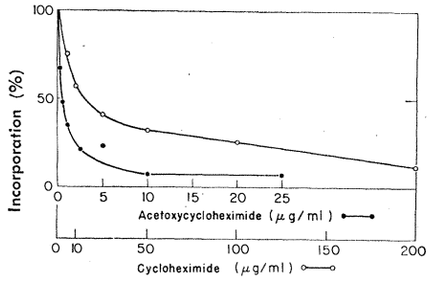

Fig. 2. Effect of cycloheximide and acetoxycycloheximide on polyphenylalanine synthesis in rat liver extracts. The reaction mixture contained in a volume of 1 ml: 100 μmole of tris-HCl (*p*H 7.8); 6 μmole of mercaptoethanol; 12 μmole of magnesium acetate; 6 μmole of disodium creatine phosphate; 20 μg of creatine kinase; 2 μmole of disodium ATP; 0.12 μmole of trisodium GTP; 100 μmole of KCl; 2.8 mμmole of C^{14}-phenylalanine (0.5 μc); 300 μg of polyU (Miles Laboratories); antibiotics as indicated; rat liver extract [Sprague-Dawley male rats; S-30 fraction, (*12*)] containing 0.43 mg RNA and 3.8 mg protein. After 30 minutes of incubation at 37°C, 0.3 ml samples were added to an equal volume of 10 percent TCA-containing nonradioactive phenylalanine (1 mg/ml). Each precipitate was centrifuged, washed once with 3 ml of 5 percent TCA and suspended in 3 ml of 5 percent TCA. The suspension was heated at 100°C for 10 minutes, and a portion was filtered through a 0.45-μ Millipore filter; the retained precipitate was washed, mounted on a planchet, and counted. Cycloheximide, (○——○); acetoxycycloheximide, (●——●).

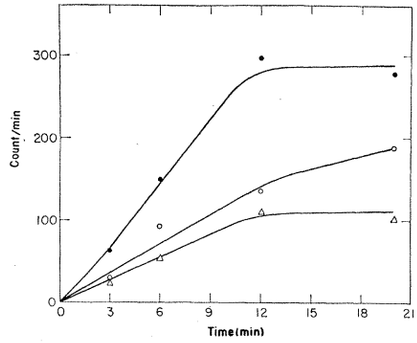

Fig. 3. Effect of acetoxycycloheximide on the kinetics of synthesis of polyphenylalanine from phenylalanyl sRNA in rat liver extract. The reaction mixture contained in a volume of 1 ml: 100 μmole of tris-HCl (*p*H 7.8); 6 μmole of mercaptoethanol; 12 μmole of magnesium acetate; 6 μmole of disodium creatine phosphate; 20 μg of creatine kinase; 2 μmole of disodium ATP; 0.12 μmole of trisodium GTP; 100 μmole of KCl; S-100 fraction (0.85 mg protein, 0.055 mg RNA); P-100 microsome fraction (0.23 mg protein, 0.15 mg RNA); C^{14}-phenylalanyl sRNA (0.28 mg, 658 count/min, prepared from rat liver sRNA); 1 μmole of C^{12}-phenylalanine; 300 μg of polyU, 37°C. Maximum transfer of counts was 45 percent. Concentration of drug: 0 μg/ml (●——●); 1 μg/ml (○——○); 10 μg/ml (△——△). Abscissa, radioactivity incorporated into polypeptide.

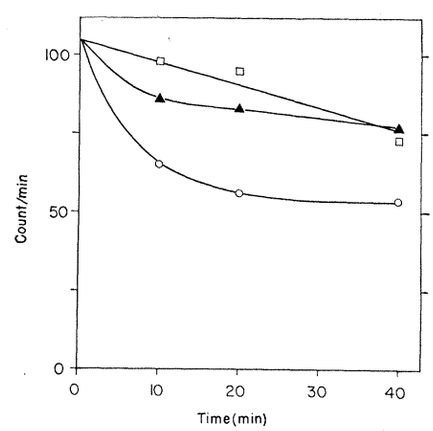

Fig. 4. Effect of acetoxycycloheximide and of puromycin on the release of labeled protein from microsomes prepared from rat liver. Microsomes were labeled by a 10-minute incubation in a complete system as given in Fig. 2, except that C^{14}-lysine (2.5 mμmole, 0.5 μc) and 19 other nonradioactive amino acids (40 μmole/ml) were added, and C^{14}-phenylalanine and polyU were omitted. The microsomes were then centrifuged at 100,000*g* for 90 minutes, and put back into a complete system containing 20 nonradioactive amino acids. Samples (1 ml) were taken at the indicated intervals, and the microsomes were again isolated by centrifugation at 100,000*g* for 90 minutes. The pellet was rinsed with medium A (*17*) and dissolved in concentrated formic acid (1 ml), and the solution was placed on a stainless steel planchet, evaporated to dryness, and counted. Control (□——□); acetoxycycloheximide, 25 μg/ml (▲——▲); puromycin, 100 μg/ml (○——○).

but not bacterial systems. In inhibiting sensitive systems, cycloheximide, acetoxycycloheximide, and chloramphenicol display the following common features: (i) protein synthesis is suppressed in intact cells before any effect on RNA synthesis appears; (ii) inhibition of protein synthesis is reversible; (iii) transfer of amino acid from sRNA to polypeptide is inhibited; (iv) the release of nascent polypeptide chains is not accelerated.

Both cycloheximide and its acetoxy derivative are unique in their high specificity for mammalian and yeast systems. They differ from puromycin in their mechanism of action and they are useful alternatives to puromycin when it is desirable to inhibit the synthesis of protein but not of RNA.

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Bone Cells: Biochemical and Biological Studies after Enzymatic Isolation

Abstract. *Short-term incubation of rat calvaria in buffered crude collagenase permitted the isolation of morphologically intact cells that absorb vital dyes, contain alkaline phosphatase, and multiply in tissue culture. Freshly harvested cells were similar to whole bone segments in aerobic glucose metabolism.*

Because calcified and noncalcified connective tissue interfere with the isolation of bone cells, almost all information concerning the metabolism of bone in vitro has been derived from the use of bone slices and segments in tissue culture or in short-term incubation. Although identifiable bone cells emigrate from bone explants in tissue culture chambers (*1*), the number of cells is inadequate for many metabolic studies. Collagenase preparations have been used to digest tissues and disperse cells effectively (*2*) without altering cell function (*3*). This report describes the dispersion of viable cells from rat bones by crude collagenase and the comparison of glucose metabolism by these cells and by intact bone.

For the isolation of cells, frontal and parietal bones were obtained under aseptic conditions from the calvaria of 17- to 21-day-old rat fetuses and newborn rats and were cleaned of superficial periosteum, dura, and cartilage along the major sutures. Approximately 10 calvaria (100 mg) were fragmented, placed in siliconized 25-ml erlenmeyer flasks containing 4.0 ml of tris-buffered saline (*p*H 7.4), glucose (5 μmole/ml), penicillin-streptomycin [50 units per milliliter (*4*)], and crude collagenase [0.1 to 6.0 mg/ml (*5*)], and shaken 90 times per minute in a Dubnoff incubator at 37°C for periods ranging from 90 to 180 minutes. After incubation, the medium was decanted into siliconized 15-ml culture tubes and centrifuged at 400*g* for 1 to 3 minutes. The pellet of cells that was obtained was washed with medium free of enzymes and centrifuged three times. Cells were finally suspended in the same medium and standardized by counting the cells in a hemocytometer.

Crude collagenase dispersed 20,000 to 50,000 cells/mg of bone from fetal and newborn rat calvaria into the incubation medium. Optimum cell release was obtained by exposure to enzyme concentrations of 1 to 3 mg/ml for 90 minutes. Histologically, collagenase-treated bone showed areas of complete cell removal, suggesting that in those areas affected by the enzyme, there was no selective removal of cell types. Centrifuged pellets contained only intact mononuclear cells. No definite hematopoietic marrow cells were recognized in coronal sections of undigested bone segments or in the cell pellets. As an indication of cell type, alkaline phosphatase activity of isolated cells was estimated histochemically by a modification of the method of Kaplow (*6*), with the use of sodium alpha naphthyl phosphate and Diazo Blue B (*7*). All cells contained detectable cytoplasmic or nuclear alkaline phosphatase, or both, and approximately 50 percent of the cells showed an intense cytoplasmic reaction (Fig. 1). Pronase (*8*), 1.0 mg/ml, produced a similar cell yield, but many cells were damaged or ruptured. Trypsin (Difco), 2.5 to 5.0 percent, without divalent cations in the incubation mixture released an insignificant number of cells.

Viability of harvested cells isolated by collagenase treatment was evaluated by vital dye uptake and multiplication in tissue culture. Over 95 percent of isolated cells appeared to be viable as evidenced by cytoplasmic staining after incubation for 30 minutes at 37°C in buffered 0.01 percent neutral red (Fig. 2). Cells suspended in standard Eagle's No. 2 medium with 20 percent fetal calf serum, glutamine at 0.25 mg/ml, and

Table 1. Glucose metabolism by isolated cells. Data are expressed as percent of glucose in the medium converted to $C^{14}O_2$ or to lactic acid-C^{14} by 10^6 isolated cells in two hours. Each figure represents the mean of four flasks. In repeat experiments relative yields of carbon dioxide and lactic acid from glucose-U-C^{14}, glucose-1-C^{14}, and glucose-6-C^{14} were constant, although small differences in absolute values were noted.

Substrate		
Glucose-U-C^{14}	Glucose-1-C^{14}	Glucose-6-C^{14}
	$C^{14}O_2$	
0.12 ± 0.02	0.20 ± 0.02	0.07 ± 0.01
	Lactic acid-C^{14}	
3.30 ± 0.18	2.56 ± 0.17	3.67 ± 0.26