Inhibitory Effects of Acetoxycycloheximide, Puromycin, and Pactamycin upon Synthesis of Protein and DNA in Asynchronous Populations of HeLa Cells

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SUMMARY

The rate of incorporation of thymidine into DNA of HeLa cells decreased within 10 min following exposure of these cells to an adequate concentration of acetoxycycloheximide, puromycin, or pactamycin. While uptake of leucine was inhibited in excess of 95% by these drugs, inhibition of incorporation of thymidine did not exceed 70% within the first hour. Incorporation of uridine into RNA was unaffected by acetoxycycloheximide but was decreased by high concentrations of puromycin and pactamycin. Concentration-effect and temporal relationships of these drug-induced phenomena indicate that interruption of protein synthesis is a primary effect; impairment of synthesis of DNA is a secondary event resulting from the disturbance in protein metabolism. Inhibition of precursor incorporation into RNA by high levels of puromycin and pactamycin seems unrelated to the action of these drugs on the biosynthesis of proteins. These results suggest the existence of a close temporal coupling between the synthesis of a protein species and the replication of DNA in an asynchronous population of mammalian cells in exponential growth.

INTRODUCTION

Relationships between the biosynthesis of protein and the replication of deoxyribonucleic acid are of current investigative interest. Since the copying of DNA is an enzyme-mediated process, requisite enzymes must obviously be made some time prior to the initiation of DNA synthesis. Experimental observations confirm that the kinases of the deoxythymidylate pathway and DNA polymerase appear at about the same time that formation of new DNA is detectable in regenerating liver (1–3) and in cultured mammalian cells in transition from stationary-phase to log-phase of growth (3). Synthesis of histones is detectable in close temporal and geographic association with replicating DNA in the macronucleus of *Euplotes* (4). Further, synthesis of certain histone varieties even seems dependent upon replication of DNA in HeLa and tobacco cells (5, 6). Conversely, the rate of synthesis of DNA decreases when formation of new protein is inhibited by means of amino acid analogs or antibiotics (7–11). This suggests that, in mammalian systems at least, normal replication of DNA may be dependent upon concurrent synthesis of protein. The studies reported herein pertain to this final point.

MATERIALS AND METHODS

Cell line and incubation techniques. HeLa cells were obtained from Microbiological Associates and maintained in monolayer culture by weekly subdivision. Eagle's minimal essential medium (12), supplemented with calf serum (10%), penicillin,

and streptomycin, was used for maintenance of cells.

Chemicals. ^{3}H- and ^{14}C-labeled thymidine, uridine, and leucine were obtained from the New England Nuclear Corporation. The following were generously provided: acetoxycycloheximide (Charles Pfizer Inc., New York, New York): pactamycin (Upjohn Co., Kalamazoo, Michigan), and puromycin (Cancer Chemotherapy National Service Center, Bethesda, Maryland).

Isotope incorporation studies. Uptake of tritium-labeled precursors into nucleic acids and protein of HeLa cells was measured by a sequential isotope technique (11). Monolayers of the HeLa cells growing on glass coverslips were first incubated in common in medium containing a carbon-14 precursor (thymidine-2-^{14}C, 0.025 μC/ml, 0.83 μM; uridine-2-^{14}C, 0.016 μC/ml, 0.53 μM; or L-leucine-1-^{14}C, 0.4 μC/ml, 16 μM[1] and 0.42 mM[2]) for 15–30 min at 37°. After a rinse with warmed medium, monolayers were placed in nonradioactive medium for 10–30 min. The monolayers were then removed in groups to medium containing the same chemical precursor previously used, now labeled with tritium (thymidine-[methyl-^{3}H] 0.42 μC/ml, 0.07 and 0.2 μM,[2] ^{3}H-uridine, 0.8 and 0.5 μC/ml,[2] 0.1 and 0.27 μM,[2] or DL-leucine-[4–5-^{3}H], 0.8 μC/ml, 0.2 μM[1] and 0.4 mM[2]). When cell exposure to ^{3}H-uridine was to be extended beyond 30 minutes, unlabeled thymidine and deoxycytidine were added to a concentration of 10 μM. Under these conditions at least 95% of the acid-insoluble radioactivity present in the cells following a 6-hr exposure to ^{3}H-uridine was found in the RNA fraction. Drugs were present in the tritium-containing medium at zero time; puromycin and acetoxycycloheximide were dissolved in medium, pactamycin required prior solubilization with ethanol before addition of medium. After incubation at 37° for varying intervals the monolayers were removed, rinsed with iced saline, fixed with iced 5% TCA, extracted with ethanol (15 min) and ether (10 min) at room temperature and air dried. In studies with leucine, nucleic acids were removed by exposing cells on the coverslips to 5% TCA at 95° for 15 min prior to lipid extraction. The coverslips were fragmented and placed in (glass) liquid scintillation counting jars. Cells were solubilized with Hydroxyide of Hyamine (Packard Instrument Co.) 1 M in methanol and heat (65° for 1–2 hr then 37° overnight) then scintillation solution (diMethyl POPOP, PPO in toluene) was added. Tritium and ^{14}C content of each sample was determined by the method of Kabara *et al.* (13) in a dual-channel liquid scintillation counter (Packard Instrument Corp., La Grange, Illinois). The $^{3}H/^{14}C$ ratio served to quantitate precursor incorporation. Deviation from the control $^{3}H/^{14}C$ ratio seen in samples which had been exposed to a drug was used as a measure of drug effect.

[1] Eagle's medium lacking leucine, supplemented with calf serum to 1%, was used in experiments with labeled leucine when incorporation periods were limited to 30 min.

[2] These concentrations of isotopically labeled precursors were utilized in those incubations that were extended beyond 30 min.

RESULTS

Inhibitory Effects of Acetoxycycloheximide, Puromycin, and Pactamycin: Specificity Relationships in 30-min Incorporation Periods

The author has previously reported (11) that cellular synthesis of protein is more rapidly and completely inhibited by acetoxycycloheximide and puromycin than are the syntheses of RNA and DNA. In the presence of increasing concentrations of acetoxycycloheximide, incorporation of leucine is almost eliminated, while accumulation of uridine in cellular RNA is unaffected. Inhibition of thymidine uptake reaches 60–70% but does not increase further in spite of a 100-fold increase in drug concentration. Puromycin inhibits leucine incorporation in excess of 95% at 10 and 25 μg/ml, while uptake of thymidine is reduced by 50–60%. Effects on uridine incorporation are slight until levels of 100 μg/ml are reached. A similar inhibitory pattern was obtained in short-term exposures with pactamycin (Fig. 1). If

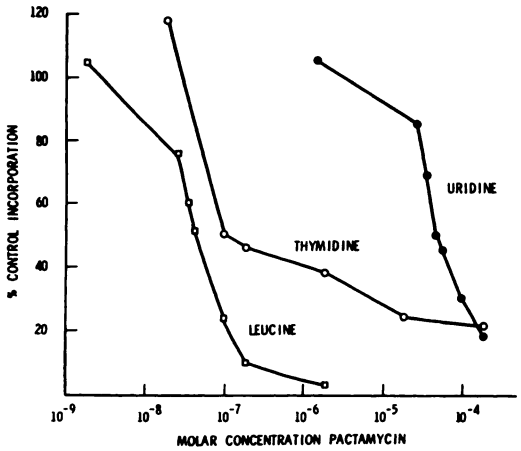

FIG. 1. *Inhibition by pactamycin of incorporation of ^{3}H-leucine, ^{3}H-thymidine, and ^{3}H-uridine into monolayers of HeLa cells. Concentration-effect relationships*

Analytic technique is described under Methods. Percentage control incorporation = (^{3}H/^{14}C experimental)/(^{3}H/^{14}C control) × 100. Drug and tritiated precursor were present at zero time; the incorporation period was 30 min. Each point represents the average of three experiments on triplicate samples.

these inhibitory effects on nucleic acid metabolism occur as a result of the drug-induced arrest in formation of new protein, the extent of inhibition of nucleic acid synthesis should correlate with the severity of impairment in synthesis of protein regardless of which drug produces this impairment. This is the case for the three drugs herein examined. The concentrations of acetoxycycloheximide, puromycin, and pactamycin, which decreased thymidine uptake by 50% (the IC_{50} in Table 1) inhibited incorporation of leucine by 76, 75, and 76%, respectively. Further, when the IC_{50}'s for uridine and thymidine are expressed as multiples of the appropriate IC_{50} for leucine (Table 1), concentration-effect relationships are almost identical when inhibition of incorporation of thymidine and leucine are examined, but are quite dissimilar when inhibition of uptake of uridine and leucine are considered. The above data are compatible with the postulate that inhibition of incorporation of ^{3}H-thymidine by these three antibiotics is a secondary event occasioned by the effect of the antibiotics on the synthesis of protein. Decreased cellular uptake of uridine in the presence of high concentrations of puromycin and pactamycin seems unrelated to drug effects on formation of protein.

Temporal Pattern of Inhibitory Events

Further support for the above postulate was obtained by examining the sequence of inhibitory events at drug concentrations which gave close to maximal inhibition of leucine uptake but had no effect on uridine incorporation within 30 minutes. Within the first hour of drug exposure (Fig. 2) acetoxycycloheximide, pactamycin, and puromycin inhibited uptake of leucine by greater than 95%; the onset of effect extrapolates to less than 2 min. Effects on incorporation of thymidine were not significant at 5 min; by 10 min inhibition was evident in the presence of each of the three drugs. When the observations were extended to 6 hr, each of the three antibiotics inhibited incorporation of leucine in excess of 95% over the entire period (Fig. 3), while no consistent inhibitory effect on uridine uptake was observed (Fig. 4). In-

TABLE 1

Drug concentrations that inhibited precursor incorporation into monolayers of HeLa cells by 50%, (IC_{50})

Thirty-minute incorporation periods were used; drugs and radioactive precursors were present at zero time.

Drug	Radioactive precursor			IC_{50} TdR/ IC_{50} Leu	IC_{50} UR/ IC_{50} Leu
	Thymidine-^{3}H	Uridine-^{3}H	Leucine-^{3}H		
Acetoxycycloheximide	0.1 μM	>0.3 mM	0.03 μM	3.3	>10,000
Pactamycin	0.1 μM	45 μM	0.04 μM	2.5	1,125
Puromycin	13.6 μM	0.21 mM	6.2 μM	2.2	34

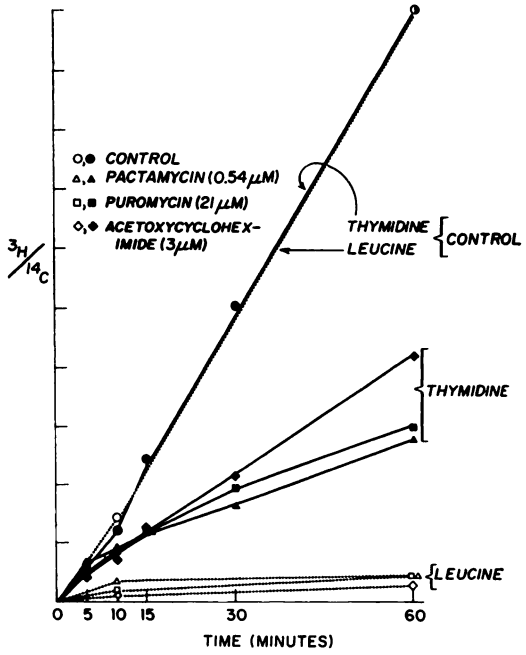

FIG. 2. *Inhibitory effects of pactamycin, puromycin, and acetoxycycloheximide on incorporation of ^{3}H-leucine and ^{3}H-thymidine into monolayers of HeLa cells*

Analytic technique is described under Methods. Drug and tritiated precursor were present at zero time. Each point represents the average of three experiments on duplicate samples. Placement on the ordinate of an individual sample within an experiment was calculated by dividing by the (^{3}H/^{14}C) of the sample by the (^{3}H/^{14}C) of the 60-min control samples.

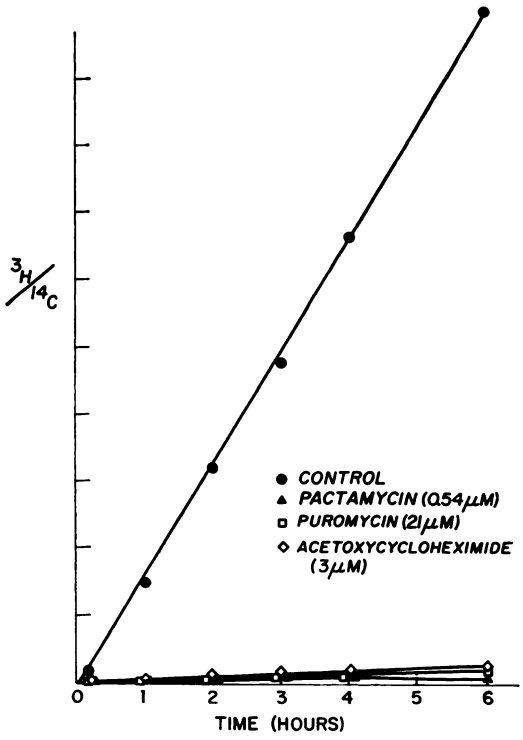

FIG. 3. *Inhibition of incorporation of ^{3}H-leucine into monolayers of HeLa cells by pactamycin, puromycin, and acetoxycycloheximide*

Drug, labeled precursor, time sequence, and graphing conventions as in Fig. 2.

corporation of thymidine continued at a linear albeit inhibited rate for 6 hr in the presence of acetoxycycloheximide. The rate of thymidine uptake decreased further after 4 hr in the presence of puromycin; in studies with pactamycin the plateau effect developed between 2 and 4 hr (Fig. 5).

DISCUSSION

Acetoxycycloheximide and related glutarimide antibiotics inhibit synthesis of protein in yeast (14, 15), and in mammalian cells (10, 11). Puromycin has been widely used as an inhibitor of the synthesis of proteins in bacterial and mammalian systems (16, 17). In cell-free systems, both acetoxycycloheximide and puromycin interfere with the transfer of amino acid from sRNA to polypeptide albeit in somewhat differing fashion (18–20).

Pactamycin is an antibiotic recovered by Bhuyan *et al.* from beers of *Streptomyces pactum* (21). Its chemical structure is yet to be elucidated; however, on the basis of color, solubility, and spectral and chemical characteristics (22) it is unrelated either to puromycin or to the glutarimide group of antibiotics. Bhuyan observed that this antibiotic rapidly inhibited uptake of amino acids into the protein of KB cells in culture; shortly after this event, uptake of thymidine into DNA also decreased (presented at the 16th annual meeting Tissue Culture Association, Miami Beach, Florida, May 1965). The results described in this communication have largely confirmed his observations.

In spite of structural dissimilarities and

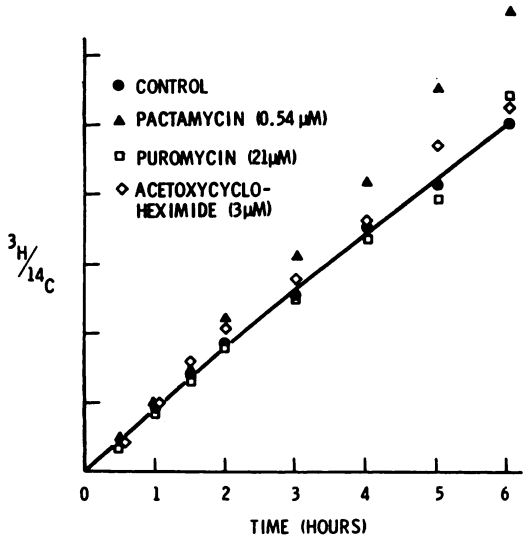

FIG. 4. *Failure of pactamycin, puromycin, and acetoxycycloheximide to inhibit incorporation of ^{3}H-uridine into monolayers of HeLa cells*

Drug, labeled precursor, time sequence, and graphing conventions as in Fig. 2.

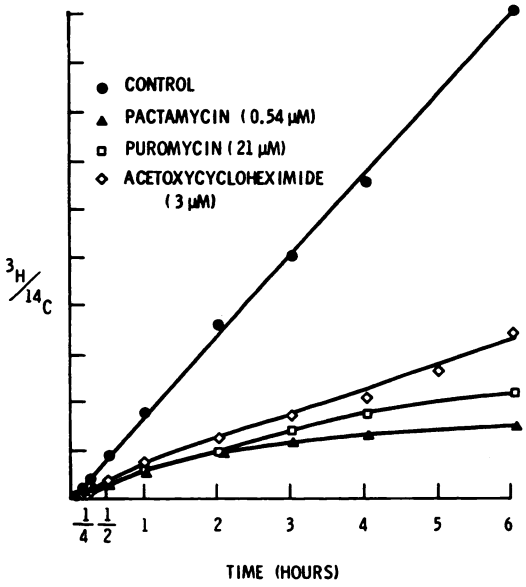

FIG. 5. *Inhibition of incorporation of ^{3}H-thymidine into monolayers of HeLa cells by pactamycin, puromycin, and acetoxycycloheximide*

Drug, labeled precursor, time sequence, and graphing conventions as in Fig. 2.

somewhat differing molecular modes of action, the three antibiotics had similar inhibitory effects on DNA metabolism[3] in HeLa cells. Although inhibition on some step in DNA formation has not been ruled out, puromycin and acetoxycycloheximide did not inhibit synthesis of DNA by cell-free extracts (9, 10); these studies excluded direct drug effect upon deoxynucleoside and deoxynucleotide phosphorylation and upon the polymerization step in DNA synthesis. The data presented can be reasonably interpreted by suggesting that concurrent protein synthesis is necessary to maintain normal rates of DNA formation in a non-synchronous population of cells in log-phase of growth.

Cessation of protein synthesis might interfere with replication of DNA through multiple mechanisms. The biologic decay of critical proteins comes readily to mind. Such proteins might include enzymes which control deoxyribonucleotide concentrations, or alter the conformation of DNA prior to replication, or proteins which stabilize newly polymerized DNA against degradation by nucleases. Other possibilities include a rise in the level of aminoacyl-sRNA or an alteration in lipoprotein metabolism. The available data do not permit a choice between these possible mechanisms, but some further comment seems reasonable.

The kinetics of acetoxycycloheximide inhibition do not fit with those that would be predicted from the biologic decay of a single critical enzyme controlling precursor concentrations. The experimentally observed initial decline in synthesis of DNA should be followed by cessation of thymidine uptake by the end of an hour, rather than by an inhibited but linear rate from 30 min to 6 hr following drug exposure.

[3] It is assumed that the decrease in incorporation of labeled thymidine induced by these drugs represents a disturbance in the biosynthesis of DNA rather than some isolated drug-induced alteration in thymidine metabolism. This seems a reasonable assumption in view of the observations by Bennett *et al.* (10) that cycloheximide inhibits incorporation not only of thymidine, but also of orotate, uridine, deoxycytidine, and adenine into DNA of HEp 2 cells.

Certainly, the extractable levels of DNA polymerase and of kinases of deoxythymidylate pathway do not decline during 6 hr of exposure to puromycin or cycloheximide (9, 10, 23).

Any proposed mechanism must account for inhibition of synthesis of DNA in those cells where replication was in process at the time of drug exposure. Under the experimental conditions herein employed this HeLa line has a doubling time of 24–27 hr with a period of DNA synthesis of 10–12 hours. The observed decrease in thymidine uptake, occurring within 10 min following drug addition, cannot be due to failure of new cells to initiate synthesis of DNA. However, if replicating subunits within chromosomes are individually activated by a process requiring protein synthesis, the failure of new units to activate (when synthesis of proteins ceased) might be detectable within 10–15 min.

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