Specificity of the Antiviral Agent Calcium Elenolate

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Calcium elenolate, an antiviral agent which inhibits reverse transcriptases, inhibits the growth of chicken embryo fibroblast cells, as well as *Escherichia coli* and *Bacillus subtilis* strains. The drug in vitro inhibits *E. coli* deoxyribonucleic acid (DNA) polymerase II and DNA polymerase III holoenzyme, as well as several unrelated enzymes. The usual DNA polymerase assay components, with the exception of spermidine, have no effect on the observed inhibition. Inhibition of DNA polymerase II by the drug appears to be due to a direct and irreversible effect on the enzyme. However, DNA synthesis in *E. coli* is no more susceptible to the drug than is the increase in cell mass. These results suggest that calcium elenolate is an inhibitor of rather low specificity.

Calcium elenolate, a monoterpene which is isolated from aqueous extracts of the olive plant (*Olea europaea*) after mild acid hydrolysis (1, 14), has been shown to be virucidal in vitro for a number of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) viruses (15). It also reduces the yields of virus from hamsters infected with parainfluenza 3 virus (16) and has minimal toxicity when administered to animals (2). Calcium elenolate inhibits the RNA-dependent DNA polymerases (reverse transcriptases) of both Moloney and Rauscher leukemia viruses, 50% inhibition occurring at calcium elenolate concentrations of 20 $\mu\text{g/ml}$ (75 μM) (5). The drug does not appear to react with nucleosides or with DNA or RNA (5, 15). It has been suggested that the drug acts directly against the reverse transcriptases (5). The drug can be inactivated by prior incubation with 2-M equivalents of the amino acids glycine, lysine, cysteine, or histidine, but not with other amino acids (15). Inactivation requires the presence of a free amino group. However, the drug does not inhibit *Escherichia coli* RNA polymerase or DNA polymerase I at drug concentrations as high as 200 $\mu\text{g/ml}$ (5). Recently the structure of elenolic acid (10) and the total synthesis of DL-methyl elenolate have been reported (6).

We decided to determine if the drug inhibited *E. coli* DNA polymerase II or III since reverse transcriptases resemble these enzymes in a number of properties (7). A specific inhibitor of either *E. coli* enzyme might prove useful in elucidating the physiological roles of these enzymes in the cell. However, we report that both enzymes are inhibited to a similar extent and, moreover, further studies indicate that the drug is rather nonspecific.

MATERIALS AND METHODS

Materials. Tissue culture dishes were from Falcon Plastics. Powdered Dulbecco modified Eagle medium, tryptose phosphate broth, calf serum, and chicken serum were obtained from Grand Island Biochemical Co. [^3H]thymine, 7.0 Ci/mmol, was purchased from Schwarz/Mann. [^3H]deoxythymidine 5'-triphosphate, 50 Ci/mmol, was obtained from New England Nuclear. Calf thymus DNA (type V) was obtained from Sigma and digested with pancreatic deoxyribonuclease (DNase) I (Worthington, EC 3.1.4.5) to give maximal DNA polymerase III activity (18). Calcium elenolate, lot 9426-JHF-2, was the generous gift of the Upjohn Company, Kalamazoo, Mich. When dissolved in water at 1 to 10 mg/ml and stored frozen, the drug appeared stable for at least 2 months with repeated freezing and thawing.

Growth of bacteria. The *E. coli* strains W3110 (*thyA36* and *dra-2*) and D110 (*polA1*, *endA*⁻, *thyA36* and *dra-2*) were obtained from the *E. coli* Genetic Stock Center, New Haven, Conn. The *Bacillus subtilis* prototrophic strain WB746, isolated by Eugene Nester, was obtained from Samuel Kaplan. Bacteria were grown in M9 minimal medium (4), supplemented with 0.2% glucose, 1 mM MgSO_4 , and 4 μg of thymine per ml. Medium for plating was solidified with 1.5% agar (Difco). Cell mass was determined by optical density at 450 nm in a Zeiss PMQII spectrophotometer. Total cell numbers were determined by counting 200 to 400 cells in a Petroff-Hauser chamber. The number of viable cells was determined by plating on M9 agar after appropriate dilutions. DNA synthesis was measured by the incorporation of [^3H]thymine into acid-insoluble counts (4).

Growth of chicken embryo fibroblasts. Primary chicken embryo fibroblasts were prepared from 10-day-old chicken embryos as previously described (17). After 4 to 5 days of growth at 38 C, the cells were removed from the tissue culture plates with 0.05% trypsin in tris(hydroxymethyl)aminomethane (Tris)-

hydrochloride plus saline (25 mM Tris-hydrochloride [pH 7.3], 140 mM NaCl, 5 mM KCl, and 0.7 mM Na_2HPO_4) and plated at 10^5 cells/35-mm tissue culture plate. Cells were grown at 41 C in Dulbecco medium containing 10% tryptose phosphate broth, 4% calf serum, and 1% heat-inactivated (56 C for 1 h) chicken serum. All experiments were begun 24 h after plating. Growth rates were determined by measuring cell number with a Coulter particle counter after removing cells from the plates with 0.25% trypsin in Tris-hydrochloride plus saline plus 0.1% glucose. Uptake of trypan blue, 0.4% in 0.9% saline, was used to estimate cell viability. In several experiments the cells were washed with phosphate-buffered saline, which contained 140 mM NaCl, 5.4 mM Na_2HPO_4 , 5 mM KCl, 1.8 mM CaCl_2 , 1 mM NaH_2PO_4 , 0.8 mM MgSO_4 , and 0.1% glucose, pH 7.3. After washing, cells were grown in conditioned medium, which is that removed from untreated plates after 24 h of cell growth.

Enzyme inhibition studies. Egg white lysozyme (A grade, EC 2.3.1.17) was obtained from Sigma. Micrococcal nuclease (NFCP, EC 3.1.4.7) and pancreatic DNase I (DPFF, EC 3.1.4.5) were obtained from Worthington. These enzymes at 40 to 50 $\mu\text{g}/\text{ml}$ were exposed to calcium elenolate for 30 min at 37 C in 5 mM Tris-hydrochloride, pH 8.0, at 37 C, and then assayed as described by the supplier. *E. coli* DNA polymerase II was purified by the method of Kornberg and Gefter (9), followed by dialysis, phosphocellulose chromatography, and Sephadex G-150 gel filtration (8). The enzyme had a final specific activity of 125 U/mg and was stored as recommended by Otto et al. (13). The standard assay for DNA polymerase II contains (0.3-ml volume): 33 mM Tris-acetate buffer (pH 7.2 at 30 C), 50 mM KCl, 16.7 mM dithiothreitol, 13.3 mM MgCl_2 , 280 μM (as nucleotide) DNase-digested calf thymus DNA, 33 μM (each) deoxyadenosine 5'-triphosphate, deoxycytosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, and [^3H]deoxythymidine 5'-triphosphate (150 counts/min per pmol), about 0.7 μg of enzyme (estimated by assuming a preparation of specific activity 270 U/mg is substantially pure [8]) and indicated concentrations of calcium elenolate. Incubations were for 30 min at 37 C; nucleotide incorporation into acid-insoluble product was measured as previously described (11). Crude extracts of DNA polymerase III holoenzyme (fraction 1) were prepared as described by Wickner and Kornberg (19) from D110 grown in M9, supplemented with 1% glucose and 0.2% Casamino Acids (Difco), to $3.5 \times 10^8/\text{ml}$. The standard assay for DNA polymerase III holoenzyme contains (0.3-ml volume): 33 mM morpholinopropane sulfonate-KOH buffer (pH 7.0), 16.7 mM dithiothreitol, 6.7 mM MgCl_2 , 5 mM NaCl, 1.4 mM (as nucleotide) DNase-digested calf thymus DNA, 1.3 mM adenosine 5'-triphosphate, 0.15 mg of bulk *E. coli* phospholipids per ml (19), 133 μM (each) deoxyadenosine 5'-triphosphate, deoxycytosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, and [^3H]deoxythymidine 5'-triphosphate (150 counts/min per pmol), about 0.01 μg of enzyme (estimated by assuming a preparation of specific activity 550 U/mg is substantially pure [19]),

and indicated concentrations of calcium elenolate. Incubations were for 5 min at 30 C; nucleotide incorporation into acid-soluble product was measured as previously described (11). DNA polymerase II activity is not detectable in crude extracts in this assay (9).

RESULTS

Spectrum of enzyme inhibition. As shown in Fig. 1, calcium elenolate inhibited both *E. coli* DNA polymerase II and DNA polymerase III holoenzyme. Consistent with the previously published report (5), control experiments (data not shown) have indicated that the inhibition of DNA polymerase II by calcium elenolate is not influenced by any of the standard DNA polymerase II or DNA polymerase III holoenzyme assay components except spermidine, which therefore was not used in the assay of DNA polymerase III holoenzyme activity in extracts. Thus the apparent differences in the susceptibility of the two enzymes cannot be explained by the differences in the enzyme assays.

Inhibition of DNA polymerase II activity by calcium elenolate could not be overcome by increasing the concentration of DNA, deoxyribonucleoside triphosphates, or magnesium, but could be overcome by increasing the concentration of enzyme (Table 1). Thus inhibition appears to be directed against the enzyme itself. Furthermore, the inhibition of DNA polymerase II by calcium elenolate appeared to be irreversible (Table 1). DNA polymerase II was exposed to 500 μg of calcium elenolate per ml for 5 min at 37 C and then diluted 30-fold into the standard assay mixture, which contained no cal-

FIG. 1. Inhibition of several enzymes by calcium elenolate. The enzymatic activity observed in the absence of the drug is taken as 100%. Symbols: Δ , micrococcal nuclease; $\circ$, egg white lysozyme; $\bullet$, *E. coli* DNA polymerase II; $\square$, *E. coli* DNA polymerase III holoenzyme; $\blacktriangle$, pancreatic DNase I.

TABLE 1. Factors which influence the inhibition of DNA polymerase II by calcium elenolate^a

Exp conditions	Observed inhibition (%)
Standard assay conditions	42
Increasing DNA concentration fivefold to 1.4 mM	40
Increasing deoxyribonucleoside triphosphate concentrations fourfold to 133 μ M each	42
Decreasing magnesium concentration fourfold to 3.3 mM	39
Increasing enzyme concentration fourfold	27
Increasing enzyme concentration 10-fold	18
Exposure of enzyme to 500 μ g of calcium elenolate per ml for 5 min at 37 C before diluting 30-fold into standard assay containing no calcium elenolate	66

^a The standard assay of DNA polymerase II was modified as indicated and the inhibition of activity by 200 μ g of calcium elenolate per ml was determined.

cium elenolate. The final concentration of calcium elenolate in the assay mix was thus 17 μ g/ml, a concentration that gave negligible inhibition of DNA polymerase II (Fig. 1). However, in this experiment, DNA polymerase II was strongly inhibited compared to a control exposed to water, indicating that the effects of exposure to 500 μ g of calcium elenolate per ml were irreversible.

The effect of calcium elenolate on the activity of several other enzymes is also shown in Fig. 1. Although pancreatic DNase I was only slightly inhibited at high drug concentrations, lysozyme and micrococcal nuclease both were more susceptible to inhibition by calcium elenolate than DNA polymerase II. Indeed micrococcal nuclease, which was 50% inhibited at 2 μ g/ml (7.5 μ M), was 10-fold more susceptible to the drug than are viral reverse transcriptases (5).

In vivo target of inhibition in *E. coli*. Since DNA polymerase III, which is essential for DNA replication in *E. coli* (3, 12), was inhibited by calcium elenolate, we wanted to determine if DNA synthesis in *E. coli* was preferentially inhibited by calcium elenolate in vivo. Preliminary experiments (data not shown) had established that the growth of *B. subtilis* WB746, as well as *E. coli* W3110, was inhibited by calcium elenolate. Thus we examined the effects of calcium elenolate on an exponentially growing culture of *E. coli* (Fig. 2). Figure 2A is the control and indicates that the cells were in balanced exponential growth as indicated by the parallel increase in all parameters.

Figure 2B shows the effects of 200 μ g (0.75 mM) of calcium elenolate per ml. The drug appeared bacteriostatic at this concentration since all parameters changed in parallel from a 60-min doubling time to a 75-min doubling time after 20 min of exposure to the drug. Note that even at a drug concentration which had only a small effect on the growth of this strain, DNA synthesis did not appear to be more susceptible to inhibition than was the increase in cell mass.

Figure 2C shows the effects of 500 μ g (1.88 mM) of calcium elenolate per ml. DNA synthesis, cell division, and cell growth ceased within 20 min of exposure to the drug. The drug appeared to be highly bactericidal at this concentration since the viable cell count dropped dramatically after the addition of the drug.

Effects of calcium elenolate on chicken embryo fibroblasts. It had been reported that calcium elenolate at a concentration of 200 μ g/ml is nontoxic to mouse embryo fibroblast cells in culture (5). We have shown that the drug at this concentration slows the growth rate of *E. coli* and inhibits a number of enzymes in vitro. Since calcium elenolate can be inactivated by amino acids (15), which are present in tissue culture medium, we have examined the effects of the drug on chicken embryo fibroblast cells in culture under several experimental conditions (Table 2). When the cells were washed with phosphate-buffered saline to remove amino acids in the medium and then exposed to calcium elenolate in phosphate-buffered saline

FIG. 2. Effects of calcium elenolate on an exponentially growing culture of *E. coli* W3110 at 37 C in M9 medium. At the time indicated by the arrow, [³H]thymine was added to 1 μ Ci/ml, and the culture was divided into three portions: (A) control; (B) 200 μ g of calcium elenolate added per ml; and (C) 500 μ g of calcium elenolate added per ml.

for 30 min, a drug concentration of 500 $\mu\text{g/ml}$ appeared to be toxic whereas 200 $\mu\text{g/ml}$ dramatically slowed the rate of growth. A drug concentration of 50 $\mu\text{g/ml}$ had no effect on the growth rate. However, as shown in Table 2, the effects of calcium elenolate on chicken embryo fibroblast cells depended strongly on the medium and the length of exposure to the drug. Even under the mildest conditions employed (30-min exposure in growth medium), the growth rate was still slightly inhibited at 200 μg of calcium elenolate per ml.

DISCUSSION

These studies with calcium elenolate have shown that the drug inhibits the growth of *E. coli* and *B. subtilis* strains. The drug inhibits *E. coli* DNA polymerase II and DNA polymerase III holoenzyme in vitro. The small difference in apparent susceptibility of the enzymes cannot be explained by differences in the assay conditions of the enzyme, but may reflect differences in the purity of the enzyme preparations since the drug appears to react directly and irreversibly with DNA polymerase II and inhibits several unrelated enzymes. Calcium elenolate does not appear to function as a sulfhydryl-blocking agent (5, 15), but is inactivated by incubation with amino acids (15) and spermidine. The drug appears to react with free amino groups (15). Although DNA polymerases II and III are inhibited by calcium elenolate in vitro, DNA synthe-

sis in *E. coli* is no more susceptible to the drug than is the increase in cell mass. These observations suggest that the primary effect of calcium elenolate in *E. coli* is inhibition of growth, probably by covalent reaction with free amino acids and proteins.

Calcium elenolate also affects the rate of growth of chicken embryo fibroblast cells in culture, the extent of the effect depending on the drug concentration and the experimental conditions. Our results suggest that care must be exercised when the antiviral effects (5, 15) of calcium elenolate are examined, since not only are a number of enzymes likely to be inhibited, but also the tissue culture cells themselves may be affected by the drug, depending on its concentration and the experimental protocol.

Calcium elenolate seems to be a drug of rather low specificity. Since it apparently reacts only with the amino group of certain amino acids (15), it seems reasonable to postulate that the drug inhibits the various enzymes by reacting with the ϵ -amino group of exposed lysine residues and the exposed N-terminal amino group of polypeptide chains. The variation in the susceptibility of enzymes (and cells) may reflect the number of such residues that are exposed to the drug and the effect of their modification on enzymatic activity. Consistent with this model is the observation that bovine serum albumin is required at bovine serum albumin to drug molar ratios of at least 2 to 1 to prevent inhibition of micrococcal nuclease by 2 μg of calcium elenolate per ml (data not shown). Thus the drug appears to react with bovine serum albumin, contrary to published results (5), but only at a small number of residues per protein molecule.

Although these results suggest that calcium elenolate has rather low specificity, the drug may still prove useful as a probe of cellular and viral surfaces and of protein structure.

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TABLE 2. *Effects of calcium elenolate on the growth of chicken embryo fibroblast cells*

Exp	Calcium elenolate (g/ml)	Ratio of doubling times (experimental culture/control culture)
1 ^a	50	1.00
	200	3.67
	500	— ^b
2 ^c	200	1.14
	500	1.21
3 ^d	200	1.71
	500	4.00

^a Cells washed twice with phosphate-buffered saline (PBS), exposed to the drug in PBS for 30 min, then washed twice with PBS before conditioned growth medium added.

^b The cell number decreased slightly over a 5-day period. Twelve hours after exposure to the drug, greater than 90% of the cells were permeable to trypan blue.

^c Cells exposed to the drug in growth medium for 30 min, then washed once with PBS before conditioned growth medium added.

^d Cells continuously exposed to the drug in growth medium.

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