A Phospholipid Derivative of Cytosine Arabinoside and Its Conversion to Phosphatidylinositol by Animal Tissue

Abstract. *We have synthesized an analog (ara-CDP-DL-dipalmitin) of cytidine diphosphate diglyceride (CDP-diglyceride) in which the antitumor drug, cytosine arabinoside, is substituted for the cytidine moiety. Enzymes in rat and human liver convert this analog to phosphatidylinositol, thereby releasing cytosine arabinoside-5'-monophosphate, an obligatory intermediate in the activation of cytosine arabinoside. Unlike cytidine diphosphate diglyceride, however, ara-CDP-DL-dipalmitin is not an efficient substrate for phosphatidylglycerophosphate synthesis in liver or phosphatidylserine in* Escherichia coli. *The antitumor activity of ara-CDP-DL-dipalmitin in mice bearing L5178Y leukemia is described.*

Cytosine arabinoside (ara-C) is a potent inhibitor of mammalian cell growth and is used extensively in cancer chemotherapy (*1*). In cell culture and in vivo ara-C is phosphorylated (*2*) sequentially to form the 5'-triphosphate and has been shown to be incorporated into RNA and DNA (*3, 4*) and to inhibit DNA synthesis (*4*). Deoxycytidine kinase (E.C. 2.7.1.74) catalyzes the first step in the activation of ara-C by converting it to cytosine arabinoside-5'-monophosphate (ara-CMP); however, the activity of the kinase present in the mammalian tissues may limit the effectiveness of the drug (*2, 5*).

As an approach to this problem, we have synthesized 1-β-D-arabinofuranosylcytosine-5'-diphosphate-DL-1,2-dipalmitin (ara-CDP-DL-dipalmitin, **3**), an ara-C analog of cytidine diphosphate diglyceride (CDP-diglyceride, **1**), a central intermediate in the biogenesis of membrane phosphoglycerides (Fig. 1). In nature, these liponucleotides are donors of phosphatidyl residues in the de novo synthesis of acidic phosphoglycerides (*6*). In eukaryotes CDP-diglyceride is a precursor of phosphatidylinositol, phosphatidylglycerophosphate, and cardiolipin, while in prokaryotes it is converted to phosphatidylserine and phosphatidylglycerophosphate (legend to Fig. 1). All of these reactions proceed with the concomitant release of cytidine 5'-monophosphate (*6*).

We now report that ara-CDP-DL-dipalmitin is an active substrate for the synthesis of phosphatidylinositol in extracts of rat and human liver. This generates ara-CMP as a by-product according to the equation: ara-CDP-DL-dipalmitin + inositol → phosphatidylinositol + ara-CMP. The activity of ara-CDP-DL-dipalmitin as a substrate for several other phosphoglyceride-synthesizing enzymes was examined; it is not readily converted to phosphatidylglycerophosphate by rat liver mitochondria or to phosphatidylserine by extracts of *Escherichia coli*. Antitumor activity of ara-CDP-DL-dipalmitin against leukemic cells L5178Y in mice also has been demonstrated.

Phosphatidic acid containing a natural mixture of fatty acids was prepared by hydrolysis of egg lecithin with phospholipase D (*7*); DL-dipalmitoyl phosphatidic acid was synthesized (chemically) (*8*). These phosphatidic acid preparations then were converted to the respective liponucleotides (**1** to **3**) by reaction with nucleoside 5'-monophosphomorpholidates in anhydrous pyridine (*9*). Phosphatidylinositol synthetase (E.C. 2.7.1.67) was measured by the procedure of Rao and Strickland (*10*) with an inositol concentration of 2 m*M* and a liponucleotide concentration of 0.5 to 1.0 m*M*. Under these conditions, there was almost no incorporation of uniformly labeled ^{14}C inositol into lipid in the absence of added liponucleotide (*10*). Phosphatidylglycerophosphate synthetase (E.C. 2.7.8.5) (of both rat liver and *E. coli*) and phosphatidylserine synthetase (E.C. 2.7.8.8) (of *E. coli*) were assayed as previously described (*11*). Isotopes were purchased from New England Nuclear. Rat liver fractions were prepared as described by Kennedy and co-workers (*12*); these were stored in 0.25*M* sucrose at −20°C prior to use. Cell-free extracts of *E. coli* were prepared by sonic disruption of exponentially growing cells (*11, 13*). The study of long-term survivals of L5178Y leukemic mice was carried out as described (*14*).

Table 1 (experiment 1) shows that various molecular species of liponucleotides are utilized as substrates for the synthesis of phosphatidylinositol in crude homogenates of rat liver. Under the assay conditions employed, there is little difference between CDP-diglyceride (**1**) derived from egg lecithin and synthetic CDP-DL-dipalmitin (**2**). The arabinoside analog (**3**) is only 40 percent less active than the riboliponucleotide (**2**). Omission of liponucleotides from the assay system results in a dramatic reduction of the rate at which uniformly labeled ^{14}C inositol is rendered chloroform-soluble (experiment 1). Washed microsomes are three times more active than crude homogenates (Table 1, experiment 2), in accordance with the known subcellular localization of the phosphatidylinositol synthetase (*6, 10*). CDP-DL-dipalmitin and ara-CDP-DL-dipalmitin are also substrates for phosphatidylinositol synthesis in homogenates of human liver and brain (autopsy specimens), although the specific activities are only 20 to 50 percent of those observed in rat liver (data not shown).

The following experiments were per-

Table 1. Phosphatidylinositol synthesis in rat liver fractions. The conditions were as described in (*10*), except that the concentration of inositol was 2 m*M*. The CDP-diglyceride (egg-derived) was included at 0.5 m*M*, while CDP-DL-dipalmitin and ara-CDP-DL-dipalmitin were employed at 1 m*M*. Conversion of uniformly labeled ^{14}C inositol to lipid was measured either by chloroform extraction (*11*) or trichloroacetic acid precipitation (*13*). Protein was measured by the method of Lowry *et al.* (*11*). The precision of the assay is approximately plus or minus 5 percent, the accuracy plus or minus 10 percent.

Source of phosphatidylinositol synthetase and liponucleotide	Specific activity (nmole min^{-1} mg^{-1} at 37°C)
Experiment 1 (homogenate)	
CDP-diglyceride (**1**)	2.0
CDP-DL-dipalmitin (**2**)	2.3
Ara-CDP-DL-dipalmitin (**3**)	1.3
Omit liponucleotide	0.05
Experiment 2 (microsomes)	
CDP-diglyceride (**1**)	6.4
CDP-DL-dipalmitin (**2**)	7.5
Ara-CDP-DL-dipalmitin (**3**)	4.9

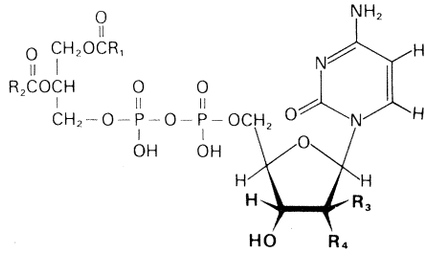

Fig. 1. A composite diagram showing the structures of three liponucleotides. **1**, CDP-diglyceride, R_1 = predominantly saturated fatty chains, R_2 = predominantly unsaturated fatty chains, R_3 = H, R_4 = OH; **2**, CDP-DL-dipalmitin, R_1 and R_2 = $(CH_2)_{14}CH_3$, R_3 = H, R_4 = OH; **3**, ara-CDP-DL-dipalmitin, R_1 and R_2 = $(CH_2)_{14}CH_3$, R_3 = OH, R_4 = H. In animal cells, CDP-diglyceride (**1**) is a phosphatidyl donor for at least three reactions: (i) CDP-diglyceride + inositol → phosphatidylinositol + CMP; (ii) CDP-diglyceride + phosphatidylglycerol → cardiolipin + CMP; and (iii) CDP-diglyceride + L-α-glycero-P → phosphatidylglycerophosphate + CMP. Bacteria generally lack enzymes that catalyze reactions (i) and (ii), but contain those that catalyze (iii). Many bacteria (especially those that are gram negative) also catalyze CDP-diglyceride + serine → phosphatidylserine + CMP (a reaction not found in eukaryotes).

Table 2. Activity of ara-CDP-DL-dipalmitin as a substrate for various phospholipid enzymes. Assay conditions were as described in (*11*), except that the liponucleotide concentrations were doubled.

Enzyme and source	Liponucleotide activity (nmole min^{-1} mg^{-1} at 37°C)	
	CDP-DL-dipalmitin (**2**)	Ara-CDP-DL-dipalmitin (**3**)
Phosphatidylglycerophosphate synthetase (rat liver mitochondria)	0.3	0.01
Phosphatidylserine synthetase (*E. coli*)	14	0.5
Phosphatidylglycerophosphate synthetase (*E. coli*)	1.2	0.6

formed in order to obtain further evidence that ara-CDP-DL-dipalmitin is converted to phosphatidylinositol and ara-CMP. The ^{14}C-labeled, chloroform-soluble compound generated in the presence of ara-CDP-DL-dipalmitin and uniformly labeled ^{14}C inositol was subjected to thin-layer chromatography at 25°C in the solvent chloroform, methanol, and acetic acid (65 : 25 : 10, by volume) on Analtech silica gel GF plates. In this system the ^{14}C-labeled lipid had an R_F value of 0.2, identical to the ^{14}C-labeled material synthesized in the presence of CDP-DL-dipalmitin, and nearly coincident with a preparation of phosphatidylinositol obtained from soybean (Sigma). Water-soluble ara-CMP (released from ara-CDP-DL-dipalmitin in the presence of inositol and rat liver microsomes) was detected by its absorption of ultraviolet light after chromatography on Whatman No. 1 paper in parallel with an authentic standard (Sigma). The solvent system consisted of ethanol with 1*M* ammonium acetate (*p*H 7.4) containing 20 m*M* sodium borate (65 : 35) by volume, and CMP separated (R_F 0.12) from ara-CMP (R_F 0.25). Ara-CMP was not rapidly degraded by the 5′-nucleotidases which contaminate the microsomal fraction (Table 1) and which may interfere with the identification of CMP (*15*).

More than 30 percent of the ara-CDP-DL-dipalmitin used in Table 1 (experiment 2) could be converted to phosphatidylinositol in the presence of inositol and microsomes. There was relatively little hydrolysis of the liponucleotides (*16*) in the absence of inositol under the conditions of Table 1. In contrast to CDP-DL-dipalmitin (and CDP-diglyceride derived from egg lecithin), ara-CDP-DL-dipalmitin was not rapidly converted to phosphatidylglycerophosphate by rat liver mitochondria or to phosphatidylserine by extracts of *E. coli* (Table 2). However, ara-CDP-DL-dipalmitin was a substrate for the phosphatidylglycerophosphate synthetase of *E. coli* which differs from the mammalian enzyme in this regard (Table 2).

In long-term animal studies of L5178Y leukemia bearing mice given ara-CDP-DL-dipalmitin at doses of 20, 40, and 50 mg/kg (injected intraperitoneally) lifespans were increased by 24, 32, and 37 percent, respectively. The percentages of cells killed in short-term studies, as determined by the dilute agar colony method (*17*) 2 hours after drug (**3**) administration, were 57, 46, and 43, when the drug was administered intraperitoneally at doses of 100, 40, and 20 mg/kg, respectively (*18*). The cytotoxicity and possible metabolic uniqueness of ara-CDP-DL-dipalmitin may serve as a basis for further investigational use of liponucleotide classes of drugs in cancer research. These could include studies of ara-C–resistant human leukemic cell lines associated with kinase deficiency or deaminase activity, and chemotherapy of meningeal leukemia or other central nervous system neoplasia.

In conclusion, the data of Tables 1 and 2 indicate that ara-CDP-DL-dipalmitin is a substrate for some phosphoglyceride-synthesizing enzymes, but not for others. The release of ara-CMP from ara-CDP-DL-dipalmitin during phosphatidylinositol synthesis is independent of kinase activity, a critical event in the activation of ara-C (*1*, *2*, *5*) as well as possibly other clinically used antineoplastic pyrimidine nucleosides (for example, 5-fluorouracil, 5-fluorodeoxyuridine, and 6-azauridine). Also, we have observed that ara-CDP-DL-dipalmitin may actually inhibit phosphatidylserine synthesis in extracts of *E. coli*, when CDP-diglyceride is used as the substrate (data not shown). If this occurs in vivo, then ara-CDP-dipalmitin itself (or other liponucleotide analogs) may alter the composition of phosphoglyceride head-groups present in the membrane. It is known from genetic and nutritional studies that such changes can interfere with cell growth (*19*).

Liponucleotide analogs such as ara-CDP-diglyceride can be conveniently altered by synthetic manipulation of the fatty acid moieties to cause wide variations in physicochemical properties, which critically influence drug absorption, plasma binding, tissue distribution, cell entry, and metabolism. The physicochemical properties of liponucleotides (that is, nature's own lipid-soluble nucleotides) and the metabolic observations reported here suggest that liponucleotides deserve further consideration as models for the design of cytotoxic drugs containing analogs of nucleoside bases or sugars.

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References and Notes

1. W. D. Kremer, *Ann. Intern. Med.* **82**, 684 (1975).
2. M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.* **14**, 333 (1965).
3. ———, *ibid.* **17**, 753 (1968); M. Y. Chu, *ibid.* **20**, 2057 (1971).
4. M. Y. Chu and G. A. Fischer, *ibid.* **11**, 423 (1962); J. T. Furth and S. S. Cohen, *Cancer Res.* **28**, 2061 (1968); F. L. Graham and G. F. Whitemore, *ibid.* **30**, 2627 (1970).
5. C. N. Coleman, D. G. Johns, B. A. Chabner, *Ann. N.Y. Acad. Sci.* **255**, 247 (1975).
6. H. van den Bosch, *Annu. Rev. Biochem.* **43**, 243 (1974); C. R. H. Raetz and E. P. Kennedy, *J. Biol. Chem.* **248**, 1098 (1973); W. Thompson and G. MacDonald, *ibid.* **250**, 6779 (1975); G. Hauser and J. Eichberg, *ibid.*, p. 105.
7. M. Kates and P. S. Sastry, *Methods Enzymol.* **14**, 197 (1969).
8. P. P. M. Bonsen and G. H. De Haas, *Chem. Phys. Lipids* **1**, 100 (1967).
9. J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.* **83**, 649 (1961); S. Roseman, J. J. Distlev, J. G. Moffatt, H. G. Khorana, *ibid.*, p. 659; B. W. Agranoff and W. D. Suomi, *Biochem. Prep.* **10**, 47 (1963); S. P. Srivastava, thesis, University of Rhode Island (1975).
10. R. H. Rao and K. P. Strickland, *Biochim. Biophys. Acta* **348**, 306 (1974).
11. C. R. H. Raetz and E. P. Kennedy, *J. Biol. Chem.* **247**, 2008 (1972); O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *ibid.* **193**, 265 (1951).
12. G. F. Wilgram and E. P. Kennedy, *ibid.* **238**, 2615 (1963); E. A. Dennis and E. P. Kennedy, *J. Lipid Res.* **13**, 263 (1972).
13. C. R. H. Raetz, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2274 (1975).
14. A. F. Ross, K. C. Agarwal, S. H. Chu, R. E. Parks, Jr., *Biochem. Pharmacol.* **22**, 141 (1973).
15. C. R. H. Raetz, unpublished observations.
16. ———, C. B. Hirschberg, W. Dowhan, W. T. Wickner, E. P. Kennedy, *J. Biol. Chem.* **247**, 2245 (1972).
17. M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.* **17**, 753 (1968).

18. M. Y. Chu and J. G. Turcotte, unpublished data.
19. E. Hawrot and E. P. Kennedy, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1112 (1975); C. R. H. Raetz, *J. Biol. Chem.* **251**, 3242 (1976); M. Glaser, K. Ferguson, P. R. Vagelos, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4072 (1974).
20. The assays of Tables 1 and 2 were carried out while C.R.H.R. was a guest worker in the Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases. This investigation was supported in part by grants CA 15898, CA 13943, and CA 15909 awarded by the National Cancer Institute, and by a grant from the University of Wisconsin Graduate School to C.R.H.R. Address reprint requests to J.G.T.

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Estradiol Shortens the Period of Hamster Circadian Rhythms

Abstract. *Continuous administration of estradiol benzoate by means of subcutaneously implanted capsules shortened the free-running circadian period of locomotor activity of blind hamsters (*Mesocricetus auratus*) that had had their ovaries removed. Estradiol also advanced the phase of the wheel running of sighted female hamsters without ovaries that were entrained to a photoperiod with 12 hours of light and 12 of darkness. These results, and findings from hamsters undergoing natural estrous cycles, indicate that endogenous estradiol is involved in the regulation of circadian periodicity.*

The period (τ) of circadian oscillations remains remarkably stable under all but a very few chemical or pharmacological challenges (*1*, *2*). Recent work indicates hitherto unsuspected and important regulatory actions of hormones on vertebrate circadian rhythms (*3*). The experiments described here confirm and extend this relationship; they demonstrate that estradiol shortens the period of female hamster circadian activity rhythms and suggest that endogenously secreted estradiol exerts a significant effect in the regulation of the circadian system.

Female hamsters were housed in standard activity apparatuses with continuous free access to food, water, and an activity wheel. Hamster locomotor rhythms were recorded in the traditional way (*2*). We initially observed that the phase angle difference (ψ), defined as the interval between lights off and the beginning of the active period (*4*), was less negative on days 3 and 4 than on days 1 and 2 of the females' estrous cycles. We have termed this pattern of cyclically earlier activity on days 3 and 4 "scalloping" (*5*). We have since observed scalloping in hamsters housed in 12 hours of light and 12 of darkness (LD 12 : 12) or constant illumination as well as in blind animals (Fig. 1) (*6*); in each case the less negative ψ's occurred on those days of the cycle during which estradiol secretion is greatest (*7*).

We next tested the influence of exogenously administered steroids (*8*) on the phase angle differences of hamsters from which the ovaries had been removed and which were entrained to the LD 12 : 12 cycle (*9*). Estradiol benzoate (EB) implanted in a capsule produced a significant phase advance in wheel running. Implantation of an empty capsule delayed the onset of running (Table 1) (*10*).

To test whether estradiol could affect τ independent of its effects on the perception of light intensity, ovariectomized hamsters were blinded by orbital enucleation and then allowed to assume their free-running periods. Subsequent implantation of empty Silastic capsules did not change the mean circadian period of the six animals tested; progesterone-filled capsules were also without consistent effect. However, each of 11 animals implanted with an EB-filled capsule shortened the period of its circadian activity rhythm (Table 2) (*11*, *12*). Furthermore, five of the animals in which the empty Silastic capsules were originally implanted were subsequently given capsules containing EB, and each of these animals responded by shortening the τ of its activity rhythm (τ = 24.10 ± .06 hours for the control period and 23.95 ± .06 hours during stimulation with EB).

Figure 2 illustrates the record of one animal with an average response to the hormone (τ changed from 24.08 to 23.87 hours). The change in τ was manifested within the first 2 days after the EB implant was in place. For 10 of the 16 animals, τ shortened within 3 days, although the latency to a stable new τ was longer ($\bar{X}$ = 9.2 ± 1.3 days). The magnitude of the change in τ induced by EB decreased with the interval elapsing between blinding and hormone treatment (r = .57, $P < .02$), and reached an asymptote about 60 to 70 days after blinding. This may reflect a decreased

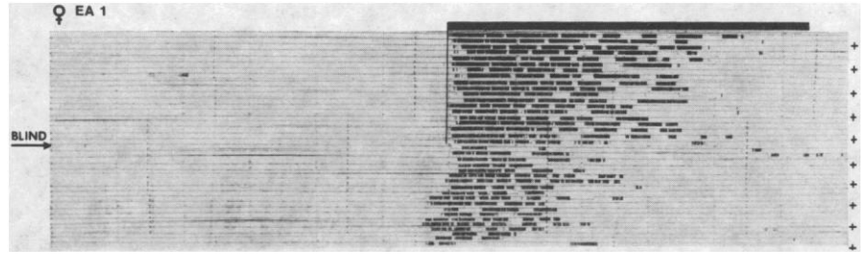

Fig. 1. "Scalloping" of wheel-running activity by a female hamster during exposure to an LD 14 : 10 photoperiod before and after orbital enucleation (arrow). A phase advance occurs on days 3 and 4 of the estrous cycle and is seen particularly clearly in the three estrous cycles that immediately preceded blinding; after blinding, the scalloping continues while the circadian rhythm is free-running. Each + at the right margin indicates detection of the postovulatory vaginal discharge. Each horizontal strip of the record represents 24 hours, with successive days pasted day below preceding day. The 10-hour daily dark period is indicated by the heavy horizontal line at the top of the figure.

Table 1. Influence of hormones on phase angle difference (ψ) of wheel running in hamsters entrained to an LD 12 : 12 cycle. The differences (Δ) between ψ's measured before and during treatment with the hormones are positive to signify a phase advance or earlier activity onset and negative to signify a later onset. The mean phase angles were calculated from the medians of 8 days of data collected before treatment began and the medians of 20 days of treatment data for each animal. Results are expressed as means ± the standard errors of the means.

Treatment	Number of animals	ψ (minutes)	Δ* (minutes)
Estradiol benzoate	14	33.2 ± 2.0	+7.1 ± 2.4
Empty capsule	11	28.9 ± 3.8	−2.8 ± 1.2
Progesterone	8	32.1 ± 4.2	−5.2 ± 2.5

*Within-group changes in Δ were significant for the EB ($P < .02$) and empty-capsule ($P < .05$) treatments, (two-tailed t-test) and were not significant ($P < .10$) for the progesterone treatment. In a between-groups comparison, EB changes differed significantly from those of the other two treatments ($P < .02$, each comparison); differences between progesterone and empty-capsule treatments were not significant.