Pergamon

Biochemical Pharmacology, Vol. 49, No. 9, pp. 1291–1302, 1995.
Elsevier Science Ltd
Printed in Great Britain.
0006-2952/95 $9.50 + 0.00

0006-2952(95)00052-6

INTRACELLULAR METABOLISM OF 2′,3′-DIDEOXYNUCLEOSIDES IN DUCK HEPATOCYTE PRIMARY CULTURES

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(*Received* 28 *April* 1994; *accepted* 21 *November* 1994)

Abstract—The intracellular fate of the potent duck hepatitis B virus (DHBV) inhibitor 2,6-diaminopurine 2′,3′-dideoxyriboside (ddDAPR), its deamination product 2′,3′-dideoxyguanosine (ddG), and the less effective DHBV-inhibitor 2′,3′-dideoxycytidine (ddC) was investigated in duck hepatocyte primary cultures. After a 1-min exposure of [^{3}H]ddDAPR to duck blood, 95% of the compound was converted to ddG. Similarly, [^{3}H]ddDAPR was converted rapidly to ddG in duck hepatocyte primary cultures, with ddG exhibiting resistance to further catabolism. The major pathway of ddG utilization in these cells was phosphorylation, yielding a concentration of 2.1 and 1.9 μM total ddG nucleotides after 5 and 26 hr, respectively, of exposure to 4 μM ddG. Removal of exogenous ddG led to a rapid ($T_{1/2}$ = 1.6 hr) decrease in the total intracellular ddG nucleotide pools. Duck hepatocytes treated with 4 μM ddC exhibited a time-dependent accumulation of ddC nucleotides, culminating in a maximum intracellular total ddC nucleotide concentration of 1.4 μM after 24–26 hr. The intracellular total ddC nucleotide level decreased with a $T_{1/2}$ of 4.4 hr following the removal of exogenous ddC. The formation of ddC nucleotides was reduced in the presence of excess 2′-dideoxycytidine implicating deoxycytidine kinase in the initial step of ddC phosphorylation. A 25-fold excess of 2′-deoxycytidine had no effect on ddG phosphorylation in duck hepatocytes. However, a 92% inhibition of ddG nucleotide formation occurred in duck hepatocytes treated for 5 hr with 4 μM [^{3}H]ddG + 100 μM adenosine in the presence of the adenosine deaminase inhibitor 2′-deoxycoformycin, suggesting that, in these cells, adenosine kinase is involved in the ddG phosphorylation process.

Key words: dideoxynucleosides; 2′,3′-dideoxyguanosine; 2′,3′-dideoxycytidine; nucleoside analog metabolism; antiviral agents; duck hepatitis B virus

HBV† infection is a major health problem in many countries of the world. There is no proven effective treatment for most HBV-infected individuals. Transmission of HBV to newborn infants leads to the establishment of chronic HBV infections in greater than 90% of the cases, whereas 90% of adult-acquired HBV infections are resolved eventually, with the establishment of lasting immunity [1]. Such persistent infection provides a reservoir of virus for the maintenance and continued spread of the disease, and persistently infected HBV carriers are at risk for the development of chronic active hepatitis, which can result in cirrhosis of the liver and death. In addition, there is an association between the frequency of chronic HBV carriers and the incidence of primary hepatocellular carcinoma in HBV-infected individuals [2, 3].

Human HBV is a member of the hepadnavirus family, a category of DNA viruses that also includes related hepatitis B viruses found in woodchucks, ground squirrels, and Pekin ducks. The animal hepatitis B viruses are similar to the human HBV in terms of morphology, genomic structure, and replication mechanism [4–9]. The limited host range exhibited by the human virus, as well as difficulty in establishing a cell culture system capable of viral propagation, has necessitated the use of the related animal hepadnaviruses as experimental models.

Potential anti-HBV compounds can be screened *in vivo* [10] or *in vitro* [11–13]. In the latter context, primary cultures of hepatocytes obtained from Pekin ducks infected with DHBV, or a clonal cell line (2.2.15) derived from the HepG2 human hepatoblastoma cell line that had been transfected with a plasmid containing HBV DNA [14] are working models. The duck system is used in the present study.

Antiviral drug development efforts have focused on two types of therapeutic agents for the control and/or elimination of HBV infection: (l) interferon,

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† Abbreviations: HBV, hepatitis B virus; MEM, Eagle's Minimal Essential Medium; NSIV, NuSerum IV; PEG, polyethylene glycol; TBAP, tetrabutylammonium dihydrogen phosphate; Ado, adenosine; ara-A, adenosine arabinoside; 2′-dAdo, 2′-deoxyadenosine; DCF, 2′-deoxycoformycin; 2′-dCyd, 2′-deoxycytidine; 2′-dGuo, 2′-deoxyguanosine; 2′-dIno, 2′-deoxyinosine; ddDAPR, 2,6-diaminopurine 2′3′-dideoxyriboside; ddC, 2′,3′-dideoxycytidine; ddCMP, 2′,3′-dideoxycytidine 5′-triphosphate; ddCDP, 2′,3′-dideoxycytidine; 5′-monophosphate; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; ddG, 2′,3′-dideoxyguanosine; ddGMP, 2′,3′-dideoxyguanosine monophosphate; ddGDP, 2′,3′-dideoxyguanosine 5′-diphosphate; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; Gua, guanine; Guo, guanosine; Hx, hypoxanthine; DHBV, duck hepatitis B virus; and PNP, purine nucleoside phosphorylase.

a naturally occurring substance that can initiate an antiviral response or exert an immunomodulatory effect [15, 16], and (2) nucleoside analogs that function as inhibitors of viral replication [12,17–22]. Clinical studies have investigated the antiviral effectiveness of these two types of compounds, alone or in combinations [17,23,24].

Ara-A and its more water-soluble monophosphate derivative ara-AMP are prominent among the purine nucleosides that have been tested clinically for anti-HBV activity in humans [17,24–26]. However, they have had limited clinical sucess, in part because of their toxic side-effects [24–27]. The sulfur-containing heterocyclic dideoxynucleoside analog 2′,3′dideoxy-3′-thiacytidine (SddC) and its 5-fluoro-substituted counterpart (FTC) have shown promising anti-HBV activity in the 2.2.15 cell test system [21]. In addition, the cytidine-deaminase-resistant (−)-enantiomers of SddC and FTC are reported to be less toxic and more active as HBV-inhibitors than are the enzyme-susceptible (+)-enantiomers [20–22]. Another dideoxynucleoside, ddDAPR, has exhibited promising antiviral activity against DHBV [12,28]. Specifically, upon treatment with ddDAPR, potent inhibition of DHBV replication was found to occur both *in vitro*, in DHBV-infected duck hepatocyte primary cultures, and *in vivo*, in ducks infected with DHBV [12,28].

Metabolism of ddDAPR to an active form within the recipient animal involves endogenous enzymes. For example, ddDAPR is known to serve as a substrate for adenosine deaminase of beef intestine [29]. In the present study we examined ddDAPR metabolism in uninfected duck tissue and observed rapid deamination of ddDAPR to ddG in both duck blood and duck hepatocyte primary cultures. These findings suggest that the antiviral effect of ddDAPR in Pekin ducks following its administration may be the result of subsequent conversion of the drug to ddG. In turn, ddG may be metabolically altered to yield the antivirally active form of the drug. The present study examines the intracellular metabolism of ddG and ddC in duck hepatocyte primary cultures.

MATERIALS AND METHODS

General Supplies. Flow Laboratories (Mississauga, ON) was the source of MEM for suspension cultures, L-15 medium with glutamine, penicillin G, and streptomycin sulfate were obtained from Gibco BRL (Burlington, ON), and the serum supplement NSIV was purchased from Collaborative Research, Inc. (Bedford, MA). TBAP was purchased from the Aldrich Chemical Company, Inc. (Milwaukee, WI). The following supplies were obtained from the Sigma Chemical Co. (St. Louis, MO): EDTA, HEPES, collagenase type IV, hydrocortisone 21-hemi-succinate, insulin, trypsin (Type I from bovine pancreas), adenosine deaminase, potato apyrase, and the nucleosides 2′-dCyd, 2′-dGuo, 2′-dAdo, Guo, and Ado. The nucleoside analogs 2,6-ddDAPR, ddG, and ddC were purchased from Terochem (Edmonton, AB), while ddGTP and ddCTP were purchased from Pharmacia (Canada), Inc. (Baie d'Urfe, PQ). Standard solutions containing the mono-, di- and triphosphate forms of ddG or ddC were prepared by treating the corresponding triphosphate with potato apyrase. DCF was a gift from Dr. A. R. P. Paterson, Department of Pharmacology, University of Alberta, and Dr. C. E. Cass, Department of Biochemistry, University of Alberta. EcoLite liquid scintillation solution was obtained from ICN Biomedicals, Inc. (Irvine, CA) and Centricon filters were from Amicon Canada Ltd. (Oakville, ON).

Radiochemicals. [2′,3′-^{3}H]ddDAPR (45 Ci/mmol) and [2′,3′-^{3}H]ddC (48 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA) and [2′,3′-^{3}H]ddG was prepared by treating [^{3}H]ddDAPR for 15 min at room temperature with adenosine deaminase, followed by Centricon-3 filtration and subsequent purification of the filtrate by HPLC (C-18 column, 10–35% methanol in 30 min, 1 mL/min, with purified ddG and any remaining ddDAPR eluting at 14 and 22 min, respectively). [^{3}H]ddG prepared in the above manner contained 94% of the radiolabel attached to the pentose sugar, while 6% was associated with the guanine moiety. [^{3}H]-Labeled nucleosides were repurified periodically by HPLC using a C-18 column and the appropriate water/methanol gradient. NEN Research Products (Markham, ON) supplied [1,2-^{3}H]PEG, while ^3H_2O (100 mCi/mL) was from ICN Radiochemicals (Costa Mesa, CA).

Experimental animals. Fertilized Pekin duck eggs were obtained from a duck colony maintained at the University of Alberta Farm. Sera from newly hatched ducklings were screened for the presence of DHBV DNA by dot hybridization [28]. The DHBV-infected and uninfected animals were maintained in separate quarters.

In vitro *conversion of ddDAPR to ddG by duck whole blood.* Blood was drawn from ducklings (1-, 16- or 19-day-old) and collected in heparin-containing Vacutainers. The blood was incubated at 42° with [^{3}H]ddDAPR (16–30 cpm/pmol; 50–67 μM). At various times, 100-μL samples were transferred to 2 mL of 90° water and heated for 2 min. The precipitated protein was removed by centrifugation, and the supernatant solution from each sample was filtered through a Centricon-3 filter (centrifuged at 5400 *g* for 3–5 hr, 10°). The filtrate was lyophilized, reconstituted in 100 μL of water, and analyzed by ion-pair reverse-phase HPLC as detailed later.

In vivo *conversion of ddDAPR to ddG in the duck circulation system.* A 4-week old duckling was anesthetized by i.v. administration of sodium pentobarbital (approximately 10 mg/kg body wt) to maintain adequate anesthesia. The animal was injected with 1.25 mL of 0.02 M [^{3}H]ddDAPR (3.68 cpm/pmol; 2 mg ddDAPR/kg body wt) in buffered saline via a cannulated left wing vein. At various times, 1-mL blood samples were withdrawn from a cannulated right wing vein; the blood was transferred to 2 mL of 90° water and boiled for 2 min. The samples were then processed and analyzed by HPLC as described above. The total sample radioactivity at each time point was quantitated by scintillation spectrometry.

Cell cultures. Primary cultures of duck hepatocytes were prepared from 2-week-old DHBV uninfected ducklings using a modified method of Tuttleman *et al.* [30]. The liver of a sodium pentobarbital

anesthetized animal (approximately 0.5 mL/kg body wt; 65 mg/mL) was perfused aseptically with medium entering through an i.v. catheter positioned in the right ventricle of the heart. The perfused fluid exited through an opening in the portal vein. A 200-mL volume of warm MEM containing 0.5 mM EGTA and 20 mM HEPES was pumped (13 mL/min) through the liver. This was followed by another 200 mL of medium supplemented with 0.5 mg/mL collagenase and 2.5 mM $CaCl_2$. The liver was removed and washed in approximately 20 mL of L-15 medium containing 15 mM HEPES, 50 IU/mL penicillin G, 10 μg/mL streptomycin sulfate, 1.2 μg/mL insulin, 1.7 μg/mL glucose, and 1.1×10^{-5} M hydrocortisone 21-hemisuccinate. Henceforth this medium will be referred to as supplemented L-15. The liver tissue was dissociated to a cell suspension by gentle mechanical teasing in two successive 50-mL volumes of supplemented L-15 medium. The cells were filtered through sterile nylon mesh (60-μm mesh size), collected in 2–4 sterile 50-mL polycarbonate centrifuge tubes, and allowed to settle for 20 min. The cells were washed three times with L-15 medium, allowing the cells to settle for 20 min after each wash. The cells were combined in one tube, resuspended to a final volume of 45 mL with supplemented L-15 medium containing 10% NSIV, and the viable cells in the preparation were quantitated using a hemocytometer and the trypan blue dye exclusion method. The cells were diluted in supplemented L-15 medium–10% NSIV to a cell concentration of 5×10^5 cells/mL and 5-mL aliquots of this suspension were pipetted into 60-mm tissue culture dishes (2.5×10^6 cells/dish). The medium was changed following 4–5 hr of incubation in a 42° humidified incubator. Thereafter, the medium was changed every other day.

Metabolism of [2′,3′-^{3}H]dideoxynucleosides in primary cultures of duck hepatocytes. Tritium-labeled ddDAPR, ddG or ddC ($[4\ \mu M]_{final}$; approximately 6×10^6 cpm/dish, 450–900 cpm/pmol) in supplemented L-15 medium was added to 60-mm tissue culture dishes containing a known number of duck hepatocytes (usually 2×10^6–3×10^6 cells) in supplemented L-15 medium with 10% NSIV. The samples were incubated at 42° for various times (0–48 hr). At the appropriate times, the incubation medium was removed and the cells were washed three times with excess PBS, pH 7.4. Cell extracts were prepared as described below.

Preparation of a hot water extract of the duck hepatocyte primary cultures. Normally, nucleotides are extracted from cells with a protein precipitant such as perchloric or trichloroacetic acid. The acid-lability of ddG required the use of alternate extraction conditions. Hot water extraction was the most reproducible alternate procedure tested, but it was inefficient in terms of ATP/ADP values when compared with acid-extraction of nucleotides. Washed cells were lysed and extracted by the addition of 2 mL H_2O, followed by a 2-min incubation in a 90–95° water bath. Maximum deproteinization of each sample was accomplished by filtration of the extract through a Centricon-3 filter (5400 *g* for 3–5 hr, 10°). The ultrafiltered samples were freeze-dried and stored at −20°. Sample recovery throughout the extraction procedure and subsequent HPLC analysis was calculated based on sample volumes.

HPLC analysis of the tissue extracts. Freeze-dried samples were reconstituted in 0.1 mL of filtered water and a known volume (70 – 95 μL) of the sample was analyzed by ion-pair reverse-phase chromatography using a Varian 5000 Liquid Chromatograph and Chromatography Data System 401 with a 3-μm C-18 column (Excalibar or Supelcosil LC-18-T; 15 cm × 4.6 mm i.d.) preceded by a 5-μm Supelcosil LC-18 guard column (2 cm × 4.6 mm i.d.). Separation of nucleosides and nucleotides was accomplished using a gradient mobile phase separation developed by Stocchi *et al.* [31], with minor modifications. The two solvent systems consisted of Buffer A: 0.1 M KH_2PO_4–8 mM TBAP, pH 6, and Buffer B: 100% methanol. The 30-min elution program began with 100% Buffer A for 2.5 min, at which point Buffer B was introduced and linearly increased to 9% by 5 min. This was followed by a linear increase in methanol composition to 18% by 10 min, and finally reaching 30% methanol by 13 min. The program continued with isocratic elution until 25 min, and there the composition was returned to the initial conditions within 30 min. The column was equilibrated with Buffer A for 1 hr before the first run of the day and subsequent runs were separated by a 30-min equilibration period with Buffer A. Sample analysis was performed at room temperature and absorbance was monitored at 254 nm. A flow rate of 1 mL/min was maintained, and during sample analysis eluate fractions (usually 0.25 mL/vial) were collected directly in mini-scintillation vials. Following the addition of 2.5 mL Tritosol [32] or EcoLite liquid scintillation solution, the radioactivity of each fraction was quantified by scintillation spectrometry.

Stability of intracellular 2′,3′-dideoxynucleotides. Duck hepatocyte primary cultures (48-hr cultures) were incubated at 42° with 4 μM [^{3}H]ddG (450–900 cpm/pmol) or [^{3}H]ddC (460–854 cpm/pmol) in supplemented L-15 with 10% NSIV. After a 5-hr incubation the medium was removed, and the cells were washed with PBS and allowed to incubate in drug-free medium. At various times thereafter, representative cell samples were extracted and the extracts were analyzed for nucleotides and their metabolites by HPLC.

Effect of an excess of naturally occurring nucleosides, deoxynucleosides, and nucleobases on ddG nucleotide formation. Duck hepatocyte primary cultures (48-hr cultures) were incubated at 42° for 5 hr in supplemented L-15 medium–10% NSIV containing 4 μM [^{3}H]ddG (450–900 cpm/pmol) with or without a 100 μM concentration of a potential competitor nucleoside: 2′-dCyd, 2′-dGuo, 2′-dAdo, 2′-dIno, Ado, or Guo. The effect of the bases Hx and Gua was also examined, although Gua was utilized at a concentration of 8 μM due to its limited aqueous solubility. The adenosine deaminase inhibitor DCF (1, 10, or 24 μM) was included in the incubation samples containing 100 μM Ado and 100 μM 2′-dAdo, and in the corresponding 4 μM ddG controls. In these samples, the cells were pretreated for 45 min with DCF before the addition

Table 1. Metabolism of ddDAPR in whole blood

Blood	Percent cpm recovered									
	0 Time*		20 Sec		1 Min		10 Min		25 Min	
	ddDAPR	ddG	ddDAPR	ddG	ddDAPR	ddG	ddDAPR	ddG	ddDAPR	ddG
In vitro†	98 ± 1.2	0.5 ± 0.05	33 ± 0.5	66 ± 0.5	3.0 ± 1.9	95 ± 2.5	1.3 ± 0	97 ± 0	ND‡	ND
In vivo§	100	0	ND	ND	1.8	95	ND	ND	1.4	92

* Zero-time samples are defined as follows: *in vitro* sample: a blood sample taken immediately following the addition of [^{3}H]ddDAPR; and *in vivo* sample: the estimated ddDAPR and ddG content of the [^{3}H]ddDAPR solution prior to injection.

† *In vitro* duck blood: Blood samples taken from a 1- or 19-day-old duckling were incubated with 50 μM [^{3}H]ddDAPR (16 cpm/pmol) at 42°. At the indicated times aliquots were removed, hot-water extracted, and the extracts analyzed by HPLC as described in Materials and Methods. Since the blood samples obtained from the 1-day-old and 19-day-old animals yielded identical results, the data reported here represent combined values obtained from the two age group samples. Values reported for the 0-time and 1-min samples are the means ± SD from four samples, whereas values for the 20-sec and 10-min time points are the means (± range) obtained from two determinations.

‡ ND = not determined.

§ *In vivo* duck blood: Blood samples were drawn at the indicated time points from a 4-week-old duckling that had been injected with 6 mg [^{3}H]ddDAPR (3.68 cpm/pmol). The samples were hot-water extracted and the extracts analyzed by HPLC. The data represents one sample per time point.

of the test drugs. In all cases, the cell samples were extracted after 5 hr of incubation and the extracts were analyzed by HPLC.

Determination of intracellular water volume. Trypsin-detached cells (5×10^5) were incubated with either ^3H_2O (to estimate total cell pellet-associated water) or [^{3}H]PEG (to measure extracellular water space) in microfuge tubes containing a 0.15-mL layer of oil (Silicon 550/paraffin oil, density ≥ 1.03 g/mL). The cells were centrifuged through the oil, the supernatant layer was removed, and the tubes were rinsed twice above the oil layer with water. After removal of the oil, the pellets were dissolved in 0.5 mL of 5% Triton X-100, 8 mL of Tritosol was added and the tritium content of the pellets was determined.

RESULTS

Conversion of ddDAPR to ddG by duck blood and hepatocytes. Fresh blood was drawn from ducklings, collected in heparin-containing Vacutainers, and incubated at duck body temperature (42°) *in vitro* with 50 μM [^{3}H]ddDAPR (16 cpm/pmol). At various time points, 0.1-mL aliquots of sample were removed and extracted with hot water, and the extract composition was analyzed by ion-pair reverse-phase HPLC. Similarly, *in vivo* metabolism of [^{3}H]ddDAPR was monitored in 1-mL blood samples drawn at various time points from a 4-week-old duckling that had been injected with 6 mg [^{3}H]ddDAPR (3.68 cpm/pmol). Table 1 illustrates that ddDAPR was converted rapidly to ddG *in vitro* and *in vivo* by duck blood. In both cases, 95% of the ddDAPR was converted to the deamination product ddG within 1 min. HPLC analysis of *in vitro* samples incubated for both 10 and 60 min indicated that there was very little glycosidic bond cleavage of the nucleoside, with <1% of the sample radioactivity eluting with HPLC retention times comparable to that of the free dideoxy-sugar and guanine moieties.

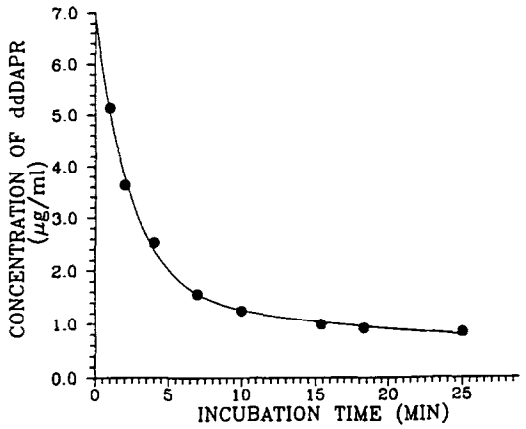

Fig. 1. *In vivo* distribution and elimination of [^{3}H]ddDAPR in the duck. An i.v. injection of [^{3}H]ddDAPR (6.25 mg in 1.25 mL buffered saline; 3.68 cpm/pmol) was administered via the wing vein of a 4-week old duckling. At various times, 1-ml blood samples were withdrawn from a cannulated vein and the blood samples were processed according to the procedure described in Materials and Methods. Values for the blood concentration of radiolabeled product at various times after injection were fitted to the biexponential equation (Equation 1) using Enzfitter, a nonlinear least squares curve-fitting program. The concentration curve illustrated is the resulting computer-generated fit to the data. The results obtained from this animal indicated that $T_{1/2\alpha} = 1.65$ min and $T_{1/2\beta} = 26$ min.

Following i.v. administration of [^{3}H]ddDAPR, the concentration of ddDAPR in duck blood samples declined over time in a biphasic manner (Fig. 1). A rapid initial or α phase of isotope elimination from the blood was followed by a more protracted β phase of elimination. Time-point values for the blood concentration of radiolabeled product were fitted to

the biexponential equation $C_b = Ae^{-\alpha t} + Be^{-\beta t}$ (Equation 1) using Enzfitter, a nonlinear least squares curve-fitting program. In Equation 1, C_b = the concentration of radiolabeled product in the blood at time t, α and β are the fitted rate constants for rapid and slow disposition, respectively, and A and B are the zero-time intercepts associated with the α- and β-phases. The concentration curve illustrated in Fig. 1 is a computer-generated fit to the data using Equation 1. The calculated rate constants yielded elimination half-life values of 1.65 min for the α-phase and 26 min for the β-phase. The biphasic kinetics of ddDAPR elimination from duck blood following an i.v. bolus injection was similar to the elimination kinetics reported for other nucleoside analogs in a variety of species [33–36].

Conversion of ddDAPR to ddG was apparent when 48-hr duck hepatocyte primary cultures were incubated with 4 μM [^{3}H]ddDAPR (407 cpm/pmol). HPLC analysis of cell extracts prepared following a 3-sec exposure of the cells to [^{3}H]ddDAPR in media indicated that significant conversion of ddDAPR to ddG had occurred, that is, 19% of the total counts in the sample were found to elute with the retention time of the ddG standard and 74% of the sample radioactivity remained unchanged from that of the original compound. Similarly, when washed cultures were lysed for several seconds in water containing [^{3}H]ddDAPR, the resulting extract was found to contain 58% ddG and 38% ddDAPR.

Metabolism of ddG and ddDAPR in duck hepatocyte primary cultures. From the above results it is apparent that deamination of ddDAPR occurs in duck blood and hepatocytes. The possibility of further metabolic conversion of the resulting nucleoside analog ddG was investigated *in vitro* using preparations of duck hepatocytes. Hepatocyte primary cultures incubated for 5 hr with 4 μM [^{3}H]-ddG (665 cpm/pmol) were hot-water extracted and the extracts were resolved by HPLC. Analysis of the cell extracts revealed an array of UV_{254} absorbing components (results not shown), while the radioactive content of the sample was confined to four discrete regions of the radiogram (Fig. 2B). The radiolabeled peaks seen in Fig. 2B exhibited elution times identical to those of ddG, ddGMP, ddGDP, and ddGTP standards (Fig. 2A). As can be seen in Fig. 2A, distinct separation of the guanine ribo- and dideoxyribonucleotide standards was achieved by the HPLC methodology employed. In the typical analysis shown in Fig. 2B, the parent nucleoside ddG comprised approximately 56% of the total hot water soluble radioactivity while the [^{3}H]-labeled metabolites corresponding to the mono-, di-, and triphosphates of ddG represented approximately 23, 12, and 3.6%, respectively, of the total radioactive content of the sample. Using data from six experiments, the mean (± SEM) percent of radioactivity recovered as ddG and its metabolites was 59.2 (± 2.8) ddG, 18.5 (± 1.5) ddGMP, 11.7 ± (1.3) ddGDP, and 3.03 ± (0.49) ddGTP, and that of the total dideoxynucleotides (ddGMP + ddGDP + ddGTP) was 33.2 (+2.6). Hepatocytes incubated with 4 μM [^{3}H]ddDAPR-supplemented medium (407 cpm/pmol) for 5 or 26 hr produced radiolabeled products that had the same HPLC retention times

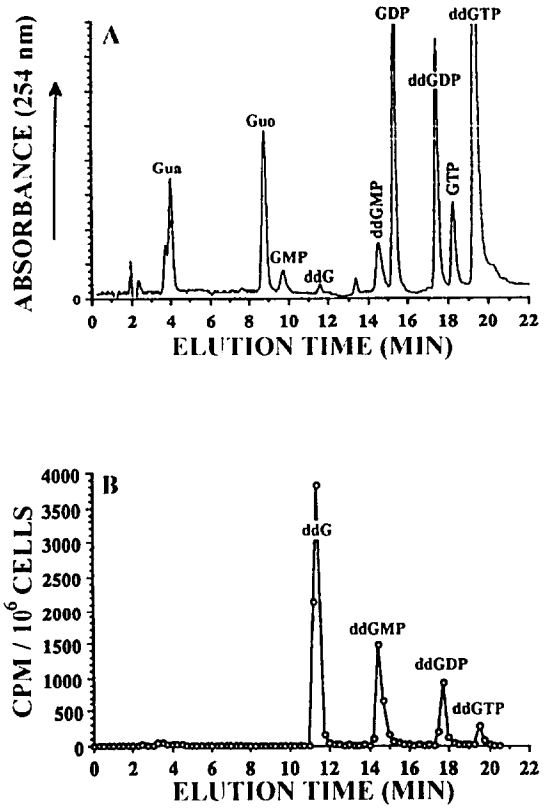

Fig. 2. Ion-pair reverse-phase HPLC analysis of cell extracts from duck hepatocyte primary cultures treated for 5 hr with [^{3}H]ddG. Duck hepatocyte primary cultures (48-hr cultures) were incubated at 42° for 5 hr with 4 μM [^{3}H]ddG (665 cpm/pmol) in supplemented L-15 medium with 10% NSIV. The cells were hot-water extracted, and the extracts were analyzed by HPLC as outlined in Materials and Methods. (A) A separation profile was obtained for the following mixture of standards: Gua, Guo, GMP, ddG, ddGMP, GDP, ddGDP, GTP, and ddGTP. (B) A radiographic profile was obtained from HPLC analysis of the cell extract. Each point in the HPLC elution profile represents an eluate sample collected for 0.25 min (flow rate = 1 ml/min), and the radioactivity in each sample is expressed as cpm/10^6 cells. Identification of the radioactively labeled metabolites as ddGMP, ddGDP, and ddGTP was made on the basis of them having elution times identical to those of ddGMP, ddGDP, and ddGTP standards.

as ddG nucleotide standards (results not shown). Although hot-water extraction of duck hepatocyte primary cultures was the most reproducible alternate nucleotide extraction procedure tested, it was inefficient in terrns of ATP/ADP values when compared with acid-extraction of nucleotides from these cells. Therefore, values representing the total dideoxynucleotide (ddGMP + ddGDP + ddGTP) content of samples have been reported in the results.

Time-dependent metabolism of ddG in duck hepatocyle primary cultures. The intracellular accumulation of dideoxynucleotides in ddG-treated duck hepatocytes was characterized further in terms of time-dependent changes in the dideoxynucleotide pool sizes. Cells treated with 4 μM [^{3}H]ddG

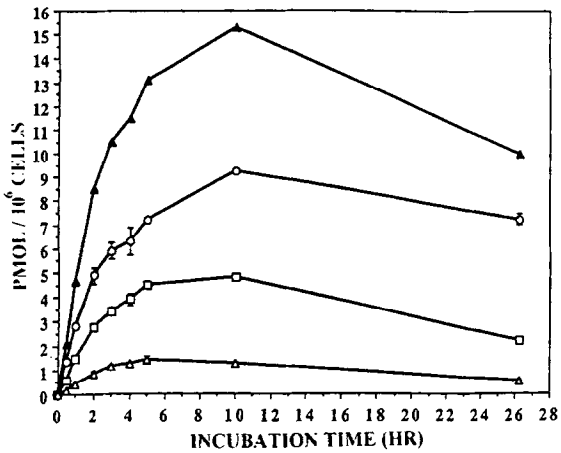

Fig. 3. Time-dependent conversion of ddG to the corresponding phosphorylated metabolites by duck hepatocytes. Duck hepatocyte primary cultures (48-hr cultures) were incubated at 42° for various times with 4 μM [^{3}H]ddG (665 cpm/pmol) in supplemented L-15 medium with 10% NSIV. The cells were extracted and the extracts analyzed by HPLC as outlined in Materials and Methods. The phosphorylated products were resolved by HPLC analysis and were expressed as pmol/10^6 cells. The levels of ddGMP (○), ddGDP (□), and ddGTP (△) as well as the total amount of dideoxynucleotides (▲) (ddGMP + ddGDP + ddGTP) recovered at each time point are indicated. Each time point represents the average of two determinations, and all samples were obtained from the same cell preparation. The mean and the range are shown for each time point. Time course data obtained from other cell preparations indicated the same relative patterns for dideoxynucleotide pool accumulation. However, the absolute amount of product formed, in terms of pmol/10^6 cells, was variable.

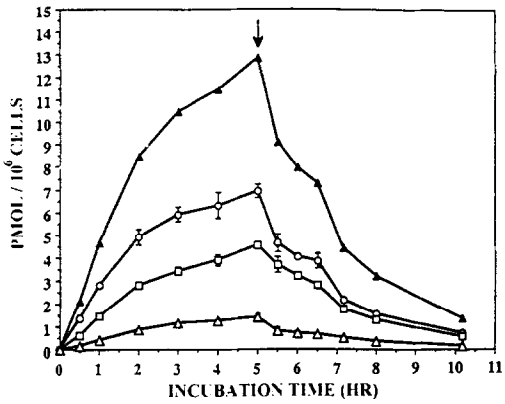

Fig. 4. Intracellular stability of radiolabeled dideoxynucleotide pools in duck hepatocytes treated with [^{3}H]-ddG. Duck hepatocyte primary cultures (48-hr cultures) were incubated at 42° for various time periods with 4 μM [^{3}H]ddG (665 cpm/pmol) in supplemented L-15 medium with 10% NSIV. After 5 hr of incubation, the ddG-containing medium was removed (indicated by arrow) and replaced with drug-free medium. At various times thereafter, the cell samples were extracted and analyzed by HPLC as described in Materials and Methods. The levels of ddGMP (○), ddGDP (□), ddGTP (△), and the sum of the three dideoxynucleotides (▲) recovered at each time point are indicated. The dideoxynucleotide levels are expressed as pmol/10^6 cells. The 5-hr time point indicates the mean value obtained from four determinations, with error bars illustrating the standard deviation. All other points represent duplicate samples with the mean and the range shown. Where not shown, error bars are contained within the time-point symbols. All samples were obtained from the same cell preparation.

(665 cpm/pmol) for various time periods were hot-water extracted and analyzed by HPLC. Under the experimental conditions, phosphorylation of ddG yielded maximal intracellular accumulation of the triphosphate metabolite within 5 hr of incubation with the drug, while maximal pool sizes detected for the mono- and diphosphate forms of the compound were achieved after 5–10 hr incubation with ddG (Fig. 3). Cellular ddG nucleotide concentrations achieved at the 5-hr time point shown in Fig. 3 were calculated to be 1.9 μM ddGMP, 1.2 μM ddGDP, 0.4 μM ddGTP, and 3.4 μM total dideoxynucleotides when a mean cell volume (N = 19 experiments) of 3.8 μL/10^6 cells was used. Analysis of the spent media following a 5-hr incubation revealed an extracellular ddG concentration of 3.5 μM, with 97% of the sample radioactivity recovered as ddG, and all other labeled products representing less than 0.5% of the total radioactivity. Repeated experiments indicated that a 5-hr incubation with 4 μM ddG produced 8.0 pmol/10^6 cells (±3.0 SD; N = 7 experiments) of total ddG nucleotides (ddGMP + ddGDP + ddGTP), or an intracellular ddG nucleotide concentration of 2.1 μM, while the internal ddG concentration at this time point was estimated to be 3.3 μM (±0.8; N = 7). Although the representative experiment shown in Fig. 3 indicates an eventual decline in ddG nucleotide levels from 4.0 μM at 10 hr to 2.6 μM at 26 hr, the dideoxynucleotide pool size differences seen at 26 hr as compared with 5 or 10 hr were insignificant when compared with the reproducibility achieved between experiments. For example, data from multiple experiments indicated that the mean (± SD; N = number of experiments) concentration of total dideoxynucleotides recovered was 2.1 μM (±0.8; N = 7) after 5 hr of incubation and 1.9 μM (±0.6; N = 4) after 26 hr of incubation. The 26-hr internal ddG concentration was estimated to be 5.2 μM (±0.6; N = 4).

Stability of intracellular ddG nucleotides formed in cell cultures. The longevity of the ddG nucleotides generated in duck hepatocyte primary cultures was examined following a 5-hr preincubation of the cells with 4 μM [^{3}H]ddG. The ddG-containing medium was removed, the cells were washed with PBS, and fresh, drug-free medium was added. Removal of external ddG led to a dramatic reduction in the amount of cell-associated ddG nucleotides (Fig. 4). Intracellular dideoxynucleotide pools appeared to decrease with respect to time according to first order kinetics. A semi-logarithmic plot of the data shown in Fig. 4 yielded $T_{1/2}$ values of 1.6, 1.6, 1.7, and 1.6 hr for ddGMP, ddGDP, ddGTP, and total

Table 2. Inhibition of ddG nucleotide formation

Samples Competitor (100 μM)	% of control* ddNTDs‡	Number of:† Experiments	Samples
None	100	3	9
2′-dCyd	103 ± 9	2	5
2′-dGuo	73 ± 9	2	5
2′-dAdo	34 ± 5	3	9
2′-dAdo + DCF§	52 ± 3	1	4
2′-dAdo + 2′-dGuo	26 ± 9	1	3
2′-dIno	67 ± 5	1	4
Guo	72 ± 26	2	5
Ado + DCF§	8 ± 8	2	7
Gua‖	88 ± 3	2	5
Hx	89 ± 8	1	4
DCF§	94 ± 7	1	4

Duck hepatocyte primary cultures were incubated at 42° for 5 hr with 4 μM ddG only (control) or with 4 μM ddG + 100 μM of a potential competitor compound. Cell extracts were prepared and analyzed by HPLC according to the procedure described in Materials and Methods.

* The amount of ddG metabolites recovered in each sample is expressed as a percent of the amount of product recovered in the control (ddG only) samples. In the control samples, 100% represents 2.1 ± 0.8 μM.

† The number of experiments performed for each condition is indicated. Results were obtained from the indicated number of experiments and the mean (± SD) for each condition was determined using the indicated number of samples.

‡ The amount of ddGMP, ddGDP, and ddGTP detected in a sample is reported as a total value (ddNTDs).

§ 1 μM DCF.

‖ 8 μM Gua.

dideoxynucleotides (ddNTDs) respectively. When this experiment was repeated with another preparation of duck hepatocyte primary cultures (not shown), the results indicated that the total intracellular ddG nucleotide pools decreased with a $T_{1/2}$ of 1.3 hr. In the experiment illustrated in Fig. 4, replacing the ddG medium with fresh medium reduced the intracellular ddG concentration from an initial concentration of 3.9 μM to 0.09 μM after 30 min in the fresh medium.

Effect of various nucleosides, deoxynucleosides, and nucleobases on ddG metabolism. Potentially competitive nucleoside substrates were used to provide insight into the enzyme pathways employed in the intracellular phosphorylation of this purine nucleoside analog. Duck hepatocyte primary cultures were incubated at 42° for 5 hr with 4 μM ddG plus 100 μM of a potentially competing nucleoside, deoxynucleoside, or nucleobase substrate. The results from this study are compiled in Table 2, with the amount of ddG nucleotides (ddGMP + ddGDP + ddGTP) recovered in cell extracts expressed as a percent of the control (4 μM ddG without a competitor) value. Analysis of cell extracts revealed that the ddG nucleotide levels achieved when the incubation medium was supplemented with 100 μM 2′-dCyd were no less than the levels attained with ddG alone. As a comparison, duck hepatocyte primary cultures were treated with 4 μM ddC with or without an excess of 2′-dCyd, a substrate for deoxycytidine kinase (Fig. 5). Extracts from the ddC-incubated cells were found to contain mono-, di-, and triphosphate forms of ddC (Fig. 5A). However, the presence of these metabolites was reduced if 100 μM 2′dCyd was included in the medium (Fig. 5B). Although 2′dCyd had no apparent effect on ddG anabolism, a marked decrease in the formation of ddG nucleotides was seen when Ado (+ the adenosine deaminase inhibitor DCF) was the competing substrate. In addition, the Ado + DCF condition was unique in its ability to induce a 5-fold increase in the relative amount of adenine nucleotides detected in the cell extracts (results not shown). A depression in the levels of phosphorylated ddG metabolites was observed when an excess of 2′-dGuo, 2′-dIno, or 2′-dAdo with or without DCF was incorporated into the medium along with ddG (Table 2). Simultaneous exposure of cells to a 100 μM concentration of both 2′-dGuo and 2′-dAdo in addition to 4 μM ddG did not produce an additive effect on the inhibition of ddG phosphorylation. In these experiments, cells treated with 2′-dAdo without DCF showed more inhibition of ddG metabolite formation than cells incubated with 2′-dAdo + DCF. However, the results from cells treated with dIno indicated that the reduction in dideoxynucleotide pools was not as pronounced as with 2′-dAdo. When the less soluble nucleobase guanine was supplied at a concentration twice that of ddG, no discernible inhibition of ddG phosphorylation was evident in the HPLC-analyzed cell extracts. Similarly, 100 μM Hx had no apparent effect on ddG nucleotide pool sizes. The dideoxynucleotide levels attained in cells treated with ddG + 1 μM DCF were no different than the levels achieved with ddG alone (results not shown). Samples tested with 1 μM DCF were also tested with 24 μM DCF, with no detectable difference in the results (results not shown).

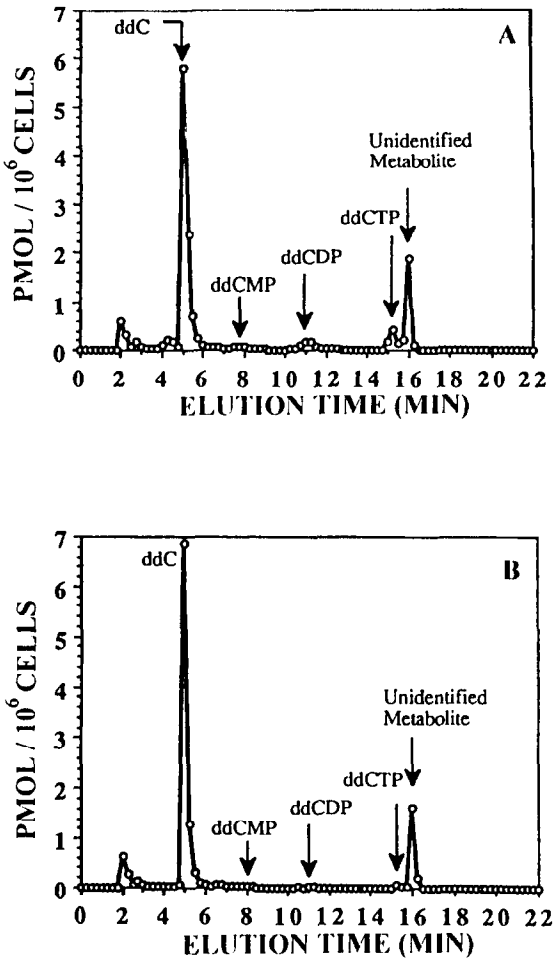

Fig. 5. Inhibition by 2′-deoxycytidine of the formation of ddC metabolites in duck hepatocytes. Duck hepatocyte primary cultures (48-hr cultures) were incubated at 37° in supplemented L-15 medium + 10% NSIV containing: (A) 4 μM [^{3}H]ddC (460 cpm/pmol) or (B) 4 μM [^{3}H]ddC (460 cpm/pmol) + 100 μM 2′-dCyd. After 5 hr of incubation, the cells were extracted and the extracts were analyzed by HPLC as described in Materials and Methods. The levels of ddC and metabolites thereof contained in the cell extracts have been quantitated as pmol/10^6 cells. Arrows indicate the elution times obtained for ddCMP, ddCDP, and ddCTP standards that were run in a separate analysis.

Comparison of ddG versus ddC metabolism in duck hepatocyte primary cultures. In vitro and *in vivo* studies have shown that DHBV is inhibited by ddG but not by ddC [12, 28, 37]. The results presented in this paper indicate that anabolic phosphorylation of ddG occurs in duck hepatocyte primary cultures. As a comparison to ddG and to augment the antiviral data obtained for ddC, the intracellular fate of ddC was explored in duck hepatocytes. Figure 5A illustrates HPLC separation of an extract from duck hepatocytes treated for 5 hr with 4 μM [^{3}H]ddC (460 cpm/pmol). Although the parent nucleoside ddC represented 60% of the total radioactivity recovered in this sample, 1.4, 3.8, and 4.6% of the radioactivity eluted with the same retention times as the standards ddCMP, ddCDP, and ddCTP, respectively. In addition, an unknown metabolite representing 14% of the total radioactivity recovered in the sample had a retention time similar to that of ddCTP. Liponucleotide metabolites of ddC such as ddCDP choline and ddCDP ethanolamine have been identified in extracts of ddC-treated human T-lymphoblasts [38, 39]. However, utilizing the HPLC separation procedures outlined in the current work, a standard of the naturally occurring biochemical cytidine 5′-diphosphocholine (CDP choline) had an elution time of 3.7 min, suggesting that a dideoxy-form of this compound would also elute in the early, rather than late, stages of the separation. Furthermore, unlike the ddC nucleotide products, accumulation of this unidentified compound was not affected by the presence of 100 μM 2′-dCyd in the incubation medium (Fig. 5B). HPLC analysis of the [^{3}H]ddC employed in these experiments indicated that 98% of the total cpm were associated with ddC, while the remaining radioactivity did not elute as discrete peaks. When the pmol/10^6 cells for each of the ddC nucleotide peaks seen in Fig. 5 were averaged from two samples for each treatment condition, the intracellular level of total ddC nucleotides (ddCMP + ddCDP + ddCTP was 1.52 pmol/10^6 cells for the ddC-treated sample (Fig. 5A) and 0.359 pmol/10^6 cells for the ddC plus 2′-dCyd condition (Fig. 5B). Comparable levels of the unknown metabolite accumulated in ddC and ddC plus 2′-dCyd samples. A comparison of the total dideoxynucleotide levels detected in duck hepatocyte primary cultures treated for 5 hr with either 4 μM ddG or ddC indicated that the hot water soluble extracts contained 8.0 pmol/10^6 cells (± 3.0 SD; N = 7 experiments) and 1.5 pmol/10^6 cells (± 0.4 SD; N = 2 experiments), respectively, of the corresponding dideoxynucleotides.

Duck hepatocyte primary cultures treated with 4 μM [^{3}H]ddC for 0–73 hr were analyzed for the intracellular content of ddC and ddC nucleotides. Figure 6 illustrates that a time-dependent intracellular accumulation of ddC occurred, with the internal concentration of ddC reaching the external concentration of 4 μM after approximately 48 hr. The intracellular level of total ddC nucleotides (ddCMP + ddCDP + ddCTP) also increased, reaching a maximum between 24 and 48 hr of incubation with ddC (Fig. 6). Using a mean (N = 19) intracellular water volume of 3.8 μL/10^6 cells and a mean pmol/10^6 cells of total ddC nucleotides calculated using the 24- and 26-hr time points of Fig. 6, the intracellular concentration of total dideoxynucleotides was 1.4 μM. In contrast, the maximum intracellular level of total ddG nucleotides achieved after 5 hr of incubation was 2.1 μM (± 0.8; N = 18), while a concentration of 4.0 μM total ddG nucleotides was detected after 10 hr in the experiment shown in Fig. 3.

When cells treated with ddC for 24 hr were incubated subsequently in drug-free medium, the intracellular dideoxynucleotide pools decreased with respect to time according to first order kinetics

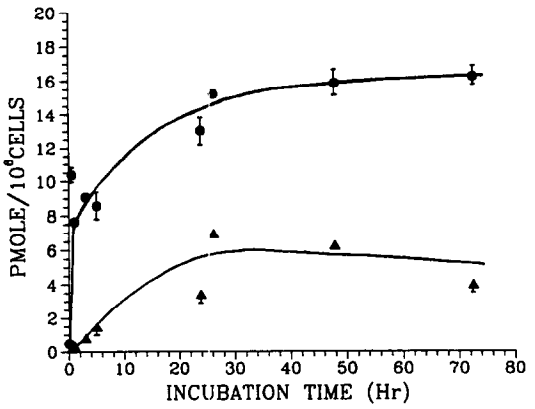

Fig. 6. Time-dependent conversion of ddC to the corresponding phosphorylated metabolites by duck hepatocytes. Duck hepatocyte primary cultures (48-hr cultures) were incubated at 37 or 42° for various times with 4 μM [^{3}H]ddC (460–854 cpm/pmol) in supplemented L-15 medium with 10% NSLV. The cells were extracted and the extracts were analyzed by HPLC as outlined in Materials and Methods. The phosphorylated products were identified by HPLC analysis and are expressed as pmol/10^6 cells. The intracellular levels of ddC (●) and ddC nucleotides (▲) (ddCMPddCDP + ddCTP) recovered at each time point are indicated. The graph represents a conglomerate of results obtained from two experiments. Except for the 5- and 26-hr time points, each data point represents the mean of 2 or 4 replicate samples from one or the other experiment. The 26-hr data were obtained from a single sample and the 5-hr time point represents duplicate samples from each of two experiments. Deviation from the mean (SD when N = 4) is indicated by error bars. The lines drawn were fitted by eye.

(results not shown). A semi-logarithmic plot of the data yielded a $T_{1/2}$ value of 4.4 hr for the disappearance of intracellular ddC nucleotides (ddCMP + ddCDP + ddCTP). Intracellular ddC nucleotide pools decreased at a 2- to 3-fold slower rate than did the ddG nucleotide levels following removal of the exogenous supply of dideoxynucleoside. After 2 hr in ddC-free medium, the internal ddC had been reduced from an initial concentration of 3.4 μM to 0.15 μM. In comparison, 30 min after duck hepatocytes were supplied with fresh medium following a 5-hr incubation with 4 μM ddG, the intracellular ddG level declined from an initial concentration of 3.9 μM to 0.09 μM.

DISCUSSION

This series of experiments was undertaken to identify the metabolic fate of the potent DHBV inhibitors ddDAPR and ddG, as well as the less effective DHBV-inhibitor ddC in the duck model. On the basis of experiments performed with both duck blood and duck hepatocyte primary cultures, it was concluded that ddDAPR acts as a prodrug to ddG in the avian system. Deamination of ddDAPR occurred rapidly in duck blood (*in vivo* and *in vitro*), as well as in duck hepatocyte primary cultures, and the resulting product, ddG, was relatively inert to further catabolism. It has been reported that ddG is a substrate for calf spleen PNP [40]. Resistance of ddG to phosphorolytic cleavage in the avian system may indicate low PNP activity in this tissue. Alternatively, there may be fundamental substrate specificity differences between the bovine and duck enzymes.

Following i.v. administration of [^{3}H]ddDAPR, the isotopic content of duck blood declined in a biphasic manner, with rapid clearance of radioactivity from the blood. The radiolabeled species detected in all samples was ddG rather than the parent compound ddDAPR. Distribution of ddG from the blood to other tissues may occur by passive diffusion along concentration gradients. Uptake of ddG by human lymphoid cells is unaffected by the nucleoside transport inhibitor NBMPR [41,42], indicating that cellular entry of ddG does not depend on the nucleoside transporter in these cells. Biphasic elimination of ddG from duck blood best fits a two-compartment kinetic model [43].

The results presented in this paper suggest that the previously reported *in vivo* and *in vitro* antihepadnaviral effect of ddDAPR in Pekin ducks and duck hepatocyte primary cultures [12, 28] is due to ddG or a metabolite thereof, rather than the parent drug ddDAPR. In the present study, duck hepatocyte primary cultures exhibited a time-dependent conversion of ddG to the corresponding dideoxynucleotides. Intracellular ddG nucleotide pools declined rapidly upon the removal of exogenous ddG, suggesting that these intracellular dideoxynucleotide pools were transient.

Potential hepatocyte enzymes involved in ddG phosphorylation were examined by providing the cells with alternate enzyme substrates in the presence of ddG. In addition to direct substrate competition for an enzyme active site, nucleoside additives and their metabolites could affect ddG metabolism by functioning as cellular enzyme regulators, leading to changes in endogenous nucleotide pools that could indirectly affect ddG phosphorylation. In human lymphoid cells, dCyd kinase is thought to be instrumental in converting dGuo to dGMP [44]. Purified human leukemic T cell dCyd kinase is capable of phosphorylating ddG [45], and deoxynucleoside competition studies in human lymphoid cell lines, as well as ddG metabolism experiments with kinase-deficient lymphoid cells [41], provide further evidence for the involvement of this enzyme in ddG anabolism in lymphoid tissue. However, the inability of excess 2′-dCyd to affect ddG anabolism in duck hepatocyte primary cultures suggested that, in these cells, dCyd kinase does not participate in the initial step of ddG phosphorylation. Evidence for a functional dCyd kinase in duck hepatocyte cultures is provided by the observation that intracellular phosphorylation of ddC was blocked by 2′-dCyd present in a 25-fold excess.

Under conditions in which the hepatocyte adenosine deaminase was blocked by DCF, an excess of adenosine produced almost complete inhibition of ddG nucleotide formation from ddG. In addition, the total adenine nucleotide pool size increased by 5-fold under these conditions. These results could indicate substrate competition at the level of Ado

kinase, an enzyme that can be generalized as having: (1) high activity in liver [46, 47]; (2) a low K_m for Ado [48]; and (3) a high K_m for 2′-dAdo [48]. Although the natural substrate guanosine is not phosphorylated by purified adenosine kinase [48], the guanosine analog ribavirin [49] and the related C-nucleoside tiazofurin [50, 51] are substrates for this enzyme. It is possible that the avian Ado kinase is able to accommodate the dideoxynucleoside analog ddG. In DCF-treated cells, a 50% suppression in ddG phosphorylation was seen in the presence of the Ado kinase substrate 2′-dAdo. This incomplete inhibition could result from a competitive interaction between two low-affinity substrates of Ado kinase. The reported high K_m of 2′-dAdo for purified rabbit liver Ado kinase [48] suggests that a concentration of 100 μM 2′-dAdo could be insufficient to saturate the enzyme, leaving a population of these macromolecules free to interact with alternate substrates. Although the adenine nucleotide pool size did not change in the presence of excess 2′-dAdo (results not shown), an increase in the characteristically low intracellular concentration of deoxyadenosine nucleotides would have gone without detection.

Substrate inhibition results obtained from duck hepatocyte cultures suggest that Ado kinase is the major enzyme involved in the initial step of ddG phosphorylation. Only a small component of the observed ddG phosphorylation was insensitive to excess Ado, while competition with a 25-fold molar excess of 2′-dGuo or 2′-dIno yielded modest inhibition of ddG nucleotide formation. Phosphorylation of ddG by other cellular enzymes could account for these results. In human lymphoid cells, both dCyd kinase and 5′-nucleotides are thought to be responsible for the conversion of ddG to ddGMP [41], while Ado kinase and 5′-nucleotidase phosphorylate the guanosine analog tiazofurin [50]. Preparations of 5′-nucleotidase from human T lymphoblast cells can catalyze the conversion of ddI [52], ddG [52], and carbovir [52, 53] to their monophosphate forms, whereas rat liver 5′-nucleotidase accepts as substrates acyclovir, ganciclovir, and ara-G, as well as Ino, Guo, and dGuo [54]. An ATP-activated, soluble 5′-nucleotidase that preferentially utilizes IMP, GMP, and their deoxycongeners as phosphate donors and accepts Ino, dIno, Guo, dGuo, and related analogs as phosphate acceptors has been purified from a wide variety of sources, including human T-cells, human placenta, chicken liver, and rat liver [52, 54–56]. In Chinese hamster ovary cells, nicotinamide ribonucleoside kinase converts the guanosine analog 3-deazaguanosine to its monophosphate form, while tiazofurin phosphorylating activity in these cells is associated with Ado kinase, nicotinamide ribonucleoside kinase, and a 5′-nucleotidase [51, 57].

Partial inhibition of ddG nucleotide formation by excess dGuo or dIno in duck hepatocytes could arise from competition with ddG for 5′-nucleotidase, an enzyme reported to be highly active in avian liver extracts [58]. Alternatively, a cellular nicotinamide ribonucleoside kinase could interact with these substrates. The potential involvement of these enzymes in ddG anabolism could be examined by treating cells with ddG plus either the IMP dehydrogenase inhibitor mycophenolic acid, or nicotinamide riboside, the natural substrate for nicotinamide ribonucleoside kinase. The preferred substrate nicotinamide riboside should produce a decrease in ddG nucleotide pools if nicotinamide ribonucleoside kinase is involved in ddG phosphorylation. However, if 5′-nucleotidase catalyzes ddG phosphorylation, elevated intracellular IMP levels precipitated by inhibition of IMP dehydrogenase should ultimately result in an increased yield of ddG nucleotides.

Anabolic conversion of ddC to its corresponding dideoxynucleotides occurred in ddC-treated duck hepatocyte primary cultures. In addition to ddC nucleotides, another [^{3}H]ddC-derived metabolite was formed. Based on the inability of excess 2′-dCyd to affect the accumulation of this unidentified metabolite, the compound is not a ddC nucleotide derivative. The identity of this unknown metabolite has yet to be elucidated.

Dideoxycytidine is ineffective as an antiviral agent when administered to either DHBV-infected Pekin ducks or primary cultures of duck hepatocytes [12, 28, 37]. Despite this inactivity, duck hepatocytes convert a portion of the intracellular ddC to ddC nucleotides, the presumptive active form of dideoxynucleoside antiviral agents. Although the maximum level of total intracellular ddC nucleotides achieved was less than that of ddG, both analogs yielded similar nucleotide concentrations (1.4 and 1.9 μM for cells treated with 4 μM ddC or ddG, respectively) after a 26-hr incubation. However, unless the hot-water extracts obtained from both the purine- and pyrimidine-treated cells are an accurate representation of the intracellular dideoxynucleotide pools, it is difficult to assess the relative levels of intracellular ddG and ddC triphosphates. In the most extreme case, an accumulated concentration of 1.9 μM total ddG nucleotides after 26 hr would be compared with an apparent ddCTP concentration of 0.3 μM after 24 hr, indicating a 6-fold excess of ddG nucleotides. However, if the accumulation of total dideoxynucleotide pools is a more accurate reflection of the intracellular ddGTP and ddCTP levels, the results suggest that the inability of ddC to inhibit DHBV is not simply a matter of low intracellular ddC nucleotide pools. Suzuki and coworkers [12] have suggested that ddG may exert its antiviral effect through a unique interaction with the viral primer protein. A covalent linkage between dGMP and the hepadnavirus (−)-strand DNA primer protein has been identified [59]. Recently, it was suggested that hepadnavirus (−)-strand DNA synthesis is primed by the formation of a covalent bond between a tyrosine residue of the virus polymerase protein and dGMP, followed by the addition of three more deoxynucleotide residues to form the complete priming complex [60]. Incorporation of a ddG nucleotide residue as the first nucleotide in the (−)-strand DNA, combined with an inability of the protein-bound, unnatural residue to be excised by a proofreading $3' \rightarrow 5'$ exonuclease activity, could selectively and irreversibly block replication of the virus [12].

Acknowledgements—This work was supported by a grant from Glaxo Canada. T. E. K. was a recipient of a Faculty of Medicine 75th Anniversary Graduate Studentship.

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