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METABOLISM OF 2',3'-DIDEOXYNUCLEOSIDES IN DUCK HEPATOCYTES AND HUMAN HEPATOBLASTOMA CELLS

BY

THERESA ELLEN KITOS

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA

SPRING, 1994

ISBN 0-612-11260-8

Canada

UNIVERSITY OF ALBERTA
RELEASE FORM

NAME OF AUTHOR: THERESA ELLEN KITOS

TITLE OF THESIS: METABOLISM OF 2',3'-DIDEOXYNUCLEOSIDES IN DUCK HEPATOCYTES AND HUMAN HEPATOBLASTOMA CELLS.

DEGREE: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: SPRING 1994

#5 - 7369 Montecito Drive
Burnaby, B.C. V5A 1R4
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Date: March 8, 1994

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled METABOLISM OF 2',3'-DIDEOXYNUCLEOSIDES IN DUCK HEPATOCYTES AND HUMAN HEPATOBLASTOMA CELLS submitted by THERESA ELLEN KITOS in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES.

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ABSTRACT

The purine nucleoside analogs 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR) and 2',3'-dideoxyguanosine (ddG) are potent inhibitors of duck hepatitis B virus (DHBV) replication, while pyrimidine analogs are less effective antiviral agents in the avian system (Lee et al., 1989; Suzuki et al., 1988). Both DHBV-infected duck hepatocyte primary cultures and human hepatoblastoma cells transfected with HBV DNA are commonly employed as *in vitro* screening systems for the identification of potential anti-hepadnaviral drugs. The work presented in this thesis was undertaken to characterize the intracellular metabolism of ddDAPR, ddG, and 2',3'-dideoxycytidine (ddC) in hepatocyte cultures and to correlate drug metabolism with the antiviral effects of these compounds.

Rapid conversion of ddDAPR to ddG occurred in duck blood, both *in vitro* and *in vivo*. After a 1 min exposure of ddDAPR to duck blood, 95% of the compound was converted to ddG. Human blood samples contained 79% ddG following a 10 min incubation with ddDAPR. Clearance of [^{3}H]ddDAPR from the duck circulatory system proceeded in a biphasic manner, yielding a $t_{1/2\alpha}$ and a $t_{1/2\beta}$ of 1.65 and 26 min, respectively. The radiolabeled species in these samples was identified as ddG.

Similarly, ddDAPR was converted to ddG in hepatocyte primary cultures, with ddG exhibiting resistance to further catabolism. The major pathway of ddG utilization in these cells was phosphorylation, yielding 1.9 μM of total ddG nucleotides after 26 hr of exposure to 4 μM ddG. Similar concentrations of the identical metabolites were detected in cells exposed to 4 μM ddDAPR for 27 hr. A 92% inhibition of ddG nucleotide formation occurred in duck hepatocytes treated with 4 μM ddG + 100 μM adenosine in the presence of the adenosine deaminase inhibitor 2'-deoxycoformycin, suggesting that adenosine kinase is involved in the ddG phosphorylation process. 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 that culminated in a maximum intracellular 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 dideoxynucleotides from ddC was significantly reduced in the presence of excess 2'-deoxycytidine, implicating the cellular enzyme 2'-deoxycytidine kinase in the initial step of ddC phosphorylation.

The predominant fate of ddG in HBV-negative (HepG2) or HBV-infected (2.2.15) human hepatoblastoma cells was glycosidic bond cleavage, resulting in the

reutilization of guanine to form guar[illegible]nd triphosphates. The lack of detectible ddG phosphorylation in [illegible] with an absence of anti-HBV activity in these cells. Conversely, micromolar quantities of ddC anabolites in HepG2 cells corresp[illegible] dependent decrease in HBV DNA in ddC-treated 2.2.15 cells.

Differences in antiviral [illegible] were observed in duck and rat hepatocyte primary cultures, human [illegible] cells and human hepatoblastoma cells, suggesting tissue-specific differences. [illegible] culture systems are to be successfully employed in the screening of [illegible] agents, it is manditory that the chosen culture system mimic the biochemical [illegible] environment of the *in vivo* human liver cell. As yet, the metabolic fate and antiv[illegible] of ddG in human liver tissue *in vivo* is not known.

ACKNOWLEDGEMENTS

This research project was conducted with the help of many people, including: Jy Huang, who invested much time and effort in the studies presented in Chapter 4 and who was my duck liver perfusion partner; Les Howard, who graciously allowed me to spend many hours with the HPLC located in his lab, who provided valuable technical assistance with the instrument, and who analyzed a group of cell extract samples; Camilla Smith, who analyzed a set of cell extracts using the HPLC in Dr. Henderson's laboratory; Donger Gong and Zarina Mohammad who coordinated the appearance of 2 week-old, doubly-confirmed DHBV-negative ducklings with the scheduled perfusion time; Andy Bennet the Chemwindows expert, who spent many hours constructing the chemical structures presented in Appendix I and II; Karl Fischer, who was my indispensable long-distance liaison with graphic services; and San Vinh, who spent time and effort printing the photographs presented in this thesis. My thanks to them all!

The work on this project would not have progressed without the support, supervision, and unwavering enthusiasm of Dr. Tyrrell. I would like to thank him for including me in his lab group, and for his continued interest and many contributions to my graduate training.

I would like to thank Dr. C.E. Cass, Dr. J.F. Henderson, Dr. M. Peppler, Dr. D.S. Shewach, and Dr. Tyrrell for the time and effort they invested on my behalf, as members of my committee. A special note of thanks goes to Dr. Cass for the friendship, advice, and support she gave me during my years in Edmonton. I would also like to express my appreciation of Dr. A.R.P. Paterson for the interest he took in my initial circumstances in Edmonton, and for his friendship throughout my academic career at the U. of A. Not only was he my first contact at the U. of A., but he very generously included me in the McEachern Cancer Research group and, in conjunction with Dr. Tyrrell, provided me with a project, lab space, and funding.

I gratefully acknowledge financial assistance received from Glaxo Canada and from the Faculty of Medicine 75th Anniversary Graduate Studentship.

My life in and out of the lab was enriched by interactions with many friends and colleagues, including Karl Fischer, Alberto Severini, the old McEachern gang, Karen King, Danuta Madej, Darlene Paine, Rob Dupuis, Weixing Luo, and Bia Boleti.

Finally, I thank my family and Andy for their support and love.

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ABBREVIATIONS AND DEFINITIONS

acyclovir	9-(2-hydroxyethoxymethyl)guanine; ACV; Zovirax
ADA	adenosine deaminase
AddMeCyt	3'-amino-5-methyl-deoxycytidine
Ado	adenosine
ALT	alanine aminotransferase
2-amino-OXT-A	9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanocyl)-2,6-diaminopurine
ara-A	9-β-D-arabinofuranosyladenine
ara-AMP	9-β-D-arabinofuranosyl adenine monophosphate
ara-5-aza-Cyd	1-β-D-arabinofuranosyl-5-azacytosine
ara-C	1-β-D-arabinofuranosylcytosine
AST	aspartate aminotransferase
ATCC	American Type Culture Collection
5-aza-Cyd	5-azacytidine
5-aza-dCyd	2'-deoxy-5-azacytidine
AZC	3'-azido-2',3'-dideoxycytidine
AZT	3'-azido-3'-deoxythymidine
AZU	3'-azido-2',3'-dideoxyuridine
AZU-MP	3'-azido-2',3'-dideoxyuridine monophosphate
(+)BCH-189	(+) 2',3'-dideoxy-3'-thiacytidine
(-)BCH-189	3TC; (-) 2',3'-dideoxy-3'-thiacytidine
BCV	(R)-9-(3,4-dihydroxybutyl)guanine; buciclovir
5-BrddC	5-bromo-2',3'-dideoxycytidine
BDU	5-bromo-2'-deoxyuridine
b.i.d.	twice daily
bp	base pairs
BSA	bovine serum albumin
buciclovir	(R)-9-(3,4-dihydroxybutyl)guanine; BCV
$CaCl_2$	calcium chloride
carbovir	9-(4α-(hydroxymethyl)cyclopent-2-ene-1α-yl) guanine
cccDNA	covalently closed, circular DNA
2'-CDG	2-amino-1,9-dihydro-9-((1α,3β,4α,)-3-hydroxy-4-(hydroxymethyl)cyclopentyl)-6H-purine-6-one
CDP choline	cytidine 5'-diphosphocholine
chloroquine	7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline
2-CldA	2-chloro-2',3'-dideoxyadenosine
CldMeCyt	3'-chloro-5-methyl-deoxycytidine
Cyd-dCyd deaminase	cytidine-deoxycytidine deaminase
2'-dAdo	2'-deoxyadenosine
3'-dAdo	3'-deoxyadenosine; cordycepin
D-ara-C	3'-deoxy-arabinofuranosylcytosine

D4C	2',3'-dideoxy-2',3'-didehydrocytidine
DCF	2'-deoxycoformycin; inhibitor of ADA
2'-dCyd	2'-deoxycytidine
dCyd kinase	deoxycytidine kinase
ddA	2',3'-dideoxyadenosine
ddC	2',3'-dideoxycytidine
ddCDP choline	2',3'-dideoxycytidine 5'-diphosphocholine
ddCTP	2',3'-dideoxycytidine triphosphate
ddDAPR	2,6-diaminopurine-2',3'-dideoxyriboside
ddG	2',3'-dideoxyguanosine
ddGTP	2',3'-dideoxyguanosine triphosphate
ddI	2',3'-dideoxyinosine
ddT	2',3'-dideoxythymidine
ddU	2',3'-dideoxyuridine
7-deazaadenosine	tubercidin
2'-dGuo	2'-deoxyguanosine
DHBV	duck hepatitis B virus
DHPG	ganciclovir; 9-(1,3-dihydroxy-2-propoxymethyl) guanine
2'-dIno	2'-deoxyinosine
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
DR1	direct repeat 1
DR2	direct repeat 2
d4T	ddeThd; stavudine; 2',3'-dideoxy-2',3'-didehydrothymidine
dThd	thymidine
dTMP kinase	thymidylate kinase
EDTA	ethylenediamino-tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
FB	Formycin B
2'-F-dd-ara-A	2'-fluoro-2',3'-dideoxyarabinosyladenine
3'-FddC	2',3'-dideoxy-3'-fluorocytidine
5-FddC	5-fluoro-2',3'-dideoxycytidine
FddMeCyt	3'-fluoro-5-methyl-deoxycytidine
3'-FddT	2',3'-dideoxy-3'-fluorothymidine
FEAU	1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil
FIAC	1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine
FIAU	Fialuridine
FMAU	1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil
formycin B	8-aza-9-deazainosine
FTC	5-FSddC; 5-fluoro-2',3'-dideoxy-3'-thiacytidine

ganciclovir	DHPG; 9-(1,3-dihydroxy-2-propoxymethyl)guanine
GHBV	ground squirrel hepatitis virus
Gua	guanine
Guo	guanosine
HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B virus e antigen
HBsAg	hepatitis B virus surface antigen
HBV	human hepatitis B virus
[^{3}H]ddC	[2',3'-^{3}H]2',3 '-dideoxycytidine
[^{3}H]ddDAPR	[2',3'-^{3}H]2,6-diaminopurine 2',3'-dideoxyriboside
[^{3}H]ddG	[2',3'-^{3}H]2',3'-dideoxyguanosine
HCO_3^-	bicarbonate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H_2G	(R)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine
[^{3}H]PEG	[1,2-^{3}H]polyethylene glycol
HPLC	high performance liquid chromatography
HPMPA	(S)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine
HPMPC	(S)-1-((3-hydroxy-2-phosphonylmethoxy)propyl) cytosine
H_3PO_4	phosphoric acid
Hx	hypoxanthine
hypericin	1,3,4,6,8,13-hexahydroxy-10,11-dimethyl-phenanthro[1,10,9,8-opqra]perylene-7,14-dione
i.m.	intramuscular
IMP	inosine 5'-monophosphate; inosinate
inosinate	inosine 5'-monophosphate; IMP
i.p.	intraperitoneal injection
i.v.	intravenous
kb	kilobases; kilobase pairs
KCl	potassium chloride
kDa	kilodaltons
KH_2PO_4	potassium phosphate monobasic
K_m	Michaelis constant; the substrate concentration at which an enzyme shows one-half its maximum velocity
MEM	minimum essential medium
$MgCl_2$	magnesium chloride
NBMPR	nitrobenzylthioinosine; 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine); inhibitor of certain nucleoside transporters
8-NH_2Guo	8-aminoguanosine
NSIV	NuSerum IV
OMP	orotidine 5'-monophosphate; orotidylate
ORF	open reading frame
orotidylate	orotidine 5'-monophosphate; OMP

OXT-A	oxatanocin-A; 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanocyl)adenine
OXT-G	9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanocyl)guanine
OXT-H	9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanocyl)hypoxanthine
PBS	phosphate-buffered saline
PCA	perchloric acid
PEG	polyethylene glycol
Pen/Strep	penicillin/streptomycin
PFA	phosphonoformic acid; trisodium phosphonoformate; Foscarnet
PMEA	9-((2-phosphonylmethoxy)ethyl)adenine
PMEDAP	9-((2-phosphonylmethoxy)ethyl)-2,6-diaminopurine
PNP	purine nucleoside phosphorylase
PRPP	5-phosphoribosyl 1-pyrophosphate
quinacrine hydrochloride	N^4-(6-chloro-2-methoxy-9-acridinyl)-N^1, N^1-diethyl-1,4-pentanediamine dihydrochloride
ribavirin	1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide
RNase	ribonuclease
RT	room temperature
s.c.	subcutaneous
S.D.	standard deviation = $\sqrt{\frac{1}{N-1}\sum(x_i - \bar{x})^2}$
SddC	(+,-)- 2',3'-dideoxy-3'-thiacytidine; the (+)-isomer is (+)-BCH-189; the (-)-isomer is (-)BCH-189 or 3TC
SddU	2',3'-dideoxy-3'-thiauridine
SDS	sodium dodecylsulfate
S.E.	standard error; standard error of the mean = $\frac{S.D.}{\sqrt{N}}$
stavudine	d4T; ddeThd; 2',3'-dideoxy-2',3'-didehydrothymidine
suramin	8,8'(carbonylbis(imino-3,1-phenylenecarbonylimino (4-methyl- 3,1-phenylene) carbonylimino))bis-1,3,5 naphthalene trisulfonic acid
TBAP	tetrabutylammonium dihydrogen phosphate
TBE	Tris/borate/EDTA buffer
3TC	(-) 2',3'-dideoxy-3'-thiacytidine
TCA	trichloroacetic acid
tiazofurin	2-β-D-ribofuranosylthiazole-4-carboxamide
Tris	tris-(hydroxymethyl)aminomethane
tubercidin	7-deazaadenosine
UMP	uridine 5'-monophosphate; uridylate
uridylate	uridine 5'-monophosphate; UMP

uv	ultraviolet
V_{max}	the maximum velocity of an enzymatic reaction
WHV	woodchuck hepatitis virus
xanthylate	xanthine 5'-monophosphate; XMP
XMP	xanthine 5'-monophosphate; xanthylate

CHAPTER 1

I. INTRODUCTION

A. Hepatitis B Virus

Hepatitis B virus (HBV) infection is a major human health problem in many parts of the world. There is no proven effective treatment for this disease. Although a vaccine has been developed to counter this infectious agent, vaccination programs are limited in their extent by the expense of the vaccine. In the United States, the current policy of preferential vaccination of high-risk populations has not affected the incidence of HBV infection in that country (Alter et al., 1990; Gerety, 1985). Most individuals with a primary HBV infection clear the virus and establish lasting immunity but 5 - 10% of these infected individuals become chronic carriers of the virus (Hollinger, 1985). Such persistent infection provides a reservoir of virus for the maintainance 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 (PHC or HCC) in HBV-infected individuals (Beasley et al., 1981; Di Bisceglie et al., 1988).

1. Infection with HBV

The human pathogen HBV has an incubation period that normally varies from 45 to 120 days before the onset of symptoms. At the end of this initial stage of the acute infection, an individual may experience flu-like symptoms and this condition is sometimes followed by the appearance of jaundice. However, not all patients develop jaundice (icterus) during the course of the disease. In anicteric hepatitis, symptoms occur without an accompanying disease-induced jaundice. Furthermore, an estimated 65% of individuals undergoing an acute HBV infection have inapparent (subclinical) hepatitis, with no symptoms or jaundice to indicate presence of disease (Hoofnagle and Schafer, 1986). Several biochemical tests are used to evaluate the liver function of a person suspected of having hepatitis, including: 1) measurement of the bilirubin content of urine and serum; 2) quantitation of serum levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST); 3) determination of the alkaline phosphatase level in the serum; 4) assessment of blood clotting systems; and 5) measurement of serum albumin and total protein concentrations (Hollinger, 1990). If hepatitis is diagnosed, the etiology of disease can be pursued through the use of serologic assays designed to detect the presence of specific viral proteins and to evaluate the immune response of the patient. In the case of inapparent or anicteric hepatitis, biochemical and serologic tests are necessary for identification of the disease. However,

inapparent hepatitis often remains clinically undetected. This cryptic form of the disease is frequently unresolved, resulting in a chronic HBV infection (Alward et al., 1985).

a. *Acute viral hepatitis*

The serologic profile of an individual with acute hepatitis type B can be used to predict the course and outcome of disease (Hoofnagle and Schafer, 1986). Early in the infection, HBV surface antigen (HBsAg) and virus products indicative of viral replication appear in the blood. Serum concentrations of these viral markers peak and begin to decline coincident with the appearance of serum liver enzymes and symptoms of the disease. An immunologic response is evident at this time, with the appearance of IgM-specific antibody to the virus core protein (HBcAg). Eventually the IgM response to HBcAg is replaced with the more permanent IgG-specific antibody anti-HBc. In most cases, the decline in serum HBsAg concentration continues until it is no longer detectable, a process that can take months. Before HBsAg dissappears completely, an antibody to the virus e protein, anti-HBe, becomes measurable in the blood. Appearance of the neutralizing antibody (anti-HBs) for HBV which is directed towards the virus surface antigen, signals the end of the infection. However, if the initially elevated serum HBsAg concentration is maintained at a relatively constant level instead of decreasing over the course of the acute infection, the disease is likely to become a chronic infection.

If liver damage occurs during an acute infection with HBV, it is usually a reversible process involving focal areas of necrosis. However, extensive necrosis can result in death (Gazzard et al., 1975; Scotto et al., 1973). Liver tissue sequelae in an individual with acute hepatitis begins with parenchymal cell degeneration. The damaged hepatocytes migrate to the sinusoids where they are phagocytosed by specialized macrophages known as Kupffer cells. Hypertrophy and hyperplasia of activated Kupffer cells in the sinusoids and portal tracts occurs as a result of the activity of this reticuloendothelial cell. In response to the viral infection, lymphocytes infiltrate the portal tracts, contributing to tissue swelling. Over the course of the disease, gradual parenchymal cell regeneration and a lessening of the inflammatory response eventually returns the liver to a healthy state.

b. *Chronic viral hepatitis*

In an estimated 5-10% of acute hepatitis type B cases, the viral infection persists in the form of chronic viral hepatitis (Hollinger, 1985). Certain factors influence the outcome of an acute HBV infection. Establishment of a chronic infection instead of permanent immunity to the virus is more likely when the primary infection occurs perinatally or at an early age in life, than if the infection occurs in adulthood (Beasley et al., 1982; McMahon et al., 1985; Stevens et al., 1975). Furthermore, the host immune

response to the virus is important in determining the outcome of acute hepatitis. Immunocompetent individuals are more likely to clear the virus than are the immunocompromised (Seeff et al., 1987; Szmuness et al., 1974). Development of the HBV carrier-state is often preceded by an anicteric or inapparent manifestation of the acute disease (Alward et al., 1985). Adult males are more likely to become chronic HBV carriers than are females, suggesting that gender predisposes viral persistence (Szmuness et al., 1978). Additional factors such as genetics and ethnicity may play a role in establishing the chronic form of hepatitis (Derso et al., 1978).

Some chronic HBV-induced liver infections exist in an asymptomatic, dormant state that is catagorized as chronic persistent hepatitis. In these cases, histologic abnormalities of the liver are usual but rarely does the condition lead to cirrhosis. Another benign form of the disease, chronic lobular hepatitis, is characterized by an oscillating condition of active disease similar to acute hepatitis, followed by a period of remission. Histologically, chronic active hepatitis is characterized by piecemeal necrosis and fibrosis in the portal-periportal regions of the liver. In the more severe forms of chronic active hepatitis, progressive cell degeneration eventually leads to confluent necrosis and multilobular manifestations of disease, a state that can eventually progress to severe cirrhosis and death (Bianchi et al., 1971).

2. HBV and Primary Hepatocellular Carcinoma (PHC or HCC)

Throughout the world, the incidence of the primary liver cancer HCC is much higher in chronic HBV carriers than in uninfected individuals (Beasley et al., 1981; Blumberg et al., 1975; Szmuness, 1978). For example, a large prospective study of Taiwanese men found that HBsAg-positive individuals were 223 times as likely to die from HCC than were the HBsAg-negative men in the study (Beasley et al., 1981). In addition, Beasley and co-workers (1981) found that the occurrence of HCC in HBsAg-positive subjects became more frequent with increasing age of the individuals. Within an HBsAg-positive group of male and female Alaskan natives there appeared to be a gender risk factor associated with the development of HCC (McMahon et al., 1990). In this particular study, the male HBV-carriers were six-times more likely to develop HCC than were the female cohorts (McMahon et al., 1990). Similar increased risk factors have been reported for the development of HCC in males from Africa, East Asia, and Western countries (Szmuness, 1978). There are numerous examples of human hepatoma tissue containing HBV sequences integrated into the host cell DNA (Brechot et al., 1980; Hada et al., 1986; Shafritz et al., 1981). There may be a causal relationship between integration of viral sequences and liver cell oncogenesis (Dejean et al., 1986; Hada et al., 1986; Wei et al., 1992).

3. Transmission of the Virus

Hepatitis type B is a communicable disease that is passed from an infected to an uninfected individual by horizontal and vertical modes of transmission. Horizontal transfer of the virus occurs by percutaneous introduction as well as through close physical contact with infected individuals. Hemophiliacs that receive plasma-derived clotting factors, intravenous drug abusers that share contaminated needles, hemodialysis patients, and medical personnel are the major groups of people that are at risk of acquiring an HBV infection that is transmitted parenterally (Hollinger, 1990; Szmuness et al., 1974; Szmuness et al., 1982). Horizontal transmission of the virus occurs through sexual contact, with spread of the disease occurring as a result of unprotected promiscuous heterosexual and homosexual behaviors (Alter et al., 1990). Close household contact, crowding, and poor hygiene may also contribute to spread of the disease (Heathcote et al., 1974; Krugman et al., 1967; Mitch et al., 1974; Szmuness et al., 1973). Vertical transmission of HBV from an infected mother to her offspring can occur perinatally during labor and delivery, and less frequently in utero, by exposure of the fetus to contaminated maternal blood via transplacental leakage (Ohto et al., 1987; Skinhoj et al., 1976; Wong et al., 1980). Virus transmission from an infected mother to her newborn child is of critical importance since 85-90% of individuals infected in this manner become chronic carriers of HBV (Stevens, 1982).

4. Geographical Distribution

Although HBV exists throughout the world, the chronic carrier state of the virally-induced disease is more prevalent in some areas than in others. In the general population of underdeveloped regions of the world including China, Southeast Asia, and sub-Saharan Africa there is a 5-15% HBsAg-positive rate, whereas other regions such as Central and South America, the Middle East, the Mediterranean regions, and Eastern Europe have a 1-5% HBsAg-positive population (Hoofnagle and Alter, 1984). In contrast, industrialized nations of North America and Western Europe exhibit an HBsAg carrier rate that is <1% of the general population (Hoofnagle and Alter, 1984). In industrialized countries, horizontal transmission is the principle mode of infection, whereas underdeveloped regions of the world experience a higher rate of vertical transmission, a mode of spread that leads to a high rate of viral persistence (Alter et al., 1990; Stevens, 1982).

B. Hepadnaviridae Family

Human hepatitis B virus (HBV) is a member of the Hepadnaviridae family of animal viruses (Gust et al., 1986). Several other viruses are categorized as hepadnaviruses based on their related structure and biology. The other viruses classified

in this group are woodchuck hepatitis virus (WHV), first identified in a *Marmota monax* colony housed at the Philadelphia Zoo, ground squirrel hepatitis virus (GSHV), found in Beechey ground squirrels (*Spermophilus beecheyi*), and duck hepatitis B virus (DHBV), found in Pekin ducks (*Anas domesticus*) (Gust et al., 1986; Marion et al., 1980; Mason et al., 1980; Summers et al., 1978). In addition, there may be other naturally occurring hepadnaviruses, such as the proposed tree squirrel hepatitis B virus (THBV) found in *Sciurus carolinensis pennsylvanicus* and an HBV-like virus found to be endemic in grey herons located in Germany (Feitelson et al., 1986; Sprengel et al., 1988). Distinguishing characteristics common to all of the hepadnaviruses include a) similar virion morphology, b) production of excess surface antigen particles, c) similar genomic size, structure, and organization, d) a characteristic mechanism of viral replication, e) a narrow host range, and f) hepatotropism (Ganem et al., 1982b; Marion et al., 1980; Mason et al., 1980; Murphy et al., 1975). In general, the mammalian Hepadnaviridae are more closely related to one another in terms of deoxynucleotide sequence and biological properties than they are to the avian form of the virus (Cote and Gerin, 1983; Galibert et al., 1982; Mandart et al., 1984; McCaul et al., 1985; Robinson et al., 1984; Stannard et al., 1983; Summers, 1981; Tiollais et al., 1985; Werner et al., 1979). Although significant differences between the viruses do exist and will be mentioned, the overall theme of the following discussion will emphasize the similarities that bind this family of viruses together.

1. Structure and Genomic Organization of Hepadnaviruses

a. *Hepadnavirus morphology*

Three types of virus particles can be found in the blood serum of HBV-infected individuals: 1) the infectious virion or Dane particle, which is a double-shelled particle approximately 42 nm in diameter and composed of an outer envelope and an electron-dense, DNA-containing inner nucleocapsid; 2) non infectious, 'empty envelope' spherical particles that are 22 nm in diameter; and 3) non infectious, filamentous envelope particles that are 22 nm in diameter and variable in length (Bayer et al., 1968; Dane et al., 1970). Both the spherical and filamentous forms of the virus are incomplete virus particles, lacking the nucleocapsid core. However these particles are often present in the serum of infected individuals in greater abundance than infectious virions (Almeida, 1972; Bond and Hall, 1972; Kim and Tilles, 1973). The mammalian hepadnaviruses produce a mixed population of spherical and filamentous forms of the envelope particles, with more numerous and lengthy filaments apparent in GSHV infection than in the other two mammalian diseases (Dane et al., 1970; Marion et al., 1980; Summers et al., 1978). The

avian virus has a larger and more pleomorphic version of the spherical envelope particle and does not produce filamentous forms (Mason et al., 1980).

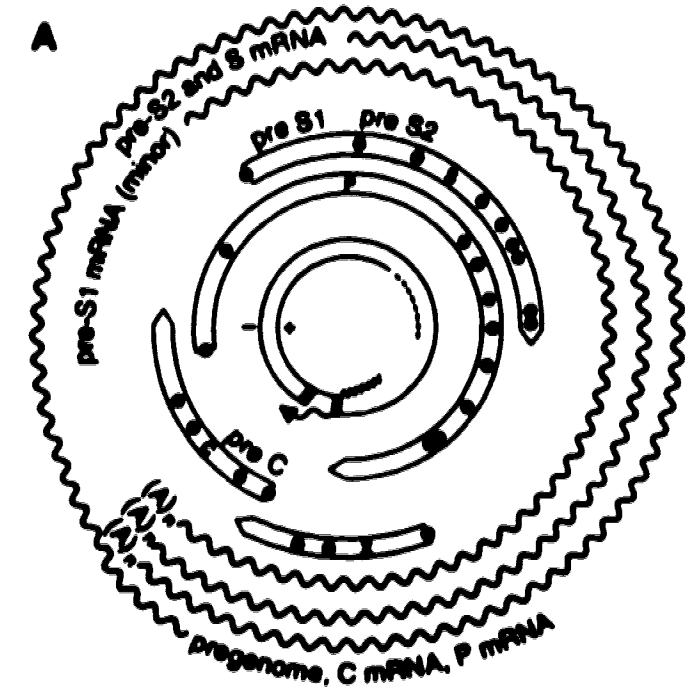
The lipid-containing envelope structures found in all three types of viral particles contain the viral surface antigen (HBsAg), an antigenically complex polypeptide. The purified 22 nm spherical envelope particles obtained from the blood of HBV carriers contains, in addition to the HBsAg, 25% lipid, having a characteristic set of host-derived phospholipids, cholesterol, cholesteryl esters, and triglycerides (Gavilanes et al., 1982).

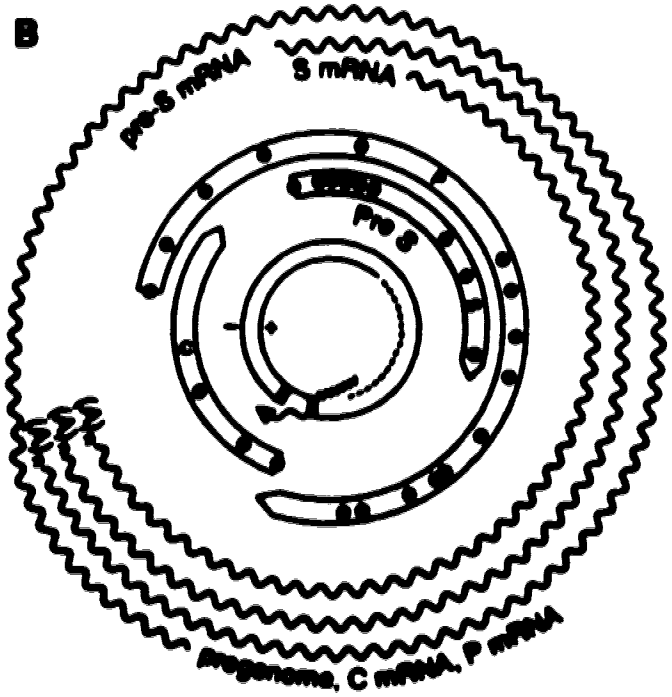
Within the envelope of the infectious virion is a 27 nm diameter nucleocapsid (or core), the major structural component of which is the viral core antigen (HBcAg) (Almeida et al., 1971; Petit and Pillot, 1985). In addition to HBcAg, the nucleocapsid has a cryptic form of the soluble hepatitis B e antigen (HBeAg) which is detected in the blood of HBV-infected patients during active viral replication (Hoofnagle and Schafer, 1986; Takahashi et al., 1979). Other constituents of the nucleocapsid include the viral DNA, the DNA-associated primer protein, a DNA-associated oligoribonucleotide, a DNA polymerase, and a protein kinase (Albin and Robinson, 1980; Gerlich et al., 1982; Gerlich and Robinson, 1980; Kaplan et al., 1973; Landers et al., 1977; Lien et al., 1986).

b. ***Structure of the hepadnavirus DNA***

The following discussion of hepadnavirus genome structure is supplemented by two representative diagrams presented in Figure 1.1. The hepadnavirus genome associated with the nucleocapsid is a relaxed, circular, partially double-stranded DNA molecule that is circularized by way of a cohesive overlap between the 5'-ends of the two DNA strands (Hruska et al., 1977; Landers et al., 1977; Sattler and Robinson, 1979). The full-length DNA strand is approximately 3200 bases long and is referred to as the (-) strand since it has a base sequence complementary to the viral mRNA, whereas the complementary but incomplete DNA (+) strand is variable in length (Delius et al., 1983; Ganem and Varmus, 1987; Landers et al., 1977; Summers et al., 1975). As a result of a terminal redundancy of approximately 9 nucleotides, the (-) strand is slightly greater than unit length (Lien et al., 1987; Seeger et al., 1986; Will et al., 1987). The location of the 5' end of the (+) strand is fixed with respect to the 5' end of the (-) strand. However, the location of the 3' end of the short strand, and hence the length of this strand, is variable (Charnay et al., 1979; Delius et al., 1983). Infectious viral particles from mammalian hepadnaviruses contain DNA that is from 50 to 80% double stranded, whereas DHBV virion DNA is often fully duplexed (Delius et al., 1983; Landers et al., 1977; Lien et al., 1987; Summers et al., 1975). Associated with the virion is a DNA polymerase activity that is capable of repairing the single-stranded gap in the viral genome, yielding a complete, double-stranded molecule (Landers et al., 1977). The 5'-end of the viral DNA

Figure 1.1 - Structural and functional elements of the hepadnavirus genome. Panel A illustrates the genetic organization of HBV, a prototypic example of a mammalian hepadnavirus. In panel B the similar genome structure of the avian hepadnavirus DHBV is shown. In each diagram, the slightly longer-than-genome-length (-)-strand DNA and the incomplete (+)-strand DNA are indicated by the inner-most concentric circles and are labeled according to their respective polarities. Attached to the 5'-end of the (-)-strand DNA is the primer protein (O) and an RNA fragment ( ) prefaces the DNA (+) strand sequences. The relaxed, circular conformation of the partially double-stranded viral DNA is maintained by base-pairing interactions of the DNA (-)- and (+)-strand 5'-ends in the area of the genome demarcated by the direct repeat sequences DR1 and DR2 illustrated as solid black rectangles. Hepadnavirus ORFs are indicated by clockwise-oriented thick arrows that encircle the DNA genome. All hepadnaviruses contain the P ORF which encodes the primer protein, reverse transcriptase, DNA polymerase, and RNAse H; the pre-C/C ORF which includes the pre-C and C gene sequences and encodes the nucleocapsid protein and the soluble e antigen; and the pre-S/S ORF which encodes a variety of surface antigens using pre-S1, pre-S2, and S gene sequences in the mammalian hepadnaviruses and pre-S and S genes in the avian form of the virus. In addition, the mammalian hepadnavirus genomes contain the X ORF which encodes a regulatory protein. Potential translation start signals (AUG) are located throughout each ORF and their positions within the ORFs are indicated by black dots. The major viral mRNAs transcribed from the DNA genomes are indicated by wavy lines that encircle the genome map. The polyadenylated 3'-end of each transcript is indicated by $(A)_n$. The three mRNA species indicated in panel A include 1) two major mRNA species: a) the genome-length mRNA that functions as the pregenome and as the mRNA for core protein and the polymerase gene products; and b) a subgenomic-sized transcript that contains the pre-S2 and S gene messages; and 2) a minor mRNA transcript that includes sequences encoded by the pre-S1 gene in addition to the contiguous pre-S2 and S gene nucleotides. The DHBV genome illustrated in panel B directs synthesis of three major mRNA species: 1) a genome-length mRNA that functions as the pregenome and the mRNA for both core and polymerase gene products; 2) a subgenomic-sized mRNA that encodes the pre-S/S gene sequences; and 3) a subgenomic-sized transcript that can function as the S gene mRNA. Information contained in this figure was obtained from the following references: Blächer et al., 1985; Ganem and Varmus, 1987; and Will et al., 1987.


A
pre-S2 and S mRNA
pre-S1 mRNA (minor)
pre S1
pre S2
pre C
pregenome, C mRNA, P mRNA


B
pre-S mRNA
S mRNA
Pre S
pregenome, C mRNA, P mRNA

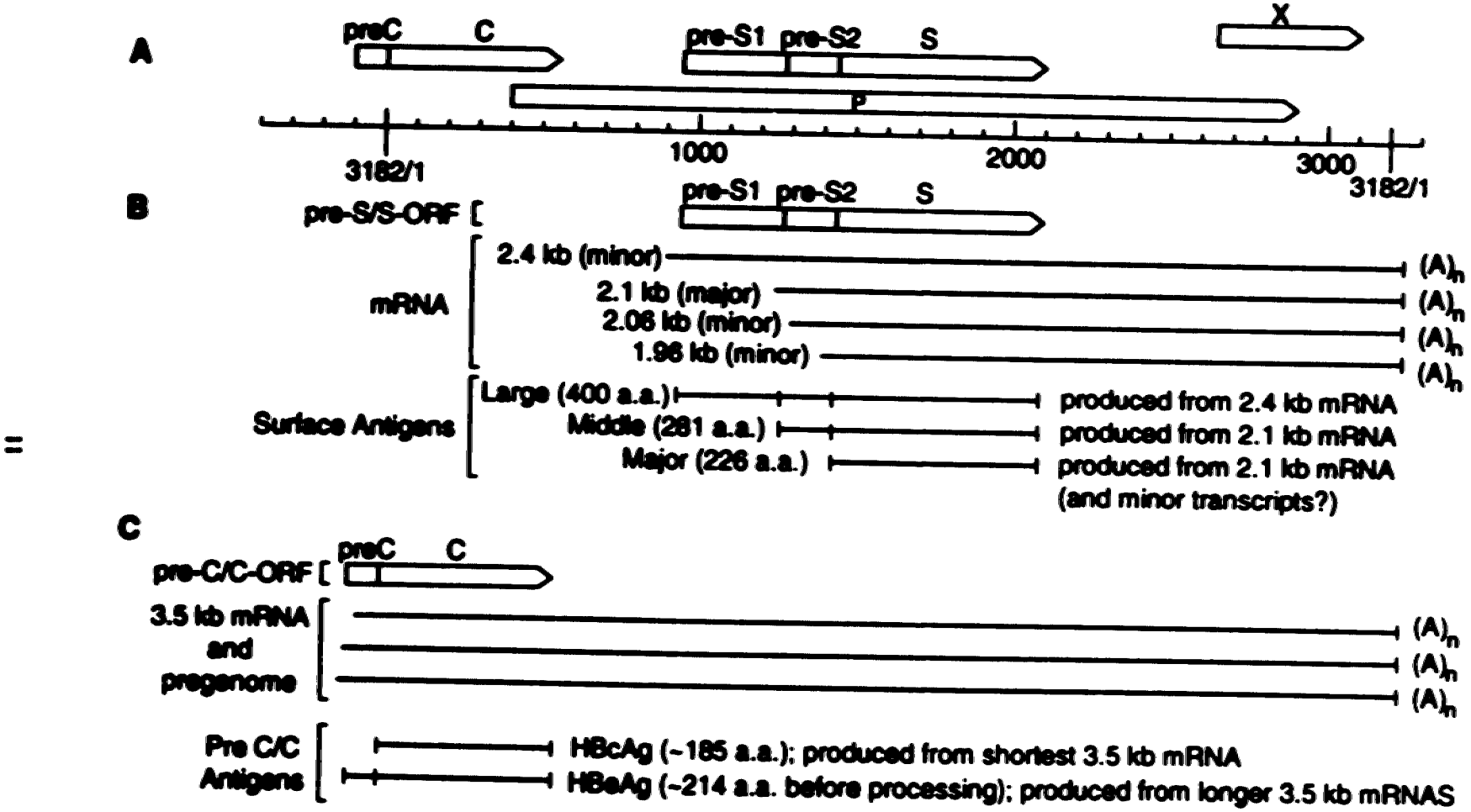
(-) strand is covalently linked to a protein which functions as a primer for (-) strand DNA synthesis (Ganum et al., 1982a; Gerlich and Robinson, 1980; Molnar-Kimber et al., 1983). A similar priming function is attributed to the capped oligoribonucleotide that is associated with the 5'-end of the DNA (+) strand (Lien et al., 1986; Lien et al., 1987; Seeger et al., 1986). Within the cohesive overlap region of the virus genome, located near the 5' ends of both strands, are 10 - 12 base pair direct repeats known as DR1 and DR2 (Molnar-Kimber et al., 1984; Seeger et al., 1986). These conserved direct repeat elements of the viral genome have been implicated in the unusual replication process employed by the virus (Seeger et al., 1986; Will et al., 1987). Section B.2. of this chapter provides a more detailed discussion of virus replication.

c. ***Genomic organization***

The genetic material from each of the four types of hepadnaviruses has been cloned and the nucleotide sequences have been determined (Galibert et al., 1979; Galibert et al., 1982; Kodama et al., 1985; Mandart et al., 1984; Ono et al., 1983; Seeger et al., 1984). Regions of nucleotide sequence homology occur in the DNA of cloned HBV, WHV, GSHV, and DHBV genomes, revealing a phylogenic relationship among the four viruses (Kodama et al., 1985; Mandart et al., 1984). As the smallest DNA virus known to infect animals, the hepadnavirus has evolved a genome that maximizes coding capacity by efficient use of the genetic coding system (Reviewed by Miller et al., 1989). In all four animal viruses, the known open reading frames (ORFs) are encoded on the (-) strand and are located at similar positions and in the same order on the strand (Galibert et al., 1981; Galibert et al., 1982; Mandart et al., 1984; Seeger et al., 1984; Siddiqi et al., 1981; Sprengel et al., 1985).

As can be seen in Figure 1.1, four ORFs are present in the genetic material of the hepadnaviruses that infect mammals whereas the avian virus has a genome that contains only 3 ORFs (Galibert et al., 1982; Seeger et al., 1984; Sprengel et al., 1985). The 3 ORFs common to all members of the hepadnavirus family are the pre-C/C ORF, the pre-S/S ORF, and the P ORF, while an additional ORF containing the X gene sequence is found exclusively in the mammalian viruses. The compact genetic organization exhibited by this virus makes use of overlapping genes that are translated in different reading frames. The structural proteins of the virus are encoded in the pre-C/C and pre-S/S ORFs. By utilizing two in-phase translation start codons in the pre-C/C ORF of mammalian hepadnavirus genomes, the nucleocapsid subunit (HBcAg) is produced from C gene-specified sequences and the related HBeAg results from translation of the pre-C and C gene regions (Figure 1.2C) (Weimer et al., 1987). Similarly, utilization of three in-phase translation start codons present in the pre-S/S ORF of the mammalian

Figure 1.2 - HBV pre-S/S and pre-C/C genes, mRNA transcripts, and pre-S/S and pre-C/C gene products. Panel A: A linear representation of a 3182 nucleotide HBV genome is illustrated, with the numbering system of Pasek et al. (1979) used to localize elements of the genome. The four overlapping ORFs are drawn as thick arrows above the nucleotide numbering scale. Panel B: Three minor (2.4, 2.06, and 1.96 kb) and one major (2.1 kb) polyadenylated RNA species are transcribed from the S-ORF region. Polyadenylation occurs at the 3'-end of the HBV transcripts and is indicated by $(A)_n$. Three viral surface antigens arise from the pre-S/S ORF: 1) the large surface antigen which is translated from the 2.4kb mRNA; 2) the middle surface antigen which is translated from the 2.1 kb mRNA; and 3) the major surface antigen which is translated from the 2.1 kb mRNA and possibly from the 2.06 and 1.96 kb minor transcripts. Panel C: Hepatitis B virus produces three slightly-larger-than-genome-length RNA transcripts (approximately 3.5 kb) that have a common 3'-end but micro-heterogeneous 5'-termini. The RNA pregenome, as well as mRNA for translation of P-gene and pre-C/C gene products arise from this pool of viral transcripts. The shortest of the 3.5 kb mRNAs is utilized for translation of the nucleocapsid (core) protein, while the two longer transcripts encode the HBeAg. The shortest genome-length RNA species also functions as the virus pregenome. Information contained in this figure was obtained from the following references: Junker-Niepmann et al., 1990; Pasek et al., 1979; and Will et al., 1987.


A
preC
C
pre-S1
pre-S2
S
X
P
1000
2000
3000
3182/1
3182/1
B
pre-S/S-ORF
mRNA
2.4 kb (minor)
2.1 kb (major)
2.06 kb (minor)
1.96 kb (minor)
(A)n
Surface Antigens
Large (400 a.a.)
Middle (281 a.a.)
Major (226 a.a.)
produced from 2.4 kb mRNA
produced from 2.1 kb mRNA
produced from 2.1 kb mRNA
(and minor transcripts?)
=
C
pre-C/C-ORF
3.5 kb mRNA
and
pregenome
Pre C/C
Antigens
HBcAg (~185 a.a.); produced from shortest 3.5 kb mRNA
HBeAg (~214 a.a. before processing); produced from longer 3.5 kb mRNAS

hepadnaviruses accommodates the production of three different envelope proteins (Heermann et al., 1984; Pasek et al., 1979; Schaeffer et al., 1986). The largest of the three envelope antigens is encoded by incorporating the pre-S1 + pre-S2 + S gene sequences, the middle-sized version of the HBsAg utilizes the pre-S2 and S gene sequences, and the S gene sequences encode the smallest, but most abundant product of the pre-S/S ORF (Figure 1.2B) (Heermann et al., 1984; Molnar-Kimber et al., 1988; Stibbe and Gerlich, 1983; Valenzuela et al., 1979; Wong et al., 1985). The resulting three surface antigens associated with mammalian hepadnaviruses are termed the large, middle, and major surface antigens. As can be seen in Figure 1.1, the pre-S/S ORF of the avian hepadnavirus contains a smaller S gene and a single pre-S gene sequence comparable in size to a contiguous pre-S1, pre-S2 gene in the mammalian form of the virus (Mandart et al., 1984; Pugh et al., 1987; Schaeffer et al., 1986). Overlapping the pre-C/C ORF and completely containing the pre-S/S ORF is the lengthy P ORF which is thought to encode the viral DNA polymerase/reverse transcriptase, the protein primer, and possibly an RNase H (Bavand et al., 1989; Bosch et al., 1988; Chang et al., 1990; Radziwill et al., 1990; Schlicht et al., 1989b; Toh et al., 1983). Although not present in the avian viral genome, the small X ORF is a constituent of the HBV, WHV, and GSHV genomes and is also overlapped by the P ORF (Galibert et al., 1979; Galibert et al., 1982; Mandart et al., 1984; Seeger et al., 1984). Detection of serum antibodies to the X gene product during HBV and GSHV infection suggests that X gene expression occurs during viral replication (Persing et al., 1986; Meyers et al., 1986). It has been shown that the X gene protein acts in *trans* to exert an effect on the transcription of numerous viral and cellular genes (Aufiero and Schneider, 1990; Spandau and Lee, 1988; Twu et al., 1989; Twu and Schloemer, 1989).

d. *Transcripts and regulatory elements of the hepadnavirus*

1) *Viral RNA transcripts*

Hepadnavirus gene expression begins with transcription of the DNA message into RNA by cellular RNA polymerase II (Rall et al., 1983). Genomic- and subgenomic-sized polyadenylated viral RNA transcripts have been found in the liver tissue of hepadnavirus-infected animals, with both classes of transcripts characterized by heterogeneous 5'-ends but a common termination site (Büscher et al., 1985; Cattaneo et al., 1984; Enders et al., 1985; Möröy et al., 1985). As shown in Figure 1.1, mammalian hepadnaviruses produce two major RNA transcripts: 1) a genome-sized RNA species with a reported length of 3.5 kb for HBV, 3.7 kb for WHV, and 3.5 kb for GSHV, and 2) subgenomic-sized RNA with a length of 2.1 kb for HBV, 2.1 kb for WHV, and 2.3 kb for GSHV (Cattaneo et al., 1983; Cattaneo et al., 1984; Enders et al., 1985; Möröy et al.,

1985; Will et al., 1987). Equal amounts of three major polyadenlyated transcripts can be detected by Northern blot analysis of mRNA obtained from DHBV-infected liver (Figure 1.1): 1) a genome-length transcript that is 3.5 kb in length, and 2) two subgenomic-sized transcripts that are 2.35 and 2.13 kb in length (Büscher et al., 1985). Terminally redundant by 120 - 270 nucleotides, the genome-sized RNA species common to all hepadnaviruses are slightly more than genome length, have heterogeneous 5'-ends, and are thought to function as the template for (-) strand DNA synthesis as well as the mRNA for the pre-C, C, and P gene translation products (Büscher et al., 1985; Cattanio et al., 1984; Will et al., 1987). The major subgenomic mRNA species are involved in the expression of viral surface antigens (Büscher et al., 1985; Cattaneo et al., 1984; Möröy et al., 1985; Will et al., 1987). Minor amounts of other hepadnavirus RNA transcripts have been detected in virus-infected livers and, in the case of the mammalian virus, are probably involved in synthesis of viral proteins present in low quantities such as the large surface antigen and the X protein (Cattaneo et al., 1984; Kaneko and Miller, 1988; Möröy et al., 1985).

2) ***Viral regulatory elements involved in gene expression***

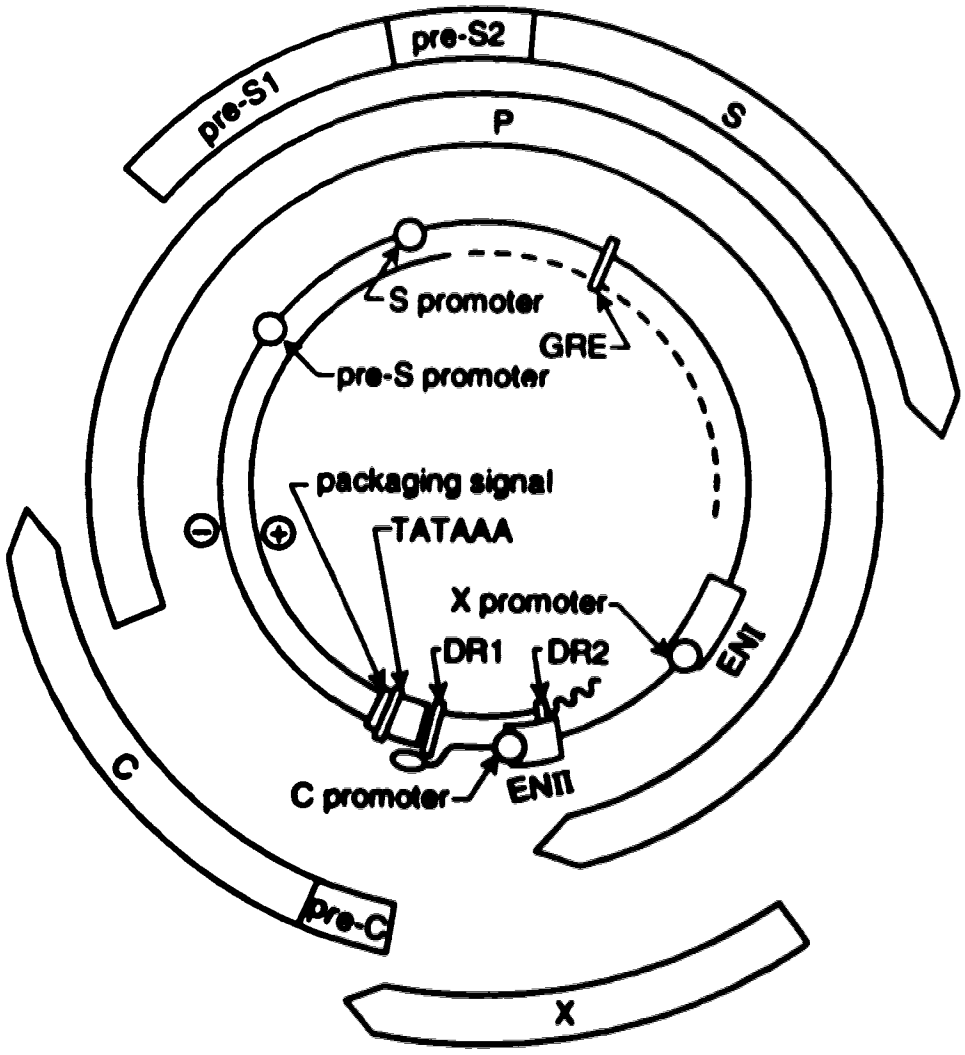
Several transcription-regulating elements have been identified in the hepadnavirus genome and are noted on the genome map shown in Figure 1.3. These functionally important elements include: 1) four promoters; 2) two regions with enhancer activity; 3) a glucocorticoid-responsive element; and 4) a polyadenylation signal (Ganem and Varmus, 1987; Laub et al., 1983; Shaul et al., 1985; Simonsen and Levinson, 1983; Rusenak and Ganem, 1990; Standring et al., 1984; Treinin and Laub, 1987; Tur-Kaspa et al., 1986; Yaginuma and Koike, 1989; Yee, 1989). A signal sequence neccessary for encapsidation of the RNA pregenome into immature core particles has been identified and is indicated in Figure 1.3 (Chiang et al., 1992). Since all areas of the DNA (-) strand function in a gene-coding capacity, the regulatory sequences necessary for virus replication and gene expression are localized within gene sequences.

e. ***Hepadnavirus gene products***

1) ***pre-S/S ORF: Surface antigens***

Several distinct forms of the viral surface antigen have been identified in serum samples from viremic carriers of HBV, WHV, GSHV, or DHBV (Feitelson et al., 1983; Schaeffer et al., 1986; Schlicht et al., 1987a). At least two glycosylation signals are encoded in the pre-S/S ORF of each virus, however the analogous carbohydrate-adorned proteins have been detected only in the mammalian viruses (Feitelson et al., 1983; Heermann et al., 1984; Schaeffer et al., 1986; Schlicht et al., 1987a; Stibbe and Gerlich, 1983). Of the four mRNAs generated from the HBV pre-S/S ORF (Figure 1.2B), the

Figure 1.3 - Regulatory elements of the HBV genome. The slightly longer-than-genome-length (-)-strand DNA and the incomplete (+)-strand DNA are indicated by the inner-most concentric circles and are labeled according to their respective polarities. Attached to the 5'-end of the (-)-strand DNA is the primer protein (O) and an RNA primer ( ) is associated with the 5'-end of the DNA (+) strand. The direct-repeat sequences DR1 and DR2 are located in the cohesive overlap region of the partially double-stranded, relaxed, circular genome. Several important regulatory elements are indicated on the HBV genome map, including: 1) TATAAA, the single polyadenylation sight present in the hepadnavirus genome; 2) ENI and ENII, two regions of the genome that exhibit enhancer activity; 3) the glucocorticoid resposive element (GRE), a region of the HBV genome involved in glucocorticoid stimulation of enhancer activity; and 4) a packaging signal necessary for the selective encapsidation of pregenomic RNA into immature nucleocapsid structures to occur. The four promoter regions identified in the HBV genome are indicated (O) on the map and labeled accordingly. Thick arrows representing the four ORFs of mammalian hepadnaviruses encircle the schematically drawn genome. The ORFs are: 1) the pre-S/S ORF which contains the pre-S1, pre-S2, and S gene sequences and encodes the virus surface antigens; 2) the P ORF which encodes the viral reverse transcriptase, DNA polymerase, and RNAse H, as well as the primer protein; 3) the pre-C/C ORF which contains the pre-C and C genes and is responsible for the nucleocapsid protein and the soluble HBeAg; and 4) the X ORF which encodes a regulatory protein. Information contained in this figure was obtained from the references listed in section I.B.1.d.2) of Chapter 1.


pre-S2
pre-S1
P
S
S promoter
GRE
pre-S promoter
packaging signal
TATAAA
X promoter
DR1
DR2
ENI
C promoter
ENII
C
pre-C
X

minor 2.4 kb species encodes the large HBsAg, whereas the major 2.1 kb mRNA component is probably a bifunctional template programmed for differential expression of the middle and major surface antigens via a ribosomal leaky scanning mechanism (Sheu and Lo, 1992). The main protein component of both the 22 nm spherical particles and the filamentous particles produced by HBV is the apparent 24 kDa major HBsAg and its glycosylated derivative, whereas the virion envelope contains a more balanced mixture of the large, middle and major surface antigens and their glycosylated analogs (Heermann et al., 1984; Peterson, 1981). In DHBV, the predominant protein in both virion envelopes and the large pleomorphic spherical envelope particles is the 17 kDa major surface antigen (Marion et al., 1983; Schlicht et al., 1987a). In addition, DHBV envelope structures contain a significant amount of a pre-S/S gene-derived protein (Pugh et al., 1987; Schlicht et al., 1987a). The two DHBV envelope proteins are translated from subgenomic-sized mRNA species with heterogeneous 5'-ends (Büscher et al., 1985). Synthesis of the 167 amino acid major surface antigen initiates at the first start codon present in the smallest of the three DHBV mRNAs while translation of the 328 amino acid preS/S protein begins at the second start codon in the intermediate-sized DHBV mRNA (Figure 1.1B) (Büscher et al., 1985; Mandart et al., 1984).

The major HBsAg can elicit a protective antibody response, with full antigenicity induced by disulfide bond-linked HBsAg dimers (Mishiro et al., 1980). Multiple HBV serotypes have been identified and are a result of the complex array of HBsAg group and subtype determinants that exist in HBV isolates (Tiollais et al., 1985). The middle and large surface antigens are also effective immunogens (Persing et al., 1986; Tiollais et al., 1985). In addition to the S gene-encoded 226 amino acids that the HBV surface antigens have in common at their carboxy-terminal ends, the amino acid sequences provided by the pre-S2 gene in the case of the middle protein, and the pre-S1 + pre-S2 genes in the case of the large protein, may have important biological consequences. The pre-S2 domain has a binding site for polymerized human serum albumin which may function as a molecular bridge indirectly attaching the virus to the hepatocyte via albumin receptors on the liver cell membranes (Machida et al., 1984; Pontisso et al., 1989a; Trevisan et al., 1982). In addition, an hepatocyte receptor recognition site has been identified in the unique pre-S1 portion of the large HBsAg (Neurath et al., 1986; Pontisso et al., 1989a; Pontisso et al., 1989b).

2) ***C ORF: Core and e antigens***

The core antigen is the structural component of the virus nucleocapsid and is a pre-C/C ORF product from translation of an mRNA containing the second of two in-frame initiation codons located in the pre-C/C ORF (figures 1.1A and 1.2B) (Weimer et

al., 1987). A characteristic set of basic amino acid residues located in the carboxy-terminal region of the 19-22 kDa core protein confers RNA- and DNA-binding affinity to this protein (Hatton et al., 1992; Nassal, 1992; Pasek et al., 1979; Petit and Pillot, 1985; Roossinck and Siddiqui, 1987). In addition to being required for RNA pregenome encapsidation, a portion of this arginine-rich carboxy-terminal region of the core protein is necessary for synthesis of the complete (+)-strand DNA, suggesting that it may be essential for HBV replication (Nassal, 1992). The core protein is phosphorylated *in vivo*. A protein kinase capable of phosphorylating this protein co-purifies with both the hepatitis B virions isolated from the plasma of HBV-infected patients and the core particles purified from HBV-infected liver tissue (Albin and Robinson, 1980; Gerlich et al., 1982; Roossinck and Siddiqui, 1987). Subcellular fractionation and indirect immunofluorescence studies of COS cells and HepG2 cells transfected with an HBV C gene-containing expression vector indicate that the phosphorylated core protein exists in the nucleus, the cytoplasm and the cytoplasmic membrane (Roossinck and Siddiqui, 1987). The 16 kDa soluble HBeAg is the other virus polypeptide known to be encoded by the pre-C/C ORF (Takahashi et al., 1983). Production of the HBeAg requires a mRNA template which includes the first of the two in-phase start codons in the pre-C/C ORF, therefore this mRNA is slightly longer than that used for production of HBcAg (figures 1.1A and 1.1B) (Weimer et al., 1987). Translation is initiated at the first AUG resulting in incorporation of amino acid residues encoded by both the pre-C and C-gene regions (Weimer at al., 1987). The pre-C region of the pre-C/C ORF encodes 29 amino acids, 19 of which function as a cleavable signal peptide that targets the e antigen to the cellular secretory pathway, thereby mediating polypeptide processing and secretion of HBeAg into the blood (Ou et al., 1986; Schlicht et al., 1987b; Standring et al., 1988). Although the presence of HBeAg in the blood is an indication of active viral replication, naturally occurring HBV variants that cannot express the pre-C gene are replication competent (Chen et al., 1992; Schlicht et al., 1987b; Schneider et al., 1991; Tong et al., 1991). The function of HBeAg in HBV infections is not known. However, evidence suggests that a more severe form of the disease is experienced by patients infected with HBV isolates unable to synthesize HBeAg (Brunetto et al., 1991; Kosaka et al., 1991; Liang et al., 1990). Undenatured core protein exhibits antigenic determinants that induce the production of antibodies by the infected host. When the core protein structure is relaxed by denaturation, an additional distinct antigenic region that reacts specifically with HBeAg antibodies is revealed (Takahashi et al., 1983). These HBeAg epitopes are present, but masked, in the core protein, and are exposed on the genetically related soluble HBeAg.

Genome-length hepadnavirus RNA is thought to function as mRNA for the separate translation of C and P gene products, and as the pregenomic RNA template required for virus replication (Will et al., 1987). Biosynthesis of the P gene product begins after ribosomal initiation at the second AUG signal in the P ORF via a leaky scanning mechanism or by *de novo* internal initiation (Figure 1.1A) (Chang et al., 1989; Lin and Lo, 1992; Schlicht et al., 1989b). When protein size is estimated using HBV nucleotide sequence data, ribosomal initiation at the second start codon would yield a protein product consisting of 786 amino acids whereas utilization of the first AUG would result in an 836 amino acid product (Mandart et al., 1984). The P gene encodes a protein moiety that acts as a primer for reverse transcription of the nascent DNA chain from the RNA pregenome (Bartenschlager and Schaller, 1988; Bosch et al., 1988; Wang and Seeger, 1992). In addition to its priming function, the P gene product is responsible for enzymatic functions that are involved in the hepadnavirus replication process, including: 1) reverse transcription of the viral DNA genome from an RNA pregenome, 2) degradation of the RNA pregenome by a viral RNase H, and 3) synthesis of a viral DNA second strand via a DNA polymerase activity (Bavand et al., 1989; Radziwill et al., 1990; Toh et al., 1983; Wang and Seeger, 1992).

3) ***P ORF: Protein primer and multifunctional nucleic acid polymerase***

On the basis of amino acid sequence homology comparisons between a deduced HBV P gene product and the known reverse transcriptase gene products of the retroviruses Moloney murine leukaemia virus and Rous sarcoma virus, Toh at al. (1983) provided circumstantial evidence that the HBV P ORF contains the nucleic acid polymerase gene. A more definitive link between the HBV P gene and a reverse transcriptase product was provided by experimental work that combined activity gel analysis of HBV nucleocapsid proteins with immunoblot analysis of the candidate protein for P gene-specific immunoreactivity (Bavand et al., 1989). Following polyacrylamide gel electrophoresis and *in situ* renaturation of the resolved nucleocapsid polypeptides, two discrete bands of reverse transcriptase activity were detected: an area of high reverse transcriptase activity in the 70 kDa region of the gel and a much lower indication of activity in the 90 kDa area of the gel (Bavand et al., 1989). When proteins from the 70 kDa region were electroeluted, re-electrophoresed, and transferred onto nitrocellulose they were found to immunoreact with antisera derived from synthetic peptides specified by the carboxy-terminal area of the P gene (Bavand et al., 1989). Similarly, an anti-polymerase monoclonal antibody recognized two proteins (93 kDa and 72 kDa) that were produced in insect cells using a baculovirus expression system that contained the HBV genome (McGlynn et al., 1992). Further evidence correlating the

hepadnavirus P gene with replication-specific catalytic activities has been obtained by mutational analysis of the gene (Chang et al., 1990; Radziwill et al., 1990). Using site-directed mutagenesis Radziwill and coworkers (1990) constructed a series of HBV mutants, introduced each mutated viral genome into the human hepatoma cell line HepG2, and analyzed the effect of the mutation by assaying for DNA polymerase activity in isolated cytoplasmic core particles. Their results suggest that the P protein consists of several functional domains, with the carboxy-terminal region active as an RNase H and the adjacent, penultimate region of the protein functioning as a reverse transcriptase/DNA polymerase (Radziwill et al., 1990). A similar arrangement of P protein functional domains was observed when the DHBV genome was explored by mutational analysis (Chang et al., 1990). Translation of a P gene product appears to result in a multifunctional polyprotein that is arranged with the primer protein sequences at the amino-terminal region of the polypeptide, physically separated from the reverse transcriptase/DNA polymerase and RNase H domains by a protease-sensitive spacer domain (Bartenschlager and Schaller, 1988; Chang et al., 1990; Radziwill et al., 1990).

Recently, two independent reports have described the synthesis of an enzymatically active nucleic acid polymerase using an *in vitro* translation system (Howe et al., 1992; Wang et al., 1992). In both cases, expression of the DHBV polymerase gene was accomplished using a cell-free rabbit reticulocyte lysate and an RNA template containing a copy of the DHBV genome. Howe and co-workers (1992) identified a 79 kDa radiolabeled protein following SDS-polyacrylamide gel electrophoresis and subsequent autoradiography of a sample of the ^{35}S-methionine-supplemented reticulocyte lysate reaction mixture. Both DNA polymerase and reverse transcriptase activities were detected when the lysate reaction mixture was assayed for the ability to incorporate radiolabeled nucleotides into DNA- or RNA-primed templates. Similarly, Wang and Seeger (1992) utilized SDS-polyacrylamide gel electrophoresis and autoradiography of an ^{35}S-methionine-labeled reticulocyte lysate reaction mixture to demonstrate clearly the presence of a major protein component of approximately 90 kDa in size and a faster migrating, minor component. In addition, nucleotide incorporation into a nascent DNA chain could be catalyzed by the *in vitro*-synthesized polymerase and this reaction was found to require magnesium and to be RNA-directed and temperature- and time-dependent (Wang and Seeger, 1992). In support of other published reports that link a P gene-encoded product to the priming of DNA synthesis, the *in vitro*-synthesized polymerase was shown to form a covalent linkage with dGTP the first nucleotide of the (-) strand DNA (Bartenschlager and Schaller, 1988; Boech et al., 1988; Wang and Seeger, 1992).

Evidence suggests that the P gene product is a required element for RNA pregenome encapsidation (Bartenschlager et al., 1990; Hirsch et al., 1990). Preferential encapsidation of the pregenomic RNA from which the P polypeptide has been translated may occur (Bartenschlager et al., 1990; Hirsch et al., 1990). Once the pregenome and the associated viral replication machinery are encapsidated, it is not known if the P gene polyprotein remains intact or if the primer protein connection to the enzymatic domains is severed. Results presented by Wang and Seeger (1992) argue that the polymerase protein retains a covalent association with the newly synthesized DNA. Once enclosed in the viral integument, the P protein is tightly associated with the genomic template and with the nucleocapsid structure itself as is shown by the inability of an enzymatically active DHBV polymerase to dissociate from permeabilized core particles or to accept exogenously supplied templates made available by permeablization of the nucleocapsids (Radziwill et al., 1988).

4) ***X ORF: X antigen***

Antibodies specific to an X gene product have been detected in the serum of some HBV-infected individuals, indicating that this viral gene is expressed during infection (Kay et al., 1985; Meyers et al., 1986). Only the mammalian hepadnavirus genomes contain the X ORF (shown in Figure 1.1) which encodes one or more proteins that can act in *trans* to regulate the transcription of a variety of cellular and viral genes that are transcribed by RNA polymerase II and III (Aufiero and Schneider, 1990; Kwee et al., 1992; Spandau and Lee, 1988; Twu et al., 1989; Twu and Schloemer, 1989; Wu et al., 1990). An expression vector containing the X gene has been used to achieve the over-production by *E. coli* of a 16.5 kDa polypeptide that reacts with antibodies raised to X gene-specified synthetic peptides (Wu et al., 1990). The *E. coli*-produced X protein was found to exhibit protein kinase activity, affecting the phosphorylation of serine and threonine residues on substrates such as the X polypeptide, histones, and casein. Similarly, an X protein with serine/threonine protein kinase activity was identified in a preparation of virions purified from the plasma of an HBV carrier (Wu et al., 1990).

Evidence suggests that the highly conserved X gene is not an essential element for virus propagation (Blum et al., 1992). Cell cultures transfected with an HBV genome modified by site-directed mutagenesis so as not to express the X protein are capable of producing wild-type levels of viral proteins and exported virions (Blum et al., 1992). Alternatively, the X protein, which is frequently found in the liver tissue of patients with chronic liver disease, may be an important aspect in the pathogenesis of chronic infection (Wang et al., 1991). There is a relationship between the presence of X antigen and markers of HBV replication in liver samples from individuals with chronic HBV

infection (Haruna et al., 1991). As yet there is little evidence correlating X gene expression to the development of hepatocellular carcinoma (HCC), a disease associated with HBV and WHV, but not the avian virus. Synthetic peptides corresponding to defined regions of the X gene are more likely to elicit an antibody response in blood from individuals with HCC than HBV-infected patients that do not exhibit HCC (Moriarty et al., 1985). In a study designed to probe the relationship of HBxAg production with development of HCC, Lee and co-workers (1990) generated transgenic mice capable of synthesizing HBV X protein in the liver. However, the level of X protein expression achieved in these animals did not result in the development of tumors. In contrast, another research group reported that transgenic mice carrying the X gene were found to develop liver cancer (Kim et al., 1991).

2. Hepadnavirus Replication

Hepadnavirus-infected cells contain several specific forms of viral nucleic acids (Mason et al., 1983; Miller and Robinson, 1984; Ruiz-Opaza et al., 1982; Weiser et al., 1983). Cell fractionation using liver tissue from patients chronically infected with HBV indicates that covalently closed, circular DNA (cccDNA), a supercoiled form of the genome, is found exclusively in the nuclei of infected cells (Miller and Robinson, 1984). In addition, a small amount of relaxed, circular DNA (rcDNA), the DNA species found in infectious virions, can be found in the nuclear fraction of these cells. All detectable viral DNA in the cytoplasm of infected cells is segregated in immature viral core particles (or nucleocapsids), which also contain RNA and a DNA polymerase activity (Enders et al., 1987; Miller et al., 1984a; Miller et al., 1984b; Miller and Robinson, 1984; Summers and Mason, 1982). Three distinct forms of viral DNA have been identified in the cytoplasmically-located core particles of hepadnavirus-infected liver tissue: 1) relaxed, circular DNA (rcDNA); 2) double-stranded, linear DNA (dsDNA); and 3) single-stranded DNA (ssDNA) (Miller and Robinson, 1984; Summers and Mason, 1982). In addition to these distinct forms of viral DNA, core particles contain a heterogeneous collection of DNA molecules that represent DNA intermediates formed during replication (Mason et al., 1982; Weiser et al., 1983).

Initially, the study of hepadnavirus replication was hampered by an inability to propagate the virus in tissue culture. As a result, innovative mechanistic studies by Summers and Mason (1982) and Mason et al., (1982) made use of immature viral core particles isolated from DHBV-infected duck liver. These studies established that replication of the avian form of the DNA virus proceeds through the use of an RNA intermediate. This unusual replication process requires a reverse transcription step similar to that employed by the RNA retroviruses and caulimoviruses, a category of plant

DNA viruses (Reviewed by Mason et al., 1987). A model for hepadnavirus replication was proposed by Summers and Mason (1982), and since that time the model has been supported by experimental evidence contributed by many research laboratories (Lien et al., 1986; Tuttleman et al., 1986a; Seeger et al., 1986; Will et al., 1987).

The complicated hepadnavirus replication cycle can be divided into four basic steps: 1) conversion of the relaxed, circular form of the incoming DNA to a covalently closed, circular DNA (cccDNA); 2) synthesis of an RNA copy of the genome (known as the pregenome), followed by encapsidation of the pregenome in cytoplasmic core particles; 3) reverse transcription of a DNA strand (the minus strand) from the RNA pregenome template, with DNA synthesis primed by a virus-encoded protein and DNA synthesis catalyzed by a viral enzyme; 4) synthesis of a complimentary (+)-strand DNA from the DNA (-)-strand, with synthesis of the second DNA strand primed by an RNA pregenome fragment (Ganum and Varmus, 1987). A more in-depth discussion of hepadnavirus replication is presented below.

a. ***Virus entry and formation of covalently closed, circular DNA***

Very little is known about the early events of hepatocyte infection with an hepadnavirus, specifically: 1) How does the virion gain entry into a susceptible host cell? 2) How and when is the virion uncoated? And 3) How is the incoming virus genome targeted to the host cell nucleus? Following virus entry into the cell, one of the first indications of viral infection is the intracellular appearance of cccDNA, a novel form of the viral DNA that is not associated with infecting virions (Mason et al., 1983; Tagawa et al., 1986; Tuttleman et al., 1986b). It is thought that the relaxed, circular form of the virus genome, which is synthesized in the cytoplasm of infected cells and found in virions, is the immediate precursor of the cccDNA species (Tuttleman et al., 1986a). Formation of cccDNA from rcDNA would require that several modifications be made to the relaxed, circular genome structure, including: 1) extension of the incomplete DNA (+)-strand; 2) excision of the two DNA primers, the protein primer attached to the (-)-strand and the RNA primer on the (+)-strand; 3) removal of the DNA (-)-strand terminal redundancy; and 4) covalent closure of the circular genome (Ganum and Varmus, 1987). It is unclear whether these alterations involve the efforts of cellular or viral components.

b. ***Transcription of the cccDNA template into pregenomic RNA and encapsidation of pregenomic RNA into core particles***

Host cell RNA polymerase II catalyzes the synthesis of mRNA from viral cccDNA (Rall et al., 1983). As discussed in Section B.1.d.1., hepadnaviruses produce subgenomic- and genomic-sized transcripts, with both classes of transcripts characterized by heterogeneous 5'-ends but a common 3'-termination site (Blächer et al., 1985;

Cattanio et al., 1984; Enders et al., 1985; Möröy et al., 1985). The larger class of transcripts are terminally redundant, exceeding the actual genome length by 120 - 270 nucleotides, depending on the type of hepadnavirus (Büscher et al., 1985; Enders et al., 1985; Möröy et al., 1985; Will et al., 1987). Synthesis of the three genome-sized transcripts begins within or immediately upstream of the pre-C gene (Figure 1.2C) with synthesis proceeding along the complete genome template and terminating at a position within the C-gene, just beyond the solitary polyadenylation site (Figure 1.3) that was ignored on the first passage around the genome. Included in all of the genome-sized RNA transcripts is a specific encapsidation signal sequence which is encoded in the pre-C region of the genome (Figure 1.3) and is located near the 5'-end of each transcript (Chiang et al., 1992; Junker-Niepmann et al., 1990). However, of the three genome-length transcripts produced (illustrated in Figure 1.2C), only the smallest RNA species is selectively packaged into immature core particles (Enders et al., 1987; Will et al., 1987). Selection of the RNA species to be encapsidated may depend on whether the encapsidation signal is obscured by translational activity in the immediate area of the signal sequence, a possibility for the two larger mRNAs which initiate upstream of pre-C gene start codons, but not for the shorter C gene mRNA which only has start codons located downstream of the encapsidation signal (Enders et al., 1987; Nassal et al., 1990). Using hepatoma cells transfected with a series of pre-C-mutated HBV genomes, Nassal and co-workers (1990) employed RNAase protection assays to demonstrate that mRNA transcripts with translating 80S ribosomes in the region of the encapsidation signal were excluded from encapsidation. Nucleic acid encapsidation occurs in the host cell cytoplasm and requires specific interaction of the viral RNA pregenome with the arginine-rich carboxy-terminal region of the viral core protein (Birnbaum and Nassal, 1990; Nassal, 1992). The virus P gene product is enclosed in the nucleocapsid structure and may also play an essential role in the selective encapsidation of pregenomic RNA (Bartenschlager et al., 1990; Hirsch et al., 1990).

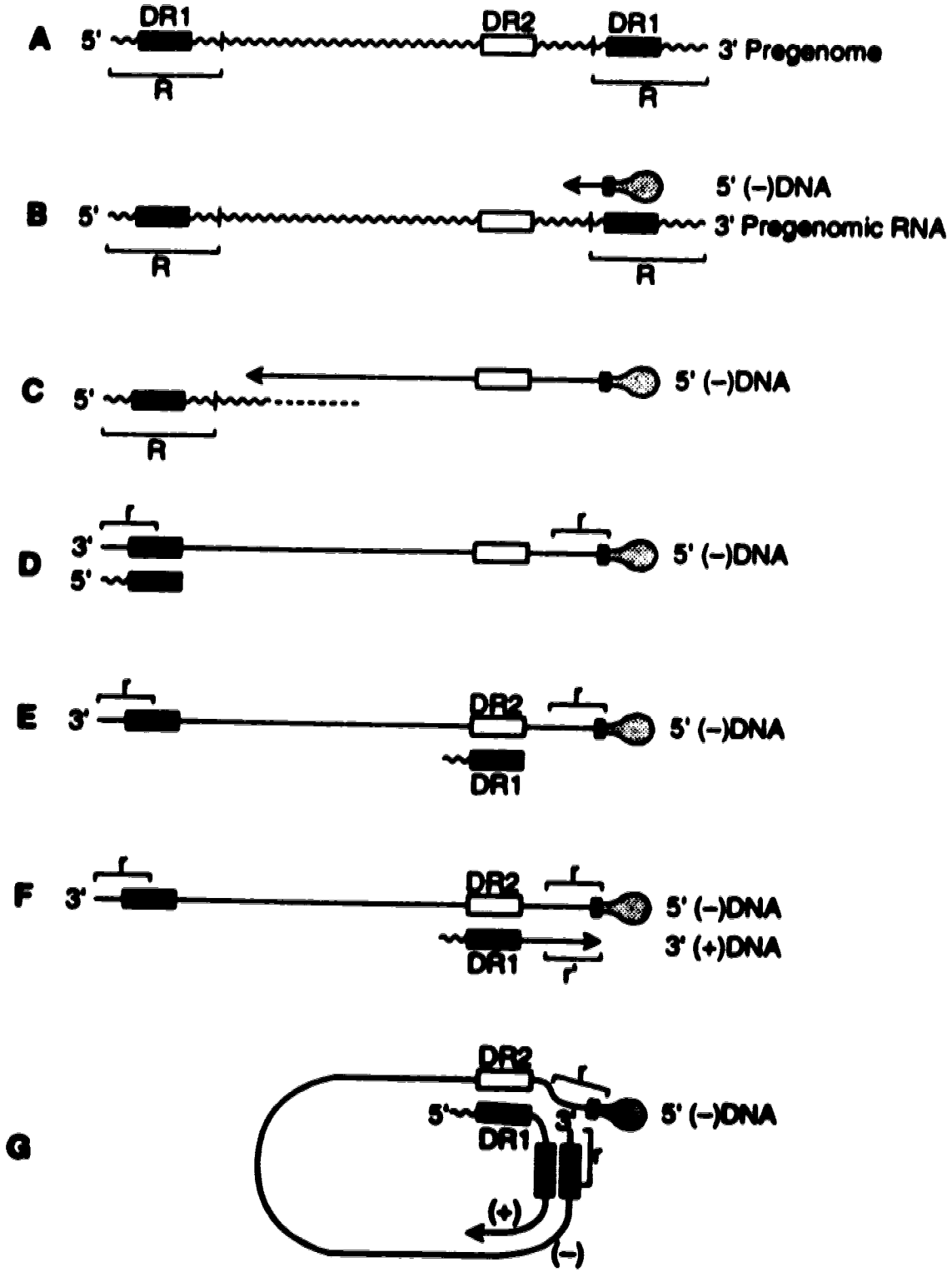
e. *Synthesis of (-)-strand DNA from the pregenomic RNA template*

Not only is the core protein the structural unit of the nucleocapsid and an important element in pregenome encapsidation, but it may also be an essential component involved in virus replication and virion maturation (Hatton et al., 1992; Nassal, 1992; Petit and Pillot, 1985; Schlicht et al., 1989a; Yu and Summers, 1991). Nucleic acid binding studies using truncated HBV core proteins expressed in *E. coli* have identified three DNA-specific binding regions as well as the RNA-binding sequence in the protamine-like carboxy-terminal region of the core protein (Hatton et al., 1992). Hatton and co-workers (1992) suggest that the core protein may be an integral part of the

replication complex, facilitating replication by interaction of the core protein DNA-binding motifs with the DNA products of replication (Hatton et al., 1992).

Enclosed within the immature virus core particle, the RNA pregenome serves as a template for synthesis of the viral DNA (-)-strand. The 5'-end of virion (-)-strand DNA has been mapped to a position within the DR1 sequence of the genome, suggesting that synthesis of the nascent DNA (-)-strand begins within one of the two copies of the DR1 sequence present in the terminally redundant RNA pregenome (Figure 1.4B) (Molnar-Kimber et al., 1984; Seeger et al., 1986; Will et al., 1987). As can be seen in Figure 1.4A and B) the 5'- and 3'-ends of pregenomic RNA contain a duplicated section of the virus genome that includes the DR1 sequence. The terminal repeat sections of the pregenome are designated "R". Although both ends of the RNA pregenome contain the DR1 sequence, initiation of DNA synthesis occurs within the DR1 located at the 3'-end of the pregenome, allowing elongation to proceed to the 5'-end of the pregenome template without interruption (Figure 1.4B-D) (Seeger and Maragos, 1990). The completed DNA (-)-strand contains a short terminal repeat (r) consisting of the 7 - 10 nucleotides located between the DR1 start position and the 5' edge of the R region (Figure 1.4D) (Lien et al., 1987; Seeger et al., 1986; Seeger and Maragos, 1990; Will et al., 1987). Negative-strand DNA synthesis is thought to be primed by a P gene-encoded protein that is covalently linked to the DNA (Bartenschlager and Schaller, 1988). In almost all hepadnavirus DNA isolates studied, the 5'-terminal nucleotide of the (-)-strand DNA is dGMP (Bartenschlager and Schaller, 1988; Lien et al., 1987; Saldanha et al., 1992). Lien and co-workers (1987) have demonstrated that the 5'-terminal deoxyguanosine nucleotide of DHBV (-)-strand DNA is covalently linked to the primer protein (Lien et al., 1987). Progressive elongation of the protein-primed DNA (-)-strand is probably accompanied by commensurate degradation of the RNA template (Figure 1.4C) (Summers and Mason, 1982). The carboxy-terminal region of the P gene-encoded polymerase contains the putative RNase H sequences, an arrangement that is similar to that found in the genetically-related retroviral reverse transcriptase (Radziwill et al., 1990; Toh et al., 1983). Kinetic studies on a retrovirus reverse transcriptase have established that DNA polymerization and RNA cleavage activities are independent of one another, and that in the native form of the protein, the corresponding enzyme domains are separated by a distance of about 19 RNA/DNA-heteroduplexed deoxynucleotides (Kati et al., 1992). Preparation and isolation of an enzymatically active hepadnavirus polymerase from virions or infected livers as well as from expression in heterologous systems has been met with difficulties. However, recent success in the expression of an enzymatically active DHBV polymerase protein will

Figure 1.4 - Replication of the hepadnavirus genome. Pregenomic RNA is transcribed from the covalently closed, circular DNA genome template in the host cell nucleus. The linear pregenome is transferred to the cell cytoplasm and, along with several other virus-specific components is sequestered within an immature core particle, the site of viral replication. Panel A: Important structural features of pregenomic RNA which is drawn as a wavy line, include: 1) R, a 120 - 270 nucleotide terminal redundancy arising from transcription of a slightly longer-than-genome-length RNA; 2) two copies of the direct repeat DR1 are located in the R region located at the 5'- and 3'-ends of the RNA transcript; and 3) one copy of the direct repeat DR2 is located in the 3-region of the RNA, but is outside of the R sequence. Panel B: Reverse transcription of the pregenome into DNA begins with synthesis of the protein-primed DNA (-)-strand initiating within the DR1 sequence located at the 3'-end of the RNA template. Panel C: Ribonuclease-catalyzed degradation of the pregenome probably occurs as DNA synthesis proceeds toward the RNA 5'-terminus. Panel D: The completed DNA (-)-strand contains "r", a short terminal repeat representing the section of nucleotides copied from the pregenome R regions. The completed DNA strand is base-paired to a short RNA oligomer, a pregenome remnant consisting of nucleotides from the RNA 5'-terminus to the 3'-edge of the DR1 sequence. Panel E: The DR1-containing RNA fragment shifts from the 3'- to the 5'-end of the DNA molecule where the RNA DR1 can base-pair with the DNA template copy of DR2. Panel F: Synthesis of (+)-strand DNA is primed by the RNA oligomer and DNA polymerization proceeds to the 5'-end of the DNA template. Panel G: The replicating complex substitutes the 3'-end of the (-)-strand DNA for the expended 5'-portion of the DNA template, thereby effecting an intramolecular template transfer. This structural rearrangement leads to the formation of a relaxed, circular DNA conformation. Prior to virion maturation and export, continued DNA polymerase activity results in (+)-strand DNA molecules of variable length. Information contained in this figure was obtained from the following references: Ganem and Varmus, 1987; Lien et al., 1987; and Seeger et al., 1986.


A
5'
DR1
DR2
DR1
3' Pregenome
R
R
B
5' (–)DNA
5'
3' Pregenomic RNA
R
R
C
5'
5' (–)DNA
R
D
3'
5'
5' (–)DNA
E
3'
DR2
5' (–)DNA
DR1
F
3'
DR2
5' (–)DNA
3' (+)DNA
DR1
G
DR2
5'
5' (–)DNA
DR1
3'
(+)
(–)

allow for enzyme characterization studies to proceed (Howe et al., 1992; Wang and Seeger, 1992).

d. *Synthesis of (+)-strand DNA from the nascent(-)-strand DNA*

The completed DNA (-)-strand contains a short terminal repeat (r), two direct repeats DR1 and DR2, and the primer protein attached to the 5'-end of the DNA strand (Figure 1.4D). An RNA fragment is generated from the capped 5'-end of the pregenome template by cleavage at the 3'-boundary of DR1 (Seeger and Maragos, 1989; Staprans et al., 1991). The DR1-containing RNA oligomer is then shifted from the 3'-end of (-)-strand DNA to base-pair with the complementary 5'-situated DR2 of the DNA template (Figure 1.4E) (Lien et al., 1986; Seeger et al., 1986; Seeger and Maragos, 1989; Staprans et al., 1991; Will et al., 1987). The mechanisms by which primer cleavage and translocation occur is unknown. However, derivation of the RNA fragment from the 5'-terminus of the pregenome is supported by evidence that the 17 - 19 base ribonucleotide has a 5'cap structure as would be expected for the pregenomic RNA and the nucleotide sequence of the RNA fragment corresponds to that of the pregenomic RNA 5'-end (Büscher et al., 1985; Enders et al., 1985; Lien et al., 1986; Lien et al., 1987; Seeger et al., 1986; Will et al., 1987). Synthesis of (+)-strand DNA is primed by the translocated RNA oligomer with nucleotide incorporation initiating at the 3'-boundary of DR2 and continuing to the protein-bound 5'-end of the (-)-strand DNA template (Figure 1.4F) (Lien et al., 1986; Seeger et al., 1986). At this point, an intramolecular transfer of the replicating complex to the 3'-end of the (-)-strand DNA template is necessary if replication is to continue. This transfer may be assisted by the base-pairing of region r at the 3'-end of the nascent (+)-strand DNA with the complementary copy of the r sequence located at the 3'-end of the (-)-strand DNA template (Ganem and Varmus, 1987). As a result of hydrogen-bonding between the r regions as well as between the RNA primer copy of DR1 and the DR2 from the (-)-strand DNA template, the replicating structure is maintained in an open circular conformation (Figure 1.4G). Continued DNA polymerase activity results in (+)-strand DNA molecules of variable length, with exported virions characterized as having partially double-stranded, relaxed, circular genomes (Delius et al., 1983; Hruska et al., 1977; Landers et al., 1977; Sattler and Robinson, 1979; Summers et al., 1975). Experimental introduction of point mutations in or around the RNA primer DR1 sequence illustrates that translocation of the primer can be prevented (Staprans et al., 1991). Elongation of the DNA (+)-strand can initiate at the 3'-end of an untranslocated primer, yielding unit-length, linear DNA with the same apparent structure as linear DNA occasionally found in preparations of wild-type virus (Staprans et al., 1991).

Hepadnavirus rcDNA from infecting virions is modified in the host cell, yielding the cccDNA form found exclusively in the cell nucleus (Miller and Robinson, 1984; Tuttleman et al., 1986a). Subsequent to infection, the nuclear pool of cccDNA is amplified more than 50-fold by intracellular *de novo* synthesis of viral DNA via the reverse transcription pathway (Tuttleman et al., 1986a; Wu et al., 1990). Measurement of the amount of viral cccDNA and RNA produced in primary duck hepatocyte cultures infected with wild-type DHBV or envelope gene-defective DHBV genomes indicates that the pre-S/S protein is responsible for regulating cccDNA amplification (Summers et al., 1990; Summers et al., 1991).

C. Immunological and Chemical Approaches to the Treatment of HBV Infection

Infection with HBV can be prevented by prophylactic immunization with a vaccine that contains HBsAg particles derived from either the plasma of asymptomatic, chronic HBV-infected individuals or yeast that has been transfected with a recombinant plasmid that carries the HBsAg gene (Heptavax or Recombivax HB, respectively) (Gerety, 1985; Maupas et al., 1978; Murphy and Chanock, 1990; Szmuness et al., 1981; Werner and Grady, 1989). Unfortunately, the availability of this expensive vaccine has been limited, with immunoprophylaxis given preferentially to high-risk populations such as health-care personnel, infants born to HBsAg-positive mothers, sexual partners of HBsAg carriers, homosexuals, and i.v. drug users (Alter et al., 1990; Gerety, 1985). An effective, inexpensive vaccine for HBV would reduce the risk of HBV infection for many but would not address the disease-induced health problems in individuals already harboring the virus. It is these HBV-positive individuals who would benefit most from antiviral agents designed to control or eradicate the virus. As yet, there is no satisfactory antiviral chemotherapy for HBV infections.

1. Human Clinical Trials

Until recently, research in the area of anti-HBV therapy has been limited by the lack of an appropriate animal or cell culture model. In fact, the earliest antiviral studies were conducted as clinical trials on chronic HBV-infected subjects. These early investigations focused on two types of potential therapeutic agents for the control and/or elimination of HBV infection: 1) immune modulators, including corticosteroids and interferons (Greenberg et al., 1976; Lam et al., 1981; Weimar et al., 1980) and 2) nucleoside analogs, especially 9-β-D-arabinofuranosyl adenine (araA), and araAMP, a more water-soluble, monophosphate form of araA (Bassendine et al., 1981; Hoofnagle et al., 1984; Scullard et al., 1981; Tyrrell et al., 1988; Weller et al., 1985).

In general, therapeutic studies in chronic hepatitis B virus infected patients have been aimed at eliminating viral replication. The therapeutic end point is determined by

measuring pre- and post-therapy serum levels of: 1) hepatitis B virus DNA (HBV-DNA), 2) the virus-associated DNA polymerase (DNAP) activity , 3) hepatitis B virus e-antigen (HBeAg), 4) hepatitis B virus surface-antigen (HBsAg), 4) antibodies to the e-antigen (anti-HBe), and 5) antibodies to the surface-antigen (anti-HBs) (Perrillo, 1989). Active liver disease is often present in chronic hepatitis B carriers that have serum markers of active viral replication including HBV-DNA, DNAP activity, and HBeAg. Cessation of viral replication in the liver is associated with loss of HBeAg from the serum and may also be accompanied or followed by development of anti-HBe (Di Bisceglie et al., 1987). These events are often preceded by a temporary exacerbation of immune-mediated liver damage, as measured by increased serum levels of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) (Alexander et al., 1987; Hoofnagle et al., 1988). Follow-up of antiviral treatment studies has shown that a certain percentage of patients that have seroconverted to an HBeAg-negative state experience reversion to an HBeAg-positive status (Korenman et al., 1991; Lok et al., 1988). However, as reported in at least one study, 65% of the treatment-induced HBeAg-negative subjects became HBsAg-negative after having been HBeAg-negative for 3 years (Korenman et al., 1991). Therefore, seroconversion to an HBeAg-negative state indicates at least a temporary hiatus in viral replication and some patients may progress to the more permanent HBsAg-negative state.

Clearance of circulating HBsAg suggests complete resolution of the viral infection, a state that is hard to achieve if the viral DNA has integrated into the host genome (Perrillo, 1989; Perrillo et al., 1990). Viral DNA integration into the host chromosomes is thought to result in preservation of an incomplete viral genome that is incapable of supporting production of complete, infectious viral particles but likely to encode for a functional, viral surface antigen (Nagaya et al., 1987; Robinson, 1990; Shaul et al., 1984). The disappearance of serum HBsAg may signal termination of the HBsAg carrier state. This concept is supported by recent evidence correlating the loss of circulating HBsAg with the disappearance of immunochemical staining for HBsAg in liver biopsy tissue (Perrillo and Brunt, 1991). Antiviral therapy may not have an impact on integrated forms of HBV but it may decrease the opportunity for random integration to occur.

a. *Immune-modulators*

The potential value of immune-modulators as antiviral agents is easy to imagine. In reality, successful application of this type of therapy is hampered by an incomplete understanding of the immune response in viral infections. It is not known what dictates the immune response that leads to resolution of an HBV infection; how does the

environment differ from that which allows the establishment of persistent infection? Effective manipulation of the host immune response to HBV with immune modifiers depends upon a detailed understanding of the immune system function in viral infections.

1) *Corticosteroid-treatment*

Although immunosuppressive therapy with corticosteroids has proven efficacious in autoimmune chronic hepatitis (HBsAg negative), these compounds are ineffective or even harmful to patients suffering from HBV-linked chronic hepatitis (Hoofnagle et al., 1986; Lam et al., 1981). When a corticosteroid treatment regime in HBsAg-positive patients is discontinued, there can be a transient reduction in HBV-DNA and DNA polymerase activity (Hoofnagle et al., 1986; Scullard et al., 1981). This observation has lead to protocols designed to "prime" the immune system by corticosteroid administration and withdrawal. The "primed" system is then subjected to a cycle of treatment with another immunoactive compound such as interferon. However, in one major study, prednisone-priming followed by interferon administration was found to be no more successful than treatment with interferon alone (Perrillo et al., 1990).

2) *Interferon-treatment*

Interferons are naturally occurring, host-coded proteins that, upon induction, can initiate an antiviral response in some cells and have immunomodulatory effects in others (Joklik, 1990). Numerous viral infections are accompanied by detectable serum levels of interferon, in contrast to the undetectable levels of interferon in the serum of healthy individuals (Levin et al., 1981). A therapeutic role for interferon in chronic hepatitis is supported by data that suggests defective production of α-interferon in patients with chronic viral hepatitis (Davis et al., 1984; Pignatelli et al., 1986; Poitrine et al., 1985).

Although more than a decade of research has been invested in clinical trials designed to evaluate the efficacy of interferon treatment of chronic HBV infections, its benefits are still uncertain (Alexander et al., 1987; Greenberg et al., 1976; Koremman et al., 1991; Lok et al., 1988; Perrillo et al., 1990; Scullard et al., 1981; Weimar et al., 1980). Evaluation of the accumulated clinical data has been difficult for a number of reasons. Many clinical trials lacked proper control groups and/or had limited numbers of patients (Perrillo, 1989). The well-documented spontaneous seroconversion from HBeAg-positive to anti-HBe-positive in untreated HBV chronic carriers makes the inclusion of patient-matched, untreated controls necessary for drug therapy studies (Dusheiko et al., 1985; Scullard et al., 1981; Viola et al., 1981). In addition, a large body of the literature on interferon therapy is hard to interpret due to the use of interferons from various sources and of differential purity, a wide range of drug dosage schedules and treatment cycles, and variation in patient characteristics such as pre-therapy levels of

HBV-DNA and circulating liver enzyme activities, as well as parameters such as patient sex, lifestyle, race, and co-infection with other agents (Davis and Hoofnagle, 1986; Perrillo, 1989). These early studies suggested that certain parameters were more often associated with long-term remission of disease (Peters et al., 1986). For instance, it was found that a positive response to interferon therapy occurred more often in females than males, that cases with active rather than persistent hepatitis were more likely to experience remission of disease, and that treatment responders exhibited lower initial serum levels of DNAP and HBV-DNA than non-responders (Peters et al., 1986; Scullard et al., 1981).

More recent studies have provided encouraging results with respect to HBV-infected patient response to treatment with recombinant α 2b-interferon (Hoofnagle et al., 1988; Perrillo et al., 1990). Hoofnagle and co-workers (1988) found that approximately one-third of the chronic hepatitis B patients treated for 4 months with α-interferon (5 million units (MU)/day or 10 MU every other day) experienced remission of disease as measured by loss of HBV-DNA, improved ALT and AST levels, and loss of HBeAg in serum samples collected for up to 8 months post-treatment. In addition, the liver histology of all but one of the responders exhibited loss of HBcAg from the tissue and a decrease in hepatic inflammation (Hoofnagle et al., 1988). Similarly, Perrillo and colleagues (1990) found that 5 MU/day of α-interferon given over a four month period resulted in 35-40% HBeAg seroconversion and a sustained loss of HBV-DNA in 40-50% of those treated. In addition, HBsAg disappeared in one-third of the responders. These results were based on follow-up examinations performed 6 months post-interferon treatment (Perrillo et. al., 1990).

Not all clinical trials with α-interferon have been as effective in achieving sustained remission of chronic type B hepatitis. In a study involving exclusively Chinese patients with chronic hepatitis B it was found that α 2-interferon (2.5, 5, or 10 MU/square meter) given 3 times a week for 12 to 24 weeks had little long-term effect on HBV suppression (Lok et al., 1988). One year after treatment had been initiated, 28% of the patients receiving the highest interferon dose exhibited a sustained clearance of HBeAg but at 24 months that number had decreased to 20% (Lok et al., 1988). Although none of the 18 positive controls showed loss of HBeAg after one year, there was spontaneous clearance in 1 out of 9 controls evaluated at the end of 2 years. Such small sample numbers make it hard to determine an accurate baseline for spontaneous clearance. However a large, 5 year retrospective study of Chinese patients with chronic hepatitis B indicated a 17% cumulative probability of spontaneous HBeAg clearance at the end of one year, with the probability increasing to 30% and 34% by the end of the

second and third years, respectively (Lok et al., 1987). It was found that the probability of spontaneous HBeAg clearance increased with patient age (Lok et al., 1987). In addition, reversion to an HBeAg-positive state occurred in 32.3% of the patients who had cleared HBeAg, but 31.8% of these reversions were only transient in nature (Lok et al., 1987). The study by Lok and co-workers (1987) emphasizes the importance of adequate controls and follow-up in order properly to evaluate antiviral treatment regimens designed for the treatment of a disease that induces such a complicated and changing serological profile.

Of the many variables that may play a role in establishing the HBV carrier-state, the mode of transmission and the age of the individual at the time of virus aquisition are important factors to consider. Most adults with a primary HBV infection clear the virus and establish lasting immunity. Although only 5 - 10% of infected adults become chronic carriers of the virus, perinatal infection leads to an overwhelming 85 - 90% carriers (Stevens, 1982). There is a strong correlation between chronic HBV infection in infants and the presence of serum HBeAg in the mother at parturition (Stevens, 1982). Infection with the virus at an early age may lead to a class of chronic hepatitis that responds poorly to antiviral drug therapy. The study of Chinese patients by Lok and associates (1988) revealed that antiviral drug therapy in an ethnic population known to have a high incidence of perinatal infection with HBV was ineffective (Derso et al., 1978; Stevens, 1982).

The antiviral activity of interferon is thought to be concentration-dependent (Perrillo et al., 1990; Scullard et al., 1981). In the Perrillo study (1990), treatment with 5 MU/day of interferon correlated with remission of HBV-induced disease, whereas treatment with 1 MU/day of interferon gave results that were not statistically different from those of the untreated controls. Maximal inhibition of HBV levels in the serum seems to require an interferon dose range of 3-20 MU/day, with no additional benefit being gained from higher doses (Peters et al., 1986). Determination of the minimal effective dose is of special interest in the case of α-interferon because maximal antiviral effect requires prolonged treatment (at least several months) and the side-effects associated with use of this compound are more pronounced at higher doses (Peters et al., 1986). Adverse effects caused by interferon include fever, influenza-like illness, fatigue, hair loss, weight loss, psychiatric problems, bone marrow suppression, leukopenia, and thrombocytopenia (Hoofnagle et al., 1988; Lok et al., 1988; Peters et al., 1986; Renaut et al., 1987). There have also been several reports linking interferon treatment with the development of autoimmune disorders (Abdi et al., 1986; Rönnblom et al., 1991).

b. *Nucleoside-analogs*

1) *Treatment with ara-A*

The purine nucleoside ara-A exerts selective antiviral activity against many DNA viruses and has proven to be an important clinical agent in the treatment of life-threatening herpesvirus infections such as herpes simplex encephalitis and neonatal herpes (Hirsch and Schooley, 1983a and 1983b; Whitley et al., 1980). Preliminary work with ara-A therapy in chronic hepatitis B patients suggested that this compound could also have a useful role in the management of HBV infections (Chadwick et al., 1978; Pollard et al., 1978). Ensuing clinical trials found a consistent, dramatic decrease in serum DNAP activity during ara-A treatment in chronic hepatitis B patients. However, in most cases there was a rebound in HBV replication upon termination of treatment (Bassendine et al., 1981; Ouzan et al., 1988; Scullard et al., 1981). In these studies there was a correlation between sustained post-treatment low DNAP activity and an initial low pre-treatment DNAP activity (Bassendine et al., 1981; Ouzan et al., 1988; Scullard et al., 1981). The subset of treatment responders also experienced persistent or transient loss of HBeAg (Bassendine et al., 1981). To certify whether a long-term antiviral effect results from the use of ara-A will require more in-depth, large-scale studies.

Two major problems that limit the use of ara-A are: 1) the low water-solubility of ara-A, which necessitates the use of a protracted drug delivery schedule, and 2) ara-A toxicity. Most drug administration protocols outline a 10-day treatment period with an ara-A dose up to 15 mg/kg/day by a 12-hr intravenous infusion of a dilute drug solution (no more than 450 mg/L) (Bassendine et al., 1981; Sacks et al., 1982; Whitley et al., 1980). Under these conditions, administration of ara-A requires hospitalization for the duration of treatment. Another consequence of ara-A therapy is dose-dependent, drug-induced toxicity that frequently accompanies treatment. Usually the toxicity is manifested in the form of mild to severe neurological side-effects which are slowly reversible following discontinuation of therapy (Bassendine et al., 1981; Sacks et al., 1982; Whitley et al., 1980). Therefore, in addition to the physical constraints imposed by the drug delivery procedure, the patient will probably experience protracted, painful side-effects.

2) *Treatment with ara-AMP*

The solubility problems encountered with ara-A are circumvented if ara-AMP, the mononucleotide form of the compound, is used. The pharmacokinetics of ara-AMP in patients with chronic active hepatitis or severe herpesvirus infections indicates that ara-AMP does not function as a reserve form of ara-A but rather, is rapidly converted to ara-A and to the more biologically stable ($t_{1/2}$ = 3.5 hr) metabolite

arabinosylhypoxanthine (ara-Hx) (Preiksaitis et al., 1981). Ara-A and ara-AMP are metabolized and eliminated in similar fashion (Glazko et al., 1975; Preiksaitis et al., 1981; Whitley et al., 1980). The serum levels of HBV DNA and the HBV DNA polymerase activity decreased during therapy with ara-AMP. However, cessation of therapy resulted in return of these two markers of viral replication to the pre-treatment levels (Hoofnagle et al., 1982; Hoofnagle et al, 1984; Lok et al., 1985; Weller et al., 1982; Weller et al., 1985). In some cases, there was improvement in liver function and histology following treatment with ara-AMP (Lok et al., 1985; Tyrrell et al., 1988; Weller et al., 1985). For example, in one study, immunochemical evaluation of liver biopsies before and after treatment with ara-AMP indicated that 33% of the patients exhibited complete clearance of HBcAg from hepatocytes (Tyrrell et al., 1988). In this paper, Tyrrell and co-workers (1988) suggested that improved liver histology in these patients was due to the removal of HBcAg, a probable target viral protein for the cytotoxic lymphocyte mediated immune response (Mondelli et al., 1982; Van Den Oord et al., 1986; Vento et al., 1985). The results of other clinical trials indicate that ara-AMP therapy provided no significant long-term benefits to chronic hepatitis B patients (Garcia et al., 1987; Hoofnagle et al., 1984; Perrillo et al., 1985). The questionable benefits of ara-A therapy, as well as its serious, transient and prolonged side-effects, have discouraged use of this antiviral agent (Garcia et al., 1987; Giulieni-Piccari et al., 1988; Hoofnagle et al., 1984).

3) ***Combination drug therapy : ara-A or ara-AMP and interferon***

Another approach to anti-HBV therapy has been the use of ara-A or ara-AMP in combination with the immune-modulator α-interferon (Garcia et al., 1987; Lok et al., 1985; Scullard et al., 1981). Results from a pilot study comparing treatment with interferon, ara-A, or both agents suggested that the combined therapy was more effective than the use of either agent alone in inducing a long-term DNAP-negative state (Scullard et al., 1981). However, the ara-A-induced side-effects were thought to be potentiated by interferon (Sacks et al., 1982; Scullard et al., 1981). Evidence provided in a more recent evaluation of ara-AMP therapy in a cohort of chronic hepatitis B patients did not support the use of ara-AMP with or without human leukocyte interferon (Garcia et al., 1987). Serious side-effects were attributed specifically to the use of ara-AMP and, as noted in the work of Sacks et al. (1982), interferon may have aggravated the ara-AMP-induced neurological sequelae (Garcia et al., 1987).

4) ***Treatment with acyclovir***

The triphosphate form of the guanine derivative 9-(2-hydroxyethoxymethyl)guanine (acyclovir) is a selective inhibitor of herpes simplex DNA

polymerase and has been reported to exert a range of inhibitory effects on other viral DNA polymerases (Elion et al., 1977; Furman et al., 1979; St. Clair et al., 1980). Although a herpesvirus-encoded thymidine kinase converts the majority of the nucleoside analog to the monophosphate derivative, cellular enzymes can also catalyze this initial phosphorylation step and are responsible for subsequent conversions to the di- and triphosphate forms of the compound (Elion et al., 1977; Fyfe et al., 1978; Keller et al., 1985). Several clinical trials have examined the effects of acyclovir on patients chronically infected with HBV, the genome of which encodes a DNA polymerase but not a nucleoside kinase (Alexander et al., 1987; Smith et al., 1980; Weller et al., 1983). These studies indicate that acyclovir treatment has only a partial and transient inhibitory effect on serum HBV DNA polymerase levels (Alexander et al., 1987; Smith et al., 1982; Weller et al., 1983). In another study, a limited number of patients was subjected to a treatment regimen of combined acyclovir and α-interferon and compared to that in which either agent was used alone (Schalm et al., 1985). All three treatments lead to decreases in serum DNA polymerase levels, but the combination therapy resulted in the greatest decrease. A gradual drop in serum HBeAg occurred only with the combination therapy (Schalm et al., 1985). Although follow-up of the patients treated with combination therapy was not reported, the decrease in DNA polymerase activity in both the acyclovir and α-interferon alone groups returned to pretreatment levels upon discontinuation of therapy (Schalm et al., 1985).

c. *Other agents*

In vitro and *in vivo* studies indicate that suramin, an agent used in the treatment of African trypanosomiasis, prevents the initial infection of Pekin ducks and duck hepatocyte primary cultures with DHBV, and human T-cells and chicken embryo fibroblasts with HIV and Rous sarcoma virus, respectively (Offensperger et al., 1993; Mitsuya et al., 1984; Petcu et al., 1988). In addition, suramin inhibits the reverse transcriptase activities associated with these viruses (Petcu et al., 1988). However, administration of this compound to three human patients with severe, chronic active hepatitis B did not reduce the level of serum HBV DNA polymerase activity during the drug treatment schedule and resulted in extreme toxicity (Loke et al., 1987). Several DNA-intercalating agents are moderate inhibitors of HBV DNA polymerase activity *in vitro* (Hirschman and Garfinkel, 1978). *In vivo* treatment with one such compound, the antimalarial drug quinacrine hydrochloride, did not have an effect on the HBV DNA polymerase activity in the serum of HBsAg-positive chronic hepatitis patients (Bodenheimer et al., 1983).

2. Antiviral Drug Screening in Animal and Tissue Culture Systems

The natural host range of HBV is restricted to man, although experimental transmission of the virus to chimpanzees and gibbons is possible (Bancroft et al., 1977; Barker et al., 1973). Common laboratory animals such as rats and mice are not permissive hosts. The discovery of related, naturally occurring animal hepadnaviruses has lead to the use of rather unconventional animal models in the study of this virus family (Marion et al., 1980; Mason et al., 1980; Summers et al., 1978). Animals that have been utilized in antiviral studies directed against the Hepadnaviridae include the *Marmata monax* species of woodchuck which can harbour woodchuck hepatitis virus (WHV), the Beechey ground squirrel (*Spermophilus beecheyi*) which is a carrier of ground squirrel hepatitis virus (GHBV), and the Pekin duck (*Anas domesticus*) which is susceptible to duck hepatitis B virus (DHBV) (Fourel et al., 1990; Hirota et al., 1987; Kassianides et al., 1989; Lee et al., 1989; Nordenfelt et al., 1982; Ponzetto et al., 1991; Sherker et al., 1986; Smee et al., 1985; Venkateswaren et al., 1987).

There are advantages and disadvantages associated with the use of each particular animal model. For example, the woodchuck is an excellent model for the study of hepadnavirus-associated hepatocellular carcinoma (HCC) because in most cases perinatal infection of the animal leads to the development of HCC by 3 years of age (Popper et al., 1987). In both the human and woodchuck, viral infection at an early age results in a higher incidence of chronic liver disease but less than 10% of adult-acquired infections lead to chronic infections (Stevens, 1982; Tennant et al., 1988). Therefore, the natural course of the disease in the woodchuck and human are comparable. In addition, in terms of nucleotide sequence homology, gene number, gene organization, virion morphology, and antigenic cross-reactivity, the mammalian hepadnaviruses WHV and HBV are more similar to each other than either one is to the avian DHBV (Cote and Gerin, 1983; Galibert et al., 1982; Mandert et al., 1984; McCaul et al., 1985; Robinson et al., 1984; Stannard et al., 1983; Summers, 1981; Tiollais et al., 1985; Werner et al., 1979). Since maintenance of woodchuck breeding colonies is both difficult and expensive, this animal model has limited usefulness in screening large numbers of antiviral compounds. A more feasible application of this *in vivo* model system is later-stage antiviral screening of agents that have shown anti-hepadnavirus activity in other less costly screening systems.

Although evaluation of antiviral compounds in GHBV-infected Beechey ground squirrels has been reported, this model is not of practical importance. The overriding disadvantage of this animal model is that infected ground squirrels are obtained by

trapping wild animals in a particular area of northern California (Marion et al., 1980; Smee et al., 1985).

The DHBV-infected Pekin duck has been used extensively as an animal model for screening of antiviral compounds (Hirota et al., 1987; Kassianides et al., 1989; Lee at al., 1989; Sherker et al., 1986). A major difference between the avian DHBV and the mammalian HBV is the X gene, which is present in the mammalian hepadnaviruses but is missing from the avian genome (Mandart et al., 1984; Tiollais et al., 1985). Recent *in vitro* work has shown that HBV X gene-minus mutants transfected into the human hepatoma cell line HuH-7, the human hepatoblastoma cell line HepG2, and primary adult rat hepatocytes produced wild-type levels of viral proteins, replicative intermediates, and virions in all three cell types (Blum et al., 1992). Therefore, although the highly conserved mammalian X gene may play a role in virus pathogenicity, it is probably not an essential element in the HBV life cycle (Blum et al., 1992). Hepatocellular carcinoma is not found in the Pekin duck although it has been observed in a type of duck obtained from Chi-tung county in China (Imazeti et al., 1988; Marion et al., 1984; Yokosuka et al., 1985). Vertical transmission of DHBV is the major natural route of infection in domestic ducks, with a significant number of the hatchlings destined to become persistent carriers (O'Connell et al., 1983; Tsiquaye et al., 1985). This is analogous to the human situation where perinatal infection is associated with a high rate of chronic HBV infection (Stevens, 1982). The DHBV-infected duck is a convenient model system for *in vivo* antiviral drug studies since the ducks are easy to breed and maintain and a persistent infection can be established either by maternal transmission or by innoculating newly hatched ducklings with DHBV obtained from the serum of persistently infected birds (Freiman et al., 1988; Marion et al., 1984).

Several *in vitro* cell culture systems capable of supporting hepadnavirus replication are now available for the screening of antiviral agents. Primary cultures of duck hepatocytes can be prepared from the livers of ducklings that are congenitally infected with DHBV (Bishop et al., 1990; Tuttleman et al., 1986a). Alternatively, duck hepatocytes obtained from uninfected animals can be infected with DHBV after plating (Pugh and Summers, 1989; Tuttleman et al., 1986b). Active hepadnavirus replication can also be maintained in primary hepatocyte cultures isolated from the livers of WHV-infected woodchucks (Thézé et al., 1987). In addition, successful *in vitro* HBV infection of human hepatocyte cultures has been reported (Gripon et al., 1988; Shimizo et al., 1986). Primary cultures of hepatocytes furnish a convenient *in vitro* system for temporary propagation of hepadnaviruses. Such nonproliferating cell culture models have the potential to provide a realistic approximation of chemotherapeutic drug

interaction with the virus in a normal liver cell environment. On the contrary, transformed cells are further removed from the natural state, with genetic and metabolic abnormalities frequently exhibited (Kletzien and Perdue, 1974; Knowles et al., 1980; Thorens et al., 1990). Under the appropriate conditions, duck hepatocyte primary cultures can be maintained in a differentiated state for several weeks (Bishop et al., 1990; Pugh and Summers, 1989). During this time however, progressive cell de-differentiation, which can eventually be observed in the hepatocyte preparation as a morphological change from polygonal- to fibroblastic-type cells, limits extended use of this cell culture system (Fourel et al., 1989; T. Kitos, personal observation; Tuttleman et al., 1986b).

Several differentiated HBV-negative cell lines derived from human hepatomas have been transfected with cloned human HBV DNA (Sells et al., 1987; Sureau et al., 1986; Tsurimoto et al., 1987). Selection of transfected clones that secrete viral antigens and infectious virus particles resulted in the establishment of stable cell lines capable of supporting HBV replication (Acs et al., 1987; Sells et al., 1987; Sureau et al., 1986; Tsurimoto et al., 1987). Characterization of these HBV-positive cell lines indicated that: 1) complete copies of the HBV genome are integrated into the cellular DNA, as well as present in an episomal form; 2) intracellular replicative intermediates and core particles can be detected; and 3) three classes of viral RNA transcripts are present in cellular poly(A) RNA extracts (Sells et al., 1988; Sureau et al., 1986; Tsurimoto et al., 1987). These HBV-producing, immortalized cell lines have provided another useful *in vitro* system for assessing the anti-HBV activity of potential chemotherapeutic agents (Jansen et al., 1993; Korba and Milman, 1991; Lampertico et al., 1991; Ueda et al., 1989). It is not known whether these transformed cells provide an accurate representation of the biochemical functions of the normal liver cell. However, genetic and physiological alterations, including chromosomal aberrations and changes in cell growth, protein production, and enzyme activities, are a common characteristic of tumor-derived cell lines (Javitt, 1990; Knowles et al., 1980; Simon et al., 1982; Thorens et al., 1990; Weber, 1977). Identification of HBV therapeutic agents using an *in vitro* cell culture system depends on retention by the cultured cells of the same metabolic pathways that function *in vivo*.

a. *In vivo drug screening*

1) *The mammalian animal models*

The antiviral effectiveness of potential therapeutic agents has been tested in WHV-infected woodchucks and in ground squirrels carrying GSHV (Fourel et al., 1990; Nordenfelt et al., 1982; Ponzetto et al., 1991; Smee et al., 1985; Venkateswaran et al.,

1987). Treatment with 20 mg/kg/day of the nucleoside analog araAMP decreased the level of WHV DNA and activity of WHV and GHBV DNA polymerase in the serum of chronically-infected woodchucks and ground squirrels (Fourel et al., 1990; Ponzetto et al., 1991; Smee et al., 1985). In both animal models, discontinuation of araAMP therapy resulted in a return to pre-treatment levels of the serum viral markers. In addition, little or no antiviral effect was observed in woodchucks treated for 4 days with 5 or 10 mg/kg/day of acyclovir monophosphate or in ground squirrels receiving a 21-day, 50 mg/kg/day regimen of a related compound 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG; ganciclovir) (Ponzetto et al., 1991; Smee et al., 1985). Comparable inhibition of WHV occurred with a five- to ten-fold lower dose of araAMP or acyclovir monophosphate when the drugs were conjugated to lactosaminated human serum albumin (Ponzetto et al., 1991). Increased *in vivo* effectiveness of the coupled versus the free form of the drugs may be due in part to extended drug stability and selective concentration of the conjugates in the liver (Ponzetto et al., 1991). Fourel and co-workers (1990) found that treatment of WHV-infected woodchucks with the 2'-fluorinated arabinosyl-pyrimidine nucleosides FIAC (1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine) and FMAU (1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil) permanently eliminated WHV DNA polymerase activity and WHV DNA from the serum. However, FIAC- and FMAU-administration resulted in severe toxicity, with all 10 animals dying from renal insufficiency or central nervous system toxicity within the 6-month period following treatment. Although the related compound FEAU (1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil) appeared to be less toxic to the animals, inhibition of WHV replication was incomplete and transient in nature (Fourel et al., 1990). Recently the substituted arabinosyl nucleoside drug known as Fialuridine or FIAU was tested in human subjects (Associated Press, 1993). Of the fifteen human volunteers involved in the 28-day clinical trial, five have died of liver and kidney failure that occurred months after termination of the drug treatment. Although the pyrophosphate analog trisodium phosphonoformate (also known as Foscarnet) is an efficient inhibitor of HBV and WHV DNA polymerase *in vitro*, the compound had no effect on serum WHV DNA polymerase activity in woodchucks treated subcutaneously twice daily with 50 or 66 mg/kg for 6 or 14 days (Nordenfelt et al., 1979; Nordenfelt et al., 1982). Venkateswaran and colleagues (1987) reported that an aqueous extract of the plant *Phyllanthus niruri* is both an effective *in vitro* and *in vivo* inhibitor of WHV DNA polymerase. The serum levels of WHV DNA polymerase activity became undetectible during treatment when extract was administered (i.p., b.i.d.;

9 mg in 0.5 ml; 72 days) to four animals that had a recently-acquired WHV infection (Venkateswaran et al., 1987).

2) ***The avian model***

Several nucleoside analogs have been tested for *in vivo* antiviral activity in DHBV-infected ducks (Haritani et al., 1989; Hirota et al., 1987; Kassianides et al., 1989; Lee et al., 1989; Luo, 1991; Suzuki et al., 1988; Zuckerman, 1987). The serum and liver tissue levels of DHBV decreased in a concentration-dependent manner when 5 month-old ducks infected with DHBV at the time of hatching were treated for 2 weeks with araA (from 20 - 80 mg/kg/day; i.v.) (Hirota et al., 1987). Discontinuation of therapy resulted in a rebound of viral DNA levels (Hirota et al., 1987). Southern blot analysis of intrahepatic viral DNA before and after treatment with the highest dose of araA indicated that the level of viral cccDNA remained unchanged after drug therapy (Hirota et al., 1987). Furthermore, preferential inhibition of viral DNA (+)-strand synthesis by araA was observed, suggesting that the drug-virus interaction may occur at the level of DHBV DNA polymerase (Hirota et al., 1987). When four or six week-old ducklings experimentally-infected at the time of hatching with DHBV were treated for 2 or 3 weeks by intramuscular injection twice daily with 10 mg/kg of the purine nucleoside analog 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR), the viral DNA content of the serum was rapidly reduced to a level that was undetectible by the dot hybridization technique (Lee et al., 1989; Luo, 1991; Suzuki et al., 1988). After completion of a two week ddDAPR treatment schedule, a low level of DHBV DNA could be detected in the post-treatment serum samples (Suzuki et al., 1988). However, the reduced serum levels of viral DNA did not increase during a 4 week period of post-therapy monitoring. The related purine 2',3'-dideoxyadenosine (ddA) was a less effective *in vivo* inhibitor of DHBV when administered at the same dose and with the same treatment schedule (Lee et al., 1989). The purine nucleoside analog ganciclovir (DHPG) exhibited potent inhibition of DHBV DNA levels present in serum and liver tissue of ducks congenitally-infected with DHBV (Shaw et al., 1991a). The only detectible viral DNA form remaining in liver tissue after 3 weeks of treatment was cccDNA (Shaw et al., 1991a). However, within the 2 weeks following drug discontinuation, there was a return of viral replicative intermediates in the tissue (Shaw et al., 1991a). This reported potent antiviral activity of ganciclovir in the avian system is in contrast to the negligible antiviral effect that ganciclovir (DHPG) had in GHBV-infected ground squirrels (Smee et al., 1985b). No appreciable reduction in serum DHBV DNA levels was seen in ducks treated for 2 weeks with 10 mg/kg b.i.d. of the synthetic pyrimidine 2',3'-dideoxycytidine (ddC) (Luo, 1991). In contrast, another group reported that ddC administration (10 mg/day) to DHBV-

infected ducks induced a partial antiviral effect that was not sustained after termination of treatment (Kassianides et al., 1989). Another pyrimidine analog 3'-azido-3'-deoxythymidine (AZT) was an ineffective inhibitor of DHBV in ducklings treated for 10 days with 15 mg/kg/day of AZT (Haritani et al., 1989). The observed selective inhibition of DHBV by purine as compared to pyrimidine analogs indicates that drug intervention may occur at a unique step in the hepadnavirus replication cycle. One possibility is that purine nucleoside analogs interact with the virus primer protein which normally forms the initial, covalent linkage to dGMP, hence blocking synthesis of the nascent DNA (-)-strand (Lee et al., 1989; Suzuki et al., 1988).

Toxicity studies performed by Tyrrell and co-workers (unpublished results) have indicated that ddDAPR is nontoxic in ducks treated for 16 weeks with 20 mg/kg/day of the compound. In contrast, animals treated for 5 weeks with ddA (20 mg/kg/day) lost more than 30% of their body weight, developed peripheral neuropathy, and in some cases died from the consequences of drug-induced toxicity (Tyrrell and Lemke, unpublished results). Ducks treated with ddC (10 mg/kg, b.i.d.) suffered from severe toxicity that often resulted in death after 2 -3 weeks of treatment (Luo, 1991).

In another *in vivo* drug therapy study, trisodium phosphonoformate administered (i.p., b.i.d.; 50 or 250 mg/kg; 10 days) to ducklings noncongenitally-infected with DHBV lead to a concentration-dependent reduction in DHBV DNA in the serum and in the liver tissue of treated animals (Sherker et al., 1986). The lower drug dose did not reduce the viral DNA content of the serum below that of the controls, a result that is in agreement with a study in which woodchucks were treated with a comparable amount of this compound (Nordenfelt et al., 1982; Sherker et al., 1986). Treatment of ducklings with the higher drug dose resulted in undetectible serum and liver tissue levels of DHBV DNA, but the viral DNA reappeared upon discontinuation of treatment (Sherker et al., 1986). Southern blot analysis of liver DNA from both the low- and high-dose treatment groups indicated that inhibition of viral (+)-strand synthesis occurred in both cases, whereas viral (-)-strand synthesis was affected only by the higher drug concentration. Therefore, the antiviral activity of trisodium phosphonoformate may result from differential interaction of the drug with both the DHBV DNA polymerase and reverse transcriptase functions of the viral enzyme (Sherker et al., 1986).

The *in vivo* duck model has been used in a number of combination drug therapy experiments (Shaw et al., 1991; Zuckerman, 1987). A significant decrease in all viral DNA forms present in the DHBV-infected liver was detected when DHBV-positive ducks were treated simultaneously with ganciclovir and a prokaryotic DNA gyrase inhibitor (nalidixic acid or coumermycin A1) (Shaw et al., 1991). When acyclovir or

suramin given individually, or a schedule of acyclovir followed by suramin, was tested for antiviral efficacy in ducks congenitally-infected with DHBV, a dramatic reduction in serum DHBV DNA polymerase activity was detected during treatment (Zuckerman, 1987). The ensuing rebound of enzymatic activity associated with discontinued therapy was rapid in animals treated with one drug and extremely protracted for the ducks that received the drug combination (Zuckerman, 1987). In another study, the infection of Pekin ducks with DHBV was prevented when the animals were pretreated with suramin (Offensperger et al., 1993).

b. *In vitro drug screening*

1) *Primary cultures of duck hepatocytes*

Successful propagation of DHBV-infected primary cultures of duck hepatocytes has allowed large-scale screening of antiviral compounds. Numerous nucleoside analogs have been tested for anti-DHBV activity. The pyrimidine analogs AZT, 2',3'-dideoxythymidine (ddT), and ddC had little or no effect on DHBV DNA synthesis in virally-infected duck hepatocyte cultures (Civitico et al., 1990; Haritani et al., 1989; Lee et al., 1989; Suzuki et al., 1988). Another study found that ddC inhibited DHBV in duck hepatocyte cultures, but that other compounds resulting from structural modifications of thymidine, cytidine, and uridine including AZT, 2',3'-dideoxy-2',3'-didehydrothymidine (d4T), 5-azacytidine (5-aza-Cyd), 3'-deoxyarabinofuranosylcytosine (D-ara-C), 2',3'-dideoxyuridine (ddU), 3'-azido-2',3'-dideoxyuridine (AZU), and 5-bromo-2'-deoxyuridine (BDU) were less effective inhibitors at noncytotoxic concentrations (Yokota et al., 1990). As was observed in WHV-infected woodchucks, phosphonoformic acid and the fluorinated pyrimidine analog FIAC were potent hepadnavirus inhibitors in DHBV-infected duck hepatocytes, whereas FMAU and FEAU were less potent inhibitors of the duck virus (Fourel et al., 1989). In contrast, the purine nucleoside analogs ddDAPR, ddG, ddA, and 2',3'-dideoxyinosine (ddI) inhibited DHBV in primary cultures of duck hepatocytes (Civitico et al., 1990; Lee et al., 1989; Suzuki et al., 1988; Yokota et al., 1990). Other purine analogs found to interfer with DHBV DNA synthesis in hepatocyte cultures include the potent inhibitors (S)-9-((3-hydroxy-2-phosphonylmethoxy)propyl)adenine (HPMPA) and 9-((2-phosphonylmethoxy)ethyl)-2,6-diaminopurine (PMEDAP), and the marginally effective agents ganciclovir, acyclovir, and ribavirin (Civitica et al., 1990; Yokota et al., 1990). Little or no viral inhibition is attributed to araA when the drug is tested in DHBV-infected hepatocytes (Civitico et al., 1990; Suzuki et al., 1988). However, in one study in which duck hepatocytes were cocultivated with rat epithelial cells, araAMP exhibited a substantial

inhibitory effect on DHBV that was not sustained post-drug treatment (Fourel et al., 1989).

A number of compounds that are active with supercoiled DNA, such as topoisomerase I or II inhibitors and prokaryotic DNA gyrase inhibitors, were found to inhibit DHBV DNA synthesis and/or production of viral surface antigen in DHBV-infected duck hepatocytes maintained in culture with human embryonic lung fibroblasts (Civitico et al., 1990). Southern hybridization analysis of intracellular viral DNA in the drug-treated cells indicated that some of the compounds reduced the amount of viral cccDNA detected while others altered the electrophoretic mobility of the DNA supercoiled species in agarose gel (Civitico et al., 1990).

Hepatocyte infection with DHBV is inhibited by several agents that possess lysosomotropic properties (Offensperger et al., 1991; Petcu et al., 1988). When uninfected duck hepatocyte primary cultures were incubated with media containing DHBV and suramin, infection of the cells was irreversibly blocked (Petcu et al., 1988). Similarly, Pekin ducks pretreated with suramin were resistant to DHBV infection (Offensperger et al., 1993). In both cases, the compound had no effect on the virus when the drug was added after infection. Similar inhibition of *in vitro* infection of duck hepatocytes with DHBV occurred with the lysosomotropic agents ammonium chloride and chloroquine (Offensperger et al., 1991). In another study, chloroquine-treatment of DHBV-infected duck primary cultures grown in co-culture with human embryonic lung fibroblasts resulted in inhibition of both viral DNA synthesis and surface antigen production (Civitica et al., 1990).

2) ***Transfected hepatoma cell lines***

Potential anti-hepadnavirus agents have been tested in the following hepatoma-derived cell lines: 1) 2.2.15 cells - an HBV-producing cell line that was selected following transfection of the human hepatoblastoma cell line HepG2 with a plasmid containing multiple copies of the HBV genome (Sells et al., 1987); 2) HB611 cells - a stable, HBV-producing cell line that resulted from transfection of the human hepatoma cell line Huh6-c15 with a plasmid carrying the viral genome (Tsurimoto et al., 1987); and 3) D2 cells - a DHBV-producing cell line derived from transfection of the chicken hepatoma cell line LMH with an expression vector containing the DHBV genome (Condreay et al., 1990).

A variety of nucleoside analogs have been tested for anti-hepadnavirus activity, including purine and pyrimidine forms of arabinosyl nucleosides, 2',3'-dideoxynucleosides, and substituted 2',3'-dideoxynucleoside derivatives (Aoki-Sei et al., 1991; Doong et al., 1991; Korba and Milman, 1991; Lampertico et al., 1991; Matthes

et al., 1990; Ueda et al., 1989; Yokota et al., 1991). Variable anti-HBV activity has been observed in 2.2.15 cells and HB611 cells treated with ara-A or ara-AMP, while ara-C was inhibitory to HBV DNA synthesis in HB611 cultures (Korba and Milman, 1991; Lampertico et al., 1991; Nagahata et al., 1988; Ueda et al., 1989; Yokota et al., 1991). Both ara-A and ara-C were cytotoxic to HB611 cells (Ueda et al., 1989). Inhibition of HBV occurred in 2.2.15 cell cultures treated with the purine dideoxynucleosides ddDAPR, ddG, ddA, or 2',3'-dideoxyinosine (ddI) (Aoki-Sei et al., 1991; Korba and Milman, 1991). In HB611 cultures, moderate to no anti-HBV activity was attributed to ddA (Ueda et al., 1989; Yokota et al., 1991). The pyrimidine dideoxynucleoside ddC exhibited moderate to potent anti-HBV activity and 2',3'-dideoxy-2',3'-didehydrocytidine (D4C) was moderately active against the virus in HB611 and 2.2.15 cells (Doong et al., 1991; Korba and Milman, 1991; Lampertico et al., 1991; Ueda et al., 1989; Yokota et al., 1990). In all but one report the thymidine analogs AZT and d4T had very little or no effect on HBV in HB611 and 2.2.15 cells (Aoki-Sei et al., 1991; Korba and Milman, 1991; Lampertico et al., 1991; Matthes et al., 1990; Ueda et al., 1989). Sub-micromolar concentrations of the modified pyrimidine dideoxynucleosides 2',3'-dideoxy-3'-fluorothymidine (3'-FddT), 3'-fluoro-5-methyl-deoxycytidine (FddMeC), 3'-chloro-5-methyl-deoxycytidine (CldMeC), and 3'-amino-5-methyl-deoxycytidine (AddMeC) effectively inhibited HBV production by 2.2.15 cells (Matthes et al., 1990). These compounds were relatively cytotoxic, with a 0.5 to 1.5 μM concentration of the drugs resulting in a 50% reduction in cell density after four days of treatment (Matthes et al., 1990). Moderate anti-HBV activity was reported for 3'-α-fluoro-2',3'-dideoxycytidine (3'-FddC) in 2.2.15 cells (Doong et al., 1991).

Other purine- or pyrimidine-containing compounds of interest in the field of anti-HBV research include acyclic and carbocyclic nucleoside analogs, heterocyclic dideoxynucleoside analogs, and oxetanosyl-N-glycosides (Chang et al., 1992; Doong et al., 1991; Furman et al., 1992; Lampertico et al., 1991; Nagahata et al., 1989; Price et al., 1989; Ueda et al., 1989). A variety of DNA viruses are inhibited by the phosphonylmethoxyalkyl-group of acyclic nucleoside analogs (De Clercq et al., 1986; Lin et al., 1987). Low concentrations of the purine phosphonylmethoxyalkyl-derivatives PMEDAP, 9-((2-phosphonyl-methoxy)ethyl)adenine (PMEA), and HPMPA inhibited HBV DNA synthesis in HB611 cells, whereas the pyrimidine derivative (S)-1-((3-hydroxy-2-phosphonylmethoxy)propyl)cytosine (HPMPC) required a 26- to 450-fold higher concentration to achieve a comparable inhibitory effect on the virus (Yokota et al., 1991). In 2.2.15 cells treated with 50 μM of the acyclic guanosine analog acyclovir there was a reduction in extra- and intracellular HBV DNA levels (Korba and Milman, 1991).

The compound 2-amino-1,9-dihydro-9-[(1α, 3β, 4α)-3-hydroxy-4-(hydroxymethyl)-cyclopentyl]-6H-purine-6-one (2'-CDG), a carbocyclic analog of 2'-deoxyguanosine, exhibited potent anti-HBV activity in 2.2.15 cells treated with sub-micromolar concentrations of the drug (Furman et al., 1992; Korba and Milman, 1991; Lampertico et al., 1991; Price et al., 1989). No 2.2.15 cell toxicity resulted from treatment with 0.4 to 19 μM of 2'-CDG, but this concentration range of 2'-CDG was toxic when the drug was tested in a human bone marrow progenitor cell assay (Furman et al., 1992; Lampertico et al., 1991; Price et al., 1989). Inhibition of HBV DNA replication occurred in 2.2.15 cells treated with 2',3'-dideoxy-3'-thiacytidine (SddC) and 5-fluoro-2',3'-dideoxy-3'-thiacytidine (FTC), whereas compounds resulting from a methyl-, chloro-, bromo-, or iodo- substitution at the 5-position of SddC were not effective antiviral agents (Doong et al., 1991). In addition, 2',3'-dideoxy-3'-thiauridine derivatives representing potential catabolic products of the 5-substituted SddCs did not reduce HBV DNA levels (Doong et al., 1991; Schinazi et al., 1992). Further work revealed that the cytidine deaminase-resistant (-)-enantiomers of SddC and FTC were less toxic and more active as HBV-inhibitors than were the enzyme-susceptible (+)-enantiomers (Chang et al., 1992; Furman et al., 1992). The natural product oxetanocin A (OXT-A; 9-(2-deoxy-2-hydroxymethyl-β-D-*erythro*-oxetanocyl)adenine) and the 2-amino and guanine derivatives OXT-G and 2-amino-OXT-A exhibited anti-HBV activity in HB611 cells (Nagahata et al., 1988; Ueda et al., 1989). However, the hypoxanthine and xanthine derivatives of oxetanocin (OXT-H and OXT-X) were not effective inhibitors of the virus (Nagahata et al., 1988).

When tested in D2 cells, the condensed anthraquinone hypericin blocked production of infectious DHBV particles at a late stage of viral morphogenesis (Moraleda et al., 1993). Although DNA-containing virions continued to be released from the cells, evidence suggested that virus particles contained pre-S proteins that were irreversibly cross-linked by this photodynamic plant pigment (Moraleda et al., 1993). The natural products interferon α and β both exhibited dose-dependent inhibition of HBV DNA synthesis in HB611 cells (Ueda et al., 1989).

D. Nucleotide Metabolism

1. Physiological nucleotides and nucleotide precursors

Purines and pyrimidines are components of nucleosides and nucleotides in all living cells. Most intracellular nucleotides are polymerized in the form of RNA and DNA, but cells also contain pools of free ribonucleotides and, to a lesser extent, free deoxyribonucleotides. The free nucleotides function as intermediates in energy

metabolism, components of group-transfer coenzymes, physiological regulators, and precursor molecules in the synthesis of histidine and the vitamins riboflavin and folate (Henderson, 1972a). They also regulate the activity of many of the enzymes involved in nucleotide metabolism (Henderson, 1972a). Nucleotides are interconvertible with their nonphosphorylated nucleoside counterparts. The purine nucleoside adenosine has diverse biological functions. In addition to its involvement in nucleotide metabolism, it is a regulator of such processes as neuronal synaptic transmission, coronary blood flow, hormone secretion, and pyrimidine biosynthesis (Fox and Kelley, 1978).

Intracellular nucleotides can be synthesized *de novo* in most animal cells. However, this purine biosynthetic pathway appears to be absent from bone marrow, blood platelets, leukocytes, erythrocytes, and some brain cells (Fontenelle and Henderson, 1969; Holmsen and Rozenberg, 1968; Lajtha, 1958; Murray, 1971; Scott 1962). In addition to *de novo* synthesis, most tissues can procure nucleotides via a salvage pathway that re-utilizes nucleosides and nucleobases released from nucleic acid and nucleotide catabolism within the cell, from dietary sources, and from endogenous production by other cells in the body, primarily the liver (Murray, 1971; Pritchard et al., 1970). Cellular utilization of exogenous nucleosides and nucleobases begins with plasma membrane permeation by the biochemicals, followed by metabolic conversion of the internalized compounds to membrane-impermeable species, primarily nucleotides.

a. ***Cellular permeation of physiological nucleosides and nucleobases***

Although animal cell plasma membranes are relatively impermeable to nucleotides, nucleosides and nucleobases traverse the membrane by way of mediated as well as nonmediated processes (Paterson and Cass, 1986; Plagemann and Wohlhueter, 1980; Plagemann et al., 1988). Physiological nucleosides and nucleobases that permeate the cell by non-mediated diffusion through the plasma membrane lipid bilayer contribute negligibly to the total amount of these biochemicals that normally enter the cell. The bulk of nucleoside and nucleobase traffic across the plasma membrane is mediated by either equilibrative, facilitated diffusion processes or concentrative, secondary-active transport systems.

1) ***Nucleoside transport***

Distinct equilibrative, facilitated diffusion processes specific for nucleosides are identified according to their differential sensitivity to nitrobenzylthioinosine (NBMPR), an inhibitor that interacts with the transporter proteins (Belt, 1983; Paterson et al., 1987; Plagemann et al., 1988). NBMPR-sensitive nucleoside transporters are present in many animal cell types, and have been extensively studied in erythrocytes (Gati and Paterson, 1989). Non-concentrative nucleoside transporters with low NBMPR-sensitivity have

been identified in certain cell lines (Belt and Noel, 1985; Paterson et al., 1987; Plagemann and Wohlhueter, 1985). NBMPR-insensitive, concentrative, secondary-active transport processes linking nucleoside transport with a Na^+ co-transport activity have been identified in a variety of tissues including mouse enterocytes, rat, rabbit, and bovine renal brush-border membranes, L1210 mouse leukemia cells, and IEC-6 intestinal epithelial cells (Dagnino and Paterson, 1990; Jakobs and Paterson, 1986; Jarvis et al., 1989; Vijayalakshmi and Belt, 1988). Two distinct Na^+-dependent nucleoside transporting activities have been characterized according to substrate specificity differences (Dagnino et al., 1991; Vijayalakshmi and Belt, 1988). Multiple nucleoside transport systems can exist in the same cell type (Belt, 1983; Dagnino and Paterson, 1990; Jakobs et al., 1990). Nucleoside transport systems exhibit broad substrate specificity, with both purine and pyrimidine ribo- and deoxyribonucleosides, as well as many synthetic nucleoside analogs, accommodated by the carriers (Paterson and Cass, 1986).

2) ***Nucleobase transport***

Nucleobase transport systems include both equilibrative, facilitated diffusion and concentrative, Na^+-dependent active transport processes (Bronk and Hastewell, 1987; Domin et al., 1988a; Plagemann and Wohlhueter, 1984). Human erythrocytes contain a purine nucleobase-specific transporter (Domin et al., 1988a). Depending on the cell type, nucleobase permeation may involve nucleoside transporters. For example, the nucleoside transporter in S49 mouse lymphoma cells accepts purine nucleobases as substrates (Aronow and Ullman, 1986; Plagemann and Wohlhueter, 1986). In two rat hepatoma cell lines, Chinese hamster ovary cells, and Ehrlich ascites tumor cells both the nucleoside uridine and the nucleobase hypoxanthine can be transported by the nucleoside carrier (Plagemann and Wohlhueter, 1984). However, hypoxanthine fluxes in other cultured cells are not associated with the nucleoside transport system (Plagemann and Wohlhueter, 1984). In rat small intestine tissue, pyrimidine base fluxes are linked to a Na^+-dependent transport activity (Bronk and Hastewell, 1987).

b. ***Metabolic pathways involved in the synthesis and degradation of physiological nucleotides***

In most cells, nucleotide synthesis proceeds by way of two separate metabolic sequences: 1) the *de novo* pathway; and 2) the salvage pathway. Although nucleotides, nucleosides, and nucleobases are the biochemical substrates recycled in the salvage pathway, these compounds are not the precursors employed in nucleotide biosynthesis *de novo*. Therefore, although both pathways result in the same nucleotide end-products, the two types of reaction sequences are independent of one another. The following

discussion of purine and pyrimidine *de novo* biosynthesis and salvage makes use of information reviewed by Henderson (1972b), Henderson and Paterson (1973), Kornberg and Baker, (1992), and Munch-Petersen, (1983). Appendix II presents the structures and numbering systems employed for the physiological purine and pyrimidine compounds discussed below.

1) *De novo biosynthesis of nucleotides*

Purine and pyrimidine nucleotides are synthesized *de novo* from chemical precursors which include CO_2, NH_3, ribose 5-phosphate, and amino acids. The final product of the purine biosynthetic pathway is inosinate (IMP; inosine 5'-monophosphate), a compound from which all other purine nucleotides can be derived. Orotate, the key nucleobase structure formed in pyrimidine biosynthesis, is converted to the nucleoside monophosphate orotidylate (OMP; orotidine 5'-monophosphate), which reacts to form uridylate (UMP; uridine 5'-monophosphate). All other pyrimidine nucleotides can be derived from UMP.

a) *De novo synthesis of purines*

Purine nucleotide biosynthesis begins with enzyme-catalyzed condensation of glutamine with 5-phosphoribosyl 1-pyrophosphate (PRPP), the activated form of ribose 5-phosphate, to form phosphoribosylamine. Subsequent reaction of phosphoribosylamine with glycine contributes C-4, C-5, and N-7 of the nucleobase structure, and C-8 is donated as a formyl group by 5,10-methenyl-tetrahydrofolate. In the next reaction, N-3 of the nucleobase is added by the transfer of an amide group from glutamine. Closure of the imidazole portion of the purine nucleobase moiety is followed by the addition of carboxylate (from HCO_3^- or CO_2) at C-6, and the introduction of purine nucleobase N-1, donated by aspartate. A formyl group supplied by 10-formyl-tetrahydrofolate becomes C-2, and the purine ring structure is completed by an enzyme-catalyzed cyclization step, yielding IMP.

b) *De novo synthesis of pyrimidines*

The *de novo* route of pyrimidine biosynthesis proceeds by step-wise construction of the nucleobase orotate, followed by condensation of orotate with PPRP to form OMP, and a final decarboxylation step to yield UMP. Synthesis of orotate is initiated by the formation of carbamyl phosphate from CO_2, ATP, and glutamine (or NH_3). The carbamyl group of carbamyl phosphate is then transferred to aspartate to give carbamyl aspartate. Enzyme-catalyzed cyclization of carbamyl aspartate yields dihydroorotate which is then oxidized to form orotate. The pyrimidine nucleotide UMP arises following orotate condensation with PPRP and subsequent decarboxylation of the nucleobase moiety. Formation of UDP and UTP from UMP, and formation of deoxyuridine

phosphates is considered in Section D.1.b.2). Biosynthesis of cytidine phosphates proceeds by amination of UTP by CTP synthetase, with glutamine as the amide donor. The thymidine nucleotide dTMP can be synthesized from dUMP by thymidylate synthetase, with the methyl group contributed by the coenzyme 5,10-methylene-tetrahydrofolate.

2) ***The salvage pathway***

Pre-existing purine and pyrimidine nucleobases, nucleosides, and nucleotides may be reclaimed for use or disposed of through an interconnecting web of metabolic reactions known collectively as the salvage pathway. Many of the enzymes involved in these metabolic conversions are regulated by nucleotides. Hence, control of the reaction network is precise, with enzyme activities adjusted in response to the availability of specific nucleotides. The complex control mechanisms that operate in intact tissue are not functional in studies that utilize cell extracts or purified nucleotide metabolizing enzymes. Therefore, *in vitro* kinetic properties of an enzyme such as K_m and V_{max} values for substrates and effective concentrations of nucleotide regulators may not be relevant *in vivo*. The relative importance of a particular arm of the salvage pathway varies depending on factors such as cell type, the state of cellular differentiation, and/or phase of the cell cycle (Hordern and Henderson, 1983). Although the following discussion considers nucleotide salvage pathway reactions in general, the specific systems in operation ultimately depend on the individual cell.

a) ***Ribonucleotide synthesis from nucleobases and ribonucleosides***

Several pathways lead to the conversion of nucleobases and ribonucleosides to the analogous phosphorylated species. Base-specific phosphoribosyltransferases utilize PPRP in the direct conversion of uracil and the purine bases adenine, guanine, hypoxanthine, and xanthine to the corresponding ribonucleoside monophosphates. Purine ribonucleosides are elevated to their monophosphate counterparts by kinases that utilize ATP as a phosphate donor. Adenosine kinase is responsible for the conversion of adenosine to AMP. Less is known about the direct phosphorylation of guanosine and inosine by kinases. Uridine and cytidine are converted to UMP and CMP, respectively, by the action of uridine-cytidine kinase, an enzyme that is specific for ribonucleosides. In addition, a two-step conversion of uracil to UMP may occur via formation of uridine from uracil by uridine phosphorylase, followed by phosphorylation of the nucleoside. Kinase-assisted, reversible interconversions between the three phosphorylation levels of nucleoside mono-, di-, and triphosphates involves energy-conserving phosphoryl group transfers. The ribonucleoside monophosphates AMP, GMP, UMP, and CMP are converted to the corresponding diphosphates by, respectively, adenylate kinase,

guanylate kinase, and pyrimidine nucleoside monophosphate kinase, which transphosphorylates CMP and UMP (Agarwal et al., 1971; Orengo and Maness, 1978; Tsuboi, 1978). The phosphate donor in these kinase-catalyzed transphosphorylations is ATP, although in some cases other deoxy- or ribonucleoside triphosphates can function in this capacity. Nucleoside diphosphate kinase catalyzes a phosphotransfer reaction between a nucleoside diphosphate substrate and a nucleoside triphosphate donor. This widely distributed, active enzyme exhibits broad substrate specificity, accepting purine and pyrimidine nucleoside di- and triphosphates, with either ribose or deoxyribose sugars.

Animal cells produce most of their ATP by mitochondrial oxidative phosphorylation. Exchange between adenine nucleotides located in the cytoplasm and within the mitochondria occurs by way of the ATP-ADP antiporter which is situated in the inner mitochondrial membrane (Harold, 1986). Mitochondrially-generated ATP enters the cytoplasm where the chemical energy conserved in this nucleotide is distributed throughout the other purine and pyrimidine nucleotide pools by kinase-directed phosphoryl group transfers.

b) ***Deoxyribonucleotide synthesis***

The major route for the formation of deoxynucleoside triphosphates is by reduction of ribonucleoside diphosphates to the corresponding deoxynucleoside diphosphate by ribonucleotide reductase, an enzyme that reduces ADP, GDP, CDP, and UDP. Deoxynucleoside diphosphates can then be readily converted to triphosphates by nucleoside diphosphate kinase or, alternatively, dephosphorylated to the monophosphate form. The sequential addition of phosphate groups to deoxynucleosides can be achieved by the action of cellular kinases. Both adenosine kinase and deoxycytidine kinase can convert deoxyadenosine to dAMP, although in this case the more physiologically relevant enzyme may be adenosine kinase (Sarup and Fridland, 1987; Ullman et al., 1981). Deoxycytidine kinase is also responsible for the conversion of deoxycytidine to dCMP, cytidine to CMP, and deoxyguanosine to dGMP (Datta et al., 1989; Kremitsky et al., 1976; Sarup and Fridland, 1987). Thymidine kinase will phosphorylate deoxynucleosides, specifically thymidine and deoxyuridine, but not deoxycytidine. Adenylate kinase converts dAMP to dADP, while guanylate kinase phosphorylates dGMP to the corresponding nucleoside diphosphate (Agarwal et al., 1971; Tsuboi, 1978). Pyrimidine nucleoside monophosphate kinase converts dCMP to dCDP (Orengo and Maness, 1978). Phosphorylation of dTMP to dTDP, and dUMP to dUDP is catalyzed by thymidylate kinase. The ubiquitous nucleoside diphosphate kinase readily converts nucleoside diphosphates to triphosphates.

c) ***Interconversion of nucleotides***

The three phosphorylation levels of ribo- and deoxyribonucleotides are metabolically connected by phosphorylation and dephosphorylation reactions that are discussed in Section D.1.b.2) a), D.1.b.2)b), and D.1.b.2)d). Purine ribonucleotide base moiety interconversions occur at the monophosphate level, with IMP functioning as the pivotal compound. Synthesis of AMP from IMP is a two-step process. In a reaction requiring GTP, adenylosuccinate synthetase converts IMP and aspartate into adenylosuccinate. This reaction is followed by the conversion of adenylosuccinate to AMP and fumarate by adenylosuccinate lyase. Conversion of AMP to IMP is accomplished by adenylate deaminase, an enzyme that also exhibits activity with dAMP. Two reactions are involved in the formation of GMP from IMP: 1) the intermediate compound XMP is synthesized from IMP by the combined action of inosinate dehydrogenase and the electron acceptor NAD^+; and 2) the substrates XMP and glutamine (or NH_3) are used by guanylate synthetase to form GMP. Guanylate reductase catalyzes the conversion of GMP to IMP. Pyrimidine ribonucleotides are interconverted by amination of UTP to form CTP. Catalyzed by CTP synthetase, this reaction utilizes glutamine as the amine donor. Recycling cytidine phosphates to uridine compounds involves catabolism to the cytosine nucleoside which can be deaminated by cytidine deaminase to form uridine.

Base moiety interconversions of adenine and guanine deoxyribonucleotides do not occur. However, pyrimidine deoxyribonucleotides may interconvert through deamination and methylation reactions. Specifically, deamination of dCMP by deoxycytidylate deaminase yields dUMP, and methylation of dUMP by thymidylate synthetase gives dTMP. Another pathway for the formation of dUMP involves deamination of deoxycytidine by cytidine deaminase to yield deoxyuridine, which can be converted to dUMP by thymidine kinase.

d) ***Nucleotide catabolism***

Various enzymes are involved in the catabolism of nucleotides. Enzyme-catalyzed reactions that function in the degradation of nucleotides include dephosphorylation reactions, deaminations, glycosidic bond cleavages, and oxidation or reduction of nucleobases.

Dephosphorylation of nucleoside triphosphates is catalyzed by metabolic enzymes that utilize nucleoside triphosphates as phosphate-group donors. Inorganic pyrophosphatase and apyrase are other dephosphorylating enzymes that act on nucleoside tri- and diphosphates. Nucleoside diphosphatase converts GDP, IDP, and UDP to monophosphates. Adenylate kinase catalyzes a reversible reaction which can produce

AMP and ATP from two molecules of ADP. Nucleoside monophosphates are converted to nucleosides by nucleotidases and phosphatases. Nonspecific acid and alkaline phosphatases accept nucleoside monophosphates with any base moiety and with 2'-, 3'-, or 5'-phosphates. Alkaline and acid 5'-nucleotidases exhibit substrate specificity, with the former nucleotidases more active with AMP, while the latter type of enzyme is selective for GMP, IMP, and xanthylate (xanthine 5'-monophosphate; XMP). Nucleotidases and phosphatases also catalyze the dephosphorylation of pyrimidine ribo- and deoxyribonucleotides.

Enzyme-catalyzed deamination reactions are involved in the catabolism of purine and pyrimidine compounds in animal cells. The purine nucleosides adenosine and deoxyadenosine can be catabolized by adenosine deaminase (ADA) yielding hypoxanthine nucleosides, while adenylate deaminase acts upon AMP and dAMP, converting these adenine nucleotides to IMP and dIMP. However, adenine deoxy- and ribo-nucleotides may utilize distinct catabolic pathways *in vivo*, as is seen in human T-lymphoblastoid cells where the breakdown of dATP proceeds mainly via the ADA pathway and catabolism of ATP usually involves the action of adenylate deaminase on AMP (Barankiewicz and Cohen, 1984). Guanylate reductase converts GMP to IMP, and hydrolytic deamination of guanine to xanthine occurs by the action of guanine deaminase. Deamination of the pyrimidine nucleosides cytidine and deoxycytidine by cytidine deaminase yields uridine and deoxyuridine. The pyrimidine nucleotide monophosphate dCMP is converted to dUMP by dCMP deaminase.

In animal cells, reversible glycosidic bond cleavage of the ribo- and deoxyribonucleosides of guanine, hypoxanthine, and xanthine is accomplished by purine nucleoside phosphorylase (PNP). Although adenine, adenosine, and deoxyadenosine are good substrates for PNP of microorganisms, adenosine phosphorolysis in animal cells is a minor degradative pathway (Hammer-Jespersen, 1983; Henderson, 1985). Instead, adenosine deaminase converts adenosine and deoxyadenosine to hypoxanthine nucleosides, which are then readily phosphorilized by PNP. In addition, the adenine-containing compounds AMP and dAMP are both substrates of adenylate deaminase, forming IMP and dIMP, respectively, which can be dephosphorylated and phosphorilized by the pathways just discussed. Pyrimidine nucleoside phosphorolysis is catalyzed by: 1) thymidine phosphorylase, which cleaves the glycosidic bonds of thymidine and deoxyuridine; and 2) uridine phosphorylase which acts on uridine, deoxyuridine, and thymidine. Direct phosphorolysis of deoxycytidine and cytidine does not occur. However, deamination of the cytosine nucleosides by cytidine deaminase yields deoxyuridine and uridine which are readily phosphorolyzed. In addition to liberating

nucleobases, phosphorolysis of deoxyribo- and ribonucleosides yields deoxyribose 1-phosphate and ribose 1-phosphate, respectively, compounds that are channeled into energy-producing reaction sequences.

The breakdown of purine and pyrimidine bases involves either oxidation or reduction of the nucleobase. The purine bases hypoxanthine and xanthine are converted by xanthine oxidase to uric acid, the end-point of purine metabolism in man, higher primates, birds, reptiles, and insects. The urinary end-product of other mammals is allantoin which arises from the action of uricase on uric acid. In some biological systems allantoin is converted to the end-products allantoic acid, urea, or ammonia. Catabolism of the pyrimidine bases uracil and thymine involves reduction of the pyrimidine ring double bond followed by oxidative cleavage of the ring structure. The resulting products are catabolized to β-amino acids, NH_3, and CO_2. The β-amino acids are utilized in other metabolic reactions.

2. Nucleoside analogs

Nucleoside analogs are used chemotherapeutically in the treatment of some viral infections and cancers. Drug development programs continue to explore this family of compounds in the search for effective antiviral agents. Therapeutic nucleosides must permeate the cell plasma membrane and interact with cellular enzymes before exerting the desired antiviral or antineoplastic effect. Drug permeation is a crucial step in ara-C therapy of adult non-lymphoblastic leukemias. Leukemic white cell sensitivity to ara-C correlates with ara-C influx rates and with the number of membrane nucleoside transporters, as determined by the number of NBMPR binding sites per cell (White et al., 1987; Wiley et al., 1982; Wiley et al., 1983; Wiley et al., 1985).

a. Cellular permeation of nucleoside analogs

Cell plasma membrane permeation of nucleoside analogs can be attributed to passive diffusion, facilitated diffusion processes, active transport, or a combination of these pathways. The equilibrative, nucleoside transport systems accept a wide range of physiologic as well as synthetic nucleosides. Nucleoside substrates that are not accepted by the equilibrative, facilitated diffusion transporters include ionized nucleosides and nucleoside analogs such as orotidine, 6-azauridine, and 3-deazauridine (Belt and Welch, 1983; Cass and Paterson, 1972; Dahlig-Harley et al., 1984). Nucleoside structure modification studies indicate that equilibrative, facilitated nucleoside transport is sensitive to loss of the 3'-hydroxyl and modification of the 3'- or 2'-hydroxyl groups on the nucleoside ribose moiety (Cass and Paterson, 1972; Gati et al., 1984; Taube and Berlin, 1972). A comparison of transport kinetics for Ado, 2'-dAdo, and 3'-deoxyadenosine (3'-dAdo; cordycepin) in human erythrocytes indicates that although

Ado and 2'-dAdo exhibit comparable kinetic parameters, loss of the 3'-hydroxyl group greatly reduces the efficiency of 3'-dAdo transport (Plagemann et al., 1990). However, removal of the 2'-hydroxyl substituent, and changes to the nucleobase moiety of nucleosides are well tolerated by facilitated diffusion-type transporters (Cass and Paterson, 1972). In mammalian cells, formycin B, a C-glycoside inosine analog with a modified base structure, is a substrate for both the equilibrative NBMPR-sensitive and -insensitive nucleoside transporters, as well as for one of the concentrative Na^+-dependent nucleoside transport systems (Vijayalakshmi and Belt, 1988). However, Na^+-dependent nucleoside transport in L1210 mouse leukemia cells exhibits increasing insensitivity to purine compounds with, respectively, F-, Br-, and Cl- substitutions at the C-2 position of the base (Dagnino et al., 1991).

Facilitated nucleoside transport systems exhibit stereoselectivity (Dagnino et al., 1991; Gati et al, 1989; Mahony et al., 1992). For example, Na^+-dependent adenosine transport in L1210 mouse leukemia cells is specific for the naturally-occurring D-enantiomer of adenosine, whereas the unphysiological L-counterpart is not a substrate for this transporter (Dagnino et al., 1991). In human erythrocytes, enantiomeric selectivity occurs in the entry of carbovir (9-[4α-(hydroxymethyl)cyclopent-2-ene-1α-yl]guanine), the carbocyclic analogue of ddG, which enters these cells by way of the nucleobase carrier and, to a lesser extent by the nucleoside transporter (Mahony et al., 1992).

The acyclic nucleoside analog acyclovir enters and exits human erythrocytes by way of the purine nucleobase carrier (Mahony et al., 1988). The human erythrocyte nucleobase transporter also accepts as substrates the structurally similar acyclic guanosine analog ganciclovir, and carbovir, a carbocyclic analog of ddG (Mahony et al., 1992; Mahony et al., 1991). In addition, the nucleoside transporter contributes to ganciclovir and carbovir permeation in human erythrocytes (Mahony et al., 1992; Mahony et al., 1991). A combination of nucleobase transport and passive diffusion is involved in 5-fluorouracil fluxes in human erythrocytes (Domin and Mahony, 1990). Entry of the nucleobase analog 3-deazaguanine and the related nucleoside analog 9-β-D-(2-deoxyribofuranosyl)-3-deazaguanine into L1210 cells occurs mainly by passive diffusion (Mian and Avarado, 1990). Although the human T-lymphoblastoid cells Molt-4 exhibit a low level (10-20% of the total influx) of ara-G transport by the nucleobase carrier, an NMBPR-sensitive nucleoside transporter is responsible for the majority of ara-G transport in these cells and is the sole transporter of ara-G in human erythrocytes (Pruss et al., 1990).

Nucleoside analogs such as the di- and tri-deoxynucleosides ddT and dddT (3-,5'-trideoxythymidine), respectively, and the 3'-azido-substituted dideoxynucleoside AZT

traverse the human erythrocyte membrane by non-facilitated diffusion (Domin et al., 1988b; Plagemann and Woffendin, 1989a; Zimmerman et al., 1987). Similarly, the influx of d4T (the 2',3'-unsaturated homologue of ddT) into human lymphocyte H9 cells proceeds by way of passive diffusion (August et al., 1991). Characteristics of ddT, d4T, and AZT influx that indicate a passive diffusion mode of entry include: 1) nonconcentrative influx; 2) nonsaturable influx that is a linear function of the permeant concentration; 3) insensitivity of the permeant to nucleoside transport inhibitors; and 4) unaltered permeant influx in the presence of a large excess of nucleoside or nucleobase transporter substrates (August et al., 1991; Domin et al., 1988b; Zimmerman et al., 1987). Expeditious cell membrane permeation of these thymidine analogs may be attributed to the inherent chemical lipophilicity associated with nucleosides containing a reduced number of hydroxyl groups on the ribose moiety and, in the case of AZT, the addition of a substituent that is a poor acceptor and a non-donor of hydrogen bonds. The octanol partition coefficient (a ratio of the nucleoside concentration in octanol to the nucleoside concentration in a balanced salt solution) values for dddT, AZT, ddT, d4T, and dThd are 2.6, 0.98, 0.25, 0.18, and 0.085, respectively, indicating that these thymidine analogs are considerably more lipophilic than the parent compound dThd (August et al., 1991; Plagemann and Woffendin, 1989a; Zimmerman et al., 1987).

Similarly, fluxes of the purine dideoxynucleosides ddA, ddI, and ddG appear to be mainly non-mediated in the mammalian cell systems examined (Agarwal et al., 1989; Ahluwalia et al., 1987; Busso et al., 1990; Plagemann and Woffendin, 1989a). Entry of ddA into L1210 and P388 mouse leukemia cells and human erythrocytes is unaffected by nucleoside transport inhibitors or by an excess of other nucleosides (Plagemann and Woffendin, 1989a). A small component of ddA permeation of peripheral blood mononuclear cells, H9 T-cells, and Namalwa B-cells may be transporter-mediated since ddA entry is weakly inhibited by both NBMPR and an excess of pyrimidine nucleosides (Agarwal et al., 1989). The relatively high lipid solubility of ddA (octanol partition coefficient = 0.609) would account for its rapid, nonmediated cellular entry, permeating human erythrocytes and mouse leukemia cells at 10% the rate of transporter-mediated nucleoside substrates (Plagemann and Woffendin, 1989a). Entry of ddA and the related purine nucleoside analog ddI into cultured human T-lymphocytes (Molt-4 and CEM cells) is unaffected by 20 μM NBMPR, suggesting that the nucleoside transporter does not play a role in ddA or ddI permeation of these cells (Ahluwalia et al., 1987). Similarly, uptake of ddG by H-9 T-lymphocytes is not affected by an excess of the nucleoside transport inhibitor NBMPR (Busso et al., 1990). CEM and Molt-4 cell permeation by ddA is 4- to 6-fold more rapid than that of ddI, reflecting the disparate

octanol partition coefficient values for these two agents (ddA = 0.603; ddI = 0.068) (Ahluwalia et al., 1987).

The pyrimidine dideoxynucleoside ddC is a substrate for the facilitated nucleoside transporter in human erythrocytes, cultured human T-lymphoblasts, and two mouse leukemia cell lines (Plagemann and Woffendin, 1989b; Ullman et al., 1988). However, cell permeation by ddC is only 1% as efficient as that of the natural substrates of the nucleoside transporter (Plagemann and Woffendin, 1989b). Although ddC has a relatively low octanol partition coefficient (0.048), non-mediated permeation contributes to ddC fluxes in human erythrocytes and mouse leukemia cells (Plagemann and Woffendin, 1989b).

b. ***Intracellular metabolism of nucleoside analogs***

In most cases, intracellular phosphorylation of nucleoside-type agents is a prerequisite to their antiviral activity (De Clercq, 1993). This essential process is usually accomplished, with varying degrees of efficiency, by the cellular enzymes that function in physiological nucleotide metabolism. The nucleoside analog is often subject to the action of other enzymes that are present in the vast cellular network of metabolic pathways. Of crucial importance to the success or failure of an antiviral agent is the relative affinity of catabolizing enzymes for the drug. As mentioned previously, the enzyme composition of a cell and the apparent enzyme activities may vary depending on the cell type. Therefore, the phosphorylated derivative of an antiviral drug may accumulate in one cell system while the same agent may follow a different metabolic pathway in another. For example, the observed selective toxicity of ara-G in several T-cell lines as compared to B-lymphoblasts may be due to differential rates of ara-GTP catabolism in the two cell types (Verhoef and Fridland, 1985).

Many nucleoside analogs are converted to the 5'-monophosphate congeners by cellular kinases. In cells of thymic origin, the dideoxynucleoside ddC is phosphorylated to the monophosphate derivative by deoxycytidine kinase (dCyd kinase) (Balzarini et al., 1987; Kierdaszuk et al., 1992; Starnes and Cheng, 1987; Ullmann et al., 1988). Conflicting reports have emerged with respect to ddA phosphorylation by dCyd kinase, an enzyme found predominantly in lymphoid tissues in humans (Carson et al., 1977; Carson et al., 1980). In several studies, partially purified dCyd kinase from normal human thymocytes or CEM cell extracts was capable of phosphorylating ddA (Johnson et al., 1987, Cooney et al., 1987; Johnson et al., 1988). In another case, purified human leukemic T-cell dCyd kinase exhibited phosphorylating activity with the dideoxynucleoside substrates ddC, ddA, and ddG (Sarup et al., 1989). More recently, a homogeneous preparation of dCyd kinase from human leukemic spleen exhibited

efficient phosphorylation of several cytosine dideoxynucleosides but was unable to phosphorylate the purine nucleoside analogs ddA, ddI, and ddG (Kierdaszuk et al., 1992). However, a nonstringent substrate specificity was apparent for purified calf thymus dCyd kinase which phosphorylates numerous purine and pyrimidine nucleosides and analogs thereof (Krenitsky et al., 1976). Experiments with kinase-deficient lymphoid cell lines and purified or partially purified dCyd kinase from several sources indicate that dCyd kinase accepts as substrates the purine analogs ara-A, 2-chloro-2',3'-dideoxyadenosine (2-CldA), 2'-fluoro-2',3'-dideoxyarabinosyladenine (2'-F-dd-ara-A), and ddG as well as the pyrimidine compounds ddC, ara-C, 5-fluoro-2',3'-dideoxycytidine (5-FddC), 5-bromo-2',3'-dideoxycytidine (5-BrddC), 2',3'-dideoxy-3'-fluorocytidine (3'-FddC), and 3'-azido-2',3'-dideoxycytidine (AZC) (Bondoc et al., 1992; Datta et al., 1989; Haertle et al., 1988; Masood et al., 1990; Sarup and Fridland, 1987; Verhoef et al., 1981). Studies utilizing partially purified adenosine kinase from the human T-lymphoblast cell line CCRF-CEM or from Chinese hamster ovary cells and experiments with normal and kinase-deficient lymphoblasts indicate that adenosine kinase can phosphorylate ddA, ara-A, ribavirin, and tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) (Fridland et al., 1986; Johnson et al., 1988; Page and Connor, 1990; Saunders et al., 1990; Verhoef et al., 1981). In another report, mono-phosphorylation of guanosine, 3-deazaguanosine, tiazofurin, and the natural substrate nicotinamide riboside was demonstrated using partially purified nicotinamide riboside kinase from Chinese hamster ovary cells (Saunders et al., 1989). Purified cytosolic thymidine kinase from H9 cells converts the pyrimidine nucleoside analog AZT to AZT-MP with kinetic parameters similar to that obtained with dThd (Furman et al., 1986). 3'-FddT, AZT, and to a lesser extent ddT, are competitive inhibitors of dThd phosphorylation by a cell extract of Molt/4F cells (Balzarini et al., 1988). Less efficient phosphorylation occurs with the substrate d4T in cell-free extracts of H9 cells and with thymidine kinase purified from H9 cells (Marongiu et al., 1990).

The role of mitochondrial kinases in the phosphorylation of nucleoside analogues has been examined in several studies. Although carbovir can enter the mitochondria of human lymphoid cells, no phosphorylated compound has been detected within these organelles (Bondoc et al., 1990). In addition, mitochondrial dGuo kinase purified from a dCyd kinase- and adenosine kinase-deficient CEM cell line was unable to phosphorylate carbovir (Bondoc et al., 1990). Purified bovine mitochondrial dGuo kinase exhibits strict phosphate-acceptor substrate specificity, with dGuo, dIno, ara-G, and 8-aza-dGuo, but not dAdo, Ado, Guo, acyclovir, 6-thio-dGuo, or dCyd accepted as substrates (Parks and Ives, 1988). Both purified cytoplasmic and mitochondrial dCyd kinase from peripheral

chronic lymphocytic leukemia cells catalyze the phosphorylation of ddC, with a higher substrate affinity measured for the mitochondrial enzyme (Starnes and Cheng, 1987). Selective mitochondrial toxicity has been implicated in the delayed cytotoxicity observed in T-lymphocytes treated with ddC and the eventual peripheral neuropathy experienced by humans treated with ddC (Chen and Cheng, 1989; Chen et al., 1991).

In some cases, the conversion of nucleoside analogs to their monophosphate derivatives does not involve the action of kinases. Utilizing IMP as a phosphate donor, cytosolic 5'-nucleotidase from human lymphoid cells, rat liver, or Chinese hamster ovary cells can act as a phosphotransferase to phosphorylate inosine and guanosine analogs such as ddI, ddG, ara-G, carbovir, acyclovir, ganciclovir, and tiazofurin, as well as inosine, guanosine, and deoxyguanosine, to their corresponding nucleoside monophosphates (Fridland et al., 1986; Johnson and Fridland, 1989; Keller et al., 1985; Saunders et al., 1990). Evidence suggests that ddA is not a substrate for the phosphotransferase in CEM cell extracts (Kierdaszuk et al., 1992). In CEM and Molt-4 cells, the major, although indirect route from ddA to ddAMP involves conversion of ddA to ddI, followed by 5'-nucleotidase-catalyzed formation of ddIMP from ddI, and subsequent conversion of ddIMP to ddAMP by the sequential action of adenylosuccinate synthetase and adenylosuccinate lyase (Ahluwalia et al., 1987; Johnson et al., 1988). Partially purified rabbit adenylosuccinate synthetase converts ddIMP to dideoxyadenylosuccinate, although this conversion occurs at only 2% of the rate obtained for IMP conversion to adenylosuccinate (Ahluwalia et al., 1987).

Other cellular enzymes have been implicated in the conversion of certain nucleoside analog monophosphates to their di- and triphosphorylated forms. An *in vitro* enzyme assay has shown that direct conversion of ara-AMP to ara-ATP can occur by the action of PRPP synthetase, an enzyme that normally functions in the pathway of IMP and UMP biosynthesis through the reversible transfer of pyrophosphate from ATP to ribose-5-phosphate, forming PRPP (Balzarini and De Clercq, 1990). Although AMP and the adenine nucleotide analogs ara-AMP, 7-deazaadenosine 5'-monophosphate (tubercidin 5'-monophosphate), PMEA, HPMPA, PMEDAP, and several other acyclic nucleoside phosphonates are substrates for PRPP synthetase, purine and pyrimidine nucleotides such as GMP, IMP, XMP, CMP, and UMP are not (Balzarini and De Clercq, 1990; Balzarini and De Clercq, 1991a; Sabina et al., 1984). Very low levels of PMEA and its phosphorylated derivatives are found in human MT-4 cells that have been exposed to the drug for 24 hr (Balzarini et al., 1991b). The monophosphates of bucicloviir ((R)-9-(3,4-dihydroxybutyl)guanine; BCV), ganciclovir and acyclovir can be phosphorylated to their diphosphate counterparts by guanylate kinase, while anabolism of ganciclovir- and

acyclovir- diphosphate to the triphosphate level can occur by the action of two glycolysis pathway enzymes, phosphoglycerate kinase and pyruvate kinase, and less efficiently by nucleoside diphosphate kinase (Smee et al., 1985a; Stenberg et al., 1986). Inefficient anabolism of AZT-MP to AZT-DP is brought about by thymidylate kinase (dTMP kinase) purified from H9 cells (Furman et al., 1986). Thymidylate kinase exhibits even lower affinity for the substrate 3'-azido-2',3'-dideoxyuridine monophosphate (AZU-MP) (Zhu et al., 1990).

Nucleoside analog modification by catabolic pathways can lead to drug inactivation and the formation of harmless or toxic metabolites that are eventually removed from the biological system. Catabolic reactions that compete with anabolic activation of various nucleoside analogs include deaminations, phosphorolytic cleavages, and dephosphorylations. The nucleoside analogs ara-A, ddA, and ddDAPR are deaminated by ADA (Balzarini et al., 1987; Carson et al., 1988). Similarly, ddAMP appears to be converted to ddIMP by AMP deaminase (Carson et al., 1988). Purified human cytidine-deoxycytidine deaminase (Cyd-dCyd deaminase) does not deaminate ddC (Starnes and Cheng, 1987). Partially purified Cyd-dCyd deaminase from peripheral human acute myelocytic leukemia cells has little effect on the cytidine analog 1-β-D-arabinofuranosyl-5-azacytosine (ara-5-aza-Cyd), but causes the deamination of 2'-deoxy-5-azacytidine (5-aza-dCyd) and ara-C (Townsend et al., 1985). Partially purified human liver cytidine deaminase converts araC, 5-aza-Cyd, 6-azacytidine, and several 5-substituted cytidine analogs to their corresponding uracil derivatives (Wentworth and Wolfenden, 1978). In addition, partially purified human cytidine-deoxycytidine deaminase accepts (+)BCH-189 and (+)FTC as substrates but is unable to deaminate the corresponding (-) enantiomers (-)BCH-189 (or 3TC) and (-)FTC (Chang et al., 1992; Furman et al., 1992). Both ddG and ddI can be catabolized to the appropriate base and a dideoxyribose phosphate sugar through the action of PNP (Ahluwalia et al., 1990; Cooney et al., 1987). However, the guanosine analog ara-G is resistant to PNP (Cohen et al., 1983). Very low bacterial thymidine phosphorylase activity is detectable with the dideoxynucleosides ddU and ddT (Balzarini et al., 1988).

E. Purpose of the Present Study

The purine nucleoside analog ddDAPR is an effective inhibitor of DHBV replication both *in vitro* and *in vivo* (Lee et al., 1989; Suzuki et al., 1988). In DHBV-infected duck hepatocyte primary cultures, a similar level of viral inhibition is achieved with comparable concentrations of ddG (Lee et al., 1989). In contrast, pyrimidine nucleoside analogs are less effective inhibitors of the virus in duck hepatocytes (Lee et

al., 1989; Yokota et al., 1990). The current project was initiated to examine the metabolic fate of these purine nucleoside analogs in duck hepatocyte primary cultures.

This body of work was undertaken with the following direction in mind:

1. The first step of the project was to determine whether the intravenously administered drug ddDAPR is deaminated to ddG by blood.
2. A comparison would then be made of the intracellular metabolism of ddDAPR and ddG, in both DHBV-infected and uninfected duck hepatocyte primary cultures.
3. If ddDAPR is converted to ddG by blood, then further characterization of intracellular metabolism in duck hepatocyte primary cultures would focus on ddG and would examine: a) time-dependent metabolism of ddG; b) the intracellular longevity of metabolites formed; and c) the inhibitory effect of various physiological nucleosides on the formation of ddG metabolites.
4. The intracellular metabolism and antiviral effectiveness of ddG would be examined in the HBV-infected human cell line 2.2.15 and the metabolic profile obtained from this human cell line would be compared with that found in the avian cells.
5. As a comparison to ddG metabolism and antiviral effectiveness in avian and human cell cultures, the intracellular metabolism and antiviral potential of the ineffective DHBV inhibitor ddC would also be examined in duck and human cells.

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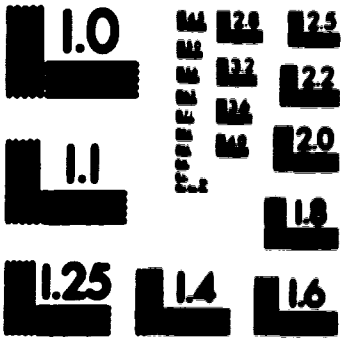

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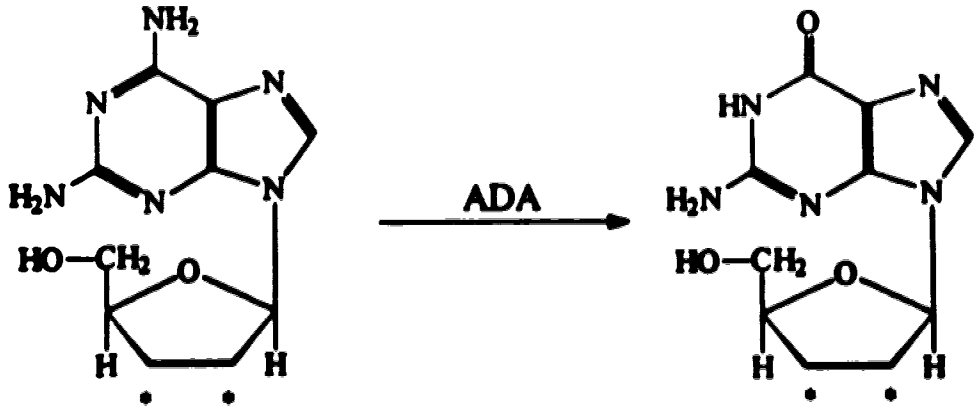
CHAPTER 2
IN VITRO AND *IN VIVO* METABOLISM OF ddDAPR IN BLOOD

I. INTRODUCTION

The nucleoside analog 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR) is a potent inhibitor of duck hepatitis B virus (DHBV) in both *in vitro* and *in vivo* test systems (Suzuki et al., 1988; Lee et al., 1989). In addition, this agent exerts selective antiviral activity in HIV-infected T4-lymphocytes (MT-4 cell line) (Balzarini et al., 1987a). The metabolic stability of ddDAPR in biological tissue is unknown. However, *in vitro* work indicates that ddDAPR is a substrate for beef intestine adenosine deaminase (Figure 2.1), exhibiting a relative initial velocity of 0.042 with respect to the natural substrate adenosine (Balzarini et al., 1987b; Pauwels et al., 1988). Although ddDAPR is a relatively poor substrate for adenosine deaminase, it can act as a competitive inhibitor of the natural substrate adenosine, indicating that the two compounds interact at the same active site of the enzyme (Balzarini et al., 1987b). These *in vitro* results indicate that it would be possible for deamination of ddDAPR to occur in a biological system. Biological modification of the compound could result in drug activation, leading to conversion of the parent nucleoside into the antivirally active form of the drug.

In vivo studies investigating the antiviral effect of ddDAPR in ducks persistently infected with DHBV indicate that intramuscular administration of 10 mg/kg drug twice a day leads to a rapid clearance of serum DHBV DNA, with no detectable viral DNA present in animals treated for 2 weeks (Lee et al., 1989). However, in these experiments, the drug form and concentration present in the duck blood at various times post-treatment was not measured (Lee et al., 1989). The body of work represented by this thesis began with a study of the metabolic fate of ddDAPR in blood.

Figure 2.1 - Conversion of ddDAPR to ddG by adenosine deaminase. Deamination of ddDAPR by adenosine deaminase (ADA) yields the purine nucleoside ddG. Studies presented in this thesis utilize [^{3}H]-labeled ddDAPR and ddG radiolabeled (*) at the 2'- and 3'- positions of the nucleoside ribose moiety.



ddDAPR

2,6-diaminopurine-2',3'-dideoxyriboside

ddG

2',3'-dideoxyguanosine

II. MATERIALS AND METHODS

A. Materials

1. General Supplies

Mini-scintillation vials were purchased from Fisher Scientific, Nepean, ON., EcoLite liquid scintillation solution was obtained from ICN Biomedicals, Inc., Irvine, CA., and sodium pentobarbitol was supplied by M.T.C. Pharmaceuticals, Cambridge, ON. Centricon-3 filters were from Amicon Canada Ltd., Oakville, ON. Tetrabutylammonium dihydrogen phosphate (TBAP) was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI., methanol was from J.T. Baker Cemical Co., Phillipsburg, N.J., and potassium phosphate monobasic (KH_2PO_4), potassium chloride (KCl), and potassium hydroxide (KOH) were from Fisher Scientific, Nepean, ON. Water was purified using a Milli Q water purification system (Millipore, Mississauga, ON.).

2. Nucleosides and Nucleoside Solutions

The nucleoside analogues 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR) and 2',3'-dideoxyguanosine (ddG) were purchased from Terochem, Edmonton, AB. The adenosine deaminase inhibitor 2'-deoxycoformycin (DCF) was a generous gift from Drs. A.R.P. Paterson and C.E. Cass, Dept. of Pharmacology and Dept. of Biochemistry, respectively, University of Alberta. In most cases, millimolar nucleoside stock solutions were prepared in water or saline and were filter-sterilized (0.22 µm Millex-GV sterile filter unit, Millipore, Mississauga, ON). An accurate concentration of each stock solution was determined by measuring the optical density of a dilution of the stock solution at the appropriate $lambda_{max}$ for the particular nucleoside (Fasman, 1975). The stock concentration was calculated by dividing the optical density by the extinction coefficient for the particular nucleoside and correcting for the dilution. The stock solutions were stored at -20°C and were utilized in the preparation of solutions containing micromolar concentrations of nucleosides and/or nucleoside analogs.

3. Radiochemicals

[2',3'-^{3}H]2,6-diaminopurine 2',3'-dideoxyriboside ([^{3}H]ddDAPR; 45 Ci/mmol) was obtained from Moravek Biochemicals Inc., Brea, CA. Radiochemicals were repurified periodically by HPLC.

4. Experimental Animals

Fertilized Pekin duck eggs were obtained from a duck colony maintained at the University of Alberta Farm and were held in a 37°C egg incubator (Cyclonic Incubator, Marsh Manufacturing Inc., Garden Grove, CA.) until hatching (approximately 28 days). Sera from newly hatched ducklings were screened for the presence of DHBV DNA by

dot hybridization (Lee et al., 1989). The DHBV-infected and uninfected animals were maintained in separate quarters.

5. Human Blood

Human blood was collected from a blood donor volunteer into a heparin-containing Vacutainer. The blood was drawn by qualified medical personnel.

B. *In Vitro* Drug Metabolism in Blood

1. *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- or sodium citrate-containing Vacutainers. The blood was incubated at duck body temperature (42°C) with [^{3}H]ddDAPR (16 - 30 cpm/pmole; 50 - 67 μM). In experiments that employed 2'-deoxycoformycin (DCF), the blood samples were preincubated for 15 min at 42°C with or without 1 μM DCF, followed by incubation with [^{3}H]ddDAPR for the appropriate times. At various times, 100-μl samples were transferred to 2 ml of 90°C water and heated for 2 minutes. The precipitated protein was removed by centrifugation and the supernatant solution from each sample was filtered through a Centricon-3 filter (centrifuged at 5400 x g for 3-5 hr, 10°C). The filtrate was lyophilized, reconstituted in 100 μl of water, and analyzed by ion-pair reverse-phase HPLC as detailed in section II.D. of this chapter.

2. *In Vitro* Conversion of ddDAPR to ddG by Human Whole Blood

Freshly drawn, heparinized human blood was incubated at 37°C with 50 μM [^{3}H]ddDAPR (16 cpm/pmole). The blood was sampled at various times and the samples were processed and analyzed as detailed in section B.1 above.

C. *In Vivo* Drug Metabolism in Blood

1. *In Vivo* Conversion of ddDAPR to ddG in the Duck Circulation System

A four-week old duckling (3 kg) was anesthetized by i.v. administration of sodium pentobarbital (approximately 10 mg/kg body weight) to maintain adequate anesthesia. The animal was injected with 1.25 ml of 0.02 M [^{3}H]ddDAPR (3.68 cpm/pmole; 2 mg [^{3}H]ddDAPR/kg body weight) in buffered saline via a canulated left wing vein. At various times, 1-ml blood samples were withdrawn from a canulated right wing vein, the blood was transferred to 2 ml of 90°C water and boiled for 2 minutes. The samples were then processed and analyzed as described in Section B.1 of this chapter.

D. HPLC Analysis of the Blood Extracts

Freeze-dried samples were reconstituted in 0.1 ml of filtered water and a known volume (70 - 95 μl) of the sample was then 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 x 4.6 mm i.d.) preceded by a 5-μm Supelcosil LC-18 guard column (2 cm x 4.6 mm i.d.). Separation of nucleosides and nucleotides was accomplished using a gradient mobile phase separation developed by Stocchi et al. (1987), with minor modifications. The two solvent systems consisted of Buffer A: 0.1 M KH_2PO_4 - 8 mM tetrabutylammonium dihydrogen phosphate (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 (Pande, 1976) or EcoLite liquid scintillation solution, the radioactivity of each fraction was quantified by scintillation spectrometry.

III. RESULTS

A. *In Vitro* Drug Metabolism in Blood

1. *In Vitro* Conversion of ddDAPR to ddG in Duck Blood

Preliminary experiments (not shown) examined the *in vitro* modification of [^{3}H]ddDAPR in blood drawn from two-week old ducklings. Blood samples containing buffered sodium citrate were incubated with [^{3}H]ddDAPR for 1 or 5 min at 37°C, diluted (1:5) with water, and heated for 1 min at 92°C. Following centrifugation to remove precipitated protein, the supernatant was freeze-dried, reconstituted in 10% methanol, and analyzed by reverse-phase HPLC according to the conditions outlined in section II.D. of Chapter 2. Results from these initial experiments indicated that both 1- and 5-min incubation periods were sufficient to convert the radiolabeled ddDAPR to a compound with an HPLC elution time identical to that of the ddG standard.

A more detailed examination of ddDAPR modification by duck blood is presented in Table 2.1. Fresh blood was drawn from ducklings, collected in heparin-containing Vacutainers, and incubated at duck body temperature (42°C) *in vitro* with 50 μM [^{3}H]ddDAPR (16 cpm/pmole). 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. Table 2.1 illustrates that ddDAPR was rapidly converted to ddG *in vitro* by duck blood. Within 1 min, 95% of the ddDAPR was converted to the deamination product ddG. After 10 min of incubation ddG accounted for 97% of the CPM recovered from the sample. HPLC analysis of 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 retention times comparable to that of the free dideoxyribose phosphate and guanine moieties. The HPLC elution profile obtained for the 10 min incubation sample is shown in Figure 2.2A. The [^{3}H]ddG product resisted further modification by duck blood despite continued incubation at 42°C for 60 min. The total pmoles of ddG product recovered in the 60 min incubation sample was 94% of the ddG that was contained in the 10 min sample, and the 10 and 60 min samples contained, respectively, 37 and 47 pmoles of compound eluting as the free dideoxyribose phosphate and guanine products associated with glycosidic bond cleavage.

If adenosine deaminase (ADA) is responsible for the apparent conversion of ddDAPR to ddG in duck blood, then inclusion of the ADA inhibitor DCF in ddDAPR-containing blood samples should reduce the amount of ddG recovered in the samples. However, the effect of DCF on time-dependent conversion of ddDAPR to ddG by duck blood was variable. Duck blood samples were pretreated at 42°C with or without 1 μM DCF, followed by incubation for various time periods with [^{3}H]ddDAPR

Table 2.1

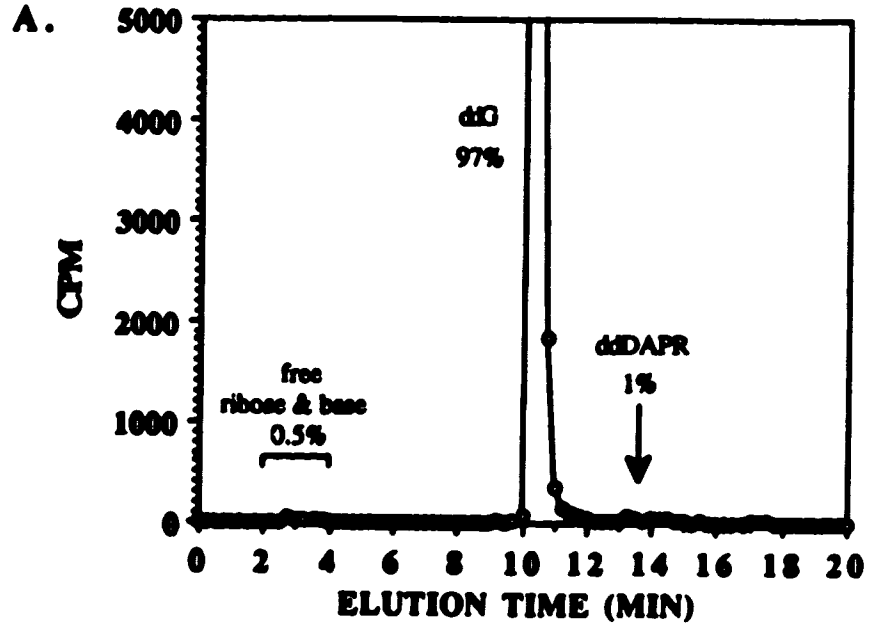
METABOLISM OF ddDAPR IN WHOLE BLOOD

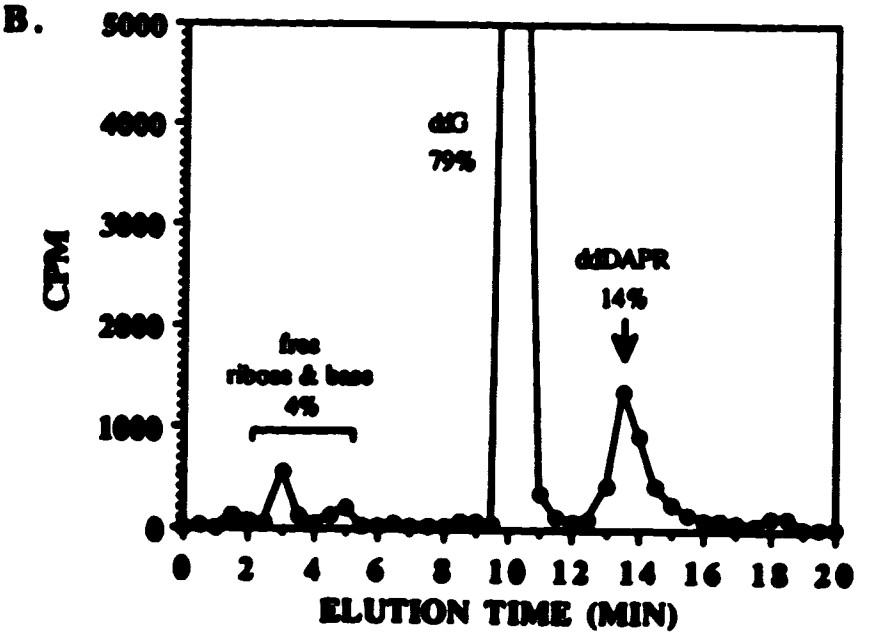
1) *in vitro* duck blood - Blood samples taken from a 1- or 19-day old duckling were incubated with 50 μM [^{3}H]ddDAPR (16 cpm/pmole) at 42°C. At the indicated times aliquots were removed, hot water extracted, and the extracts analyzed by HPLC as described in Materials and Methods. Since the blood samples obtained from the one-day old and 19-day old animals yielded identical results, the data reported here represents combined values obtained from the two age group samples.

2) *in vitro* human blood - Freshly drawn blood was incubated with 50 μM [^{3}H]ddDAPR (16 cpm/pmole) at 37°C. At the indicated times samples were removed, extracted, and the extracts analyzed by HPLC.

Percent CPM Recovered								
	0 TIME		20 SEC		1 MIN		10 MIN	
BLOOD	ddDAPR	ddG	ddDAPR	ddG	ddDAPR	ddG	ddDAPR	ddG
DUCK	98	0.5	33	66	3.0	95	1.3	97
HUMAN	-	-	96	3.8	-	-	14	79

Figure 2.2 - Comparison of ddDAPR conversion to ddG by duck blood and human blood. Heparinized blood samples incubated *in vitro* for 10 minutes with 50 μM [^{3}H]ddDAPR were extracted and the extracts analyzed by ion-pair reverse-phase HPLC as detailed in Materials and Methods. A) Duck blood incubated for 10 minutes at 42^{o}C and B) human blood incubated for 10 minutes at 37^{o}C. The percentage of total sample radioactivity recovered in each peak is indicated.


A.
5000
4000
3000
2000
1000
0
CPM
ddG
97%
ddDAPR
1%
free
ribose & base
0.5%
0 2 4 6 8 10 12 14 16 18 20
ELUTION TIME (MIN)


B.
5000
4000
3000
2000
1000
0
CPM
ddG
79%
ddDAPR
14%
free
ribose & base
4%
0 2 4 6 8 10 12 14 16 18 20
ELUTION TIME (MIN)

(16 - 30 cpm/pmole; 50 - 67 μM ddDAPR) in the presence or absence of 1 μM DCF. The results presented in Figure 2.3 illustrate that samples incubated for 10 min with and without DCF contained, respectively, 93% and 96% of the total sample radioactivity in the form of ddG. Blood samples incubated for 1 min with [^{3}H]ddDAPR + DCF had converted 21% of the [^{3}H]ddDAPR to [^{3}H]ddG, whereas 97% of the total sample radioactivity was associated with ddG in analogous 1-min samples without DCF (Figure 2.3). However, in a similar experiment (results not shown), conversion of ddDAPR to ddG was essentially complete after 1 min under both conditions, with 93% sample radioactivity present as ddG in the DCF-containing samples compared with 95% ddG in samples without the inhibitor. In addition, 1 μM DCF did not appreciably retard the conversion of ddDAPR to ddG in samples taken at time points under 1 min.

The effect of sample processing on the radiolabel recovery was minimal as can be see by Control A and Control B in Figure 2.3. HPLC analysis of an untreated [^{3}H]ddDAPR sample (Control A) indicated that 97% of the CPM were recovered as ddDAPR. When a [^{3}H]ddDAPR sample (Control B) was diluted in saline instead of blood, incubated for 600 sec, and processed in the same manner as the experimental samples, 92% of the CPM were recovered as ddDAPR.

2. *In Vitro* Conversion of ddDAPR to ddG in Human Blood

Freshly drawn human blood was incubated *in vitro* with 50 μM [^{3}H]-ddDAPR (16 cpm/pmole) at the human body temperature of 37^{o}C. At various times samples were removed, extracted, and the extracts analyzed by HPLC. *In vitro* conversion of ddDAPR to ddG was found to occur in human blood, with 79% of the radiolabeled ddDAPR converted to ddG within 10 min (Table 2.1 and Figure 2.2B). A human blood sample that was lysed by sonication and then incubated for 20 sec at 37^{o}C contained the same distribution of radioactivity as the 20-sec sample of nonlysed blood. In contrast to the results obtained from duck blood, a 10-min incubation of ddDAPR with human blood resulted in approximately 4% of the total sample radioactivity exhibiting elution times corresponding to that of guanine and the free ribose moieties (Figure 2.2).

B. *In Vivo* Drug Metabolism in Blood

Rapid conversion of ddDAPR to ddG was observed in blood samples taken from a 4-week old duck that had been injected intravenously with 2 mg [^{3}H]-ddDAPR (4 cpm/pmole) per kilogram body weight. Figure 2.4A presents the HPLC separation profile for an extract obtained from duck blood sampled 1 min after injection of the animal with [^{3}H]ddDAPR. One minute after injection, 95% of the sample radioactivity was present in the form of ddG. [^{3}H]-ddG was still detectible as the major radiolabeled product in a sample taken 25 min after [^{3}H]-ddDAPR was introduced into the blood

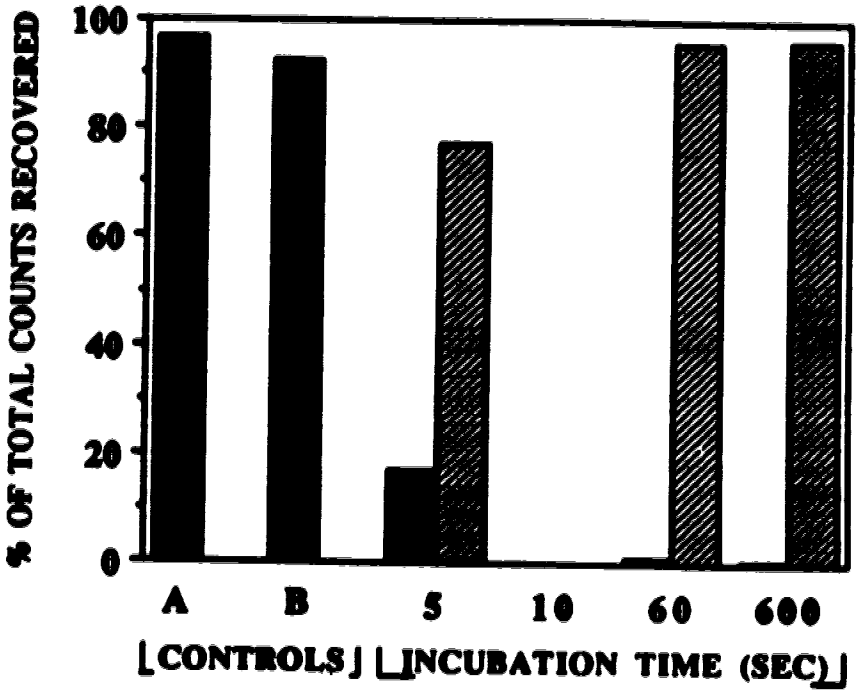
Figure 2.3 - *In vitro* metabolism of ddDAPR in freshly drawn duck blood. Fresh duck blood from three 16 day-old ducks was pooled in a tube containing buffered sodium citrate as an anticoagulant. Aliquots (0.1 ml) of the blood were preincubated at 42°C for 15 min with or without 1 μM of the adenosine deaminase inhibitor 2'-deoxycoformycin (DCF). The samples were then incubated for various times at 42°C with 67 μM [^{3}H]ddDAPR (30 cpm/pmole). At the appropriate times the reaction was terminated by the addition of 2 ml hot water, followed by heating for 1.5 min in a boiling water bath. Precipitated protein was removed by centrifugation and the supernatant samples were lyophilized, resuspended in a small volume of water and analyzed by reverse-phase HPLC according to the following specifications: 1) Spectra Physics 8000 HPLC; 2) Whatman partisil-10 ODS column; 3) column temperature = 42°C; 4) flow rate = 1 ml/min; 5) a three solvent system: Buffer A: 0.1 M KH_2PO_4, pH 4.8; Buffer B: 100% methanol; Buffer C: 0.01 M KH_2PO_4 - 5 mM tetrabutylammonium dihydrogen phosphate (TBAP), pH 4.9; and 6) a 65 min gradient mobile phase separation program:

Time	Buffer A	Buffer B	Buffer C
0	0	0	100
10	0	0	100
15	0	10	90
30	15	15	70
40	35	25	40
55	45	30	25
65	0	0	100

Absorbance was monitored at 254 nm and 0.5-ml eluate fractions were collected and quantified by scintillation spectrometry.

A preincubation sample of the [^{3}H]ddDAPR stock solution is identified as Control A. Control B represents a sample of saline-diluted [^{3}H]ddDAPR stock solution that was incubated for 600 sec and processed in the same manner as the experimental samples. All of the control and experimental samples were analyzed by HPLC and the cpm recovered as ddDAPR and as ddG are expressed as a percentage of the total radioactivity recovered during the HPLC separation. The recoveries of ddDAPR and ddG are indicated by black bars and hatched bars, respectively. Panel A illustrates the time-dependent conversion of [^{3}H]ddDAPR to [^{3}H]ddG upon ddDAPR incubation with duck blood in the absence of DCF. The time course shown in panel B represents the incubation of duck blood + ddDAPR in the presence of 1 μM DCF. Panel A does not have a 10-sec time point and panel B is missing the 5-sec sample.

A.


% OF TOTAL COUNTS RECOVERED
100
80
60
40
20
0
A
B
5
10
60
600
CONTROLS
INCUBATION TIME (SEC)

B.

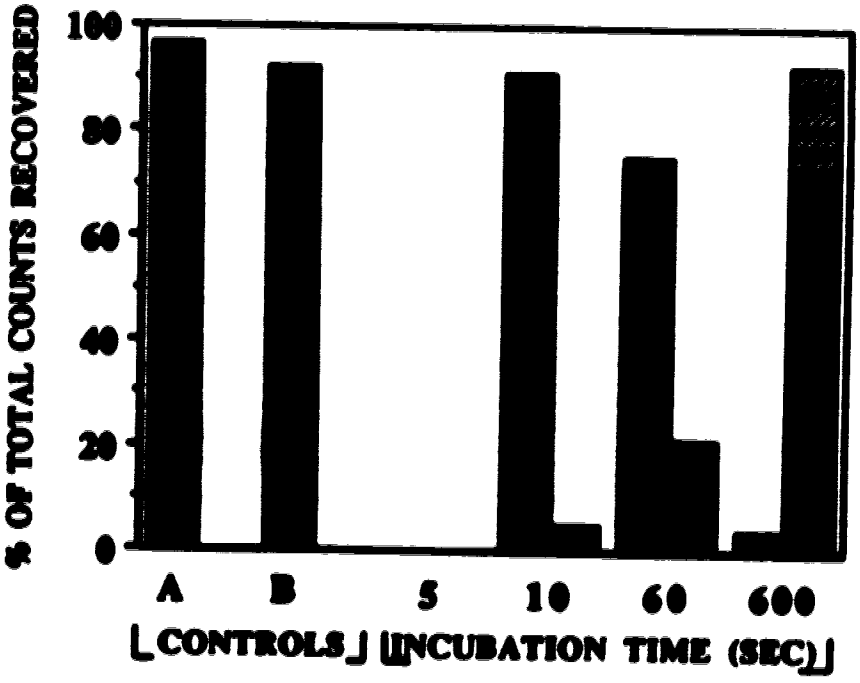
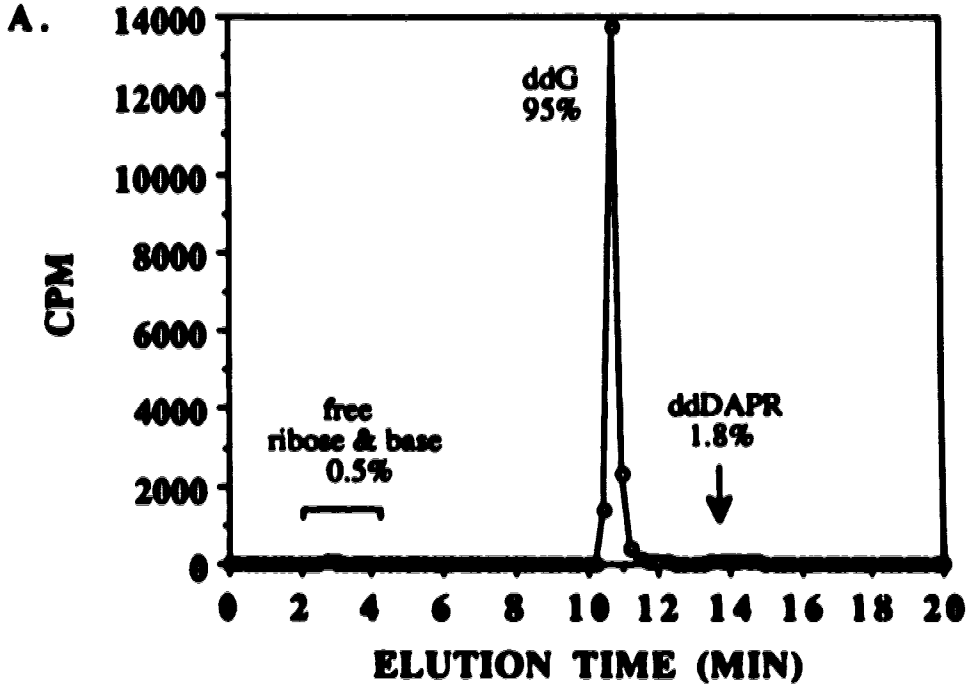
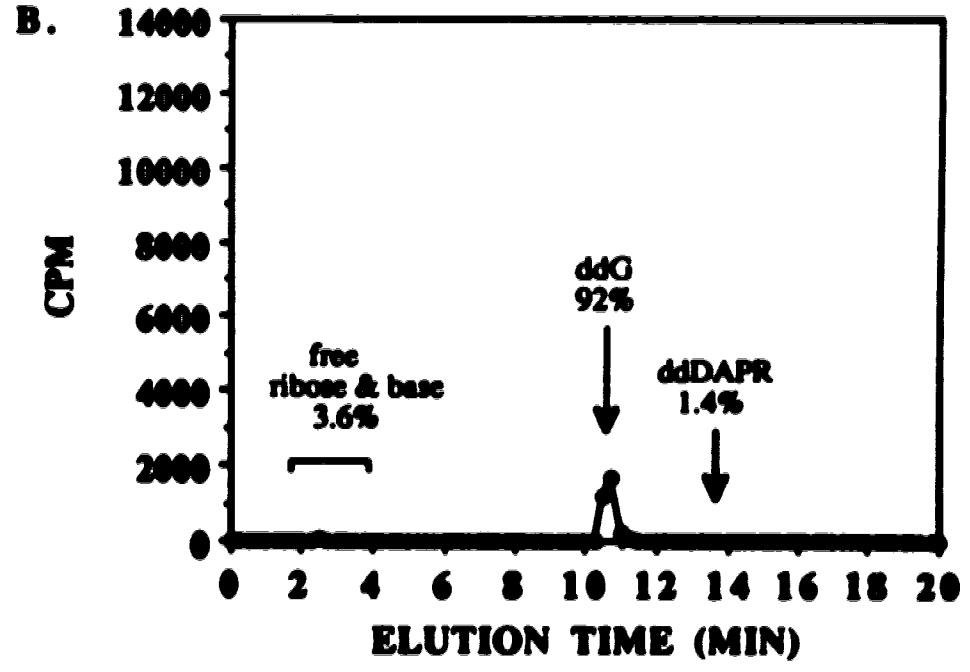

% OF TOTAL COUNTS RECOVERED
100
80
60
40
20
0
A
B
5
10
60
600
CONTROLS
INCUBATION TIME (SEC)

Figure 2.4 - *In vivo* metabolism of [^{3}H]ddDAPR in a one month old Pekin duck. An i.v. injection of [^{3}H]ddDAPR (6.25 mg in 1.25 ml buffered saline; 3.68 cpm/pmole) was administered via the wing vein of a four-week old duck. One-ml blood samples were withdrawn from a cannulated vein and the samples were processed and analyzed according to the Materials and Methods. This figure illustrates the HPLC elution profiles of the radioactivity present in duck blood extracts obtained from A) a 1-min time point blood sample, and B) a 25-min time point sample. The percentage of total sample radioactivity recovered in each peak is indicated.


A.
14000
12000
10000
8000
6000
4000
2000
0
CPM
ddG
95%
free
ribose & base
0.5%
ddDAPR
1.8%
0 2 4 6 8 10 12 14 16 18 20
ELUTION TIME (MIN)


B.
14000
12000
10000
8000
6000
4000
2000
0
CPM
ddG
92%
free
ribose & base
3.6%
ddDAPR
1.4%
0 2 4 6 8 10 12 14 16 18 20
ELUTION TIME (MIN)

stream of the animal (Figure 2.4B). Following i.v. administration of [^{3}H]-ddDAPR, the total radioactivity in the blood declined over time in a biphasic manner (Figure 2.5). 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:

$$C_b = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Equation 1)}$$

using Enzfitter, a nonlinear least squares curve-fitting program. In the equation, C_b = the concentration of radiolabeled product in the blood at time t, α and β are the fitted rate constants for, respectively, rapid and slow disposition, and A and B are the zero-time intercepts associated with the α- and β-phases. The concentration curve illustrated in Figure 2.5 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 half-life values obtained in this study for ddDAPR distribution and elimination in the Pekin duck, as well as half-life data for other nucleoside analogs derived from animal models are summarized in Table 2.2. 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 (Chabot et al., 1983; Kelley et al., 1987; Schinazi et al., 1992; Wientjes and Au, 1992).

Figure 2.5 - *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/pmole) was administered via the wing vein of a four-week old, 3-kg duck. 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.

IN VIVO DISTRIBUTION AND ELIMINATION OF [^{3}H]ddDAPR IN THE DUCK

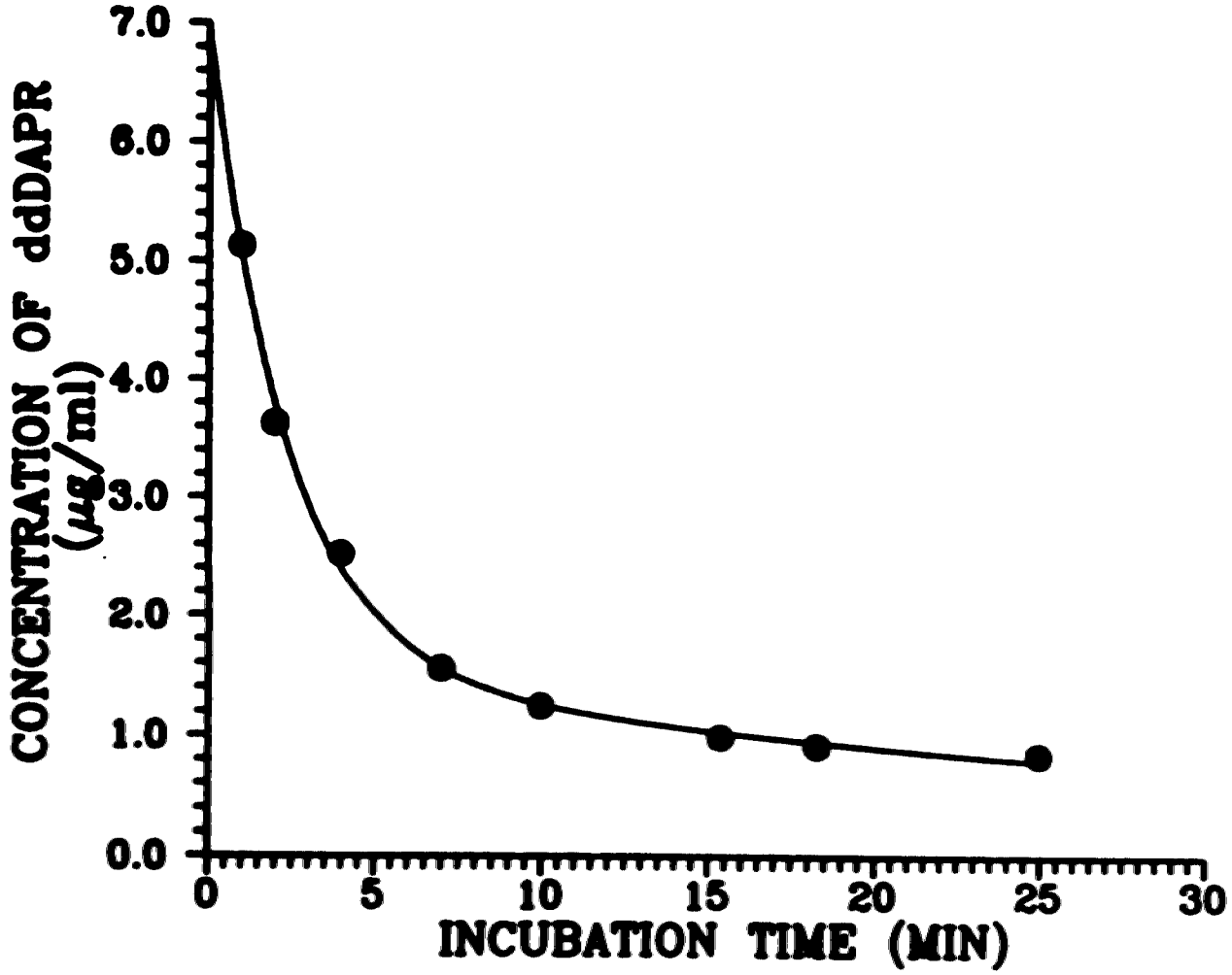


Table 2.2
REPORTED PLASMA HALF-LIFE VALUES FOR *IN VIVO* DRUG ELIMINATION

Drug	$T_{1/2\alpha}$ (min)	$T_{1/2\beta}$ (min)	Animal	Dose (mg/kg)	Administered	Reference
Acyclovir	-----	120	Rat	25	s.c.	Stahle & Oberg, 1992
H2G	-----	28	Rat	25	s.c.	Stahle & Oberg, 1992
ddDAPR	1.7	26	Duck	2.1	i.v.	This thesis
ddI	3	35	Rat	250	i.v., 2 hr infusion	Anderson et al., 1990
ddI	-----	33.4	Rat	40	i.v.	Wientjes & Au, 1992
ddI	5	45	Dog	500	i.v.	Stoltz et al., 1988
ddI	-----	38	Human	0.2 - 6.4	i.v., 1.5 hr infusion	Hartman et al., 1990
ddC	-----	102 - 114	Monkey	27	i.v.	Kelley et al., 1987
ddC	-----	69	Mice	100	i.v.	Kelley et al., 1987
FTC	-----	77	Monkey	33	i.v.	Schinazi et al., 1992
d4T	-----	18	Mice	25	i.v.	Russell et al., 1989
d4T	-----	50; 55	Monkey	15	i.v.; oral	Kaul & Dandekar, 1993
d4T	-----	150; 138; 96	Monkey	25	i.v.; oral; s.c.	Cretton et al., 1993
AZT	-----	21	Mice	25	i.v.	Russell et al., 1989
AZT	-----	26	Rats	40	i.v.	Wientjes & Au, 1992

IV. DISCUSSION

The dideoxynucleoside ddDAPR has been tested both *in vivo* and *in vitro* for activity against the duck hepadnavirus DHBV in ducks and duck hepatocyte primary cultures, respectively (Lee et al., 1989; Suzuki et al., 1988). The work presented in this chapter clearly indicated that ddDAPR in concentrations similar to those used in testing for *in vivo* antiviral activity was rapidly converted to ddG by duck blood. In the experiments herein, conversion of ddDAPR to the purine dideoxynucleoside ddG was essentially complete after 1 min of exposure to duck blood, both *in vitro* and *in vivo*. *In vitro* conversion of ddDAPR to ddG also occurred in the human blood samples that were tested. Human blood catabolizes 10 μM adenosine through the action of ADA and PNP to hypoxanthine with a $t_{1/2}$ of <15 sec for the disappearance of adenosine (Dawicki et al., 1988). The adenosine analog ddA is rapidly converted to ddI in human blood during i.v. infusion of the compound (Hartman et al., 1990). Analysis of serum samples taken from AIDS patients during ddA infusion revealed the presence of the deamination product ddI but did not detect the parent drug (Hartman et al., 1990). In the present study, deamination of ddDAPR was slower in the human than in the duck blood samples. However, kinetic characterization of ddDAPR deamination by both human and duck blood would allow an accurate comparison of the conversion rates in the two systems. Since a sample of lysed human blood gave the same results as blood in which the cells had not been disrupted, the observed slower conversion of ddDAPR to ddG by human blood is probably not mediated by intracellular compartmentalization of the drug or the enzyme.

Previous work has shown that, *in vitro*, ddDAPR is a substrate for beef intestine ADA (Balzarini et al., 1987b; Pauwels et al., 1988). Presumably ADA is responsible for the conversion of ddDAPR to ddG in duck and human blood. However, the present equivocal results with respect to the effect of the ADA inhibitor DCF on ddDAPR deamination in blood samples fail to clarify this issue. Conversion of ddDAPR to ddG by duck blood was retarded by the presence of 1 μM DCF in one instance, but it had little effect on ddDAPR deamination in another experiment. It is possible that a higher concentration of DCF was needed to saturate the ADA in the samples. The number and the extent of experiments performed with DCF were restricted due to the commercial unavailability of this inhibitor. At the drug concentration used, DCF binding to ADA and to other blood proteins may have rendered the inhibitor inaccessible to the total population of ADA molecules contained in the blood samples. Non-specific binding of the nucleoside transport inhibitor NBMPR to plasma proteins and erythrocytes in whole blood has been suggested as a possible reason for the significantly higher amount of

NBMPR required to inhibit transport in whole blood than is required in preparations of isolated cells (Dawicki et al., 1988). In addition, slight differences in experimental design may have contributed to the inconsistent results, including: 1) inhibitor concentration: 1.03 μM DCF was used in the experiment shown in Figure 2.3, whereas 0.934 and 0.943 μM DCF were the calculated concentrations used in the second experiment; 2) blood volume present during the incubation: in the first experiment, 0.1-ml aliquots of blood were incubated in separate tubes, whereas in the second experiment, 0.1-ml samples were removed at the appropriate times from each of 2 incubation tubes containing 0.5 or 0.6 ml of blood obtained from 2 different animals; and 3) anticoagulant: the blood used in the first experiment was collected in a Vacutainer that contained buffered sodium citrate, whereas in the second experiment, Vacutainers with heparin were utilized. The variation in DCF concentration and blood volume to which it was exposed are critical conditions if the inhibitor concentration is not present in excess. The amount of free DCF in the samples may not have been sufficient to inhibit all of the ADA molecules present. In addition, it is possible that the activity of ADA could be affected by heparin or sodium citrate. Measurement of ADA activity with and without increasing amounts of DCF in both duck and human blood samples would confirm the presence of and help characterize the enzyme derived from these two different tissue sources. El Dareer et al. (1989) have reported that mice pretreated with DCF exhibit transiently lower plasma levels of ddI following intraperitoneal injection of ddA as compared to animals not treated with the inhibitor.

Although ddDAPR underwent chemical modification by blood, the subsequent product ddG exhibited resistance to further degradation in this environment. After 10 and 60 min of incubation, the total amount of ddG product present in duck blood samples remained constant. Conversion of ddDAPR to ddG was incomplete after 10 min of incubation in human blood. However, human blood incubated for 10 min contained more than seven times the amount of glycosidic bond cleavage products than was found in duck blood samples incubated for 10 min. High activity levels of the catabolic enzyme PNP are present in human erythrocytes (Agarwal et al., 1971). The apparent differential metabolism of the dideoxynucleoside(s) ddDAPR and/or ddG in the two systems suggests that these nucleoside analogs are more susceptible to catabolism by the components of human blood than by duck blood constituents.

Following i.v. administration of [^{3}H]ddDAPR, the isotopic content of duck blood declined in a biphasic manner, with rapid ($t_{1/2\alpha}$ = 1.65 min and $t_{1/2\beta}$ = 26 min) clearance of radioactivity from the blood. High performance liquid chromatography

analysis of the samples confirmed that the radiolabeled species detected in all samples was ddG rather than the parent compound ddDAPR. Distribution of ddG from the blood to other tissues probably occurs by passive diffusion along concentration gradients. Biphasic elimination of ddG from the blood best fits a 2-compartment kinetic model (Riegelman et al., 1968).

The results presented in this chapter indicate that ddDAPR was rapidly converted in blood to the more biologically stable compound ddG. Therefore, the previously reported *in vivo* antihepadnaviral effect of ddDAPR in Pekin ducks is more likely to be due to ddG or a metabolite of this nucleoside rather than to the parent drug ddDAPR (Lee et al., 1987; Suzuki et al., 1988). Furthermore, in the duck, the pharmacokinetic profile of ddDAPR content of the blood over time suggests that the resulting metabolite ddG was rapidly distributed to and eliminated from other tissues of the body.

V. BIBLIOGRAPHY

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CHAPTER 3
METABOLISM OF DIDEOXNUCLEOSIDES IN DUCK HEPATOCYTE PRIMARY CULTURES

I. INTRODUCTION

The study of hepadnaviruses has been influenced by the restricted host range exhibited by the members of this virus family. Much of what is known about the virus was determined through use of the convenient Pekin duck animal model in the study of DHBV. In lieu of an *in vitro* human test system capable of propagating HBV, pharmacological agents were screened both *in vivo* and *in vitro* for antihepadnaviral activity using the duck model. At the time that the following studies were initiated, *in vitro* drug screening utilized DHBV-infected duck hepatocyte primary cultures.

Potent inhibition of DHBV replication occurs in DHBV-infected duck hepatocyte primary cultures treated with ddDAPR or ddG (Lee et al., 1989; Suzuki et al., 1988). Infected hepatocytes treated for 18 days with 0.004 - 40 μM of ddDAPR or ddG exhibited a dose-dependent decrease in the amount of detectible intracellular DHBV DNA (Lee et al., 1989). The identical dose-response curves generated from treatment of hepatocytes with these compounds indicated that little or no viral DNA could be detected when a dose of 4 or 40 μM was used. The purine dideoxynucleosides ddA and ddI were less potent, exhibiting IC_{50} values of 0.51 and 0.64 μM, respectively, as compared to an IC_{50} of 0.28 μM for both ddDAPR and ddG (Lee et al., 1989). In contrast, the pyrimidine nucleoside analogs ddC and ddT were ineffective inhibitors of the virus in the concentration range tested (Lee et al., 1989).

The work presented in Chapter 3 was initiated to characterize the intracellular metabolism of ddDAPR and ddG in duck hepatocyte primary cultures. The results from Chapter 2 indicate that, in blood, ddDAPR acts as a prodrug for ddG. Therefore, the characterization studies presented in Chapter 3 focus on the intracellular metabolism of ddG. The ability of duck hepatocytes to metabolize the ineffective anti-DHBV agent ddC was examined as a comparison to the effective antiviral agent ddG.

II. MATERIALS AND METHODS

A. Materials

1. General Supplies

Flow Laboratories, Mississauga, ON. was the source of Minimal Essential Medium Eagle (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 NuSerum IV was purchased from Collaborative Research, Inc., Bedford, MA. Tetrabutylammonium dihydrogen phosphate (TBAP) was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. The following supplies were obtained from Sigma Chemical Co., St. Louis, MO.: ethylenediaminotetraacetic acid (EDTA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), collagenase type IV, hydrocortisone 21-hemisuccinate, insulin, trypsin (Type I from bovine pancreas), and the nucleosides 8-aminoguanosine (8-NH_2Guo), 2'-deoxycytidine (2'-dCyd), 2'-deoxyguanosine (2'-dGuo), 2'-deoxyadenosine (2'-dAdo), guanosine (Guo), adenosine (Ado), and thymidine (dThd). The nucleoside analogues 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR), 2',3'-dideoxyguanosine (ddG), and 2',3'-dideoxycytidine (ddC) were purchased from Terochem, Edmonton, AB., while 2',3'-dideoxyguanosine 5'-triphosphate (ddGTP) and 2',3'-dideoxycytidine 5'-triphosphate (ddCTP) were purchased from Pharmacia (Canada), Inc., Baie d'Urfe, PQ. Formycin B (FB), 2'-deoxycoformicin (DCF), and NBMPR were gifts from Dr. A.R.P. Paterson, Dept. of Pharmacology, University of Alberta, and Dr. C.E. Cass, Dept. of Biochemistry, University of Alberta. Sodium pentobarbitol was supplied by M.T.C. Pharmaceuticals, Cambridge, ON., and calcium chloride ($CaCl_2$), perchloric acid (PCA), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), magnesium chloride ($MgCl_2$), phosphoric acid (H_3PO_4), glucose, and mini-scintillation vials were purchased from Fisher Scientific, Nepean, ON. Methanol was purchased from J.T. Baker, Inc., Phillipsburg, N.J., EcoLite liquid scintillation solution was obtained from ICN Biomedicals, Inc., Irvine, CA., Diff-Quik Stain Set was supplied by Harleco, Gibbstown, NJ., Alamine 336 was from Henkel Co., Kankakee, IL., Freon TF was from Du Pont Canada Inc., and Centricon filters (3 and 10) were from Amicon Canada Ltd., Oakville, ON. Drs. A.R.P. Paterson and C.A. Cass provided the paraffin oil (Saybolt viscosity 125-135) and silicone 550 oil. Water was purified using a Milli-Q water purification system (Millipore, Mississauga, ON.) and double distilled water was used in all tissue culture reagents.

2. Nucleoside and Nucleotide Solutions

Standard solutions containing the mono-, di-, and triphosphate forms of ddG or ddC were prepared by treating 1 mM of the corresponding triphosphate for 1.5 minute at 30°C with 0.4 mg potato apyrase (Sigma Chemical Co., St. Louis, MO.; activity/mg: 1.9 units 5'-ATPase, 0.56 units 5'-ADPase, and 0.01 units 5'-AMPase) in 25 mM succinate (pH 6.5) and 5 mM $CaCl_2$. This reaction mixture was diluted (1:4) in 100°C water, boiled for 2 minutes, filtered through a 3000 molecular weight cut-off Centricon-3 filter (Amicon Canada Ltd., Oakville, ON.), and filtrate aliquots were stored at -20°C. Millimolar nucleoside stock solutions were prepared in water or saline and were filter-sterilized. An accurate concentration of each stock solution was determined as described in Chapter 2. The stock solutions were stored at -20°C and were utilized in the preparation of solutions containing micromolar concentrations of nucleosides and/or nucleoside analogs. Most of the physiological bases, nucleosides, and nucleotides utilized in these studies were generously provided by Dr. A.R.P. Paterson.

3. Radiochemicals

[2',3'-^{3}H]2,6-diaminopurine 2',3'-dideoxyriboside ([^{3}H]ddDAPR; 45 Ci/mmol) and [2',3'-^{3}H]2',3 '-dideoxycytidine ([^{3}H]ddC; 48 Ci/mmol) were obtained from Moravek Biochemicals Inc., Brea, CA. and [2',3'-^{3}H]2',3'-dideoxyguanosine ([^{3}H]ddG) was prepared by treating [^{3}H]ddDAPR for 15 minutes at room temperature with adenosine deaminase (Sigma Chemical Co., St. Louis, MO.), 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). HPLC-purified [2,8-^{3}H]-β-D-adenosine in 27% methanol/water was provided by Dr. A.R.P. Paterson. Purified, [^{3}H]-labeled nucleosides were stored in the water/methanol eluate at 4°C and 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]polyethylene glycol ([^{3}H]PEG), while ^3H_2O (100 mCi/ml) was from ICN Radiochemicals, Costa Mesa, CA.

4. Experimental Animals

Fertilized Pekin duck eggs were obtained from a duck colony maintained at the University of Alberta Farm and were held in a 37°C egg incubator (Cyclonic Incubator, Marsh Manufacturing Inc., Garden Grove, CA.) until hatching (approximately 28 days). Sera from newly hatched ducklings were screened for the presence of DHBV DNA by dot hybridization (Lee et al., 1989). The DHBV-infected and uninfected animals were maintained in separate quarters.

B. Cell Cultures

Primary cultures of duck hepatocytes were prepared from two week old DHBV uninfected ducklings using a modified method of Tuttleman et al. (1986). Over the course of this project, minor changes have been made in the primary culture cell preparation procedures. The methods described below represent the evolved procedure.

The liver of a sodium pentobarbital anesthetized animal (approximately 0.5 ml/kg body weight; 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, resulting in a rapid blanching of the liver tissue. An additional 200 ml of medium supplemented with 0.5 mg/ml collagenase (type IV) and 2.5 mM $CaCl_2$ was passed through the liver. After treatment with the collagenase-containing medium, the liver tissue was soft and on the verge of disintegration. The liver was carefully 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 x 10^{-5} M hydrocortisone 21-hemisuccinate. Henceforth this medium will be referred to as supplemented L-15. The liver tissue was dissociated into 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 minutes. The cells were washed 3 times with L-15 medium, allowing the cells to settle for 20 minutes after each wash. The cells were combined in one tube and resuspended to a final volume of 45 ml with supplemented L-15 medium containing 10% NuSerum IV (NSIV). A hemocytometer and the trypan blue dye exclusion method were used to quantify the viable cells in the preparation (Procedure described in section II.D.2.a., Chapter 3). The cells were diluted in supplemented L-15 medium + 10% NSIV to a cell concentration of 5 x 10^5 cells/ml and 5-ml aliquots of this suspension were pipetted into 60-mm tissue culture dishes (2.5 x 10^6 cells/dish). The medium was changed following 4-5 hr incubation in a 42°C humidified incubator. Thereafter, the medium was changed every other day.

C. Dideoxynucleoside Drug Metabolism Studies

1. Metabolism of [2',3'-^{3}H]-dideoxynucleosides in Primary Cultures of Duck Hepatocytes

Tritium-labeled ddDAPR, ddG or ddC ([4 μM]$_{final}$; approximately 6 x 10^6 cpm/dish, 450 - 900 cpm/pmole) in supplemented L-15 medium was added to 60-mm

tissue culture dishes containing a known number of duck hepatocytes (usually 2 x 10^6 - 3 x 10^6 cells) in supplemented L-15 medium with 10% NSIV. The samples were incubated at 42°C for various times (0 - 48 hr). At the appropriate times, the incubation medium was removed and the cell monolayers were washed 3 times with excess dPBS. Cell extracts were prepared as described below.

2. Preparation of a Hot Water Extract of the Duck Hepatocyte Primary Cultures

Washed cells were lysed and extracted by the addition of 2 ml H_2O, followed by a 2-min incubation in a 90 - 95°C water bath. Maximum deproteinization of each sample was accomplished by filtration of the extract through a Centricon-3 filter (centrifuged at 5400 x g for 3-5 hr, 10°C). The ultrafiltered samples were then freeze-dried and stored at -20°C. Sample recovery throughout the extraction procedure and subsequent HPLC analysis was calculated based on sample volumes.

3. HPLC Analysis of the Cell Extracts

Freeze-dried samples were reconstituted in 0.1 ml of filtered water and a known volume (70 - 95 µl) of the sample was then analyzed by ion-pair reverse-phase chromatography as detailed in Chapter 2, section II.D. A flow rate of 1 ml/min was maintained and during sample analysis 0.25-ml eluate fractions were collected directly in mini-scintillation vials. Following the addition of 2.5 ml Tritosol (Pande, 1976) or EcoLite liquid scintillation solution, the radioactivity of each fraction was quantified by scintillation spectrometry.

4. Stability of Intracellular ddG Nucleotides

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

5. Effect of an Excess of Naturally Occurring Nucleosides and Free Bases on ddG Nucleotide Formation

Duck hepatocyte primary cultures (48-hr cultures) were incubated at 42°C for 5 hours in supplemented L-15 medium - 10% NSIV containing 4 µM [^{3}H]ddG (450 - 900 cpm/pmole) with or without 100 µM of a potential competitor nucleoside: 2'-dCyd, 2'-dGuo, 2'-dAdo, 2'-deoxyinosine (2'-dIno), Ado, or Guo. The effect of the bases hypoxanthine (Hx) and guanine (Gua) was also examined, although Gua was utilized at a concentration of 8 µM due to its limited aqueous-solubility. The adenosine deaminase inhibitor 2'-deoxycoformycin (DCF) (1, 10, or 24 µM) was included in the incubation

samples containing 100 μM adenosine and 100 μM 2'-dAdo, and in the corresponding 4 μM ddG controls. In these samples, the cells were pretreated for 45 minutes with DCF before the addition of the test drugs. The effects of 100 μM of the nucleoside analogs formycin B (FB) and 8-aminoguanosine (8-NH_2Guo) on ddG phosphorylation were also tested. In all cases, the cell samples were extracted after 5 hours of incubation and the extracts were analyzed by HPLC.

D. Other Procedures

1. Determination of Intracellular Water Volume

Trypsin-detached cells (5 x 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 5% Triton X-100, 8 ml of Tritosol was added and the tritium content of the pellets was determined.

2. Dye Exclusion Method to Determine Cell Viability

a. *Enumeration of hepatocytes in primary culture preparations*

An estimate of the number of viable hepatocytes in a fresh preparation of primary cultures was determined by enumerating the trypan blue dye-excluding cell population in a representative sample of the preparation. A 50-μl sample of the concentrated cell suspension was incubated for 5 min with 0.95 ml of 0.04% trypan blue in PBS. A small volume of this suspension was applied to a hemocytometer and eight grids (16 squares/grid) were counted at a 10 X magnification. Typically, a total of 200 - 300 cells were counted.

b. *Enumeration of viable cells grown in tissue culture dishes*

The number of viable hepatocytes in a culture was estimated according to the following procedure: 1) The adherent cells were washed with PBS and incubated at 42°C for 5 - 8 min with 2 ml of trypsin solution; 2) The cells were gently dispersed by drawing the suspension up and down in a 5-ml pipette 3) 0.1 ml of the cell suspension was incubated for 5 min with 0.04% trypan blue solution (1:4 or 1:5 dilution of cells to trypan blue solution); 4) A sample of the cell dilution was transferred to a hemocytometer and the viable cells were counted as described above (Chapter 3, section II.D.2.a.).

3. Determination of Protein Concentrations

Adherent cultures were washed 4 times with 2 ml dPBS and solubilized with 1.25

ml of 0.5 M KOH for 30 min at R.T. with gentle agitation. The cells were detached from the plate surface, the mixture was neutralized with 0.625 ml of 1.0 M HCl and mixed by gentle trituration. Protein concentrations were measured by the Bio Rad protein assay (Bio-Rad Laboratories, Mississauga, ON) according to the manufacturer's instructions with bovine serum albumin as the standard protein.

4. Fixation and Staining of Cell Monolayers

Cell cultures grown in 60-mm tissue culture dishes were fixed directly onto the plastic dishes and stained using a commercially available differential staining kit (Diff-Quik Stain Set) that produces an effect similar to that of Wright-Giemsa stain. Medium was removed from the 60 mm tissue culture plate and the cells were washed with 5 ml of warm PBS. The wash was removed and 2 ml of fixative (1.8 mg/L triarylmethane dye in methanol) added and allowed to incubate for 45 sec at room temperature. This solution was then replaced by 2 ml of Solution I (1 g/L xanthene dye) and was incubated for 45 sec. Following the removal of Solution I, 2 ml of Solution II (1.25 g/liter thiazine dye mixture, 0.625 g/L Azure A, and 0.625 g/L methylene Blue) was added, incubated for 45 sec, and removed. The stained cells were rinsed 5 times with 4 ml of water, air-dried by inverting the dish on a paper towel, and the plates were stored indefinitely at room temperature. The fixed, stained cells were photographed at 10X and 40X magnification using an Olympus photographic system (model PM-10 AD) which consisted of an exposure control unit and a BH-2 microscope with an attached C-35 AD camera.

5. Identification of Radiolabeled Metabolites in Extracts from ddG-Treated Duck Hepatocytes

Extracts from ddG-treated hepatocytes were subjected to potato apyrase according to the procedure described in II.A.2. of Chapter 3, except for the following modifications: 1) Three lyophilized, ddG-treated cell extract samples were reconstituted, combined, and preincubated for 5 min at 30°C in a 0.4-ml final reaction volume containing 0.025 M succinate buffer, pH 6.5, 1.25×10^{-4} M ddGTP, and 5 mM $CaCl_2$; 2) "0"-time sample - Upon the addition of 4 mg of apyrase, a 0.18-ml sample of the reaction mixture was diluted into 2 ml of preheated water, heated in a boiling water bath for 2 min, and transferred to an ice bath; 3) 5 min sample - After 5 min of incubation at 30°C, a 0.18-ml sample of the reaction mixture was processed according to the procedure described for the "0"-time sample; 4) The samples were filtered through Centricon-3 filters, the filtrates were lyophilized, and stored at -20°C; 5) The samples were reconstituted in a small volume of water and analyzed by HPLC according to the method described in section II.C.3 of this chapter.

6. Determination of the Degree of ^3H Labeling in the Ribose vs Base Moiety of [^3H]ddG

Four 0.03-ml aliquots of a reaction mixture containing 0.001 M [^3H]ddG (13 - 16 cpm/pmole) were incubated at room temperature for 2 or 25 hr with or without 0.1 M_{final} H_3PO_4. The acid-treated 0.03-ml aliquots were neutralized with an equimolar amount of KOH and the untreated samples received the equivalent volume of H_2O.

a. Thin layer chromatography (TLC) analysis of samples

Using a calibrated glass capillary tube, a 5-μL volume of each sample was applied to a small silica gel TLC plate (Silica gel 60, F254, plastic backed, 3 cm x 7 cm) and allowed to air-dry. The plate was run twice in SSE solvent, allowing the plate to dry after each run. SSE solvent consisted of ethyl acetate + isopropanol + H2O in a 4:1:2 proportion (This mixture was combined by several cycles of vigorous shaking followed by settling, and after allowing the layers to separate overnight, the top (organic) layer was collected and stored at room temperature for an indefinite amount of time). The plate was examined under short-wave ultraviolet light and the visible spots were circled with pencil. The plate was sprayed with 5% sulfuric acid in ethanol, placed on a hot plate (100^{0}C), and heated until the ribose portion of each sample became visible as a brown spot. Each sample lane of the plate was cut into 6 sections and the pieces were placed in scintillation vials. Total count samples were prepared by spotting duplicate 5-μL aliquots of each sample onto 1-cm^2 pieces of silica gel plate, and, in addition, two 5-μL aliquots of each sample were pipetted directly into scintillation vials. Twelve milliliters of scintillation cocktail was added to each vial and the radioactivity in each sample was quantitated by scintillation spectrometry.

b. HPLC analysis of samples

A 10-μL volume of each sample was analyzed by reverse-phase chromatography using a Varian 5000 Liquid Chromatograph with a 3-μm C-18 column (Supelcosil LC-18-T; 15 cm x 4.6 mm i.d.) preceeded by a 5-μm Supelcosil LC-18 guard column (2 cm x 4.6 mm i.d.). The 25-minute gradient mobile phase separation program began with 100% water for 3 minutes, at which point methanol was introduced and linearly increased to 5% by 10 minutes. This was followed by a linear increase in methanol composition to 30% by 20 minutes. The methanol composition was then reduced, returning the column to initial conditions by 25 minutes. Sample analysis was performed at room temperature, absorbance was monitored at 254 nm, and a flow rate of 1 ml/min was maintained. During sample analysis, eluate fractions (0.4 or 0.5 ml/vial) were collected in mini-scintillation vials and, following the addition of EcoLite liquid scintillation fluid, the radioactivity of each fraction was quantified by scintillation

counting.

7. Comparison of Extraction Methods for the Extraction of Nucleosides and Nucleotides From Duck Hepatocyte Primary Cultures

Duck hepatocyte primary cultures were prepared as described in section II.B. of this chapter. Immediately before extraction of the cells, each 60-mm tissue culture dish was washed by dunking the plate in 1 liter of ice-cold dPBS and the plate was drained on a paper towel for 15 seconds before extraction. The following extraction procedures were performed:

a. ***Hot water extraction***

The washed cells were lysed and extracted by the addition of 2 ml of room temperature H_2O, followed by immediate incubation of the plate for 2 minutes in a 90°C water bath. The extract was filtered through a Centricon-10 filter (5000 x g; 3 hr; 4°C) and the filtrate volume was measured and stored at -20°C.

b. ***Cold water extraction***

The washed cells were extracted with the addition of 2 ml of ice-cold H_2O, followed by incubation in an ice bath for 5 minutes. The sample was processed according to the procedure described for hot water extraction (Chapter 3, section II.D.7.a.).

c. ***60% Methanol extraction (4 min)***

To the washed cells was added 2 ml cold 60% methanol (the methanol was chilled in an acetone-dry ice bath). The tissue culture dish was put on a metal tray and incubated for 4 minutes in a -20°C freezer. The extract volume was measured, transferred to a glass conical tube, and stored at -20°C.

d. ***60% Methanol extraction (30 min)***

The cell monolayer sample was treated in the same manner as described in section II.D.7.c. above, except that the sample was incubated in a -20°C freezer for 30 minutes.

e. ***70% Methanol - 25 mM Tris/HCl, pH 7.4***

Two ml of cold 70% methanol - 25 mM Tris/HCl, pH 7.4 (chilled in an acetone-dry ice bath) was added to the sample plate. The plate was placed on a metal tray and incubated in a -20°C freezer for 30 minutes. The sample was processed as described in section II.D.7.c. above.

f. ***0.4 M Perchloric acid (PCA) extraction***

The washed cells were detached from the plate by a 5-min incubation at 37°C with 1.5-ml trypsin solution. The cells were transferred to a microfuge tube and centrifuged at 12,000 g x 1 min. The cell pellet was washed with PBS, extracted for 20

min on ice with 0.1 ml of cold 0.4M PCA, and centrifuged (12,000 g x 1 min). The supernatant fraction supplemented with 1 µl of the pH indicator Bromcresol Purple (0.1%) was neutralized by the addition of an equal volume of 0.5 M Alamine/freon. Following a 15-min incubation at room temperature, the extract was centrifuged (12,000 g x 1 min) and the upper layer was collected and stored at -20°C.

g. HPLC analysis of extracts

The cell extract samples were concentrated by lyophilization and were resuspended in a small volume of H_2O. The extracts were analyzed by ion exchange chromatography using a Whatman Partisil 10 SAX column (25 cm x 4.6 mm i.d.) preceeded by a guard column packed with Whatman pellicular anion exchanger. Separation of di- and triphosphate nucleotides was accomplished by a 45-minute isocratic elution program with 0.25 M KH_2PO_4 - 0.5 M KCl, pH 4.5, using a flow rate of 2 ml/min. During sample analysis, the eluate was monitored for absorbance at 254 nm. The area counts obtained from the ATP and the ADP absorbance peaks in a particular sample were used to calculate an ATP/ADP ratio. The ATP/ADP values from the various samples were compared to assess the quality of the extraction procedure, with the ATP/ADP ratios indicative of whether or not the nucleotide concentrations in the extract were representative of the *in vivo* ratios.

8. Adenosine Uptake Measurements in Duck Hepatocyte Primary Cultures

The composition of solutions utilized in this section is itemized in Appendix III. Twenty-four-hour cell cultures were treated for 2 hr at 37°C with 1 µM DCF before adenosine uptake was initiated either at room temperature, following a 15-min equilibration at ambient temperature, or in a room maintained at 37°C. For samples not treated with DCF, solutions were prepared without the adenosine deaminase inhibitor. Immediately prior to the addition of 1.5 ml of 10 µM [^{3}H]adenosine (35 - 37 cpm/pmol) in sodium buffer, the sample plate was washed twice with 2 ml of sodium buffer containing 1 µM DCF. Two seconds before the adenosine incubation period had ended, the nucleoside solution was removed by aspiration, and the adherent cell layer was washed by submerging the plate in 3 successive volumes (1.4 L, 1 L, and 1 L) of ice-cold dPBS. For each time point, correction for cell-associated radioactivity attributed to nonspecific binding and NBMPR-insensitive nucleoside fluxes was made using uptake values obtained for samples treated with sodium buffer and adenosine solutions supplemented with 10 µM NBMPR. The washed cells were extracted with 1.5 ml of 0.4 M TCA for 20 min on ice, the extract transferred to a microcentrifuge tube in an ice bath, and after 20 samples had been collected, they were centrifuged (12,000 x g) for 2 min in a 4°C room. The extracts were transferred to glass tubes (13 x 100 mm) containing 1.5

ml 0.5 M alamine/freon, and the tubes were vortexed for 15 sec and centrifuged (1000 x g, 2 min) in a cold table top centrifuge. A 1-ml volume of the aqueous layer was transferred from each tube to a 10-ml glass conical tube, and the extracts were stored at -20°C until they were lyophilized. The percent recovery achieved after sample processing was determined by comparing the amount of radioactivity left in 4 samples treated with 0.4 M TCA containing [^{3}H]adenosine with the known amount of TCA-[^{3}H]adenosine added to the 4 plates. Lyophilized extracts were reconstituted in 0.05 ml water immediately prior to sample analysis by thin layer chromatography (TLC) or HPLC.

a. ***Determination of the radiolabeled nucleoside and nucleotide content of hepatocyte extracts by TLC***

Fifteen microliters of extract plus 1 µL of a standard mixture (1 µL contained 4 x 10^{-8} moles each of Ado, Ino, AMP, ADP, and ATP) was combined in a microcentrifuge tube and spotted, 5 µL at a time, onto a plastic-backed polyethyleneimine cellulose TLC plate (Polygram MN CEL 300, PEI/UV$_{254}$, 20 x20 cm; Macherey-Nagel, Düren, Germany) that had been prewashed with water and 50% methanol. The microfuge tube was rinsed with 10 µL of water and this volume was applied to the plate in the same manner. A duplicate sample of each extract containing standards was applied to a 1 inch x 0.5 inch piece of plate and was used to determine the total amount of radioactivity in the sample. Inosine and adenosine were separated from the nucleotide metabolites when the sample-loaded plates were developed to within 1.5 cm of the top with 0.05 M acetic acid and then redeveloped for 10 cm with 2.6 M $(NH_4)_2SO_4$ (R_f = 0.73, 0.58, and 0.22 for Ino, Ado, and adenine nucleotides, respectively). To resolve the nucleoside mono-, di-, and triphosphates, another set of samples and plates fitted with filter paper wicks (Whatman #1) were developed overnight with 50% methanol, and then developed in a stepwise manner without intermediate drying of the plates: Development 1 - ascending chromatography with 0.66 M acetic acid, proceeding to a point 2 cm from the lower edge of the plates; Development 2 - continued development with 0.66 M acetic acid-0.33 M LiCl to a point 10 cm from the bottom; and Development 3 - 0.66 M acetic acid-0.66 M LiCl to within 1.5 cm of the top (R_f = 0.59, 0.25, and 0.056 for AMP, ADP, and ATP, respectively). Dried plates were viewed under shortwave UV light, the visible spots corresponding to nucleosides and nucleotides were marked with pencil, and sections of plate containing the compounds of interest were excised. The plate sections were transferred to 20-ml scintillation vials, incubated with elution buffer overnight at 37°C with gentle mixing, the pH was adjusted to approximately pH 8 with 0.06 ml of 12 N HCl, and 12 ml of Triton-X was added. The radioactivity in each sample was quantitated by scintillation spectrometry.

b. *HPLC analysis of hepatocyte extracts*

A 0.05-ml volume of cell extract supplemented with standards (0.89×10^{-9} to 3.6×10^{-9} moles each of Hx, Ino, Ado, AMP, ADP, and ATP) was analyzed by reverse-phase HPLC according to the following specifications: 1) Spectra Physics 8000 HPLC; 2) Whatman partisil-10 ODS column; 3) column temperature = 42°C; 4) flow rate = 1 ml/min; 5) a 3 solvent system: Buffer A: 0.1 M KH_2PO_4, pH 4.75; Buffer B: 100% methanol; Buffer C: 0.02 M KH_2PO_4 - 5 mM TBAP, pH 4.8; and 6) a 65-min gradient mobile phase separation program:

Time	Buffer A	Buffer B	Buffer C
0	0	0	100
10	0	0	100
15	0	10	90
30	15	15	70
40	35	25	40
55	45	30	25
65	0	0	100

Absorbance was monitored at 254 nm and 0.5-ml eluate fractions were collected and quantified by scintillation spectrometry. Both the internal standards and an independent analysis of a standard mixture were used to identify the radiolabeled metabolites.

III. RESULTS

A. Growth Conditions and Properties of Duck Hepatocyte Primary Cultures

Prior to beginning the drug metabolism studies, a series of experiments was performed to optimize the growth conditions of duck hepatocyte primary cultures. The results reported in sections III.A.1 through III.A.3 below were obtained from experiments conducted by Darlene McClure, Andrew Ng, and myself, under the guidance of Dr. C. Cass and Dr. A.R.P. Paterson. The incubation temperature study reported in section III.A.4 was the result of my efforts. Two people were involved in the preparation of duck hepatocyte primary cultures. Beginning with section III.A.4, the experiments reported in this chapter relied on hepatocyte isolation and culture set-up performed by Jy Huang and myself.

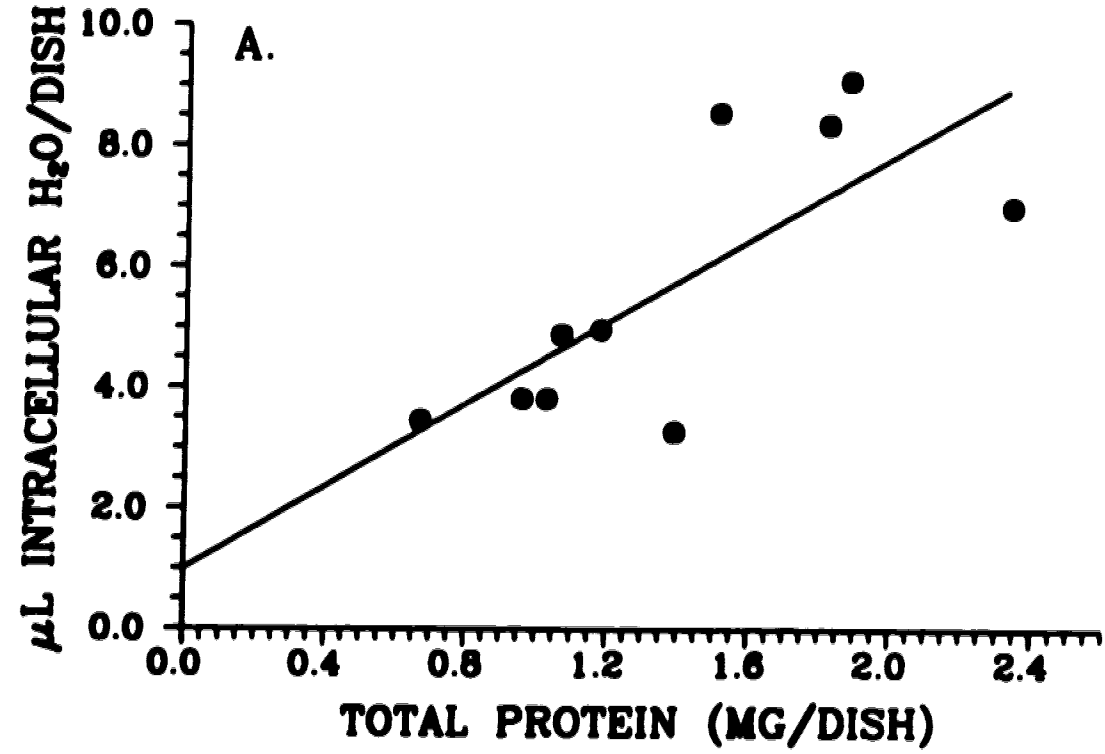
1. Evaluation of Cell Number, Intracellular Water Volume, and Protein Content of Cultures

Isolation of hepatocytes from the liver of 2 week-old ducklings typically yielded 5.5×10^8 ($\pm 0.8 \times 10^8$; n = 5) cells/liver. When 24-hr duck hepatocyte primary cultures were analyzed for total milligrams of protein and total number of cells per 60-mm tissue culture dish, there was a positive correlation between these parameters as they increased in value (Figure 3.1B). Similarly, an increase in protein content of 24-hr monolayer cultures was associated with an increase in the total intracellular water content of the cultures (Figure 3.1A). As can be seen in Figure 3.2B, primary cultures of duck hepatocytes experienced a time-dependent decrease in the population of viable cells. In this particular experiment, the hepatocyte population decreased with a $t_{1/2}$ of 4.6 days (Figure 3.2B). Time-dependent attrition of the cell population is in contrast to the uniform protein content maintained in the cell cultures (Figure 3.2A and B). Over a span of 2 weeks, the protein content of cultured hepatocytes ranged from 1.37 to 2.00 mg protein/dish, with a mean (± S.E.) value of 1.71 ± 0.091 mg protein/dish calculated for this time period. The estimated intracellular water volume of duck hepatocytes maintained in culture for 1- to 6-days was 3.84 ± 0.31 $\mu L/10^6$ cells (mean ± S.E., n = 19), with values ranging from 1.99 to 6.57 $\mu L/10^6$ cells. Intracellular water volume measurements determined for 1- and 2-day cultures were, respectively, 3.71 ± 0.40 $\mu L/10^6$ cells (n = 8) and 3.81 ± 0.63 $\mu L/10^6$ cells (n = 5).

2. Adenosine Uptake by 24 Hr Cultures

The ability of 24-hr duck hepatocyte primary cultures to salvage and metabolize an exogenous supply of adenosine was examined as a measure of the integrity of cell metabolic function *in vitro*. Adenosine was incorporated into cellular nucleotides when 24-hr duck hepatocyte primary cultures were incubated at room temperature or at 37°C

Figure 3.1 - Relationship of cell sample protein content to cell number and intracellular water volume in 24-hr duck hepatocyte primary cultures. Hepatocyte monolayers contained in 60-mm tissue culture dishes were analyzed for protein content, cell number, and intracellular water volume according to the methods described in Chapter 3, sections II.D.1, 2, and 3. Panel A) Relationship of the total protein content of each culture with the intracellular water content of each dish. Panel B) Relationship of the total protein content of each sample with the total number of cells present in each culture. Each point represents the mean value calculated from 3 to 4 replicate samples. The data were obtained from single experiments for each protein concentration-to-cell number or protein concentration-to-intracellular water volume value shown.


A.
μL INTRACELLULAR H2O/DISH
10.0
8.0
6.0
4.0
2.0
0.0
0.0
0.4
0.8
1.2
1.6
2.0
2.4
TOTAL PROTEIN (MG/DISH)

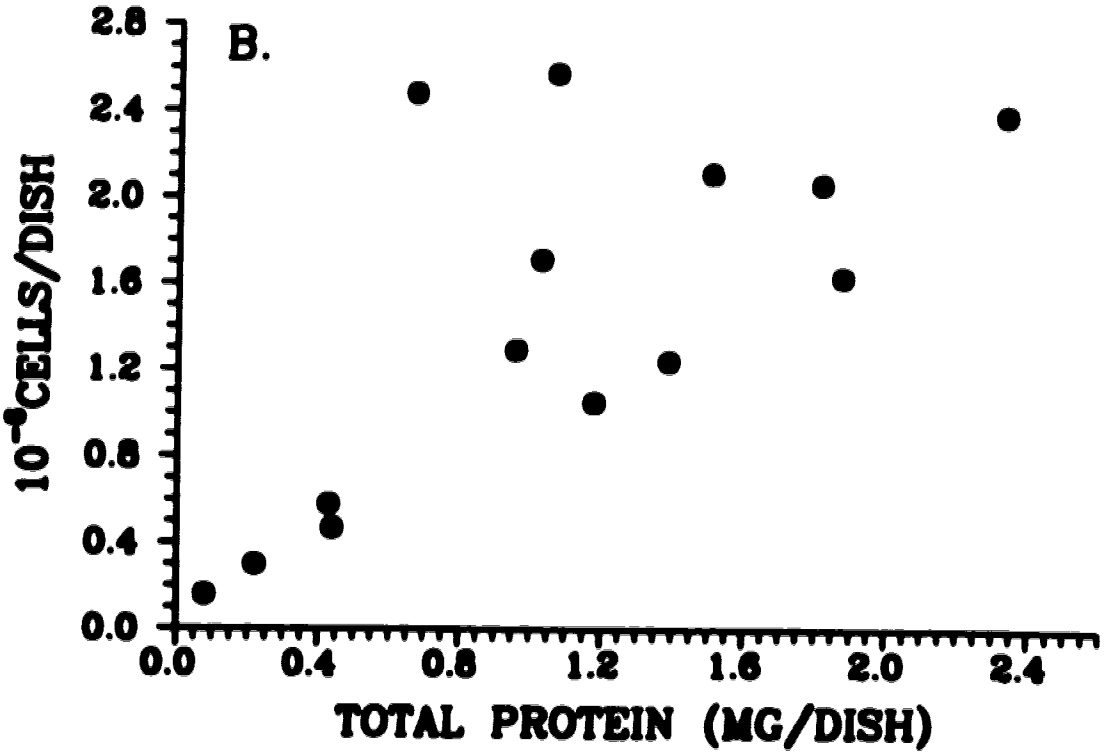
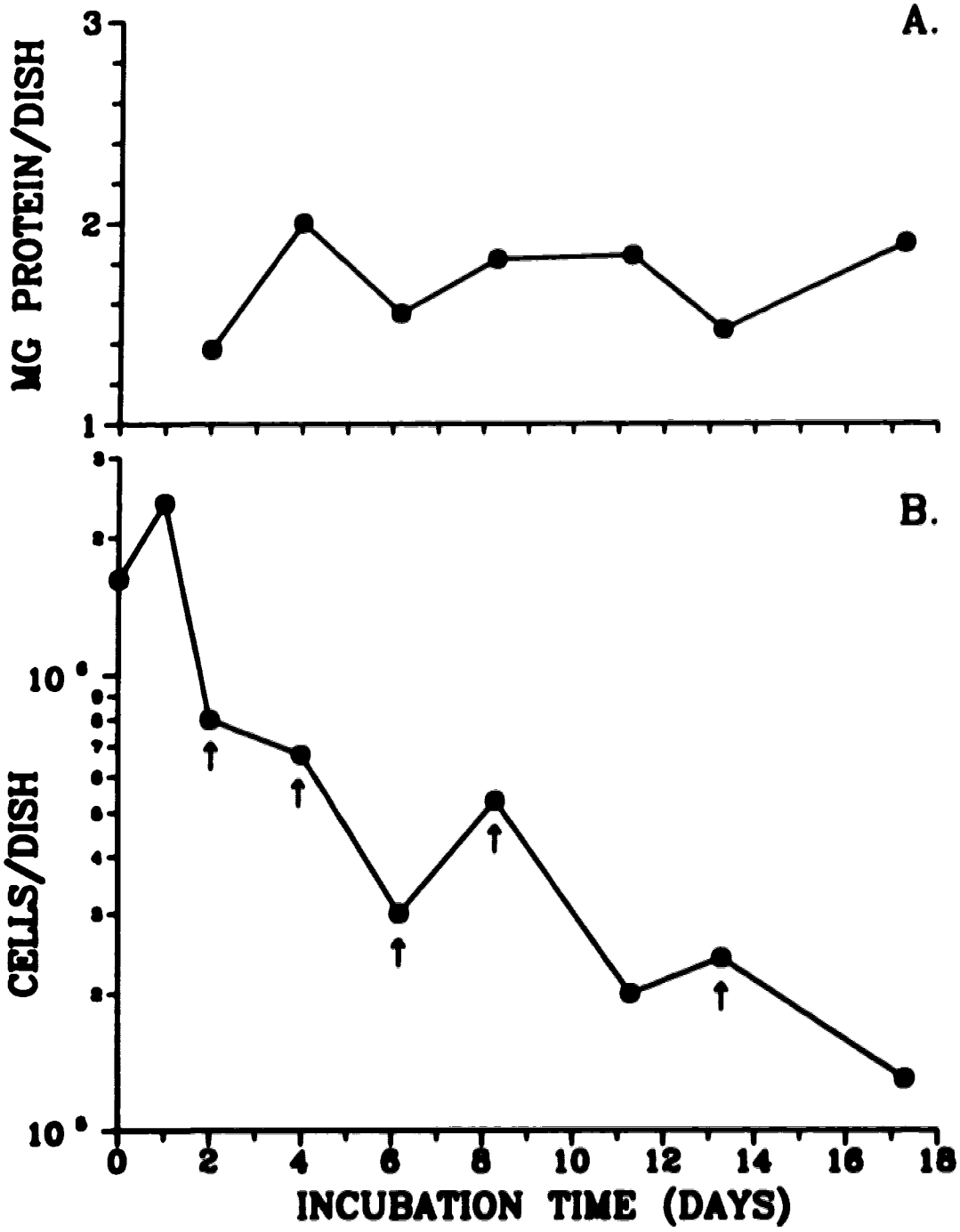

B.
10⁻⁶CELLS/DISH
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0.0
0.4
0.8
1.2
1.6
2.0
2.4
TOTAL PROTEIN (MG/DISH)

Figure 3.2 - Time-dependent alterations in the total protein content and number of viable cells present in a population of duck hepatocyte primary cultures. Hepatocytes obtained from the livers of two 13 day-old Pekin ducklings were plated at an estimated 1.6×10^6 cells/plate and incubated at $37^{\circ}C$. Panel A) The protein content of hepatocyte monolayers was determined in cultures incubated for extended periods of time. Panel B) The population of viable hepatocytes was enumerated for monolayer samples maintained in culture for 1 to 18 days. In Panels A and B, each point represents the mean value obtained from 3 or 4 replicate samples. Media changes are indicated by arrows.


A.
MG PROTEIN/DISH
3
2
1
B.
CELLS/DISH
10 6
10 5
0
2
4
6
8
10
12
14
16
18
INCUBATION TIME (DAYS)

with 10 μM [^{3}H]Ado (37 cpm/pmol) (Table 3.1). Analysis of cell extracts by TLC indicated that after a 1-min exposure at room temperature, approximately 50% of the extract radioactivity was recovered in the form of nucleotides, while Ado and Ino were present at much lower levels (Table 3.1). Of the radiolabeled nucleotides found in cells incubated for 1 min with Ado, approximately 80% were in the form of ATP, while 13-14% and 3% were present as ADP and AMP, respectively (Table 3.1). When untreated cell samples were extracted with TCA containing a known quantity of [^{3}H]Ado, the recovery of radioactivity post-extraction and extract processing was estimated to be 70.6% ± 1.9 (n=4). Radiolabeled nucleotide levels continued to increase in hepatocytes exposed to adenosine for extended periods of time. HPLC analysis of an extract from duck hepatocytes incubated for 2 hr with 10 μM adenosine (132 cpm/pmole) in the presence of 1 μM DCF resulted in 89% of the sample radioactivity present as nucleotides, while 0.3%, 0.4%, and 1.2% co-eluted with, respectively, adenosine, inosine, and hypoxanthine internal standards. In addition, an unidentified metabolite eluting 2 min prior to AMP contained 6.6% of the sample radioactivity and was detected as a large peak by UV absorbance monitoring. The intracellular accumulation of adenine nucleotides following a 2-hr exposure to adenosine was 3609, 512, and 59 pmol/10^6 cells of ATP, ADP, and AMP, respectively. Using these concentration values, the ATP/ADP ratio was 7.

3. Evaluation of Cultures Maintained on Positively- Versus Negatively- Charged Tissue Culture Plates and in Media Containing Serum Supplements

There was no difference in the behavior of duck hepatocyte cultures maintained for 7 days on 60-mm tissue culture plates that had a net positive (Falcon Primaria) or net negative (Nunc) charge to the growth surface. For example, cells maintained comparable amounts of protein. The mean (± S.D.) mg protein/plate measured during the 7-day period was 2.10 ± 0.19 for the negatively-charged and 2.08 ± 0.17 for the positively-charged surface. Tissue culture plates with a net negative charge (Nunc) were used in all subsequent experiments.

Duck hepatocyte cultures were maintained in L-15 medium supplemented with a) 5% fetal calf serum (FCS); b) 10% Nu-Serum, a commercial serum product (Collaborative Research, Bedford, MA) containing 25% new-born calf serum plus growth factors, hormones, and undefined additives; or c) 10% Nu-Serum IV (NSIV), a low-serum formulation containing 25% FCS plus the additives described in b) above. The cell viability curves generated and sample protein concentrations measured over a 7-day time period did not differ for the 3 groups of samples. Therefore, the more economical NSIV replaced FCS as a medium supplement in all future experiments.

Table 3.1

UPTAKE OF ADENOSINE BY DUCK HEPATOCYTE PRIMARY CULTURES

Twenty-four-hour duck hepatocyte primary cultures were pre-incubated for 2 hr at 37°C with or without 1 μM DCF. Cultures were then incubated at RT or 37°C for 1 min with 10 μM Ado (37 cpm/pmol; [2,8-^{3}H]-β-D-Ado). The samples were extracted, and the extracts were processed and analyzed according to the procedure outlined in sections II.D.8. and II.D.8.a. of Chapter 3. The amount of radiolabeled Ino, Ado, and total nucleotides (total NTDs) detected in each sample was expressed as a percent of the total radioactive content of the sample. The amount of AMP, ADP, and ATP contained in the extracts was expressed as a percent of the total nucleotide content of the extract. The ATP/ADP ratio achieved for each sample condition is indicated. The results shown represent duplicate determinations from one experiment.

Sample	% CPM Recovered			% Total NTDs Recovered			ATP/ADP
	Ino	Ado	Total NTDs	AMP	ADP	ATP	
RT; - DCF	1.2	13	55	3.4	14	79	5.8
RT; + DCF	0.6	13	42	3.1	14	80	5.8
37°C; + DCF	1.1	4.0	56	2.9	13	82	6.4

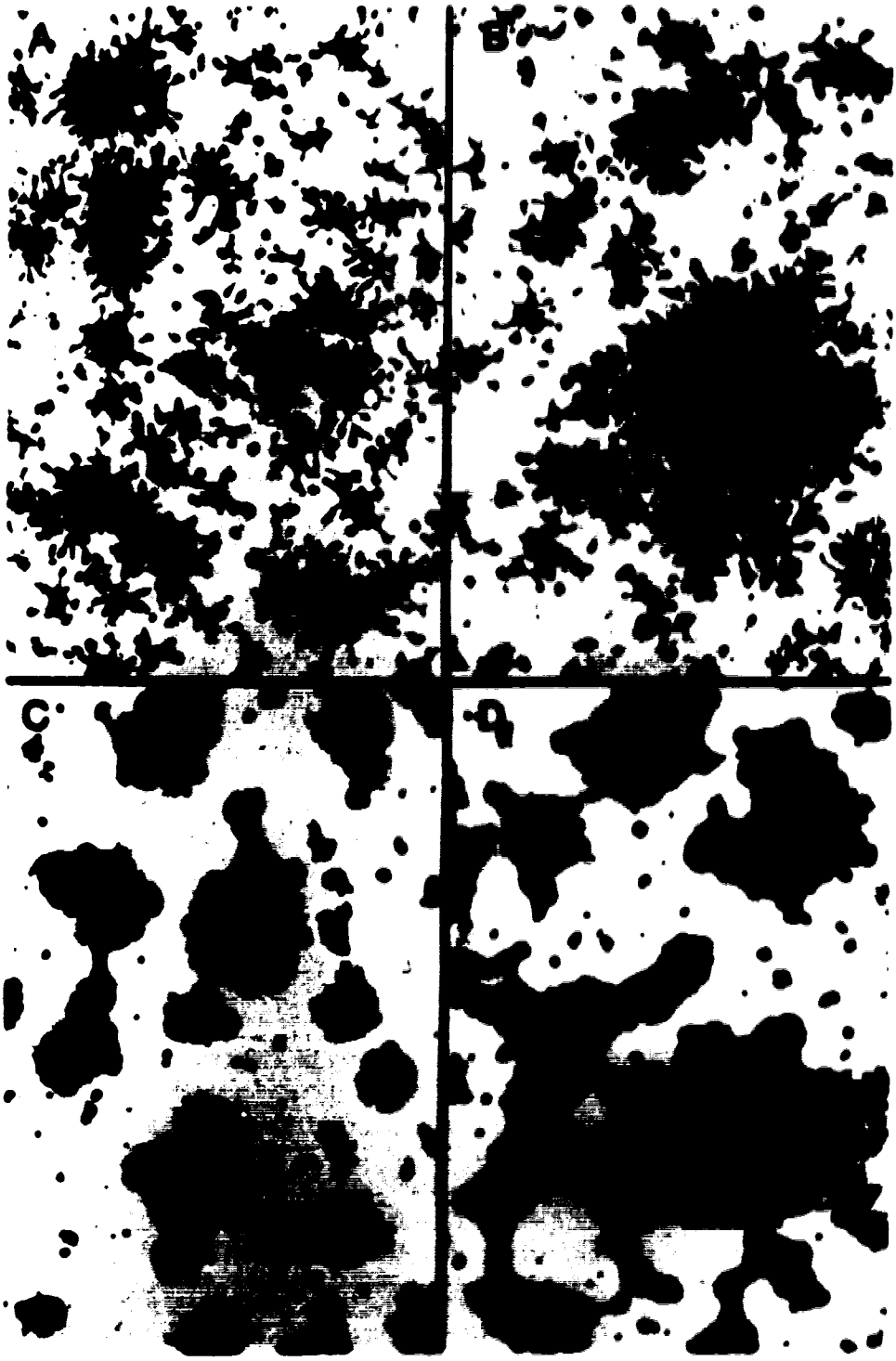
4. Morphological Comparison of Cultures Grown at 37°C versus 42°C

Duck hepatocyte primary cultures obtained from the liver of a DHBV-negative, 2 week-old duck were seeded at 1.4 x 10^6 cells/60-mm dish and incubated at either 37°C or 42°C for 39 days. During this time, representative monolayer cultures were periodically fixed and stained according to the procedure outlined in Chapter 3, section II.D.4. Within 4 hours of plating, the cells attached to the tissue culture dish surface and the medium was changed. Figure 3.3 presents a visual comparison of 1, 2, 4, 8, 18, and 39 day-old monolayers grown at the 2 temperatures. In both samples, cell aggregation is apparent at 26 hr and is more defined after 2 days in culture. During the first 2 days in culture, cells incubated at 42°C (Figure 3.3, B and D) became firmly established as granular epithelial cell aggregates while colony-formation in 37°C samples (Figure 3.3, A and C) appeared to be retarded. The number of cells per plate from both incubation conditions was the same at 26 hr (1.06 x 10^6 and 1.02 x 10^6 cells for 37°C and 42°C samples, respectively) and similar at 4 days (0.39 x 10^6 and 0.54 x 10^6 cells for 37°C and 42°C samples, respectively). The characteristic polygonal morphology of liver cells was apparent in both sets of cultures through days 4 to 6 (Figure 3.3, E and F; 6-day time point not shown), although slight dissolution of the organized mosaic of cells is visible in the 37°C, 4-day sample. As can be seen in Figure 3.3, G and H, drastic degeneration of the culture morphology occurred by 8 days in culture, with the 37°C monolayer appearing fibroblastic and the 42°C sample presenting less defined, irregular-shaped cells. This alteration in cell morphology occurred within a 2-day period and involved the whole culture. At 8 days in culture, both incubation temperatures produced cells that were less granular in appearance than the cells incubated for 4 days or less (Figure 3.3, A - H). Continued maintenance of the cell cultures resulted in progressive degeneration of the cells (Figure 3.3, I - L). Unless otherwise noted, the ensuing metabolism experiments employed 2 day-old duck hepatocyte primary cultures that were incubated at 42°C.

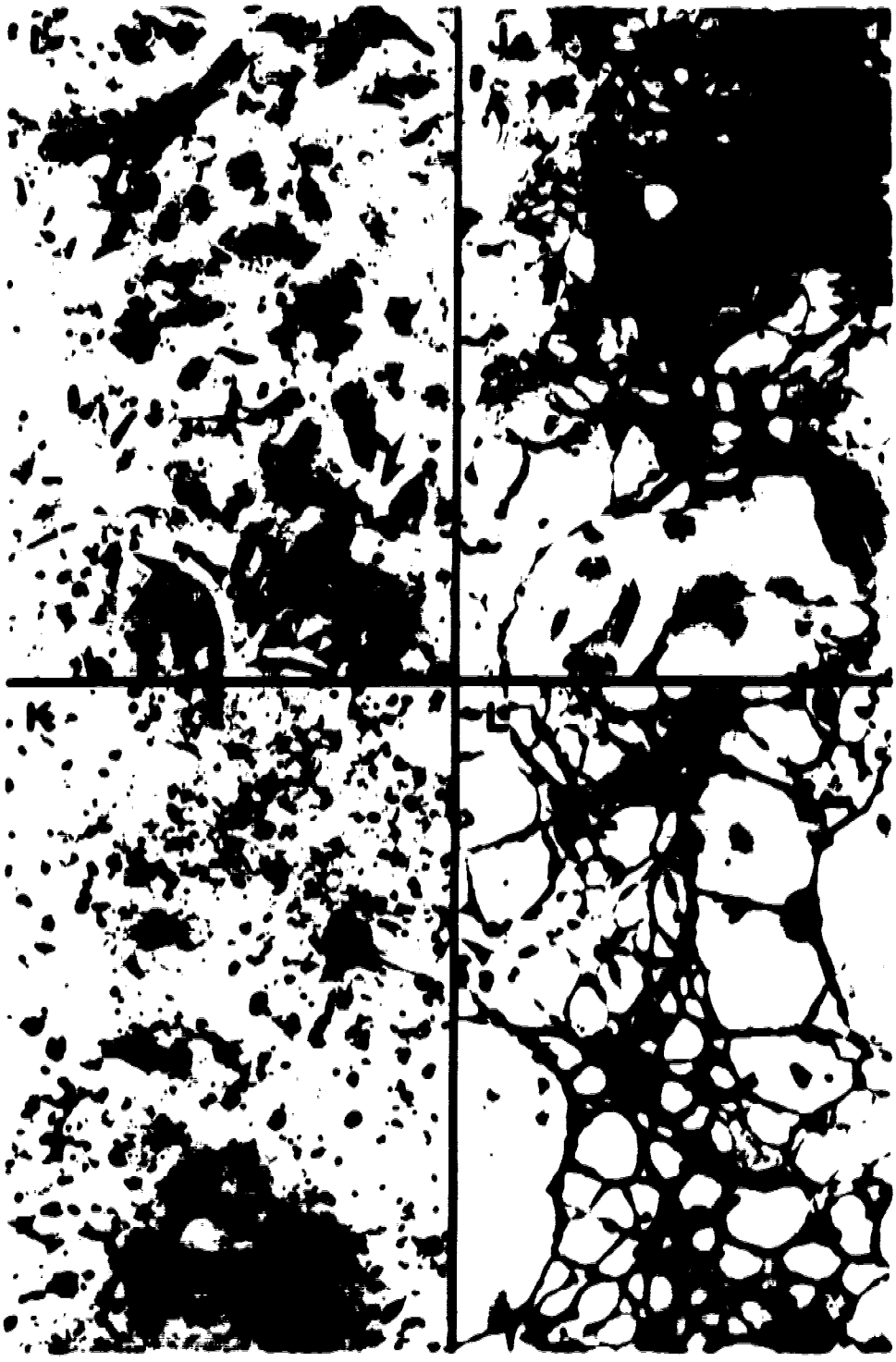
B. Comparison of Extraction Methods

Normally, nucleobases, nucleosides, nucleotides and related compounds are extracted from cells with a protein precipitant such as perchloric acid (PCA) or trichloroacetic acid (TCA). The acid extracts must then be neutralized to prevent breakdown of the biochemicals during storage. Purine 2'-deoxynucleosides and 2',3'-dideoxynucleosides are especially susceptible to acid-catalyzed hydrolysis of the molecular N-glycosidic bond, the end result of which is liberation of a purine base and ribose sugar (York, 1981). Conversely, pyrimidine nucleosides and their derivatives are more resistant to acid-induced hydrolysis. The acid-lability of ddG was tested by treating [8-^{3}H]ddG samples for 20 min and 4 hr with 0.3M TCA. HPLC analysis of

Figure 3.3 - Comparison of duck hepatocyte primary cultures grown at two different incubation temperatures. Duck hepatocytes were obtained and plated as described in Materials and Methods. The plates were incubated at either 37°C or 42°C in an humidified environment. At various times, plates were removed from the incubators, stained, and photographed according to the procedure outlined in Materials and Methods. The cell monolayers presented in this figure are shown at 10X magnification. A) 26-hr incubation, 37°C; B) 26-hr incubation, 42°C; C) 2-day incubation, 37°C; D) 2-day incubation, 42°C; E) 4-day incubation, 37°C; F) 4-day incubation, 42°C; G) 8-day incubation, 37°C; H) 8-day incubation, 42°C; I) 18-day incubation, 37°C; J) 18-day incubation, 42°C; K) 39-day incubation, 37°C; and L) 39-day incubation, 42°C.


A
B
C
D

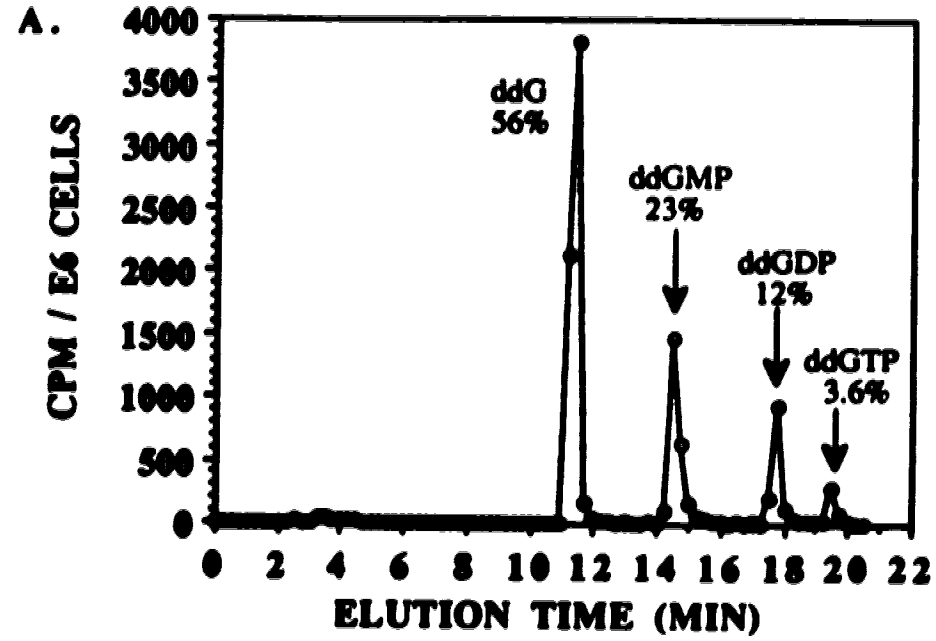

F

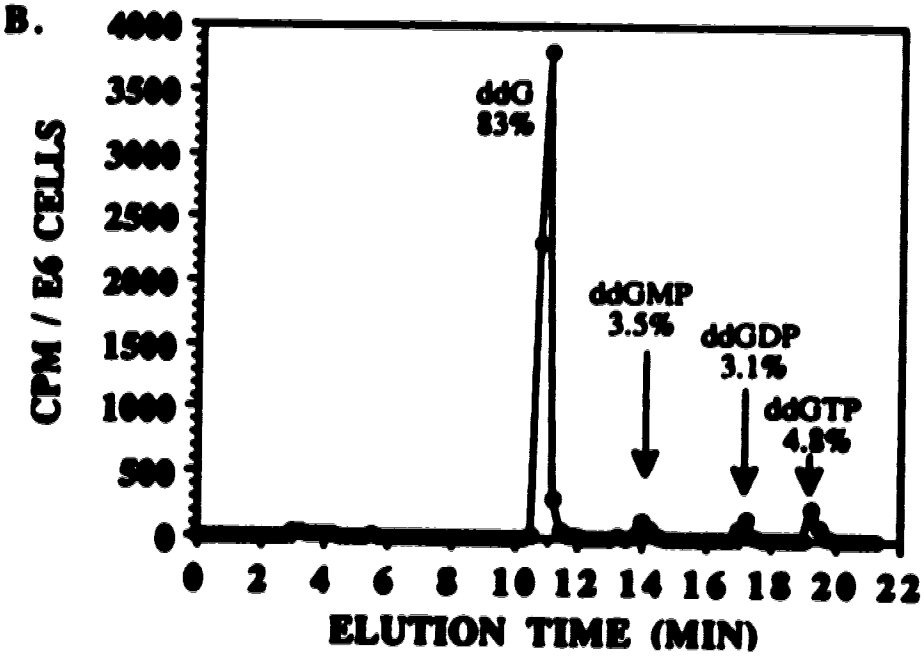

K

alamine/freon neutralized samples revealed that, after a 20-min exposure, approximately 50% of the ddG had cleaved, releasing the radiolabeled guanine base. Less than 1% of the ddG remained intact following a 4-hr exposure to the acid environment.

Alternate nucleotide extraction conditions yielded a poorer quality of nucleotide extraction from duck hepatocyte primary cultures than the acid extraction procedure. In the acid extraction process, hepatocytes were first trypsin-detached from the tissue culture plate and concentrated by centrifugation before being exposed to a small volume of PCA. Following neutralization of the acid extract, the sample was stored at -20°C. An ATP/ADP value of 1.14 was calculated after HPLC analysis of the extract. Direct application of 2 ml of 0.4 M PCA to hepatocyte monolayers, neutralization with an equal volume of 0.5 M alamine/freon, and lyophilization of the extract resulted in a syrupy concentrate that could not be analyzed by HPLC. The other extraction conditions tested involved direct application of 2 ml of the appropriate extractant solution to hepatocyte monolayers. The resulting extracts were reduced in volume by lyophilization before chromatographic analysis was performed. Hepatocyte monolayers treated with H_2O for 2 min at 90°C yielded an ATP/ADP value of 0.374 ± 0.044, whereas exposure to ice-cold water for 5 min resulted in a value of 0.019 ± 0.021. Similar to the hot water extraction results, a sample treated with 50 mM Tris - 2mM $MgCl_2$, pH 7.2 for 2 min at 90°C produced a ratio of 0.32. Extraction of cells with 60% methanol for 4 min or 30 min at -20°C produced individual ATP/ADP values that varied wildly for replicate samples. Hepatocytes extracted with 60% methanol for 4 min produced a mean (n = 5) ATP/ADP ratio of 1.09 ± 1.44, with values ranging from 0 to 3.63, while extraction for 30 min yielded a ratio of 0.119 ± 0.23 (n = 4). A comparison was made of duck hepatocyte primary cultures incubated for 5 hr with 4 μM isotopically-diluted ddG and extracted by either the hot water method or by treatment with 60% methanol on ice for 4 min. Figure 3.4. illustrates that although similar amounts of ddG were recovered under both conditions, there was a difference in both the amounts and proportions of the radiolabeled metabolites recovered. A comparatively small quantity of ddG nucleotides was recovered in the methanol extract. However, the methanolic condition resulted in ddGTP recovery in excess of ddGDP and ddGMP, while the hot water extract contained dideoxynucleotides in the proportion of ddGMP > ddGDP > ddGTP. The best ATP/ADP ratio obtained for a non-acid extraction method, a value of 2.59 ± 0.415 (n = 3), was achieved when hepatocytes were extracted with 70% methanol - 25 mM Tris, pH 7.4 for 30 min at -20°C. When these results were compared with the hot water extraction data, the two procedures yielded similar amounts of ADP but differed in the amount of ATP recovered. Unfortunately, the 70% methanol-25 mM Tris condition was not examined

Figure 3.4 - Comparison of two methods employed in the extraction of nucleosides and nucleotides from duck hepatocyte primary cultures. Duck hepatocyte primary cultures (48-hr cultures, 2.80 x 10^6 cells/plate) were incubated at 42°C for 5 hr with 4 μM [^{3}H]ddG (665 cpm/pmole) in L-15 medium supplemented with 10% NSIV. The cells were: A) Hot-water extracted according to the method outlined in Chapter 3, II.C.2.; or B) Extracted for 4 min on ice with cold 60% methanol, followed by extract filtration (Centricon-10 filter), and lyophilization. The dried extracts were reconstituted in 0.1 ml water and 0.09 ml of each sample was analyzed by ion-pair reverse-phase HPLC. 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 radiolabeled metabolites as ddGMP, ddGDP, and ddGTP was made on the basis of having elution times identical to those of ddGMP, ddGDP, and ddGTP standards. The percentage values noted for each of the major peaks shown in panels A and B indicate the percent of the total eluted radioactivity that was contained in the individual peaks detected in each sample. The data shown in panels A and B are representative of the results obtained from replicate samples in the same experiment. (The mean (± S.D.) percent of the total counts recovered in 4 replicate samples of hot water extracted cells was 58 (± 3) for ddG, 20 (± 2) for ddGMP, 14 (± 2) for ddGDP, and 3.7 (± 0.7) for ddGTP, while duplicate samples of methanol-extracted cells resulted in an average percent of 82 for ddG, 3.5 for ddGMP, 3.5 for ddGDP, and 5.1 for ddGTP).


A.
CPM / E6 CELLS
4000
3500
3000
2500
2000
1500
1000
500
0
ddG
56%
ddGMP
23%
ddGDP
12%
ddGTP
3.6%
0 2 4 6 8 10 12 14 16 18 20 22
ELUTION TIME (MIN)


B.
CPM / E6 CELLS
4000
3500
3000
2500
2000
1500
1000
500
0
ddG
83%
ddGMP
3.5%
ddGDP
3.1%
ddGTP
4.8%
0 2 4 6 8 10 12 14 16 18 20 22
ELUTION TIME (MIN)

until late in the thesis work. Although inefficient in terms of ATP/ADP values, the reproducible hot water extraction procedure was adopted as the extraction method for removal of acid-labile nucleosides and nucleotides from duck hepatocyte primary cultures.

Subjection of [8-^{3}H]ddG in H_2O (pH 6.0) to a 97°C water bath for 1 min did not result in appreciable degradation of the nucleoside. A 5-min exposure of [8-^{3}H]ddG to the elevated temperature caused an 8.5% reduction in the radiolabeled ddG peak. This was accompanied by a slight increase in the guanine product and an increase in the radioactivity associated with the column void volume that was approximately double that found in the control sample. When a sample of ddGTP in water was lyophilized, rehydrated, and analyzed by HPLC, breakdown of the ddGTP was apparent by the ddGTP/ddGDP value of 25 compared to a ratio of 67 for the untreated standard. However, there was no difference between the ddGTP/ddGDP value obtained for the sample that was lyophilized and a sample that was heated for 2 min at 90°C before lyophilization.

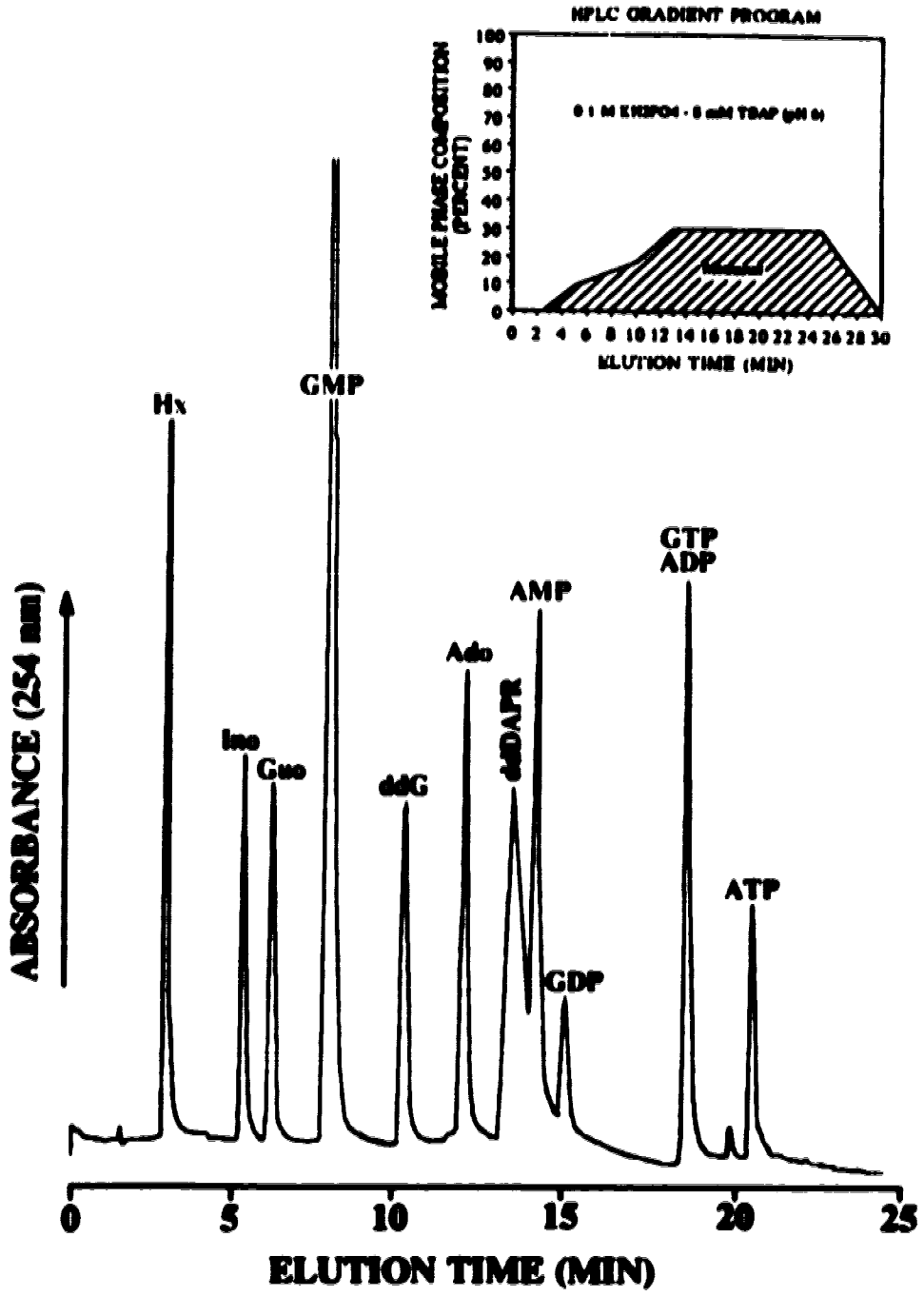
C. Drug Metabolism in Duck Hepatocyte Primary Cultures

Cell extracts prepared from duck hepatocyte primary cultures were analyzed by HPLC according to the procedure described in section II.D. of Chapter 2. Figure 3.5 presents a typical separation profile obtained for a mixture of standards containing a nucleobase, nucleosides, and nucleotides. The mobile phase conditions employed in ion-pair reverse-phase HPLC provide for a charge-dependent interaction of nucleotides with the column, resulting in column retention of these compounds for longer than their corresponding nucleosides and bases. The elution profile shown in Figure 3.5 illustrates the typical early recovery of a base (Hx), followed by a grouping of nucleosides (Ino, Guo, ddG; Ado, and ddDAPR), and an adjacent, overlapping region that contains nucleotides (GMP, AMP, GDP, ADP, GTP, and ATP) eluted in an order that correlates increasing column retention time to an increasing number of phosphate groups.

1. Conversion of ddDAPR to ddG in Duck Hepatocyte Primary Cultures

Conversion of ddDAPR to ddG was apparent when 48-hr duck hepatocyte primary cultures were incubated with 4 μM [^{3}H]ddDAPR (407 cpm/pmole). HPLC analysis of cell extracts prepared following a 3-sec exposure of the cells to isotopically diluted ddDAPR in media indicated that significant conversion of ddDAPR to ddG had occurred, that is, ~9% 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

Figure 3.5 - Ion-pair reverse-phase HPLC separation profile obtained for a mixture of base, nucleoside, and nucleotide standards. The standard mixture was separated according to the procedure outlined in section II.D. of Chapter 2. The sample was resolved on a 3 μm C-18 column (Excalibar Spheresorb ODS, 15 cm x 4.6 mm i.d.) preceded by a 5 μm Supelcosil LC-18 guard column (2 cm x 4.6 mm i.d.) and the column effluent was monitored for absorbance at 254 nm. Separation was achieved using 0.1 M KH_2PO_4-8mM TBAP, pH 6, and methanol in the proportions indicated in the inset graph. The mixture of standards applied to the column contained from 0.7 to 5.0 nmoles of each of the following components: hypoxanthine (Hx), inosine (Ino), guanosine (Guo), guanosine monophosphate (GMP), 2',3'-dideoxyguanosine (ddG), adenosine (Ado), 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR), adenosine monophosphate (AMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP).


HPLC GRADIENT PROGRAM
MOBILE PHASE COMPOSITION (PERCENT)
ELUTION TIME (MIN)
Hx
GMP
Ino
Guo
ddG
Ado
dADPR
AMP
GTP
ADP
ATP
GDP
ABSORBANCE (254 nm)
0
5
10
15
20
25
ELUTION TIME (MIN)

found to contain 58% ddG and 38% ddDAPR.

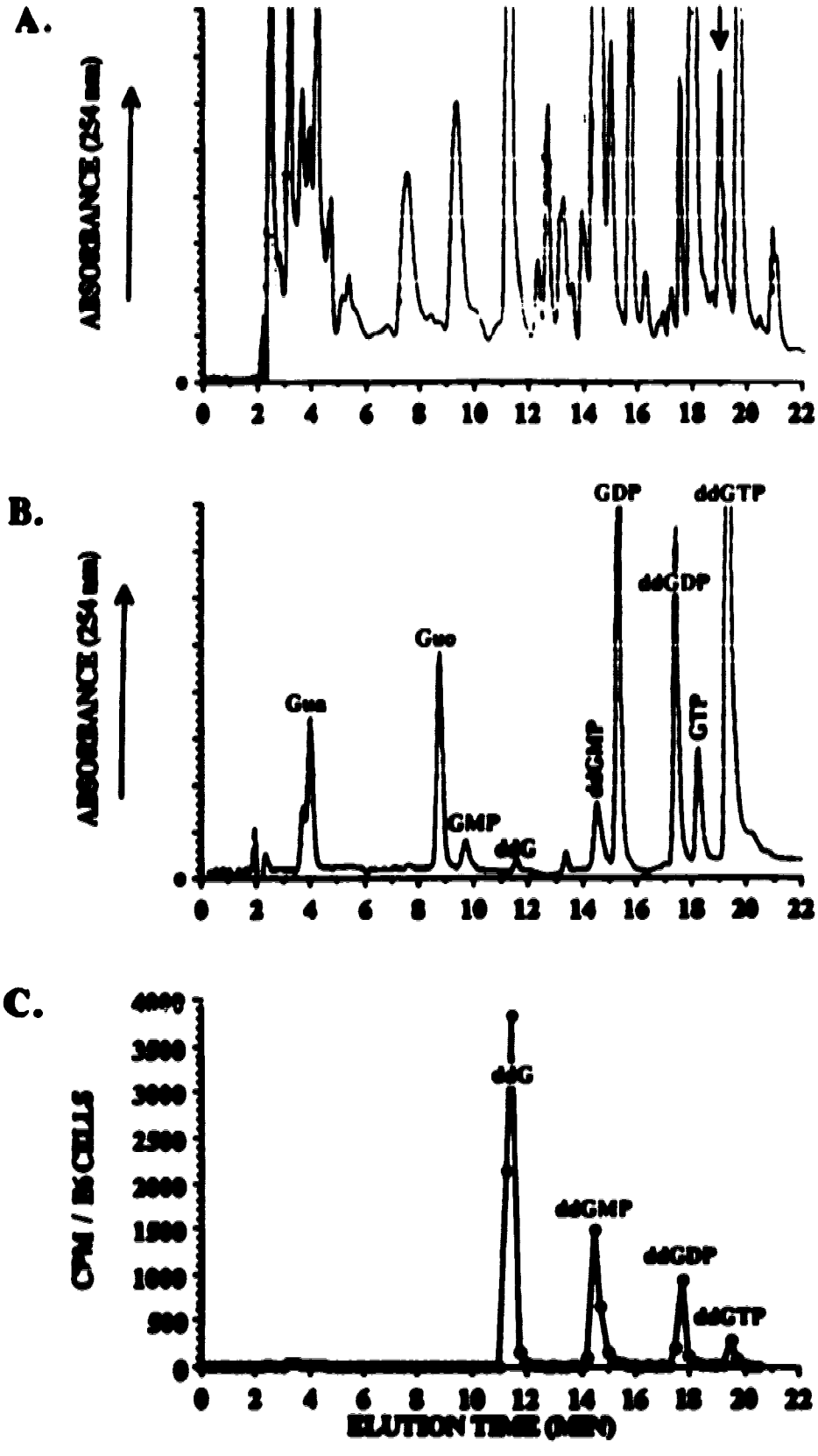
2. Metabolism of ddG and ddDAPR in Duck Hepatocyte Primary Cultures

From the above results and the data presented in Chapter 2 it is apparent that deamination of ddDAPR occurs in duck hepatocytes and blood. 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 (451 cpm/pmole) were hot water extracted and the extracts were resolved by HPLC. Analysis of the cell extracts revealed an array of UV_{254} absorbing components (Figure 3.6A), while the radioactive content of the sample was confined to 4 discrete regions of the radiogram (Figure 3.6C). The radiolabeled peaks seen in Figure 3.6C exhibited elution times identical to those of ddG, ddGMP, ddGDP, and ddGTP standards. As can be seen in Figure 3.6B, distinct separation of the guanine ribo- and dideoxyribonucleotide standards was achieved by the HPLC methodology employed. In the typical analysis shown in Figure 3.6C, 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 6 experiments, the mean (± S.E.) 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, 3.03 (± 0.49) ddGTP, and total dideoxynucleotides (ddGMP + ddGDP + ddGTP) were 33.2 (± 2.6).

Identification of the radiolabeled metabolites as nucleotides was accomplished by subjecting ddG-treated cell extracts to a preparation of potato apyrase containing 5'-ATPase, 5'-ADPase, and 5'-AMPase activity in a ratio of 190:56:1. When compared to an extract sample treated with the enzyme for the shortest possible time ("0"-time sample), a 5-min exposure resulted in a decrease in the cpm recovered in the ddGTP and ddGDP peaks, and an increase in the amount of radiolabeled product present in the ddGMP peak. When expressed as a percent of the total counts in the sample, the ddGTP peak decreased from 0.51% to 0.29%, the ddGDP peak was reduced from 4.5% to 0.89%, and the ddGMP region increased from 18.0% to 23.7%, while the percent of the total counts in the ddG peak remained similar (65.7% and 64.1% for the "0"-time and 5 min samples, respectively).

Hepatocytes incubated with 4 μM [^{3}H]ddDAPR-supplemented medium (407 cpm/pmole) for 5 or 27 hr produced radiolabeled products that had the same HPLC retention times as ddG nucleotide standards. Quantitation of the amount of ddGMP produced after 5 hr was not possible because this nucleotide and ddDAPR had

Figure 3.6 - 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^{o}C for 5 hr with 4 μM isotopically diluted ddG (665 cpm/pmole) in L-15 medium supplemented with 10% NSIV. The cells were hot-water extracted and the extracts were analyzed by HPLC as outlined in Materials and Methods. A) The cell extract absorbance profile was monitored at 254 nm. An arrow indicates the peak contributed by 0.7 nmoles of ddGTP standard that was added to the cell extract immediately before HPLC analysis commenced. B) A separation profile was obtained for the following mixture of standards: guanine (Gua), guanosine (Guo), guanosine monophosphate (GMP), 2',3'-dideoxyguanosine (ddG), 2',3'-dideoxyguanosine monophosphate (ddGMP), guanosine diphosphate (GDP), 2',3'-dideoxyguanosine diphosphate (ddGDP), guanosine triphosphate (GTP), and 2',3'-dideoxyguanosine triphosphate. C) 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. In this typical elution profile, ddG comprised 56% of the total eluted radioactivity while the corresponding mono-, di-, and triphosphates contributed 23%, 12%, and 3.6%, respectively. 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.


A.
ABSORBANCE (254 nm)
0 2 4 6 8 10 12 14 16 18 20 22
B.
ABSORBANCE (254 nm)
Gua
Guo
GMP
ddG
ddGMP
GDP
ddGDP
GTP
ddGTP
0 2 4 6 8 10 12 14 16 18 20 22
C.
CPM / 8K CELLS
4000
3500
3000
2500
2000
1500
1000
500
0
ddG
ddGMP
ddGDP
ddGTP
0 2 4 6 8 10 12 14 16 18 20 22
ELUTION TIME (MIN)

overlapping elution times. However, the pmol/10^6 cells of ddG, ddGDP, and ddGTP formed from ddDAPR increased over a 27-hr incubation period, eventually reaching the same dideoxynucleotide levels that were achieved during a 5-hr incubation with ddG (Table 3.2).

3. Comparison of the Metabolism of ddG and ddDAPR in DHBV-infected versus DHBV-uninfected Duck Hepatocyte Primary Cultures

Exposure of both uninfected and DHBV-infected duck hepatocyte cultures to 4 μM [^{3}H]ddG (451 cpm/pmole) for 5 hr resulted in the formation of similar quantities of ddG metabolites (Table 3.2). The total amounts of dideoxynucleotides (ddNTDs) formed after a 5-hr exposure to ddG were 8.8 pmol/10^6 cells for the uninfected tissue and 10.2 pmol/10^6 cells in the DHBV-positive cultures. In both cases, the ddG metabolite pool sizes increased with continued exposure to the drug, with levels reaching a maximum after 5 hours of exposure and decreasing thereafter (Figure 3.7). In an experiment using 11 day-old DHBV-positive and DHBV-negative hepatocyte cultures (data not shown), a similar pattern of time-dependent accumulation of dideoxynucleotides was seen. However, the amount of radiolabeled nucleotide metabolites detected in both the infected and noninfected tissues was less than in the 15-day cultures. Infected and uninfected hepatocytes treated with 4 μM [^{3}H]ddDAPR (407 cpm/pmole) generated comparable amounts of ddG metabolites (Table 3.2). However, the accumulation of radiolabeled metabolites generated from ddDAPR was delayed when compared to ddG-treated samples. After a 27-hr incubation with 4 μM ddDAPR, the total amount of dideoxynucleotides recovered in the uninfected and infected samples was 8.2 and 8.3 pmol/10^6 cells, respectively.

4. Time-Dependent Metabolism of ddG in Duck Hepatocyte Primary Cultures

The intracellular accumulation of dideoxynucleotides in ddG-treated, DHBV-uninfected duck hepatocytes was further characterized in terms of time-dependent changes in the dideoxynucleotide pool sizes. Cell monolayers treated with 4 μM [^{3}H]ddG (665 cpm/pmole) for various time periods were extracted and analyzed by HPLC. Under these 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 upon 5-10 hr incubation with ddG (Figure 3.8). Cellular ddG nucleotide concentrations achieved at the 5-hr time point 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 3.84 μl/10^6 cells was used. In this experiment, the intracellular concentration of ddG remained constant at 3.9 μM between

Table 3.2

METABOLISM OF ddDAPR AND ddG IN DHBV-NEGATIVE AND DHBV-POSITIVE DUCK HEPATOCYTE PRIMARY CULTURES

Duck hepatocyte primary cultures were prepared from the livers of 15 day-old ducklings that were confirmed by dot hybridization of serum samples to be either free of DHBV DNA (DHBV-negative) or carriers of DHBV DNA (DHBV-positive). Forty-eight hour uninfected and DHBV-infected hepatocyte cultures were incubated with 4 μM [^{3}H]ddG (451 cpm/pmole) or [^{3}H]ddDAPR (407 cpm/pmole) for 5 or 27 hr at 37°C. At the appropriate times, cell monolayers were hot-water extracted, and the extracts were processed and analyzed by HPLC according to the procedure outlined in section II.C.2 and II.C.3 of Chapter 3. The radiolabeled products resolved by HPLC were quantitated in terms of pmole of product per one million cells. Identification of the radiolabeled metabolites of ddG and ddDAPR as dideoxyguanosine nucleotides was made on the basis of elution times that corresponded to the column retention times of ddGMP, ddGDP, and ddGTP standards. Due to incomplete separation of ddDAPR and ddGMP in the 5-hr ddDAPR-treated samples, the amount of radiolabeled product formed in this area of the chromatogram was combined and expressed as one value. The total amount of dideoxyguanosine nucleotides (Total ddNTD) recovered in a sample represents the sum of the pmole/10^6 cells obtained for ddGMP, ddGDP, and ddGTP. The values shown represent single or duplicate determinations from one experiment.

Sample	pmole Per One Million Cells											
	5 Hr Incubation						27 Hr Incubation					
DHBV Negative	ddG	ddDAPR	ddGMP	ddGDP	ddGTP	Total ddNTD	ddG	ddDAPR	ddGMP	ddGDP	ddGTP	Total ddNTD
ddDAPR	15.4	←—— 9.3	——→	1.7	0.3	——	24.8	——	5.3	2.2	0.7	8.2
ddG	20.0	——	5.0	3.0	0.8	8.8	20.7	——	3.7	1.7	0.5	5.9
DHBV Positive												
ddDAPR	13.9	←—— 6.4	——→	1.4	0.4	——	22.6	——	5.4	2.3	0.6	8.3
ddG	16.3	——	6.4	2.8	0.9	10.2	21.7	——	4.6	1.9	0.6	7.1

Figure 3.7 -Time-dependent metabolism of ddG in DHBV-negative and DHBV-positive duck hepatocyte primary cultures. Duck hepatocyte primary cultures were prepared from the livers of 15 day-old ducklings that were either DHBV-negative or DHBV-positive. Forty-eight hour cultures were incubated with [^{3}H]ddG (451 cpm/pmole) for 0 to 27 hr at 37^{o}C. Cell monolayers were hot-water extracted, the extracts processed, and analyzed by HPLC according to the procedure discussed in sections II.C.2. and II.C.3. of Chapter 3. The radiolabeled products were quantitated in terms of pmole/10^6 cells. Intracellular accumulation of dideoxynucleotides is indicated as follows: 1) ddGMP, uninfected (–●–) and infected (– – O – –) cells; 2) ddGDP, uninfected (–●–) and infected (– –□– –) cells; 3) ddGTP, uninfected (–▲–) and infected (– – Δ – –) cells; and 4) total ddG nucleotides (ddGMP + ddGDP + ddGTP), uninfected (· · ♦ · ·) and infected (· · ◇ · ·) cells. The data points represent single or duplicate determinations (n = 2 for DHBV-uninfected, 5-hr time point and DHBV-infected, 3-hr time point) from one experiment.

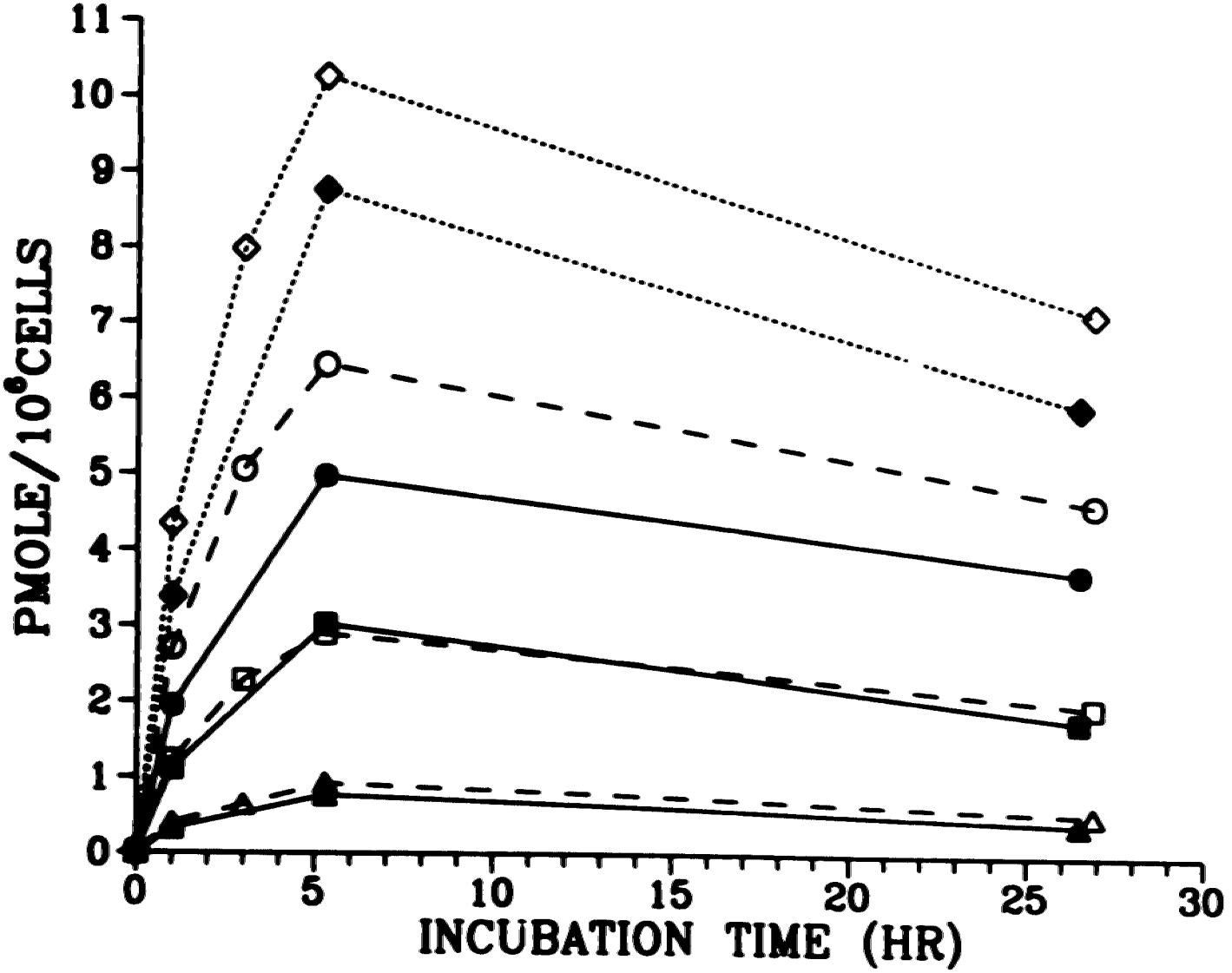

PMOLE/10⁶CELLS
INCUBATION TIME (HR)
0
1
2
3
4
5
6
7
8
9
10
11
0
5
10
15
20
25
30

Figure 3.8 - Time-dependent conversion of ddG to the corresponding phosphorylated metabolites by duck hepatocytes. Duck hepatocyte primary cultures (48 hr cultures) were incubated at 42°C for various times with 4 μM isotopically diluted ddG (665 cpm/pmole) 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 pmoles per one million cells. The level of ddGMP (O), ddGDP (□), and ddGTP (Δ) as well as the total amount of dideoxynucleotides (▲) (ddGMP + ddGDP +ddGTP) recovered at each time point is indicated. Each time point represents the average of two determinations, and all samples were obtained from the same cell preparation. Deviation from the mean is indicated by error bars. 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 pmole/10^6 cells was variable.

PMOLES / E6 CELLS
INCUBATION TIME (HR)
total deoxynucleotides
dCMP
dGTP
dGTP

2 and 5 hrs of incubation. However, cell samples incubated for 10 and 26 hr contained, respectively, 4.3 and 5.5 μM ddG in the extracts. Other cell preparations showed similar levels of ddG accumulation. The mean (± S.D., n = number of experiments) concentration of intracellular ddG calculated from several experiments was 3.2 μM (± 0.5; n = 2) at 0.5 hr, 3.7 μM (± 1.0; n = 4) at 1 hr, 4.0 μM (± 0.7; n = 3) at 3 hr, 3.3 μM (± 0.8; n = 7) at 5 hr, and 5.2 μM (± 0.6; n = 4) at 26 hr. After a 5-hr incubation of duck hepatocytes with 4 μM ddG, the spent medium contained an estimated 3.5 μM (n = 2 samples) ddG. In addition, 97% of the total radioactivity recovered in the sample of spent media was associated with ddG, while all other labeled products represented less than 0.5% of the total radioactivity. When ddG in fresh medium was incubated at either $4^{o}C$ or $42^{o}C$ for 5 hrs without cells, 97% of the sample radioactivity was recovered as ddG under both conditions. Figure 3.8 illustrates an experiment in which incubation of cells with 4 μM ddG for up to 26 hr lead to an eventual decrease in the metabolite pool sizes, with the total dideoxynucleotide concentration decreasing from 4.0 μM at 10 hrs to 2.6 μM at 26 hrs of incubation. Data from multiple experiments indicated that the mean (± S.D.; n = number of experiments) concentration of total dideoxynucleotides recovered at 5 hr was 2.1 μM (± 0.8; n = 7), while 26 hr of incubation with 4 μM ddG resulted in 1.9 μM (± 0.6, n = 4) of intracellular dideoxynucleotides.

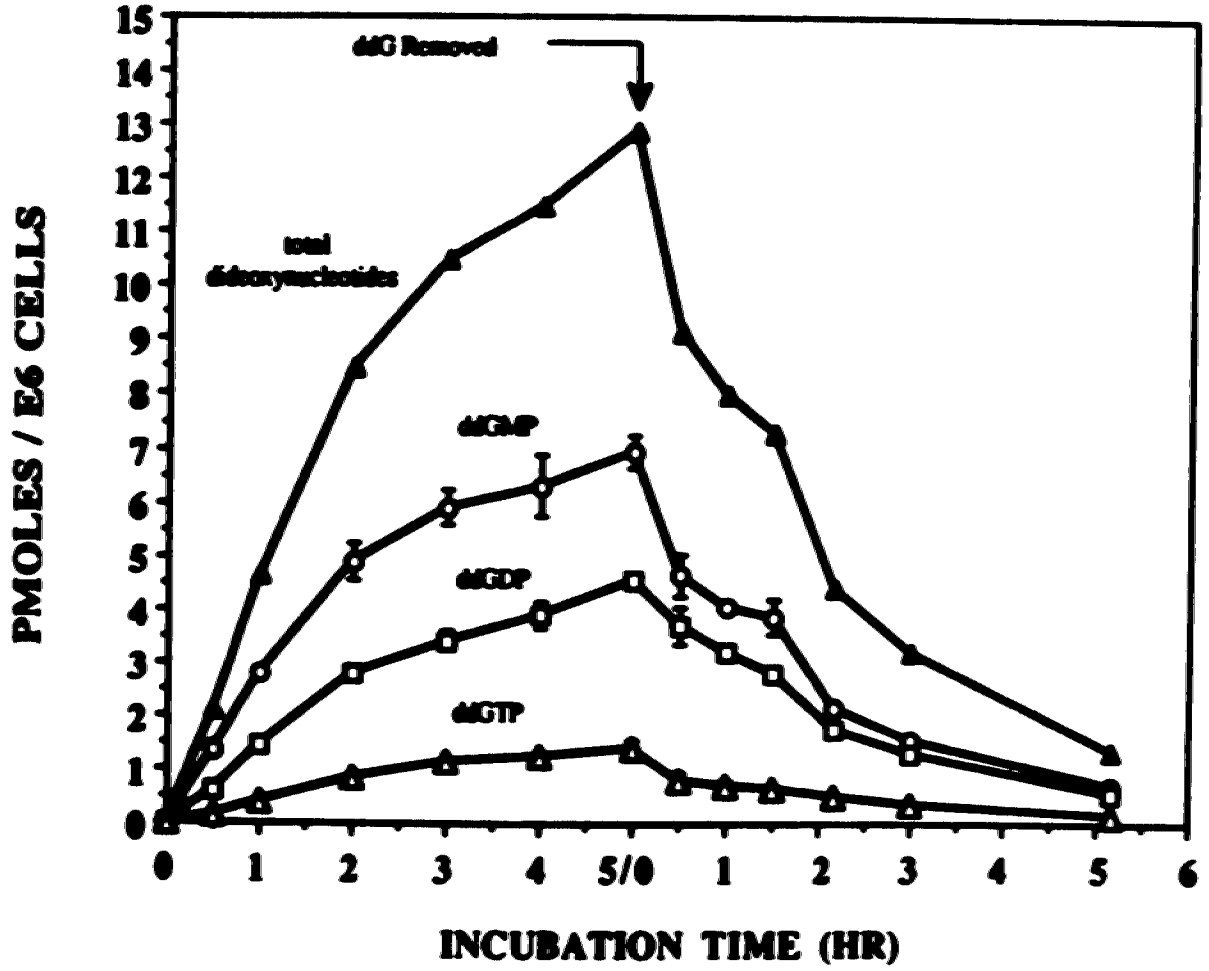
5. Stability of Intracellular ddG Nucleotides Formed in Cell Cultures

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 monolayers were washed with PBS, and fresh, drug-free medium was added. Removal of external ddG lead to a dramatic reduction in the amount of cell-associated ddG nucleotides (Figure 3.9). Intracellular dideoxynucleotide pools appeared to decrease with respect to time according to first order kinetics. A semi-logarithmic plot of the data shown in Figure 3.9 yielded $t_{1/2}$ values of 1.6 hr, 1.6 hr, 1.7 hr, and 1.6 hr for ddGMP, ddGDP, ddGTP, and total 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 Figure 3.9, 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.

6. Effect of Various Nucleosides and Nucleobases on ddG Metabolism

In duck hepatocyte primary cultures, ddG is phosphorylated by the action of endogenous enzymes. Potentially competitive nucleoside substrates were used to provide

Figure 3.9 - 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^{o}C for various time periods with 4 μM [^{3}H]ddG (665 cpm/pmole) in L-15 medium supplemented with 10% NSIV. After 5 hours 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 (O), ddGDP (□), ddGTP (Δ), and the sum of the three dideoxynucleotides (▲) recovered at each time point are indicated. The dideoxynucleotide levels are expressed as pmoles/10^6 cells and all but the 5-hr time point represent the average of two determinations. The 5-hr time point indicates the mean value obtained from 4 determinations, with error bars illustrating the standard deviation. For all other points, the error bars indicate deviation from the average value obtained from duplicate samples. Where not shown, error bars are contained within the time point symbol. All samples were obtained from the same cell preparation.


dG Removed
total deoxynucleotides
dGMP
dGDP
dGTP
PMOLES / E6 CELLS
INCUBATION TIME (HR)
0
1
2
3
4
5/0
1
2
3
4
5
6
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

insight into the enzyme pathways employed in the phosphorylation of this purine nucleoside analog. Duck hepatocyte primary cultures were incubated at 42°C for 5 hr with 4 μM ddG plus 100 μM of a potentially competing nucleoside or nucleobase. The results from this study are compiled in Table 3.3, with the amount of ddG and 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 (Figure 3.10). Extracts from the ddC-incubated cells were found to contain mono-, di-, and tri-phosphate forms of ddC (Figure 3.10A). However, the presence of these metabolites was significantly reduced if 100 μM 2'-dCyd was included in the medium (Figure 3.10B). Although 2'-dCyd had no apparent effect on the formation of ddG nucleotides, 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 included in the medium along with ddG (Table 3.3). Simultaneous exposure of cells to 100 μM 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. A marked decrease in the formation of ddG nucleotides was seen when Ado in the presence of the adenosine deaminase inhibitor DCF was the competing substrate. 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 (Table 3.3). When the less soluble nucleobase guanine was supplied at a concentration twice that of ddG, no discernible pattern of ddG nucleotide inhibition was evident in the HPLC-analyzed cell extracts. Similarly, 100 μM Hx had no apparent effect on ddG nucleotide pool sizes. The dideoxynucleotide, GMP, and adenine nucleotide levels attained in cells treated with ddG + 1 μM DCF were no different than the levels achieved with ddG alone. Sample conditions that employed 1 μM DCF were also tested at 24 μM DCF, with no difference detected in the results (results not shown).

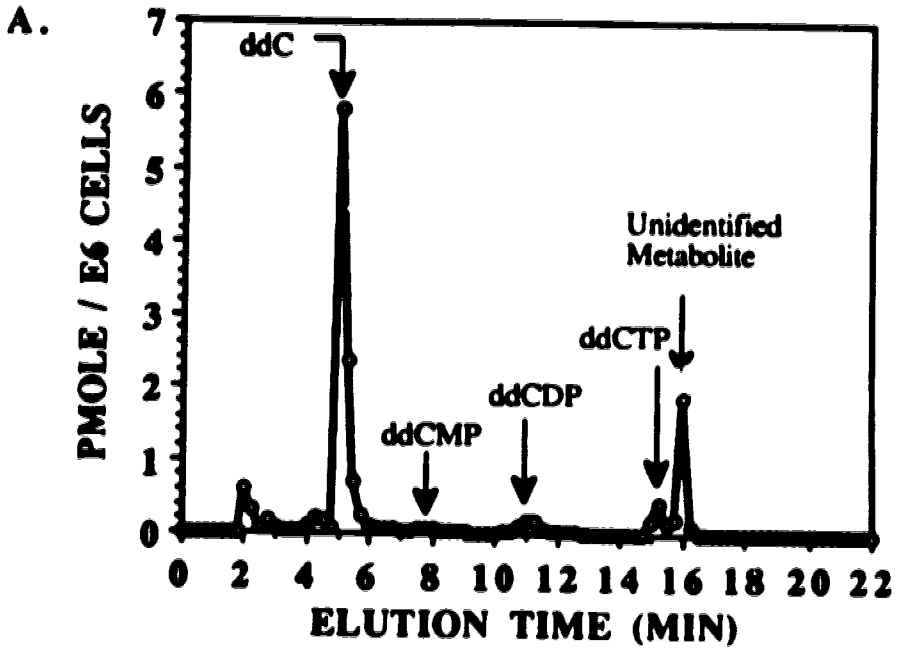
TABLE 3.3
INHIBITION OF ddG NUCLEOTIDE FORMATION

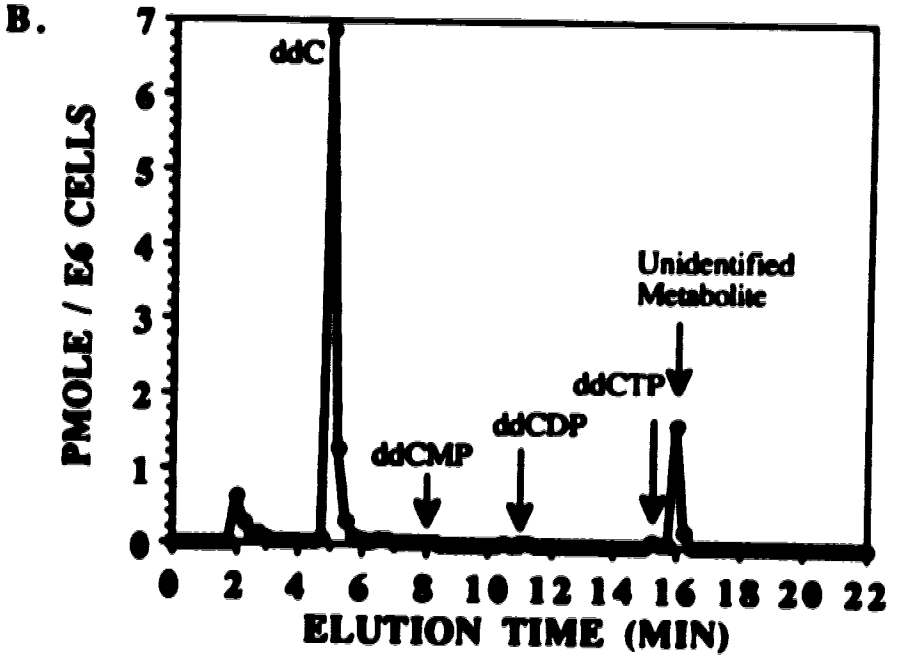
Duck hepatocyte primary cultures were incubated at 42°C for 5 hrs with 4 µM ddG only (Control) or with 4 µM ddG + 100 µM of a potential competitor compound. Cell extracts were prepared and analyzed by HPLC according to the procedure described in Materials and Methods. The amount of ddG and ddG metabolites recovered in each sample is expressed as a percent of the amount of product recovered in the control (ddG only) samples. The amount of ddGMP, ddGDP, and ddGTP detected in a sample is reported as a total value (ddNTDs). The adenine nucleotides (Ade NTDs) AMP, ADP, and ATP and the guanine nucleotide GMP detected in the HPLC absorption profile from each sample are expressed as a ratio of the area counts for the specified nucleotides to the area counts of an unrelated compound present in the cell extract. The number of experiments performed for each condition is indicated. Results were obtained from the indicated number of experiments and the mean (± S.D.) value for each condition was determined using the indicated number of samples.

SAMPLES	% OF CONTROL		RELATIVE AMOUNT		NUMBER OF:	
Competitor	ddG	ddNTDs	GMP	Ade NTDs	Exper.	Samples
None	100	100	7 ± 3	43 ± 9	3	9
2'-dCyd	97 ± 6	103 ± 9	7 ± 2	40 ± 1	2	5
2'-dGuo	103 ± 4	73 ± 9	8 ± 1	44 ± 6	2	5
2'-dAdo	99 ± 10	34 ± 5	7 ± 3	43 ± 7	3	9
2'-dAdo + DCF[1]	101 ± 2	52 ± 3	8 ± 3	40 ± 5	1	4
2'-dAdo + 2'-dGuo	95 ± 3	26 ± 9	6 ± 1	37 ± 3	1	3
2'-dIno	108 ± 5	67 ± 5	8 ± 1	36 ± 9	1	4
Guo	104 ± 7	72 ± 26	11 ± 5	45 ± 9	2	5
Ado + DCF[1]	94 ± 3	8 ± 8	6 ± 2	223 ± 129	2	7
Guo[2]	87 ± 4	88 ± 3	8 ± 2	42 ± 5	2	5
Hx	111 ± 8	89 ± 8	9 ± 6	50 ± 2	1	4
DCF[1]	105 ± 5	94 ± 7	7 ± 3	42 ± 9	1	4

[1] = 1 µM DCF; [2] = 8 µM Guo.

Figure 3.10 - Inhibition by 2'-deoxycytidine of the formation of ddC metabolites in duck hepatocytes. Duck hepatocyte primary cultures (48-hr cultures) were incubated at 37^{o}C in L-15 medium supplemented with 10% NSIV containing: A) 4 μM [^{3}H]ddC (460 cpm/pmole) or B) 4 μM [^{3}H]ddC (460 cpm/pmole) + 100 μM 2'-dCyd. After 5 hours of incubation, the cells were extracted and the extracts were analyzed by HPLC. Each point in the HPLC profiles represents an eluate sample collected for 0.25 min (1 ml/min flow rate). The levels of ddC and metabolites thereof contained in the cell extracts have been quantitated as pmoles/10^{6} cells. Arrows indicate the elution times obtained for ddCMP, ddCDP, and ddCTP standards that were run in a separate analysis. When the pmole/10^{6} cells for each of the indicated peaks was averaged from two samples for each treatment condition, the amount of ddC, ddCMP, ddCDP, ddCTP, and unknown metabolite contained in each of the corresponding peaks was: Panel A) 9.33, 0.247, 0.740, 0.784, and 2.09 pmole/10^{6} cells, and the total amount of the three nucleotides was 1.52 pmole/10^{6} cells, and Panel B) 8.95, 0.086, 0.133, 0.141, and 2.05 pmole/10^{6} cells, and the total amount of the three nucleotides was 0.359 pmole/10^{6} cells.


A.
PMOLE / E6 CELLS
ddC
ddCMP
ddCDP
ddCTP
Unidentified Metabolite
0 1 2 3 4 5 6 7
0 2 4 6 8 10 12 14 16 18 20 22
ELUTION TIME (MIN)


B.
PMOLE / E6 CELLS
ddC
ddCMP
ddCDP
ddCTP
Unidentified Metabolite
0 1 2 3 4 5 6 7
0 2 4 6 8 10 12 14 16 18 20 22
ELUTION TIME (MIN)

7. 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 (Lee et al., 1989; Luo, 1991; Suzuki et al., 1988). The results presented in this thesis 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 heretofore unknown intracellular fate of ddC was explored in duck hepatocytes. Figure 3.10A illustrates HPLC separation of an extract from duck hepatocytes treated for 5 hr with 4 μM [^{3}H]ddC (460 cpm/pmole). 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. Unlike the ddC nucleotide products, this unidentified compound was not affected by an excess of 2'-dCyd in the 4 μM ddC incubation medium (Figure 3.10B). Duck hepatocyte primary cultures treated with 4 μM [^{3}H]ddC for 0 to 73 hr were analyzed for the intracellular content of ddC and ddC nucleotides. Figure 3.11 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 (Figure 3.11). Using a mean (n = 19) intracellular water volume of 3.84 μL/10^6 cells and a mean pmole/10^6 cells of total ddC nucleotides calculated using the 24 and 26-hr time points of Figure 3.11, the intracellular concentration of total dideoxynucleotides was 1.4 μM. In contrast, the maximum intracellular level of ddG nucleotide 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 hrs in the experiment shown in Figure 3.8.

When cells treated with ddC for 24 hr were subsequently incubated in drug-free medium, the intracellular dideoxynucleotide pools decreased with respect to time according to first order kinetics (Figure 3.12). A semi-logarithmic plot of the data yielded $t_{1/2}$ values of 3.5 hr, 4.2 hr, 5.4 hr, and 4.4 hr for ddCMP, ddCDP, ddCTP, and total dideoxynucleotides (ddCMP + ddCDP + ddCTP), respectively. According to these data, intracellular ddC nucleotide pools decreased at a 2- to 3-fold slower rate than did the ddG nucleotide levels reported in section III.C.5. 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, results presented in Figure 3.9 and section III.C.5. of this chapter indicate

Figure 3.11 - 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^{o}C for various times with 4 μM [^{3}H]ddC (460 - 854 cpm/pmole) in L-15 medium supplemented with 10% NSIV. The cells were extracted and the extracts analyzed by HPLC as outlined in Materials and Methods. The phosphorylated products were identified by HPLC analysis and are expressed as pmoles/10^{6} cells. The intracellular levels of ddC (●) and ddC nucleotides (▲) (ddCMP + ddCDP +ddCTP) recovered at each time point are indicated. The graph represents a conglomerate of results obtained from 2 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 2 experiments. Deviation from the mean (standard deviation when n = 4) is indicated by error bars. The lines drawn were fitted by eye.

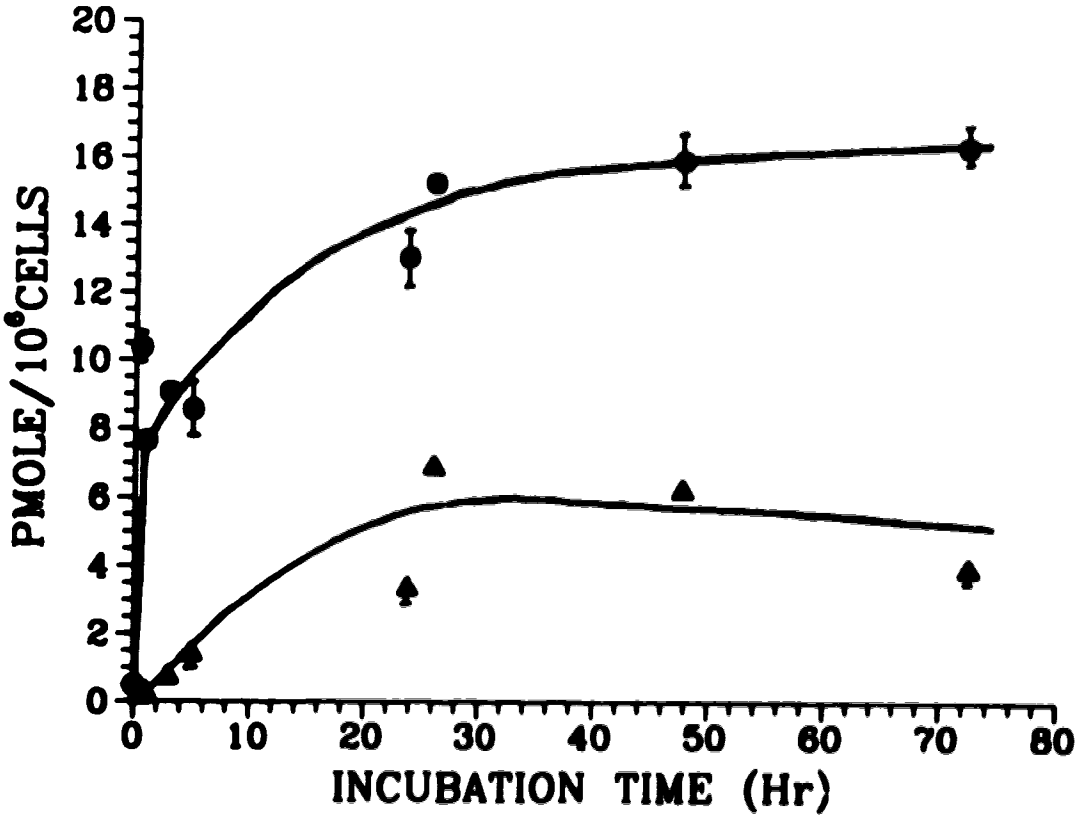
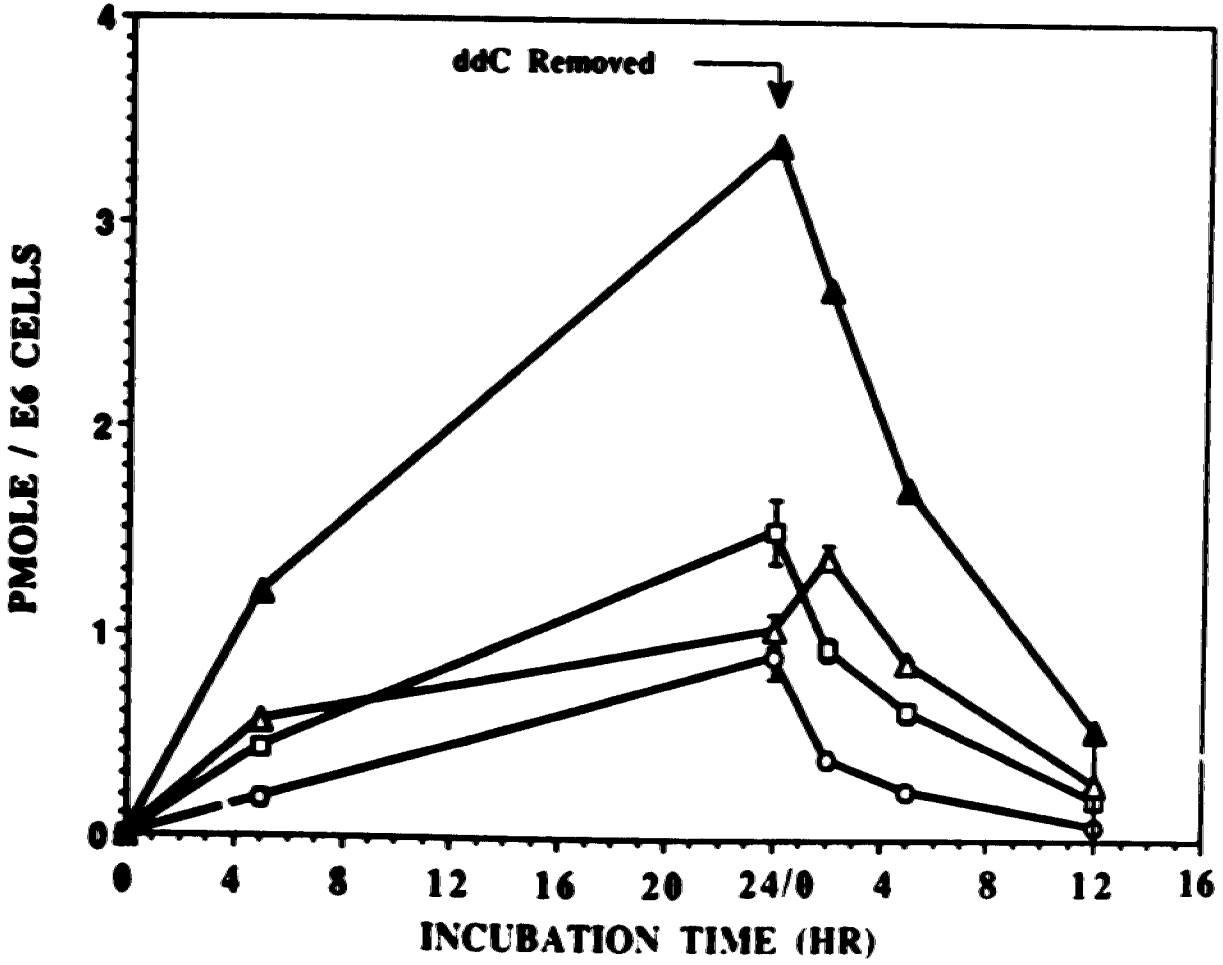

PMOLE/10⁶CELLS
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
60
70
80
INCUBATION TIME (Hr)

Figure 3.12 - Intracellular stability of radiolabeled dideoxynucleotide pools in duck hepatocytes treated with [^{3}H]ddC. Duck hepatocyte primary cultures (48-hr cultures) were incubated at 42^{o}C for various time periods with 4 μM [^{3}H]ddC (854 cpm/pmole) in L-15 medium supplemented with 10% NSIV. After 24 hours of incubation, the medium was removed (ddC removal indicated with arrow) and replaced with drug-free medium. At various times thereafter, the cultures were extracted and analyzed by HPLC as described in Materials and Methods. The levels of ddGMP (O), ddGDP (□), ddGTP (Δ), and the sum of the three dideoxynucleotides (▲) recovered at each time point are indicated. The dideoxynucleotide levels have been quantitated as pmoles/10^6 cells and each time point shown represents the mean of two replicate samples (n = 4 for the 24 hr time point). All measurements were made with cultures from the same liver preparation. Error bars indicate deviation from the mean (standard deviation when n = 4).


ddC Removed
PMOLE / E6 CELLS
0
1
2
3
4
0
4
8
12
16
20
24/0
4
8
12
16
INCUBATION TIME (HR)

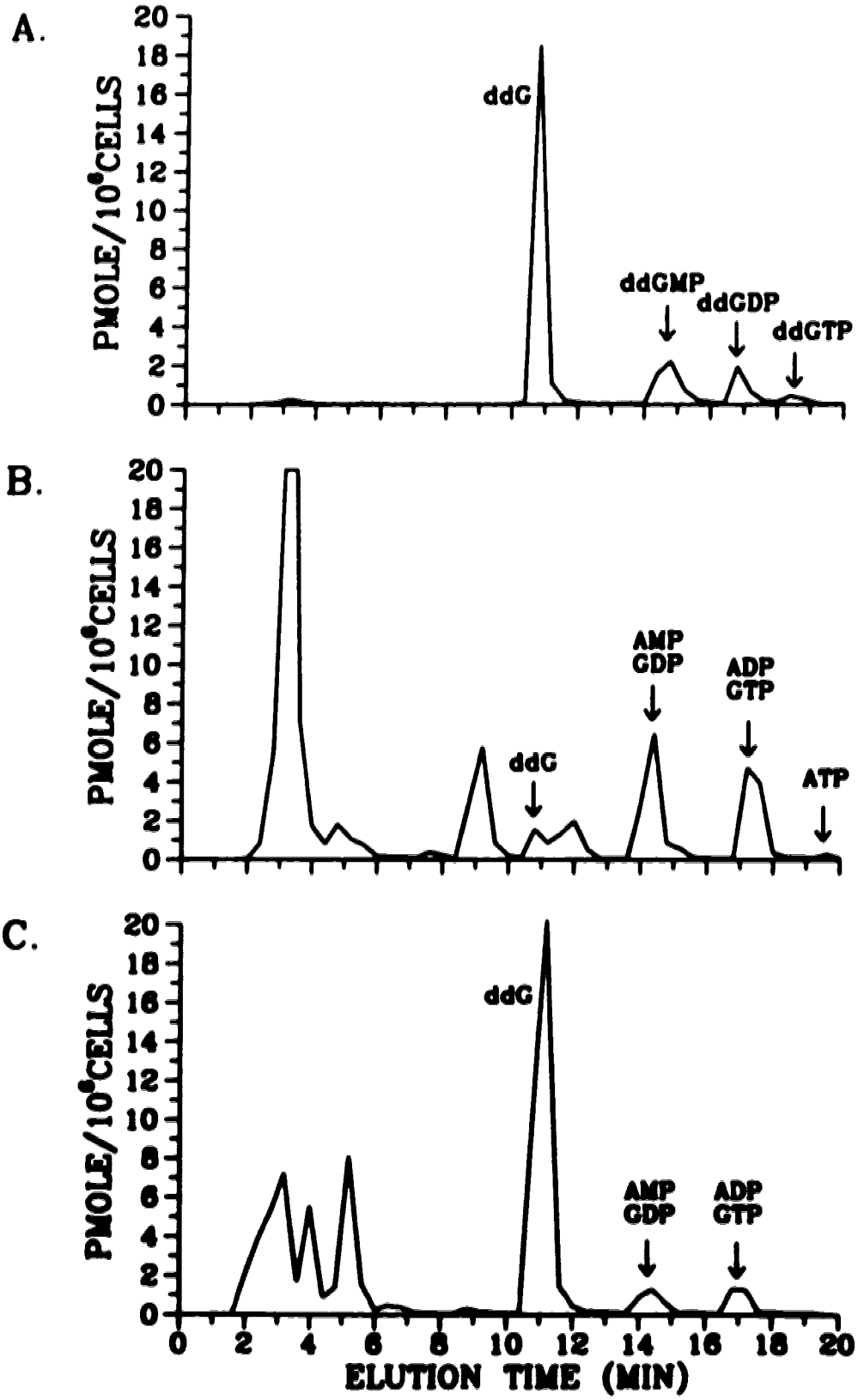
that 30 min after duck hepatocytes were supplied with fresh medium following a 5 hr incubation with 4 μM ddG, the intracellular ddG level declined from an initial concentration of 3.9 μM to 0.09 μM.

8. Comparison of ddG Metabolism in Duck Hepatocytes, Rat Hepatocytes, and CEM Cells

Duck hepatocyte primary cultures (48-hr cultures), rat hepatocyte primary cultures (72-hr cultures), and actively proliferating cultured human lymphoblastoid CEM cells were incubated with 4 μM [^{3}H]ddG (451 cpm/pmole) for several time periods at 37^{o}C. Extracts from cells treated with ddG for as little time as possible ("0"-time) contained radiolabeled ddG, as well as a small peak of radioactivity that had a short retention time (2 to 4 min) when analyzed by HPLC. The radioactive content of this early eluting product(s) represented 2%, 7%, and 2% of the total radioactivity in the extract of duck hepatocyte, rat hepatocyte, and CEM cell extracts, respectively. Analysis of rat hepatocyte extracts following a 27 hr-incubation with ddG revealed the formation of multiple radiolabeled peaks, with very little ddG remaining (Figure 3.13B). The radiolabeled extracts from CEM cells treated with ddG for 5 hr contained a large amount of radioactivity eluting from 2 to 6 min, followed by a sizable ddG peak and 2 possible nucleotide products (Figure 3.13C). A 5-hr incubation of duck hepatocytes with ddG did not result in significant quantities of early-eluting radiolabeled metabolites (Figure 3.13A). However, the duck cell extracts contained substantial quantities of ddG, and 3 peaks of radioactivity that elute with the same retention times as ddGMP, ddGDP, and ddGTP standards.

Although the tritiated ddG employed in these experiments was labeled in the ribose portion of the nucleoside molecule, a small amount of the label was associated with the nucleobase moiety. Acid hydrolysis of a sample of the [^{3}H]ddG stock, followed by HPLC separation of the neutralized reaction mixture indicated that 94% of the radiolabel was attached to the pentose sugar, while 6% was associated with the guanine moiety.

[illegible] Comparison of ddG metabolism in duck hepatocytes, rat hepatocytes, and [illegible]ells. A) Duck hepatocyte primary cultures (48-hr cultures) were incubated at [illegible]r with 4 μM [^{3}H]ddG (451 cpm/pmole) in L-15 medium supplemented with [illegible] NSIV. A hot-water extract was prepared from the cells. B) Percoll gradient isolated rat hepatocytes were prepared by Emmanuel Awumey (a graduate student under supervision of Dr. A.R.P. Paterson, Dept. of Pharmacology) from the collagenase-treated liver of a Sprague-Dawley rat (200-250 g animal). Cells were plated in 60-mm tissue culture dishes at a cell density of 2 x 10^6 cells/plate and incubated in L-15 medium supplemented with 10% NSIV at 37°C. A hot water extract was prepared from 72-hr rat hepatocyte primary cultures that had been incubated with 4 μM [^{3}H]ddG (451 cpn./pmole) for 27 hr at 37°C. C) Actively proliferating CEM cells were cultured at 37°C in roller bottles in MEM supplemented with 10% fetal calf serum in 5% CO_2. Cells (generation number 23) were harvested, resuspended in a small volume of MEM with 10% FCS, and 2-ml aliquots containing 2 x 10^6 cells were incubated in a total volume of 4 ml growth media with 4 μM [^{3}H]ddG (450 cpm/pmole) for 5 hr in an humidified, 37°C, 5% CO_2 environment. The samples were centrifuged (1000 x g, 3 min) in a 4°C table top centrifuge, the cell pellet was hot water extracted, and cell debris was removed by centrifugation. The duck hepatocyte, rat hepatocyte, and CEM cell extracts were processed and analyzed by HPLC according to the procedure outlined in section II.C.2 & 3 of this chapter. Panels A, B, and C present the radiographic profiles obtained from HPLC analysis of the cell extracts, with the radioactivity in each sample expressed as pmole/10^6 cells. In each panel, the elution times of relevant nucleotide standards are indicated by arrows. The position of ddG is also noted.


A.
PMOLE/10⁶CELLS
ddG
ddGMP
ddGDP
ddGTP
B.
PMOLE/10⁶CELLS
AMP
GDP
ADP
GTP
ddG
ATP
C.
PMOLE/10⁶CELLS
ddG
AMP
GDP
ADP
GTP
ELUTION TIME (MIN)

IV. DISCUSSION

Previous work has shown that the purine nucleoside analog ddDAPR inhibits DHBV both *in vivo* and *in vitro* (Lee et al., 1989; Luo, 1991; Suzuki et al., 1988). Conversion of ddDAPR to ddG by duck blood was evident in the results presented in Chapter 2 of this thesis. In Chapter 3, metabolism of the pro-drug ddDAPR and its subsequent deamination product ddG was explored using duck hepatocyte primary cultures. Optimization of cell culture conditions and selection of an appropriate cell extraction methodology prefaced the study of dideoxynucleoside metabolism in these cells.

A. Growth Conditions and Properties of Duck Hepatocyte Primary Cultures

Primary cultures of duck hepatocytes experienced a consistent loss in cell viability over several weeks in culture. At 24 hr in culture, the protein content of a cell sample was correlated with the number of cells present. However, as the number of viable cells decreased with increasing time in culture, the protein content of the sample remained constant. Therefore, in terms of sample quantitation, cell number was a more meaningful reflection of culture status than was protein content. A mean intracellular water space of 3.8 $\mu L/10^6$ cells was obtained for duck hepatocytes. However the range of values among cultures was quite variable, evidently reflecting the fragile nature of hepatocyte primary cultures. In comparison to duck hepatocytes, the estimated intracellular water volume of Percoll-fractionated rat hepatocyte primary cultures is 4.5 - 5.5 $\mu L/10^6$ cells (Personal communication, Emmanuel Awumey, 1989).

After 24 hr in culture, duck hepatocytes retained the ability to salvage and metabolize adenosine. Following a 1-min exposure of cells to [^{3}H]Ado, more than one-half of the cell-associated radioactivity consisted of nucleotides, Ado, and Ino. Radiolabel incorporation into other probable metabolites such as hypoxanthine, xanthine, and uric acid was not examined. After a 2-hr incubation, 89% of the total sample radioactivity was present as adenine nucleotides, suggesting that, under these conditions, phosphorylation was the major anabolic fate of internalized adenosine.

Duck hepatocyte primary cultures retained morphologic characteristics of liver tissue for less than one week under the growth conditions used in this thesis. Cells incubated at the avian body temperature of 42°C were more prompt to establish monolayers than their 37°C cohorts, suggesting that cell recovery from the initial isolation process and monolayer formation in the *in vitro* environment was assisted by the warmer temperature. However, by 8 days in culture, cells maintained under both conditions experienced morphological changes indicative of decreased biochemical function (Reid and Jefferson, 1984). Under normal tissue culture conditions, hepatocyte

primary cultures are unable to divide, they undergo progressive dedifferentiation, become agranular, and progressive biochemical dysfunction quickly leads to cell death (Reid and Jefferson, 1984). Increased viability of hepatocyte primary cultures has been achieved by a variety of culture conditions, including modified cell attachment surfaces (collagen substrata, floating collagen strata, extracellular matrix substrata), medium additives (1-2% dimethyl sulfoxide (DMSO), insulin, hormones, serum-free medium), and co-culture of hepatocytes with another cell type (Enat et al., 1984; Guguen-Guillouzo et al., 1983; Isom et al., 1985; Michalopoulos and Pitot, 1975). The medium additive DMSO is reported to maintain hepatocyte primary cultures in a differentiated state for at least 6 weeks, to confer upon duck hepatocyte cultures the ability to support DHBV replication for longer periods of time, and to induce the susceptibility of noninfected duck hepatocytes to infection with DHBV for up to 2 weeks (Galle et al., 1989; Isom et al., 1987; Pugh and Summers, 1989). However, the possibility of DMSO-induced membrane perturbations discouraged the use of this compound with duck hepatocyte primary cultures to be used in drug uptake studies.

B. Comparison of Extraction Methods

Nucleobases, nucleosides, and nucleotides are normally extracted from biological tissue using perchloric acid or trichloroacetic acid. Typically, a ratio of the amount of ATP to ADP present in the extract functions as a monitor of the extraction process, with ATP/ADP values of approximately 7 indicating adequate preservation of liver cell ATP levels during the extraction procedure. The susceptibility of purine 2',3'-dideoxynucleosides to acid-catalyzed hydrolysis of the molecular N-glycosidic bond necessitated the use of an alternative extraction condition in the experiments performed in this thesis. After comparing several types of extraction conditions, the inefficient, but reproducible, hot water extraction procedure was selected as the best method for removal of bases, nucleosides and nucleotides from duck hepatocyte primary cultures treated with the acid-labile dideoxynucleosides. When compared to PCA extraction results, the hot water procedure yielded lower ATP/ADP results. The low ATP/ADP values obtained for PCA extraction of trypsinized duck hepatocytes suggests that this sample preparation procedure not optimal. Direct extraction of [^{3}H]Ado-treated hepatocyte monolayers with 0.4 M TCA produced cell extracts with ATP/ADP values ranging from 5.8 to 7. The mechanical manipulations involved in trypsinization of a fragile cell type may have resulted in a loss or breakdown of intracellular nucleotides in the PCA-treated hepatocyte samples. Acid extraction of tissues such as liver can yield ATP/ADP values below 1, although higher ratios are attainable (Perrett, 1986; Smith et al., 1977). Variable extraction results were obtained when duck hepatocytes were exposed to 60% methanol.

However, treatment with 70% methanol - 25 mM Tris (pH 7.4) produced high, reproducible ATP/ADP ratios, suggesting that maintenance of a stable pH environment may be crucial to nucleotide preservation during methanol extraction. Although the 70% methanol - 25 mM Tris condition yielded superior extraction results to the hot water method, it was not tested until late in the thesis work. If it had been used, this methanol extraction method might have produced cell extracts containing nucleotide ratios that were a closer approximation to the actual intracellular nucleotide composition.

Exposure of [8-^{3}H]ddG to a 97°C water bath for 5 min resulted in a slight decrease in radiolabeled ddG and an increase in the amount of tritium-labeled product eluting in the area of the column void volume. This increase in radioactivity probably represents tritiated water formed by H-^{3}H atom exchange between H_2O and the ^{3}H located at the 8-position of the purine base. In most experiments, this H-^{3}H exchange would be of no consequence since the ribose-labeled dideoxynucleoside [2',3'-^{3}H]ddG was employed.

C. Drug Metabolism in Duck Hepatocyte Primary Cultures

Conversion of ddDAPR to ddG was evident in duck hepatocyte primary cultures treated briefly with ddDAPR in either cell culture medium or water. These results, as well as the results from Chapter 2 indicate that, in the avian system, ddDAPR acts as a short-lived prodrug for the more biologically stable nucleoside analog ddG. In terms of commercial production, ddDAPR is the more economical of the two related agents. In addition, the greater lipid solubility of ddDAPR relative to ddG may provide for more rapid diffusion of ddDAPR into cells (Robins et al., 1989). However, in Pekin ducks it is likely that expeditious plasma membrane permeation of the more lipid soluble ddDAPR is pre-empted by deamination of ddDAPR by blood tissue enzymes.

Intracellular metabolism of ddG was characterized in DHBV-negative duck hepatocytes for two reasons: 1) infected and noninfected hepatocyte monolayers treated with [^{3}H]ddG or [^{3}H]ddDAPR produced similar quantities of radiolabeled ddGMP, ddGDP, and ddGTP; and 2) due to a high demand for DHBV-positive ducks in the laboratory, DHBV-negative animals were more available for use.

An important aspect of ddG metabolism in duck hepatocytes was the relative absence of drug catabolism. After several hours of incubation, the internal and external ddG concentration of the cells had reached apparent equilibrium, with a portion of the intracellular nucleoside analog converted to phosphorylated derivatives. In cultures exposed to 4 μM ddG, the nucleotide products ddGMP, ddGDP, and ddGTP accumulated in a time-dependent manner to a level of 2 - 4 μM after 5 to 26 hr. Recovery of dideoxynucleotides from ddG-treated duck hepatocytes varied from one cell preparation

to the next, making it difficult to determine precisely the internal dideoxynucleotide levels achieved. Other researchers have reported similar variability in the recovery of ddG nucleotides from cell extracts obtained from replicate experiments (Ahluwalia et al., 1990). For example, Molt-4 cells treated with 5 μM ddG for 5 hr contained 0.126 to 0.265 pmole/10^6 cells of ddGDP and 0.055 to 0.120 pmole/10^6 cells of ddGTP (Ahluwalia et al., 1990). In comparison, duck hepatocytes exposed to 4 μM ddG for 5 hr contained 1.5 to 4.5 pmole/10^6 cells of ddGDP and 0.29 to 1.4 pmole/10^6 cells of ddGTP in cell extracts obtained from 7 separate experiments.

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 may affect nucleoside analog metabolism by competing for uptake or by functioning as cellular enzyme regulators. Therefore, the results from these types of studies are often difficult to interpret.

In human lymphoid cells, dCyd kinase is thought to be instrumental in converting dGuo to dGMP (Sarup and Fridland, 1987). Purified dCyd kinase from human leukemic T cells was capable of phosphorylating ddG, yielding a V_{max}/K_m of 0.08 (Sarup et al., 1989). Deoxynucleoside competition studies in human lymphoid cell lines, as well as ddG metabolism experiments with kinase-deficient lymphoid cells indicated that dCyd kinase is involved in ddG anabolism (Bondoc et al., 1992). 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.

Under conditions in which the hepatocyte adenosine deaminase (ADA) 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. The results could indicate substrate competition at the level of Ado kinase, an enzyme that can be generalized as having: 1) high activity in liver; 2) a low K_m for Ado; and 3) a high K_m for 2'-dAdo (Carson et al., 1977; Krenitsky et al., 1974; Miller et al., 1979). Although the natural substrate guanosine is not phosphorylated by purified adenosine kinase, the guanosine analog ribavirin and the related C-nucleoside tiazofurin are substrates for this enzyme (Fridland et al., 1986; Miller et al., 1979; Saunders et al., 1990; Willis et al., 1978). 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 Ado kinase suggests that a concentration of 100 μM 2'-dAdo would be insufficient to saturate the enzyme, leaving a population of these macromolecules free to interact with alternate substrates (Miller et al., 1979). Although the adenine nucleotide pool size did not change in the presence of excess 2'-dAdo, an increase in the characteristically low intracellular concentration of deoxyadenosine nucleotides would have gone without detection.

Results from the current study indicated that dideoxynucleotide formation in hepatocytes was inhibited to a greater extent by 2'-dAdo without DCF, than by 2'-dIno, the immediate product of 2'-Ado deamination. This result is surprising if 2'-dAdo, a normally good substrate for ADA, is readily deaminated in duck hepatocytes (Balzarini et al., 1987; Ma and Fisher, 1968; Pfrogner, 1967). If efficacious deamination of 2'-dAdo occurs, both 2'-dAdo and the resultant deamination product 2'-dIno should have the same overall effect on ddG phosphorylation. Another relevant catabolic product of nucleoside metabolism, Hx, did not affect ddG nucleotide pool sizes. A pivotal compound in nucleotide metabolism, Hx is released by catabolism of Ino or dIno and is either degraded to waste products or linked to the adenine and guanine nucleotide pathways by conversion to IMP. That Hx had no effect on ddG metabolism or on the intracellular levels of GMP and adenine nucleotides suggests that the observed effect of 2'-dAdo and 2'-dIno on ddG metabolism does not involve their reutilization through the Hx salvage pathway described.

Guanosine analog metabolism to monophosphate derivatives has been explored in several cell culture systems. In CHO cells, 3-deazaguanosine is converted to its monophosphate form by nicotinamide ribonucleoside kinase, while tiazofurin phosphorylating activity in these cells is associated with Ado kinase, nicotinamide ribonucleoside kinase, and a 5'-nucleotidase (Saunders et al., 1989; Saunders et al., 1990). Similarly, human lymphoblastoid cells contain an Ado kinase and a 5'-nucleotidase that phosphorylate tiazofurin (Fridland et al., 1986). Partially purified 5'-nucleotidase from human T lymphoblast cells catalyzes the conversion of ddI, ddG, and carbovir to their monophosphate forms, while highly purified rat liver 5'-nucleotidase accepts as substrates acyclovir, ganciclovir, and ara-G, as well as Ino, Guo, and dGuo (Bondoc et al., 1990; Johnson and Fridland, 1989; Keller et al., 1985). An ATP-activated, soluble 5'-nucleotidase that preferentially utilizes IMP, GMP, and their deoxy-congeners 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 (Itoh et al., 1978; Johnson and Fridland, 1989; Keller et al., 1985; Spychala et al., 1988).

Substrate inhibition results obtained from duck hepatocyte cultures indicate that, in addition to Ado kinase, other enzymes could contribute to ddG phosphorylation. Modest inhibition of ddG nucleotide formation occurred when either Guo, 2'dGuo or 2'-dIno was provided in a 25-fold molar excess to ddG. Presumably, these nucleosides could act as substrates for a 5'-nucleotidase as well as for a cellular nicotinamide ribonucleoside kinase. To assess the potential involvement of either of these two enzymes in hepatocyte phosphorylation of ddG, competition of ddG with an excess of ribavirin or nicotinamide riboside should be examined. In the case of nicotinamide ribonucleoside kinase as a possible candidate for ddG phosphorylation, inclusion of the natural substrate nicotinamide riboside should cause a reduction in ddG nucleotide pools. In CHO cells, the 3-deazaguanosine nucleotide pool was reduced 6-fold by the presence of an equimolar concentration of nicotinamide riboside (Saunders et al., 1989). If 5'-nucleotidase is responsible for the conversion of ddG to ddGMP, then an increase in the intracellular IMP pool size induced by inhibition of IMP dehydrogenase by the monophosphate derivative of ribavirin should ultimately result in an increased yield of ddG nucleotides. Anabolism of ddG and ddI in a human T-lymphocyte cell line is reported to be initiated by a cytosolic 5'-nucleotidase (Ahluwalia et al., 1990; Hartman et al., 1991). When Molt-4 cells were exposed to [^{3}H]-labeled ddG, ddA, or ddI in the presence of ribavirin, the cells contained elevated levels of radiolabeled ddG and ddA nucleotides (Ahluwalia et al., 1990; Hartman et al., 1991). In another study, phosphorylation of ddG by several human lymphoid cell lines was attributed to both dCyd kinase and a cytosolic 5'-nucleotidase (Bondoc et al., 1992). However, contrary to the results obtained with duck hepatocytes, accumulation of ddGTP in CEM or Molt-4 cells treated with 5 or 10 μM ddG was enhanced by 100 μM 2'-dAdo, uneffected by 100 μM 2'-dGuo, and antagonized by 100 μM 2'-dCyd (Bondoc et al., 1992). Similar substrate inhibition results were reported for ddA-treated Molt-4 cells: 100 μM 2'-dAdo enhanced the formation of ddATP, whereas 100 μM 2'-dCyd effected a decrease in ddATP levels (Cooney et al., 1987).

Dideoxycytidine is ineffective as an antiviral agent when administered to either DHBV-infected Pekin ducks or primary cultures of duck hepatocytes (Lee et al., 1989; Luo, 1991; Suzuki et al., 1988). 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 μM and 1.9 μM for cells treated with 4 μM ddC or ddG, respectively) after a 26-hr incubation. These results indicate that the inability of ddC to inhibit DHBV is not due to an inability of hepatocytes to phosphorylate ddC. However, the results give no indication of the relative intracellular availability of ddC and ddG nucleotides. For instance, selective intracellular compartmentalization or utilization of one agent over the other could influence the outcome of the antiviral results. Alternatively, as suggested by Suzuki and co-workers (1988), ddG may exert its antiviral effect through a unique interaction with the viral primer protein.

In vivo experiments with Pekin ducks have indicated that ddC is extremely toxic, often resulting in animal death 2 to 3 weeks into treatment, whereas ddDAPR the prodrug of ddG is well tolerated by these animals (Luo, 1991). In humans, peripheral neuropathy has been observed in patients after prolonged (1.5 - 3 months) treatment with ddC (Yarchoan et al., 1988). Both mitochondrial and cellular toxicities are associated with inhibition of mitochondrial DNA synthesis in ddC-treated human T lymphoblastic cells (Chen and Cheng, 1989). Furthermore, ultrastructural abnormalities are reported to occur in the mitochondria of human T lymphoblasts treated for 12 days with 4 μM ddC (Lewis et al., 1991). Starnes and Cheng (1987) have shown that although ddCTP is a competitive inhibitor of dCTP for the purified human DNA polymerases α, β, and γ, the K_i values obtained for ddCTP inhibition of the three enzymes indicate that DNA polymerase γ is the most sensitive of the three enzymes to this agent. These data support the proposal that selective mitochondrial toxicity in ddC-treated cells is a result of a high affinity of mitochondrial DNA polymerase for ddCTP (Chen et al., 1991).

Hepatocytes retained ddC nucleotides for a longer time ($t_{1/2}$ = 4.4 hr) following the removal of external ddC than they did ddG nucleotides ($t_{1/2}$ = 1.6 hr) when exogenous ddG was withdrawn. Dideoxycytidine nucleotides that are biochemically employed could contribute to a slower mobilization of the intracellular stores of ddC metabolites. When the time-dependent, intracellular accumulation of exogenous ddC and ddG is compared, more rapid equilibration of the internal and external concentrations occurs with the more lipophilic ddG, suggesting that lipid-solubility may play a role in plasma membrane permeation of these compounds (Plagemann et al., 1988; Robins et al., 1989).

It is clear that the outcome of ddG metabolism is highly dependent on the cell type. A comparison of ddG metabolism in duck and rat hepatocytes revealed a very different pattern of drug utilization in these two tissues. Catabolism of ddG is of minor importance in duck hepatocytes, whereas glycosidic bond cleavage products accounted

for much of the radiolabel recovered in rat hepatocyte cell extracts. Duck hepatocytes produced micromolar quantities of ddG nucleotides. In comparison, phosphorolytic cleavage of ddG, followed by reutilization of guanine in the formation of adenine and guanine mono-, di-, and triphosphates appeared to be the primary anabolic result in rat hepatocytes treated with ddG. The human T-lymphoblastoid cell line CEM did not produce detectible quantities of dideoxynucleotides when treated with ddG. However, the presence of glycosidic bond cleavage products in cell extracts indicates that catabolism of ddG was the fate of a large portion of the intracellular ddG in these cells. Since a small percentage of the [^{3}H]-label in ribose-labeled ddG was associated with the guanine moiety, subsequent reutilization of purine nucleoside phosphorylase (PNP) - liberated guanine probably accounted for the two discrete radiolabeled peaks having elution times similar to AMP/GDP and ADP/GTP. Bondoc and co-workers (1992) reported the detection of low ddG nucleotide levels (ddGDP + ddGTP = 222 pmol/10^9 cells) in CEM cells treated for 6 hr with 10 μM ddG. In another human lymphoid cell line (WI-L2) treated with 10 μM ddG, the accumulation of radiolabeled guanosine ribonucleotides was drastically reduced in the presence of an excess of the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) substrate Hx (Bondoc et al., 1992). This result supports the suggestion that upon cleavage of ddG by PNP, the sparcely radiolabeled guanine moiety is recycled into purine ribonucleotides via HGPRT (Bondoc et al., 1992). Anabolic conversion of ddG in another human lymphoid cell (Molt-4) results in the accumulation of sub-micromolar dideoxynucleotide levels when the cells are treated with 5 μM ddG for 5 hr (Ahluwalia et al., 1990). As a consequence of active ddG catabolism in Molt-4 cells, radiolabeled guanine and adenine nucleotides make up a significant portion of the radiolabeled compounds recovered in these cell extracts (Ahluwalia et al., 1990). Species-dependent metabolism differences have been reported for other dideoxynucleoside analogs (Balzarini et al., 1988). In addition, quantitative differences in nucleoside drug metabolism have been observed in different cell types within a single species (Balzarini et al., 1988; Carson et al., 1988; Cooney et al., 1986).

The results presented in Chapter 3 illustrate that metabolism of the effective anti-DHBV agent ddG in duck hepatocytes leads to the formation of intracellular ddG nucleotide pools. However, a comparison of ddG metabolism in duck hepatocytes, rat hepatocytes and T-lymphocytes reveals that the intracellular fate of this drug varies according to the host tissue. In the next chapter, metabolic characterization and anti-HBV activity of the promising pharmacological agent ddG is explored in the human hepatoblastoma cell line HepG2 and the HBV gene-carrying derivative cell line 2.2.15.

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CHAPTER 4
METABOLISM AND ANTIVIRAL EFFECTIVENESS OF DIDEOXYNUCLEOSIDES IN HepG2 and 2.2.15 CELLS

I. INTRODUCTION

The development of therapeutic agents for hepatitis B virus (HBV) has been complicated by the narrow host range specificity exhibited by the hepadnaviruses. Until recently there was no known *in vitro* tissue culture system capable of supporting HBV replication. Accurate screening of potential antiviral agents for HBV will depend on the utilization of a test system that will best represent the *in vivo* HBV infection. Several *in vitro* cell culture systems have been utilized for evaluation of the anti-HBV effectiveness of therapeutic agents, including: 1) duck hepatocyte primary cultures prepared either from the livers of DHBV-infected ducks, or from DHBV-uninfected livers, with subsequent experimental infection of the cultures with DHBV from the sera of DHBV-infected ducks; and 2) a clonal cell line (2.2.15) derived from the human hepatoblastoma cell line HepG2 that has been transfected with a plasmid containing HBV DNA (Lee et al., 1989; Matthes et al., 1990; Price et al., 1989; Suzuki et al., 1988;Yokota et al., 1990).

As can be seen in Chapter 3, there were marked differences in the intracellular metabolism of the nucleoside analog ddG in duck hepatocytes, rat hepatocytes, and a human T-lymphocyte cell line (CEM). Duck hepatocyte primary cultures converted ddG to the corresponding dideoxynucleotides and exhibited very little catabolism of the parent compound. In contrast, cell extracts from rat hepatocyte primary cultures or CEM cells exposed to ddG contained radiolabeled products consistent with dideoxynucleoside catabolism and subsequent reutilization of the guanine base moiety. These results suggest that cell-dependent differential drug metabolism may drastically influence the apparent antiviral potential of a prospective drug. Hence, it is imperative that selection of an *in vitro* drug screening model should focus on a system that mimics the biochemical and metabolic composition of the *in vivo* human liver.

In Chapter 4, the metabolism and antiviral effectiveness of ddG and ddC was examined in the human hepatoblastoma-derived HepG2 cell line and its HBV-infected, derivative cell line 2.2.15.

II. MATERIALS AND METHODS

A. Materials

1. General Supplies

Flow Laboratories, Mississauga, ON. was the source of AutoPow MEM, while fetal calf serum, geneticin sulfate, Penicillin G, Streptomycin sulfate, BamHI, EcoRI, Hind III, and React 2 and 3 buffers were obtained from Gibco BRL, Burlington, ON. Tetrabutylammonium dihydrogen phosphate (TBAP) was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. The following supplies were obtained from Sigma Chemical Co., St. Louis, MO.: ethylenediaminotetraacetic acid (EDTA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), trypsin, and the nucleosides 2'-deoxycytidine (2'-dCyd), 2'-deoxyguanosine (2'-dGuo), 2'-deoxyadenosine (2'-dAdo), guanosine (Guo), and adenosine (Ado). The nucleoside analogues 2',3'-dideoxyguanosine (ddG), and 2',3'-dideoxycytidine (ddC) were purchased from Terochem, Edmonton, AB., while 2',3'-dideoxyguanosine 5'-triphosphate (ddGTP) and 2',3'-dideoxycytidine 5'-triphosphate (ddCTP) were from Pharmacia (Canada), Inc., Baie d'Urfe, PQ. Calcium chloride ($CaCl_2$), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), magnesium chloride ($MgCl_2$), and mini-scintillation vials were from Fisher Scientific, Nepean, ON. EcoLite liquid scintillation solution was obtained from ICN Biomedicals, Inc., Irvine, CA., Diff-Quik Stain Set was supplied by Harleco, Gibbstown, NJ. and Centricon filters (3 and 10) were from Amicon Canada Ltd., Oakville, ON. Water was purified using a Milli-Q water purification system and distilled water was used in all tissue culture reagents.

2. Nucleoside and Nucleotide Solutions

Standard solutions containing the mono-, di-, and triphosphate forms of ddG or ddC were prepared as described in Chapter 3, section II.A.2. Millimolar nucleoside stock solutions were prepared in water or saline and were filter-sterilized. An accurate concentration of each stock solution was determined as described in Chapter 2, section II.A.2. The stock solutions were stored at -20°C and were utilized in the preparation of solutions containing micromolar concentrations of nucleosides and/or nucleoside analogs.

3. Radiochemicals

[2',3'-^{3}H]2,6-diaminopurine 2',3'-dideoxyriboside ([^{3}H]ddDAPR; 45 Ci/mmol) and [2',3'-^{3}H]2',3 '-dideoxycytidine ([^{3}H]ddC; 48 Ci/mmol) were obtained from Moravek Biochemicals Inc., Brea, CA. and [2',3'-^{3}H]2',3'-dideoxyguanosine ([^{3}H]ddG) was prepared from [^{3}H]ddDAPR according to the procedure described in Chapter 3, section II.A.3. These radiochemicals were repurified periodically by HPLC using a C-18 column and water/methanol gradient. NEN Research Products, Markham, ON. supplied

[1,2-^{3}H]polyethylene glycol ([^{3}H]PEG), while ^3H_2O (100 mCi/ml) was from ICN Radiochemicals, Costa Mesa, CA.

B. Cell Cultures

1. HepG2 Cell Cultures

The hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The monolayer cultures were grown in MEM supplemented with 750 mg/L sodium bicarbonate, 300 mg/L L-glutamine, 50 IU/ml penicillin G, 10 µg/ml streptomycin sulfate, and either 10% fetal calf serum or 10% NuSerum. At each passage the confluent monolayers in a 75-cm^2 flask were washed sequentially with 5 ml warm PBS and 5 ml warm trypsin solution. An additional 5 ml of trypsin solution was then added and all but 1 ml of this volume was removed. The flask was placed in the 37^{o}C incubator and the cell monolayer was allowed to incubate in the trypsin for approximately 6 min. The cells were resuspended in 10 ml of medium, transferred to a 15-ml polystyrene tube, and the tube was centrifuged for 5 min x 500 g. The resulting cell pellet was resuspended in 10 ml of fresh medium and 2 ml of this cell suspension was added to each of three to five 75-cm^2 flasks containing 25 ml of medium. The flasks were returned to the incubator and the medium was changed every 3 to 5 days.

Cells to be utilized in an experiment were grown as described above and were harvested when the monolayers had reached approximately 80% confluency. Cell pellets obtained from several 75-cm^2 flasks were resuspended, combined, and brought to a total volume of 200 ml with fresh medium. An hemocytometer and the trypan blue dye exclusion method were used to quantify the viable cells in the suspension. Alternatively, cells were counted with a Coulter Electronics model Z_F electronic particle counter fitted with a particle size analyzer (Channelyzer II). The following settings were used when counting HepG2 cells by this method: 1) amplification = 4; 2) threshhold = 10; and 3) aperture current = 4. The concentrated cell suspension was diluted with medium so that the cell concentration was 300,000 cells/ml. The cells were plated in 60-mm tissue culture dishes at an initial density of 1.2 x 10^6 cells/dish (4 ml of the cell suspension per dish). Thereafter, the medium was changed every other day.

2. 2.2.15 Cell Cultures

The HBV-producing 2.2.15 cell cultures utilized in the following studies were obtained from Dr. M. A. Sells (Mount Sinai School of Medicine, New York). These cultures were derived from HepG2 cells that were transfected with a plasmid vector containing G418-resistance sequences (a selectable marker that confers resistance to the antibiotic geneticin [G418]) and 2 head-to-tail dimers of the HBV genome (Sells et al,

1987). The 2.2.15 clone was selected in medium containing G418 and the cells were found to: 1) produce elevated levels of both HBeAg and HBsAg; 2) secrete infectious virions into the culture medium; and 3) contain chromosomally integrated HBV DNA sequences, as well as relaxed circular, covalently closed circular, and incomplete episomal copies of the genome (Acs et al., 1987; Sells et al., 1987; Sells et al., 1988).

The 2.2.15 cells were maintained in a Level C tissue culture facility. The cultures were grown in MEM containing 900 mg/L sodium bicarbonate, 300 mg/L L-glutamine, 380 mg/L geneticin sulfate, and 10% fetal bovine serum and were maintained in a 37°C humidified incubator with a 5% CO_2 atmosphere. Passage and plating of the cells were conducted in the same manner as described for HepG2 cells (section II.B.1. of this chapter).

C. Dideoxynucleoside Drug Metabolism Studies

1. Metabolism of Dideoxynucleosides in HepG2 and 2.2.15 Cells

Cell cultures were seeded at an initial density of 1.2×10^6 cells per 60-mm tissue culture dish and were allowed to grow for 5 days with medium changes every other day. On day 5 the medium was replaced with medium containing 4 μM of [^{3}H]ddG (300 - 800 cpm/pmole) or [^{3}H]ddC (800 cpm/pmole) and the cells were incubated at 37°C for various time periods in the presence of drug. The drug-containing medium was replenished every 2 days in the case of extended time points. At the appropriate times, incubation medium was removed, the monolayers were washed with PBS, and cell extracts were prepared by the addition of 2 ml H_2O, followed by a 2-min incubation in a 90 - 95°C water bath. The cell lysates were transferred to microfuge tubes and centrifuged (12,000 x g) for 1 min to remove cell debris. Maximum deproteinization of the clarified supernatants was accomplished by filtration of the extract through a Centricon-10 filter (centrifuged at 5000 x g for 3 - 5 hr, 10°C). The ultrafiltered samples were freeze-dried and stored at -20°C. Sample recovery throughout the extraction procedure and subsequent HPLC analysis was calculated based on sample volumes.

2. HPLC Analysis of the Cell Extracts

Freeze-dried samples were reconstituted in 0.1 ml of filtered water and a known volume (70 - 95 μl) of the sample was then analyzed by ion-pair reverse-phase chromatography as detailed in Chapter 2, section II.D. A flow rate of 1 ml/min was maintained and during sample analysis 0.25 ml eluate fractions were collected directly into mini-scintillation vials. Following the addition of 2.5 ml Ecolite scintillation cocktail, the radioactivity of each fraction was quantified by scintillation spectrometry.

D. Isolation and Analysis of DNA

The composition of the reagents employed in this section is itemized in Appendix III.

1. Isolation of Whole-Cell Nucleic Acids

HepG2 or 2.2.15 cell monolayers were washed with warm PBS and incubated for 20 min at room temperature with 1 ml TE-Sarkosyl (TES) solution. The cell extract was transferred to a glass tube (12 x 75 mm), the cell culture plate washed with an additional 0.5 ml of TES, and the wash combined with the extract. The sample was digested with 500 µg/ml of Pronase E (Sigma type XXV; self-digested) overnight at 37°C and was then deproteinized by 3 - 6 extractions with an equal volume of Tris-saturated phenol : chloroform (1:1) followed by a final extraction with chloroform alone. Samples concerned with the selective extraction of the cccDNA form of the viral nucleic acid of the viral genome were not treated with Pronase E, but were subjected to 2 phenol/chloroform extractions, followed by 1 chloroform extraction. DNA precipitation was induced by adjusting the salt content of the sample to 0.2 M NaCl, adding 2 volumes of ice-cold 95% ethanol, and allowing the sample to remain on ice for 30 min. The DNA pellet obtained after centrifugation in a Sorvall swinging bucket rotor (2190 g x 20 min; 4°C) was washed with 70% ethanol, centrifuged at 2190 g x 5 min, and air-dried at room temperature overnight. The DNA sample was dissolved in 0.15 ml of TE buffer, pH 7.5 and stored at -20°C.

2. Isolation of Cytoplasmically-Located HBV Core Particles from Cell Extracts

The procedure by Staprans et al. (1991) for the isolation of cytoplasmically-located viral nucleic acids from cell extracts was used, with some modifications. Cell monolayers were washed with 5 ml PBS (37°C) and incubated at room temperature for 20 min with 1 ml lysis solution per plate. Cells were resuspended in the lysis solution and the extract was transferred to microcentrifuge tubes and centrifuged at 12,000 g x 3 min (4°C) to remove debris and cell nuclei. After adjusting the clarified extracts to 0.006 M $MgCl_2$, the samples were treated for 45 min at 37°C with 20 U DNase plus 20 µg RNase A per ml sample. The viral particles contained in each 1 ml sample were precipitated with the addition of 0.33 ml of 26% PEG - 1.4 M NaCl - 0.025 M EDTA. After 30 min on ice, the samples were centrifuged at 12,000 g x 4 min at 4°C, the resulting pellets were resuspended in 0.2 ml of 0.01 M Tris/HCl - 0.005 M EDTA - 1% SDS (pH 7.5), and the samples were incubated at 37°C overnight with 500 $\mu g/ml_{Final}$ Proteinase K. DNA extraction was carried out in the same manner as described previously (section II.D.1. of this chapter), except that the samples were contained in microfuge tubes. After adjusting the samples to 0.1 M NaCl, 20 µg of yeast tRNA was

added as a carrier and DNA precipitation was induced by the addition of 2 volumes of ice-cold 95% ethanol and a 30 min incubation on ice. The samples were centrifuged (12,000 g x 10 min, 4°C), the pellets washed with ice-cold 70% ethanol, and centrifuged again, this time for 2 min. The resulting DNA pellets were air-dried overnight and resuspended in 0.02 ml TE buffer, pH 7.5 and stored at -20°C.

3. Restriction Endonuclease Digestion of DNA

a. *Hind III digestion of lambda (λ) DNA*

A microcentrifuge tube containing a final concentration of 10 μg λ DNA (Pharmacia), 1X React 2 buffer, and 30 U Hind III was incubated for 1 hr at 37°C. The reaction was terminated by the addition of 0.01 M_{Final} EDTA and $1X_{Final}$ gel loading buffer. The standard was stored at -20°C. Hind III-cleavage of λ DNA generated fragments of the following kilobase pairs (kbp) in size: 23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 0.56, and 0.13. The thawed standard was heated at 60°C for 2 min, cooled immediately on ice, and a 5 to 10 μL aliquot (200 - 400 ng DNA) was loaded into an agarose gel sample well immediately prior to electrophoresis. The λ DNA fragments were visualized by ultraviolet light transillumination of the Ethidium Bromide-stained gel.

b. *BamHI digestion of pAM 6 DNA*

Approximately 1 μg of pAM 6 DNA was incubated for 1 hr at 37°C with $1X_{Final}$ React 3 buffer, and 5 U BamHI. The reaction was terminated with the addition of 0.01 M_{Final} EDTA. An aliquot of the sample was diluted with $1X_{Final}$ gel loading buffer to achieve a concentration of 7 pg DNA/μL and 5 to 10 μL of this standard solution was loaded into an agarose gel sample well immediately prior to electrophoresis. The standard was stored at -20°C. BamHI-cleavage of pAM 6 DNA generated fragments of the following kilobase pairs (kbp) in size: 4.36, 1.9, and 1.3.

c. *EcoRI digestion of whole cell DNA extracts*

Five to 20 μg of cellular DNA, 1X React 3 buffer, and 10 U EcoRI were incubated for 1 hr at 37°C. The reaction was terminated with the addition of $1X_{Final}$ gel loading buffer, the sample was loaded into an agarose gel sample well, and the sample was electrophoresed according to the procedure discussed in II.D.6. of this chapter.

4. Preparation of Radiolabeled HBV DNA

The radiolabeled HBV DNA probe utilized in these studies was the plasmid pAM 6 which carries one copy of HBV (subtype adw) cloned into a Bam HI site of the *E. coli* plasmid vector pBR322 (Moriarty et al., 1981). The pAM 6 DNA (originally obtained from ATCC) was radiolabeled utilizing the nick translation method of Rigby et al. (1977), with modifications. Stock solutions of unlabeled deoxynucleotides, nick

translation buffer, and enzymes (*E. coli* DNA-polymerase I and DNase I) were obtained from a Nick Translation Kit (Boehringer Mannheim).

A 20 µl reaction mixture containing 1x nick-translation buffer, 400 pmoles each of dGTP, dATP, and dTTP, 220 ng pAM 6, 16.7 pmoles (50 µCi) [α-^{32}P]dCTP (specific activity = 3000 Ci/mmole), and 2 µl of the enzyme mixture (DNA polymerase I + DNase I) provided with the Nick Translation Kit was incubated at 15^{o}C for 1 hr. The reaction was terminated by the addition of 20 µl of 37^{o}C stop solution (0.1 M EDTA, pH 7.5 - 1% SDS - 100 µg/ml herring sperm DNA), followed by heating at 65^{o}C for 5 min.

Specific incorporation of radiolabeled deoxynucleotide into the DNA was quantitated using a trichloroacetic acid (TCA) precipitation assay (Sambrook et al., 1989c). To obtain an estimate of the total amount of radioactivity in the reaction mixture 1 µl of an 1:10 dilution of the sample was spotted onto a Whatman GF/C filter disk and allowed to air dry. To determine the radioactive content of the acid-insoluble portion of the sample, 1 µl of the diluted reaction mixture was added to 5 ml of ice-cold 5% TCA and, following the addition of 50 µg salmon sperm DNA and a 5 min incubation on ice, the content of the tube was filtered through a GF/C filter. The tube was rinsed twice with 5 ml of 5% TCA and twice with cold 95% ethanol, filtering each successive rinse through the GF/C filter. The filter was allowed to dry and then the filter disks were placed into scintillation vials, scintillation cocktail added, and the radioactive content of each sample determined.

The proportion of the [α ^{32}P]dCTP incorporated into the pAM 6 DNA was calculated by dividing the cpm obtained from the acid-precipated sample by the cpm obtained from the sample that was not treated with acid. When expressed as a percent, the amount of label incorporated into the DNA ranged from 18 to 60%. Specific activity of the radiolabeled DNA was calculated by dividing the cpm representative of the acid-precipitable fraction of the total reaction mixture by the total amount of plasmid DNA present in the sample. Typically, the specific activity was in the order of 3 x 10^{8} cpm/µg DNA. The radiolabeled DNA probe was stored at -70^{o}C and was utilized within 1 week of preparation.

5. Dot Hybridization

DNA-containing samples (cellular DNA extracts or cell-conditioned medium) were denatured in 0.2 N_{Final} NaOH for at least 1 hr at room temperature. The samples were then applied to a nylon filter (Hybond-N; Amersham) that had been soaked in water and immobilized in a microfiltration apparatus (Bio-Dot; Bio-Rad Laboratories). Once the samples had filtered, the individual wells were rinsed with a volume of 0.2 N NaOH equal to the sample volume that had been applied. The apparatus manifold was removed,

the well positions marked with pencil, and the filter transferred to a dish containing 100 ml of 2X SSC (pH 7.0). Following neutralization with 2 successive 100-ml volume washes with 2X SSC, the filter was dried at 37°C, exposed to ultraviolet irradiation for 3 min (UV Transilluminator, UVP, Inc.; 254 nm; total exposure = 1.44 J/cm^2), and either stored in the dark at room temperature until ready to process at a later date, or immediately treated according to the hybridization procedure outlined by Sambrook et al., (1989b; 1989c) with modifications. Prior to hybridization of the radiolabeled DNA probe to the nylon membrane-immobilized DNA, the filter was treated with prehybridization solution as follows: the dry filter was rehydrated, placed in a heat-sealable bag, 10 ml of freshly prepared prehybridization solution (42°C) added, air bubbles removed from the bag, and the bag sealed. The bag was submerged in a 42°C water bath and incubated for 2 hr. A recently prepared (within 1 week) ^{32}P-labeled pAM 6 DNA probe (Sp. Act. = 2-4 x 10^8 cpm/μg DNA) was denatured by heating at 96°C for 2 min, immediately cooled in an ice bath, and a sufficient amount of the probe added to the prehybridization solution so that the solution contained 1 x 10^6 cpm/ml. The bag was resealed, the contents mixed, the bag submerged in the 42°C water bath, and incubated overnight.

Removal of nonspecific-bound probe from the filter was accomplished by a brief rinse of the filter with 100 ml of 1X SSC - 0.1% SDS followed by 2 successive 45-min washes with 200 ml of the same solution at room temperature with gentle, constant agitation. Any remaining probe that was not bound to the immobilized target DNA with a high percentage of base pair match was removed by washing the filter twice for 45 min at 65°C in 500 ml changes of 0.1X SSC - 0.1% SDS.

The damp filter was protected by Saran Wrap and a sheet of X-ray film (Kodak X-OMAT AR) was sandwiched between the immobilized filter and an intensifying screen in a film cassette. Film exposure was carried out at -70°C for 16 hr to 5 days, depending on the experiment. The autoradiogram was developed using a Kodak X-OMAT M20 Proc automatic developer. After an autoradiographic image had been obtained, the filter was cut into 1-cm^2 pieces containing the individual sample dots and the radioactive content of each sample was quantitated by liquid scintillation spectrometry.

6. Agarose Gel Electrophoresis and Southern Transfer

Separation of a population of DNA by agarose gel electrophoresis and subsequent transfer of the DNA to a nylon filter employed procedures outlined by Sambrook et al. (1989a; 1989b), with minor modifications. Extracted DNA samples diluted in 1X gel-loading buffer were electrophoresed (Bio-Rad Wide Mini-Sub Cell and 200/2.0 power

supply; 4-5 V/cm; 2.5-3 hr) in an horizontal slab gel of 0.8% agarose in 0.5% TBE. Upon completion of electrophoresis, the gel was gently agitated for 40 min in 200 ml of 0.5 μg/ml Ethidium Bromide in TE buffer. Removal of excess stain from the gel was accomplished with a 15-min wash with water. DNA bands were visualized and recorded on film when the gel was transilluminated with ultraviolet light (Polaroid CU-5 Land Camera; polaroid 667 black & white film, 3000 ISO; F 4.7; 1/15- or 1/8-sec exposure; 30 sec development time). Partial depurination of the DNA was effected by incubating the gel for 12 min at room temperature in a solution of 0.25 M HCl. This solution was then replaced with 0.4 M NaOH and hydrolysis of the depurinated DNA was allowed to proceed for 1 hr, with gentle mixing. The gel was neutralized for 30 min with neutralizing solution, changing the solution once during this time and then the gel was equilibrated in 6X SSC for 30 min. The DNA was transferred from the gel to a nylon membrane by the capillary movement of 6X SSC buffer through the gel and nylon filter, respectively. The transfer was allowed to proceed for 12 to 15 hr after which time the nylon filter was washed briefly in 2X SSC and then exposed to ultraviolet light for 3 min (1.44 J/cm^2). Hybridization of the cross-linked nylon-DNA to a radiolabeled DNA probe was performed in the same manner as previously described for dot hybridizations (section II.D.4. of this chapter).

III. RESULTS

The labor-intensive experiments presented in this chapter were completed through the joint efforts of Jy Huang and myself. This partnership was employed throughout various stages of the experiments, including: a) cell preparation, plating, and maintenance; b) cell counts and staining; and c) DNA extractions, DNA sample preparation, and radiolabeled HBV DNA probe preparation.

A. Dideoxynucleoside Metabolism in HepG2 and 2.2.15 Cells

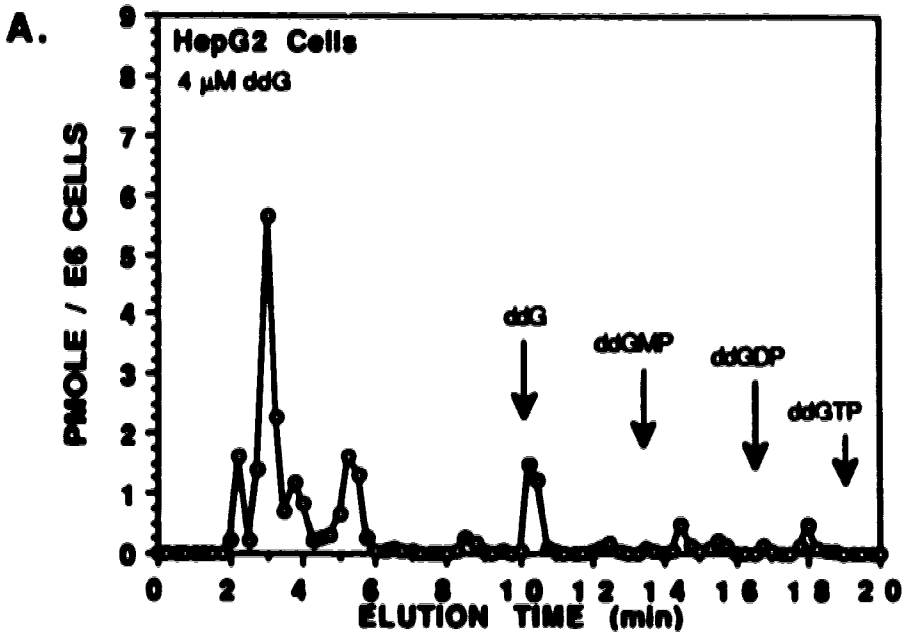
1. Growth Conditions of HepG2 and 2.2.15 Cell Cultures

The HBV-uninfected human hepatoblastoma cell line HepG2 exhibited a doubling time (± S.D.) of 34.9 ± 9.3 hr when grown in MEM - 10% NSIV compared to 39.9 hr in MEM - 10% FCS. A doubling time of 36.5 ± 1.9 hr was determined for the HBV-infected 2.2.15 cell line grown in MEM - 10% FCS. When the cells from either cell line were plated in 60-mm tissue culture dishes, exponential cell growth occurred in a cell population range of 1×10^5 to 7×10^6 cells/dish.

2. Metabolism of ddG and ddC in HepG2 Cells

HepG2 cell monolayers in a logarithmic growth phase were treated for 5 or 24 hr at 37°C with 4 μM [^{3}H]ddG with or without 100 μM of the PNP inhibitor 8-aminoguanosine (8-NH_2Guo). After 5 hrs of incubation with ddG, 11% of the cell extract radioactivity was associated with ddG, metabolites eluting between 2 and 6 minutes in HPLC-analyzed extracts represented 75% of the sample radioactivity, and a scattering of small peaks contributed a collective 13% to the total sample radioactivity (Figure 4.1A). In contrast, inhibition of the catabolic enzyme PNP with 8-NH_2Guo resulted in the retention of 82% of the sample radiolabel as ddG, 10% as early-eluting species, and a limited number of small "peaks" representing a total of less than 4% of the sample radioactivity (Figure 4.1B). Continued incubation of cells with ddG for 24 hr resulted in 9% of the sample radiolabel eluting with ddG, 66% eluting between 2 and 6 min, and additional accumulations of radiolabeled products in regions of the chromatogram that did not correspond to the elution times of ddG nucleotide standards (Figure 4.2A). A 24-hr incubation of cells with ddG and 8-NH_2Guo produced a cell extract radio-elution profile identical to that obtained after a 5-hr exposure (results not shown). Radiolabeled ddG that was incubated for 24 hr at 37°C in MEM-10% NSIV without cells represented 96% of the total sample radioactivity. The amount of product eluting at the 8-9 min, 14-15 min, and 17.5-18.5 min regions in Figure 4.1A was found to increase 2- to 3-fold after a 24-hr incubation with ddG. These three regions correspond to the elution times of, respectively, GMP, GDP, and GTP standards. Radiolabeled ATP represented 0.1% of the cpm recovered in the 5 hr HepG2 cell

Figure 4.1 - Metabolism of ddG in HepG2 cells. Actively proliferating HepG2 cells were incubated for 5 hr at 37°C in MEM-10% NSIV containing: A) 4 μM [^{3}H]ddG (772 cpm/pmole); or B) 4 μM [^{3}H]ddG (772 cpm/pmole) + 100 μM 8-aminoguanosine (8-NH_2Guo). Cell extracts were prepared and analyzed by HPLC according to the procedure outlined in sections II.C.1. and 2. of this chapter. Each point in the HPLC profiles represents an eluate sample collected for 0.25 min (1 ml/min flow rate). The levels of ddG and ddG metabolites contained in the cell extracts have been quantitated as pmoles/10^6 cells. Arrows indicate the elution times detected by absorbance for a ddGTP standard added to the experimental sample just prior to HPLC analysis, and for ddG, ddGMP and ddGDP standards that were run in a separate analysis.


A.
HepG2 Cells
4 μM ddG
PMOLE / E6 CELLS
ddG
ddGMP
ddGDP
ddGTP
0 2 4 6 8 10 12 14 16 18 20
ELUTION TIME (min)

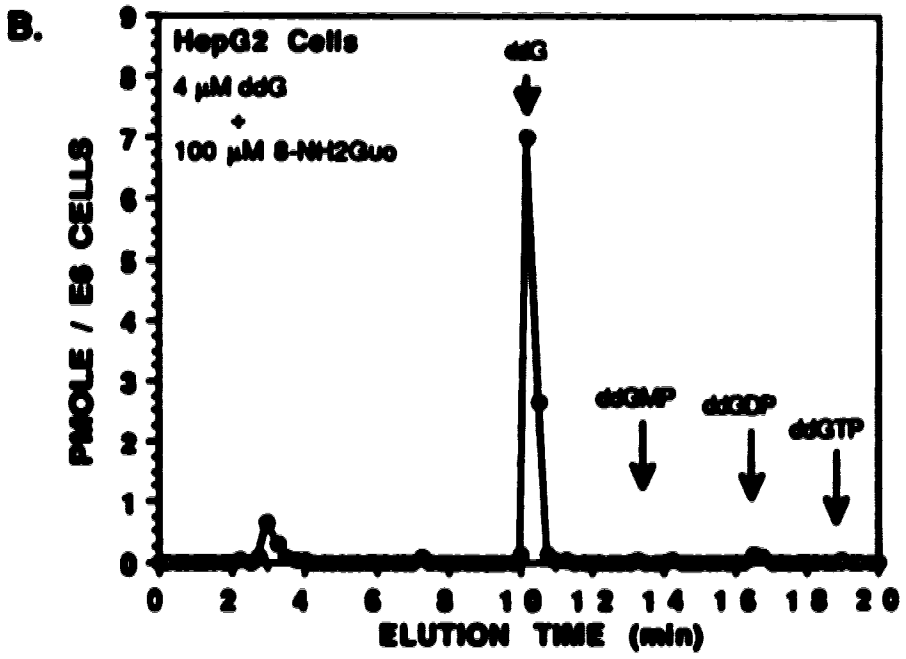
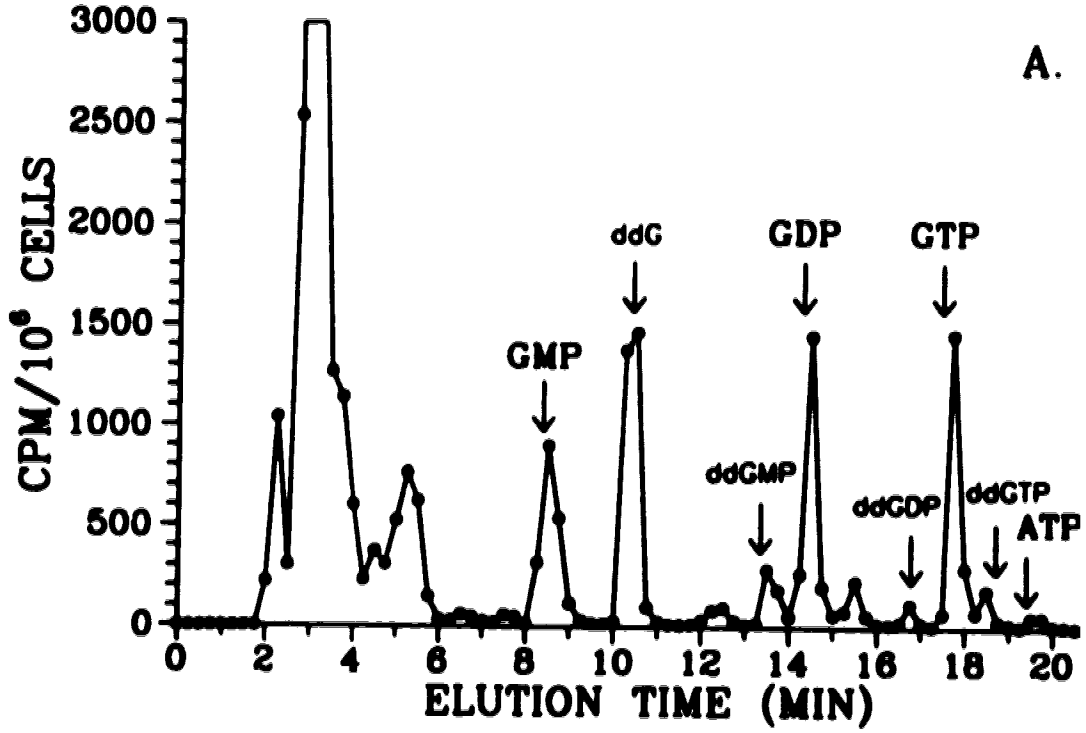
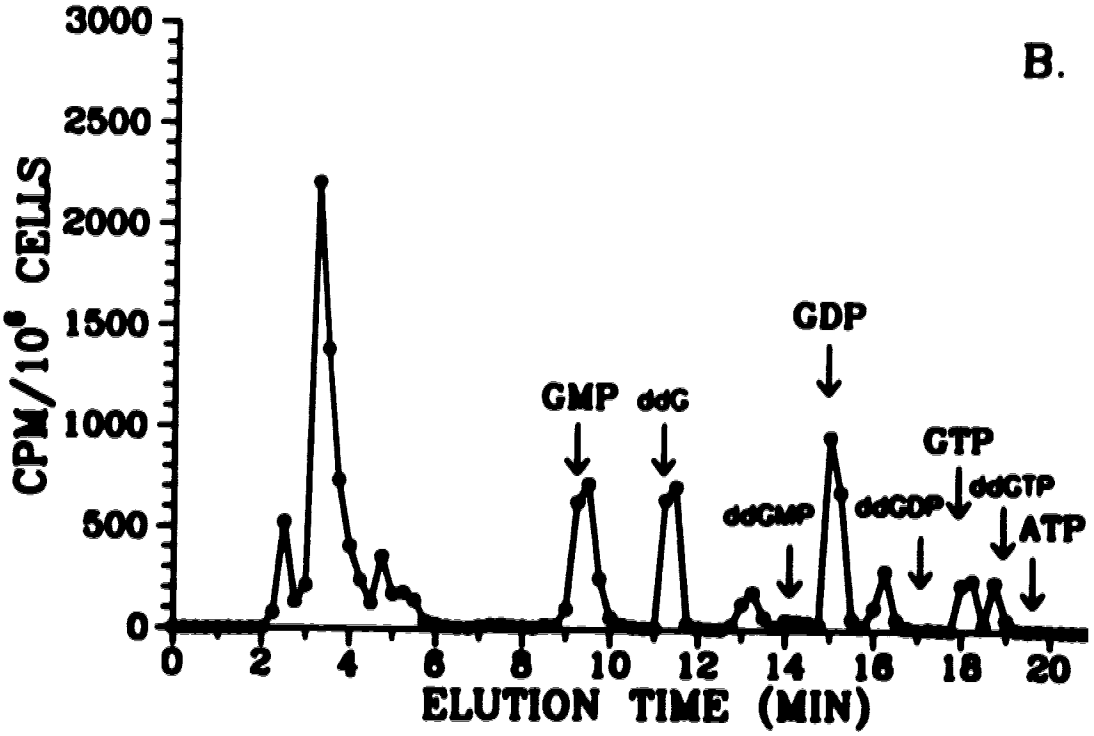

B.
HepG2 Cells
4 μM ddG
+
100 μM 8-NH2Guo
PMOLE / E6 CELLS
ddG
ddGMP
ddGDP
ddGTP
0 2 4 6 8 10 12 14 16 18 20
ELUTION TIME (min)

Figure 4.2 - A comparison of ddG metabolism in HepG2 cells and 2.2.15 cells. A) Actively proliferating HepG2 cells were incubated with 4 μM ddG (759 cpm/pmole) in MEM-10% NSIV for 24 hr at 37°C. B) Actively proliferating 2.2.15 cells were exposed to 4 μM ddG (315 cpm/pmole) in MEM-10% FCS for 26 hr at 37°C. Cell extracts were prepared and analyzed by HPLC according to the procedure outlined in sections II.C.1. and 2. of this chapter. The sample radioactivity is expressed as cpm/10^6 cells. Arrows indicate the elution times obtained for standards run in separate analyses. The elution time for ddGTP was determined from the absorbance peak contributed by an aliquot of ddGTP standard added to each cell extract immediately prior to HPLC analysis.


A.
3000
2500
2000
1500
1000
500
0
CPM/10⁶ CELLS
GMP
ddC
GDP
GTP
ddGMP
ddGDP
ddGTP
ATP
0 2 4 6 8 10 12 14 16 18 20
ELUTION TIME (MIN)


B.
3000
2500
2000
1500
1000
500
0
CPM/10⁶ CELLS
GDP
GMP
ddC
GTP
ddGMP
ddGDP
ddGTP
ATP
0 2 4 6 8 10 12 14 16 18 20
ELUTION TIME (MIN)

samples and 0.4% after a 24 hr incubation (Table 4.1). The total amount of product eluting with the same elution times as ddGMP, ddGDP and ddGTP standards was 0.26 pmole/10^6 cells in the 24 hr, ddG + 8-NH_2Guo sample, while ddGDP + ddGTP was 0.22 pmole/10^6 cells in the 24 hr, ddG only sample.

A 24-hr incubation of HepG2 cells with 4 μM ddC resulted in the formation of three ddC metabolites with elution times that corresponded to that of ddCMP, ddCDP, and ddCTP standards, as well as an unidentified metabolite (Figure 4.3B). After 24 hr, the total accumulation of intracellular ddC nucleotides (ddCMP + ddCDP + ddCTP) was 17.8 pmole/10^6 cells. When the experimentally determined intracellular H_2O volume of 3.33 μL/10^6 cells was considered, the intracellular ddC nucleotide concentration was 5.35 μM. Although ddC was metabolized by duck hepatocytes (Figure 4.3A), there were apparent differences in the products detected in the avian and HepG2 cell systems. Under the cell culture conditions employed, HepG2 cells produced almost 18 pmole/10^6 cells of ddC nucleotides after a 24-hr incubation with ddC, while duck hepatocytes accumulated 3.4 ±0.3 pmole/10^6 cells for the same ddC exposure time. Both duck hepatocytes and HepG2 cells produced an additional ddC-derived, unknown metabolite. However, judging by the disparate elution times exhibited by the unknown compound particular to each sample, the unidentified metabolites were not the same chemical species (Figure 4.3).

3. Metabolism of ddG in 2.2.15 Cells

2.2.15 cell extracts were analyzed for the presence of radiolabeled drug metabolites after a ddG treatment regime that continued for extended periods of time. Actively proliferating 2.2.15 cells were grown for 5 days in drug-free medium before the introduction of 4 μM [^{3}H]ddG. The cells were then grown in the presence of ddG for up to 10 days, with media changes occuring after 2, 4, 6, and 8 days of treatment. After a 26-hr incubation, 2.2.15 cell extracts revealed a similar HPLC elution profile of radiolabeled metabolites to that seen from HepG2 cells incubated with ddG for 24 hr (Figure 4.2). In both cases, a high percentage of the sample radioactivity eluted between 2 and 6 min, while other significant peaks of radioactivity coincided with the elution times of guanosine nucleotides (Figure 4.2). Analysis of a ddG sample in MEM-10% FCS incubated for 48 hr at 37°C indicated that 92% of the sample radioactivity was associated with ddG. Little or no indication of the presence of ddG nucleotides is found in the 2.2.15 cell extracts incubated with ddG for 0.25 to 10 days. Although an unidentified peak of radioactivity in the extract exhibited a similar retention time to ddGTP, the elution of this unknown did not coincide with the elution time of an internal ddGTP standard (Figure 4.2B). As can be seen in Table 4.1, the percent of the

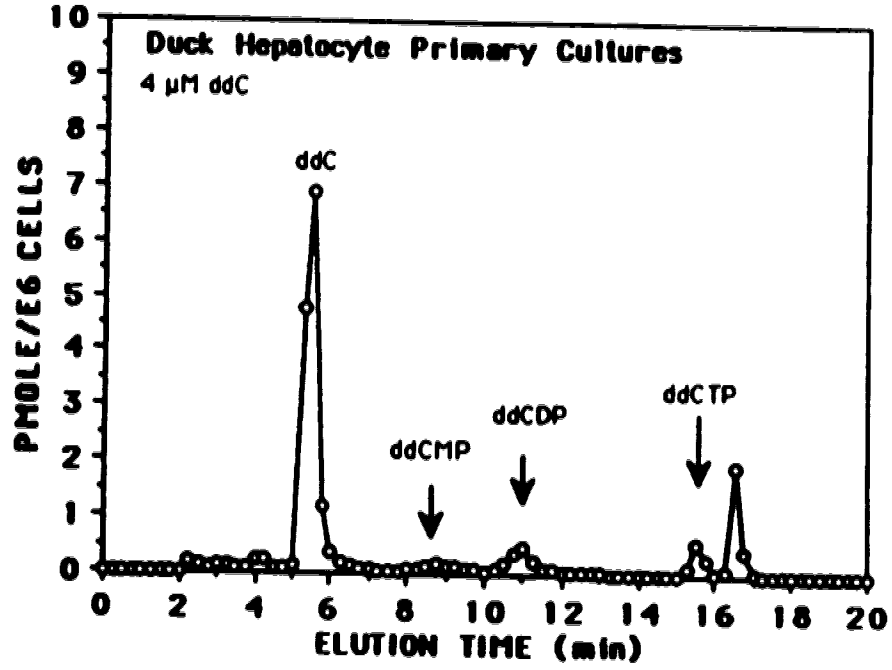
Table 4.1

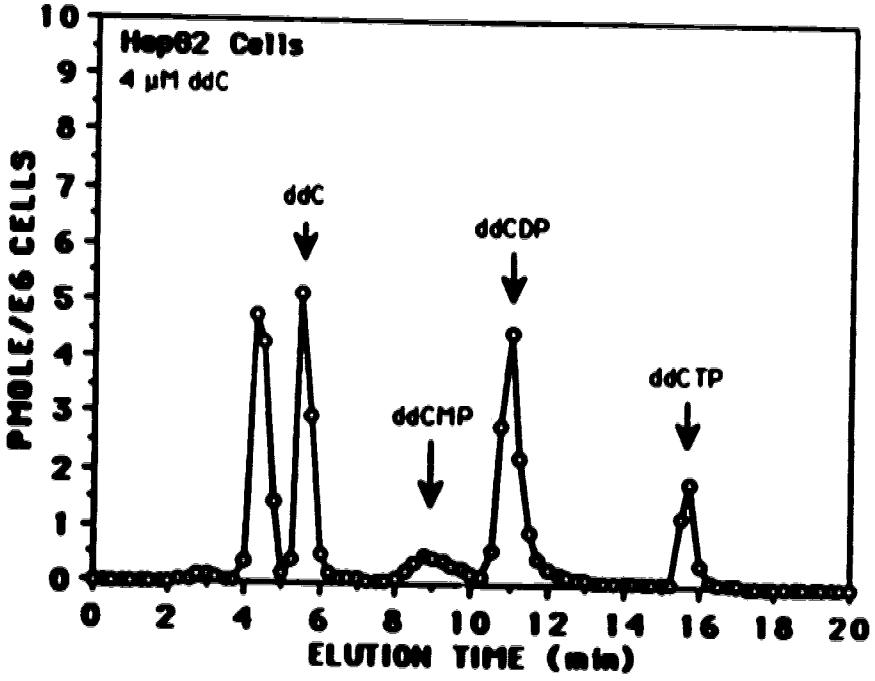
RADIOLABELED METABOLITES RECOVERED FROM ddG-TREATED HepG2 AND 2.2.15 CELLS

HepG2 cells were incubated with 4 μM ddG (759 - 772 cpm/pmole) in MEM-10% NSIV and 2.2.15 cells were treated with 4 μM ddG (315 cpm/pmole) in MEM-10% FCS for various time periods. Media changes were performed after 2, 4, 6, 8, and 10 days. Cell extracts were prepared and analyzed according to the procedure outlined in section II.C.1. and 2. of Chapter 4. Metabolite identification is based on the comparative elution times obtained from standards run in separate HPLC analyses. The cumulative values obtained from compounds eluting between 2 and 6 min are reported under the "early eluting compounds" heading. The results presented as GTP may also contain contributions from ADP due to coincidental elution of these two compounds. The HepG2 cell results reported for 0.21 days represent the average of duplicate samples from one experiment, while the 1 day results for these cells represent the mean of 6 samples from replicate experiments. The values reported for 0.25 to 6 day 2.2.15 cell samples represent the average of duplicate samples from one experiment, while the 8 and 10 day time point data was obtained from single samples.

				% of the Total CPM Recovered/10^6 Cells						
		Pmole/10^6 Cells		Guanosine Nucleotides				Adenosine Nucleotides		
Cells	Incub Time (days)	Early Eluting Compounds	ddG	GMP	GDP	GTP	Total	AMP	ATP	Total
HepG2	0.21	20.4	3.0	2.1	2.6	2.8	7.5	0.5	0.1	0.6
HepG2	1	25.6	3.5	6.0	6.1	5.8	17.9	1.5	0.4	1.9
2.2.15	0.25	23.7	7.2	3.9	3.8	1.3	9.0	0	0.02	0.02
2.2.15	1	21.8	4.9	11.8	11.7	3.9	27.4	0	0.04	0.04
2.2.15	2	18.0	2.3	13.2	8.0	6.4	27.6	1.2	0.7	1.9
2.2.15	6	14.4	1.4	28.6	11.4	4.4	44.4	3.8	0.3	4.1
2.2.15	8	13.1	1.8	28.7	13.5	6.4	48.6	4.0	0.8	4.8
2.2.15	10	16.2	2.2	28.3	11.2	5.1	44.6	7.1	0.6	7.7

Figure 4.3 - A comparison of ddC metabolism in duck hepatocyte primary cultures and HepG2 cells. A) Forty-eight hour duck hepatocyte primary cultures were incubated with 4 μM ddC (854 cpm/pmole) in supplemented L-15 medium - 10% NSIV for 24 hr at 42°C. Cell extracts were prepared and analyzed by HPLC according to the procedure described in sections II.C.2. and 3. of Chapter 3. B) Actively proliferating HepG2 cells were treated with 4 μM ddC (786 cpm/pmole) in MEM - 10% NSIV for 24 hr at 37°C. Cell extracts were prepared and analyzed by HPLC according to the method described in sections II.C.1. and 2. of this chapter. The levels of ddC and ddC metabolites contained in the cell extracts have been quantitated as pmoles/10^6 cells. Arrows indicate the elution times obtained for ddC, ddCMP, ddCDP, and ddCTP standards that were run in separate analyses.


Duck Hepatocyte Primary Cultures
4 µM ddC
ddC
ddCMP
ddCDP
ddCTP
PMOLE/E6 CELLS
ELUTION TIME (min)


Hep62 Cells
4 µM ddC
ddC
ddCDP
ddCTP
ddCMP
PMOLE/E6 CELLS
ELUTION TIME (min)

total cpm/10^6 cells recovered in each sample as guanosine nucleotides increased over time to a maximum level of 44 - 49% after 6 days of incubation. Radiolabeled adenosine nucleotides were also detected in 2.2.15 cell extracts (Table 4.1). A time-dependent, gradual increase from 0.04 to 4.4% was observed for a peak tentatively identified as IMP (results not shown). The ddG content of 2.2.15 cells plateaued at 1.4 to 2.3 pmole/10^6 cells for samples treated with ddG for 2, 6, 8, and 10 days (Table 4.1). If 2.2.15 cells have an intracellular H_2O volume of 3.33 μL/10^6 cells, the pmole/10^6 cell values obtained from the 2 to 10 day time points would translate into an intracellular ddG concentration of 0.4 to 0.7 μM. The 2 - 10 day time point samples all have in common that they were collected immediately prior to a scheduled media change.

4. The Effect of ddG and ddC on 2.2.15 Cell Culture Growth and Morphology

The cell population of 2.2.15 cell cultures treated for 27 days with 0, 4, 20, and 50 μM ddG or ddC is shown in Figure 4.4. In panel A, the number of cells present in cell culture samples treated with 4 and 20 μM ddG were comparable to the cell population of untreated cultures. In addition, except for the 18 day sample, the cells incubated with 50 μM ddG did not differ in cell number from that of the control (Figure 4.4A). Treatment of cell cultures with three ddC concentrations produced consistently lower cell numbers through 18 days of treatment (Figure 4.4B). After 26 days of treatment, the two higher drug concentrations maintained a lower cell number than the control, while 4 μM ddC samples exhibited a cell population equal to that of the untreated cells. Removal of ddC from the media resulted in cell culture populations that were in the same range as the untreated samples, which experienced gradual cell attrition after 18 days in culture (Figure 4.4B).

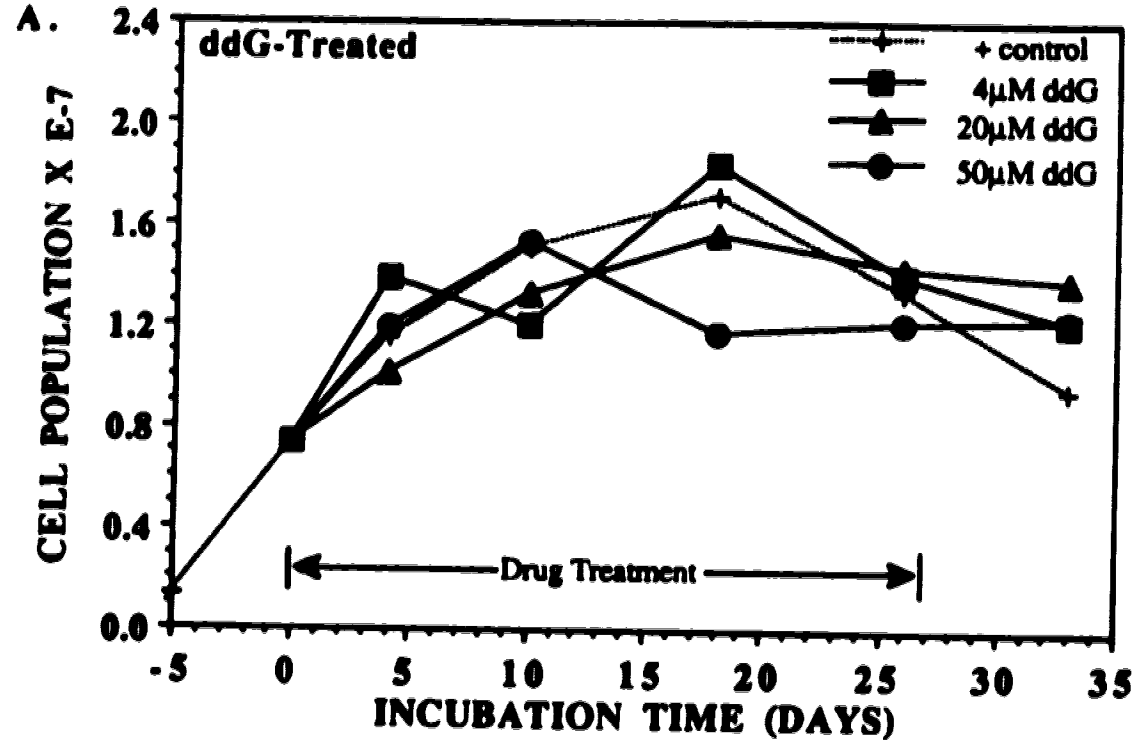
A morphological comparison of 2.2.15 cells treated for 4 and 26 days with 50 μM ddG or ddC is shown in Figure 4.5. At both 10X and 40X magnifications, no drug-induced changes in cell culture morphology could be discerned. In general, cell cultures maintained for 26 days had replaced the typical tight, round, often double nuclei structures (visible in Figure 4.5b,c, and d) with a more diffuse, single structure (Figure 4.5f,g, and h). This morphological change was less apparent in the HepG2 cell cultures grown in drug-free medium for 4 and 26 days (Figure 4.5a and e). The 4 day cell samples contained evenly distributed monolayers, while the 26 day cultures had more open spaces and large areas of dark-staining cell debris (Figure 4.5A-H).

B. Antiviral Effectiveness of ddG and ddC in 2.2.15 Cells

1. The Effect of ddG and ddC on Intracellular Levels of HBV DNA

The intracellular content of HBV DNA in whole cell nucleic acid extracts prepared from 2.2.15 cells treated for up to 10 days with 0 or 4 μM ddG or ddC was

Figure 4.4. - Comparison of 2.2.15 cell growth over time for cells that were grown in media supplemented with 0, 4, 20, or 50 μM ddG or ddC. Cells were plated at an initial density of 1.25 x 10^6 cells/60 mm dish and were incubated at 37°C in an humidified environment containing 5% CO_2. The media was replenished on the third day following plating, and every 2 days thereafter. On the fifth day after plating, the cells were given media containing 0, 4, 20, or 50 μM ddG or ddC. The first day of drug administration corresponds to Day 0 on the abscissa. The cell population x 10^7 cells is indicated on the ordinate. From Day 27 until Day 33 all cells received drug-free media. A) Cell growth curves generated by untreated cells (+ control) and cells treated with 4 μM ddG (■), 20 μM ddG (▲), or 50 μM ddG (●). B) Cell growth curves generated by untreated cells (+control) and cells treated with 4 μM ddC (□), 20 μM ddC (Δ), or 50 μM ddC (○). Each sample value represents the average cell count from duplicate samples.


A.
ddG-Treated
+ control
4μM ddG
20μM ddG
50μM ddG
CELL POPULATION X E-7
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Drug Treatment
-5
0
5
10
15
20
25
30
35
INCUBATION TIME (DAYS)

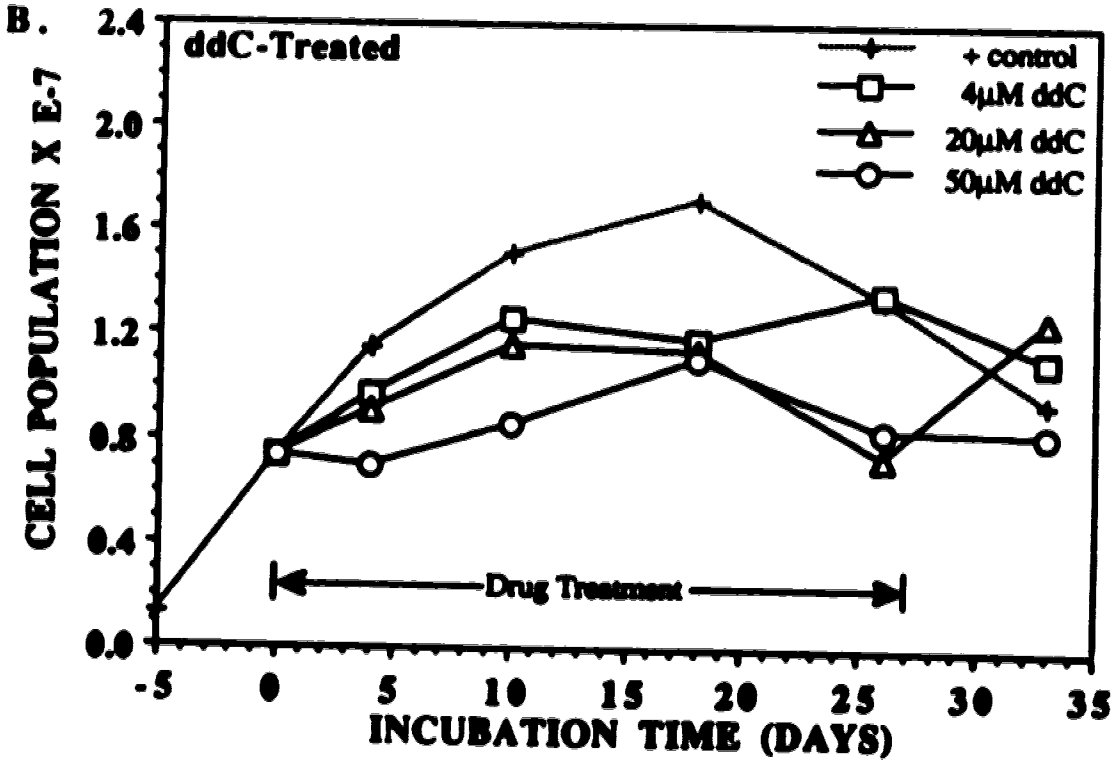
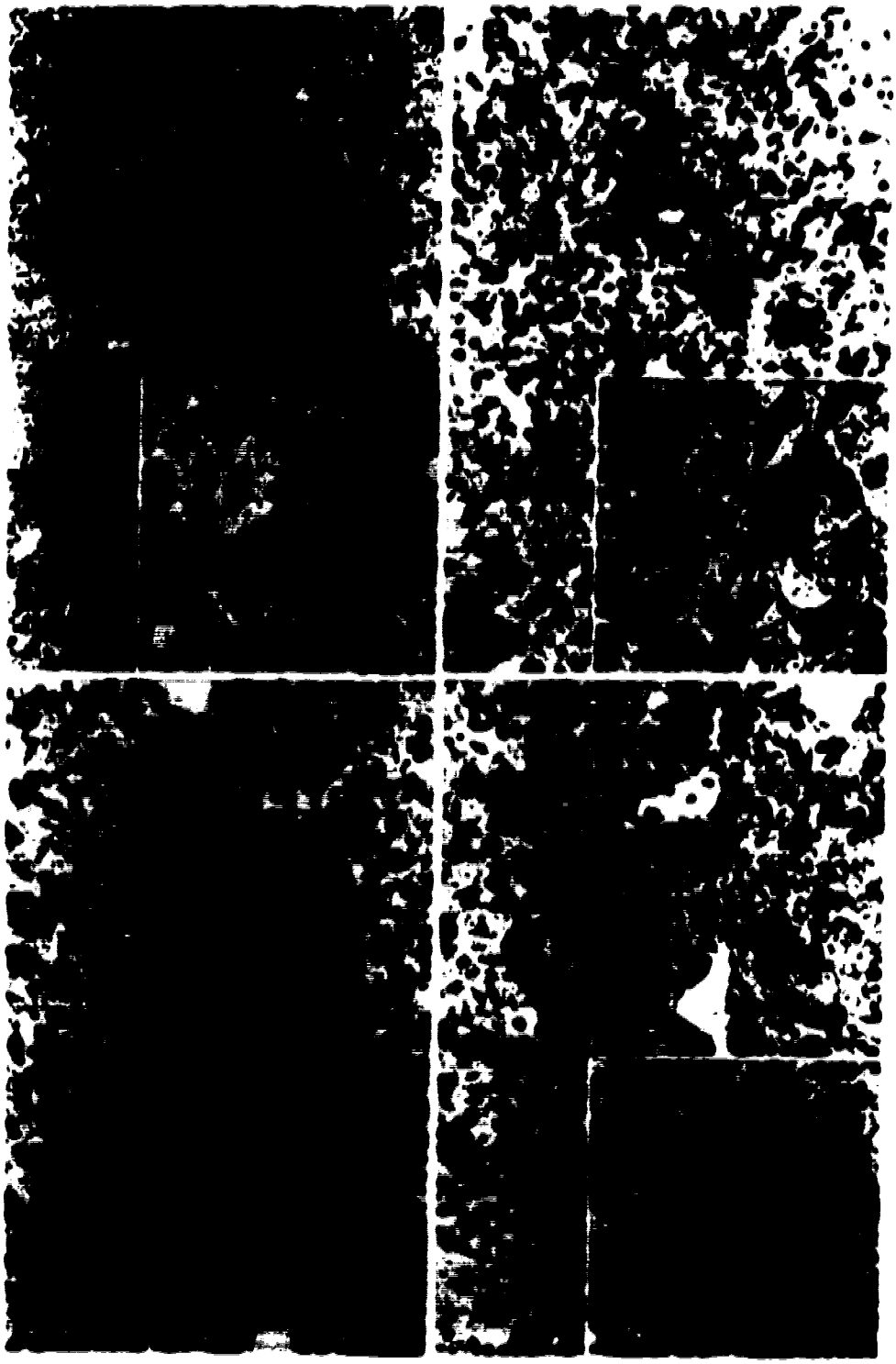
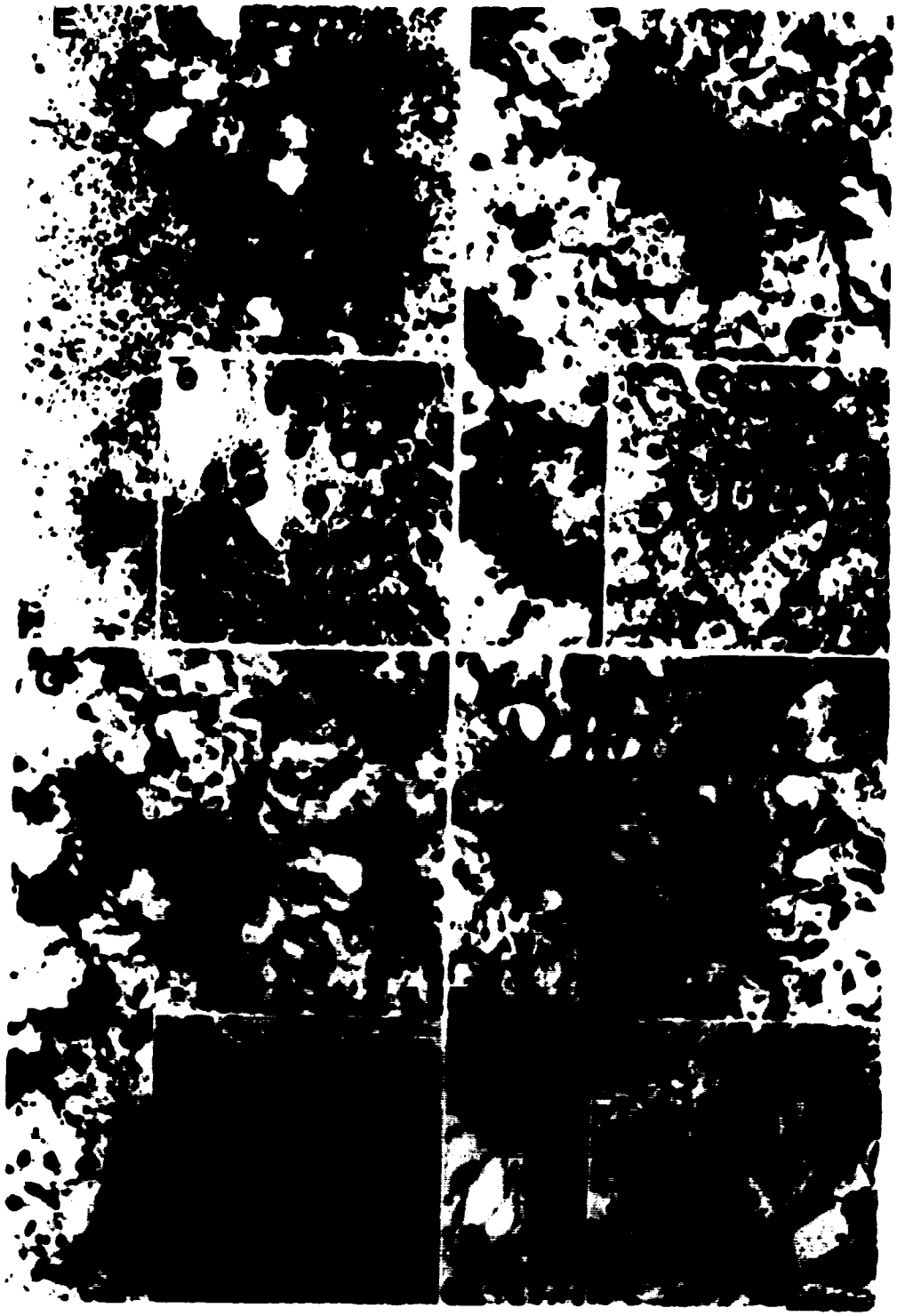

B.
ddC-Treated
+ control
4μM ddC
20μM ddC
50μM ddC
CELL POPULATION X E-7
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Drug Treatment
-5
0
5
10
15
20
25
30
35
INCUBATION TIME (DAYS)

Figure 4.5 - Morphological comparison of 2.2.15 cells grown for 4 or 26 days in media supplemented with 50 μM ddG or 50 μM ddC. Cells were plated at an initial density of 1.25 x 10^6 cells/60-mm dish and were incubated at 37°C in an humidified environment containing 5% CO_2. The media was replenished on the third day following plating. On the fifth day after plating, 2.2.15 cells were given media containing 50 μM ddG, 50 μM ddC, or no drug (+ control) and incubated for 4 or 26 days, with fresh media provided every 2 days thereafter. HepG2 cells plated at an initial density of 1.25 x 10^6 cells/60-mm dish and grown in drug-free MEM-10% NSIV for 4 or 26 days are included as an example of uninfected cells (negative control). After the appropriate incubation period, cell samples were fixed, stained, and photographed according to the procedure described in Chapter 3, section II.D.4. A 10X magnification of the monolayer samples is presented in Panels A - H, and a 40X magnification of each sample is shown in the inset photographs a - h. A) 4 days, - control; B) 4 days, + control; C) 4 days, 50 μM ddG; D) 4 days, 50 μM ddC; E) 26 days, - control; F) 26 days, + control; G) 26 days, 50 μM ddG; and H) 26 days, 50 μM ddC.





analyzed by dot hybridization. As can be seen in Figure 4.6, both compounds induced an eventual decrease in the intracellular content of HBV DNA. In this particular analysis, ddC treatment produced elevated levels of viral DNA relative to the control during the first 4 days of drug therapy. After an initial decrease in HBV DNA to about 90% of the control value, virus DNA production in ddG-treated cultures rebounded to 100%, and then consistantly decreased to 44% of the control by 10 days of treatment.

In another experiment, whole cell nucleic acid extract samples containing 4.6 to 18.5 μg of isolated, nondeproteinated DNA were analyzed by agarose gel electrophoresis. When transilluminated with ultraviolet light, the ethidium bromide stained gel contained a diffuse distribution of DNA in each sample lane in addition to an high molecular weight region of heavy staining. After a Southern transfer of the DNA from the gel to a nylon filter, the restained gel revealed that only a portion of the high molecular weight DNA remained in the agarose, indicating that most of the DNA had transferred to the filter. The presence of HBV-specific DNA on the nylon filter was visualized using a radiolabeled HBV DNA probe and subsequent autoradiography of the filter. High molecular weight HBV DNA sequences and two faster migrating HBV DNA species of 3.6 and 3.1 kbp were evident in the autoradiogram of whole cell nucleic acid extract samples prepared from 2.2.15 cells. Furthermore, when a sample was treated with the restriction enzyme EcoRI, the diffuse high molecular weight species disappeared and was replaced by a prominent band localized at a position corresponding to 3.6 kbp, as well as two faint, faster migrating species located in the 1.6 to 2.0 kbp range. Although the isolated DNA used in this experiment was not deproteinated, the presence of covalently closed, circular HBV DNA (cccDNA) was not apparent. Analysis of DNA isolated from HBV virions that were collected from spent cell culture medium revealed 4.1 kbp and 3.3 kbp bands in the autoradiogram, representing, respectively, the relaxed circular and the double-stranded, linear forms of the HBV genome present in virions.

2. The Effect of ddG and ddC on Cytoplasmic HBV DNA

The HBV DNA macromolecules isolated from cytoplasmically-located, replicating core particles in 2.2.15 cells were separated by agarose gel electrophoresis, Southern transferred to a nylon membrane, and probed with ^{32}P-labeled HBV DNA. Figure 4.7 presents the autoradiographic image produced from core particle HBV DNA samples obtained from cells treated for various time periods with 0 or 4 μM ddG or ddC. In panel A, the virus content of 2.2.15 cell samples is shown to increase with time in culture. During this time, the cell number increased from 5.8×10^6 cells/sample at 0 time to a confluent monolayer (2.0×10^7 cells/sample) on day 4, indicating that part of the increase in HBV DNA detected over time is due to the corresponding expansion in

Figure 4.6 - The effect of ddG and ddC on the intracellular HBV DNA content of 2.2.15 cells. Cells were plated at an initial density of 1.25 x 10^6 cells/60 mm dish and were incubated at 37°C in an humidified environment containing 5% CO_2. The media was replenished on the third day following plating, and on the fifth day post-plating, the cells were given media containing 4 μM ddG, 4 μM ddC, or no drug (control). Fresh media was provided every 2 days thereafter. At the indicated times, nucleic acids were isolated from cell samples according to the procedure outlined in section II.D.1. of this chapter. Nucleic acid samples equivalent to 0.072 of each monolayer sample were analyzed by dot hybridization (Chapter 4, section II.D.5.) and the radioactive content of each 1 cm^2 dot hybridization sample was determined by scintillation spectrometry. The HBV DNA detected in each drug-treated sample is expressed as a percentage of the HBV DNA detected in the corresponding untreated control. Data from the ddG-treated (●) and the ddC-treated (▲) samples have been normalized to represent a cell number equal to that of the control sample at each time point. Each data point represents one cell extract sample analyzed in duplicate. The results presented are from a single experiment.

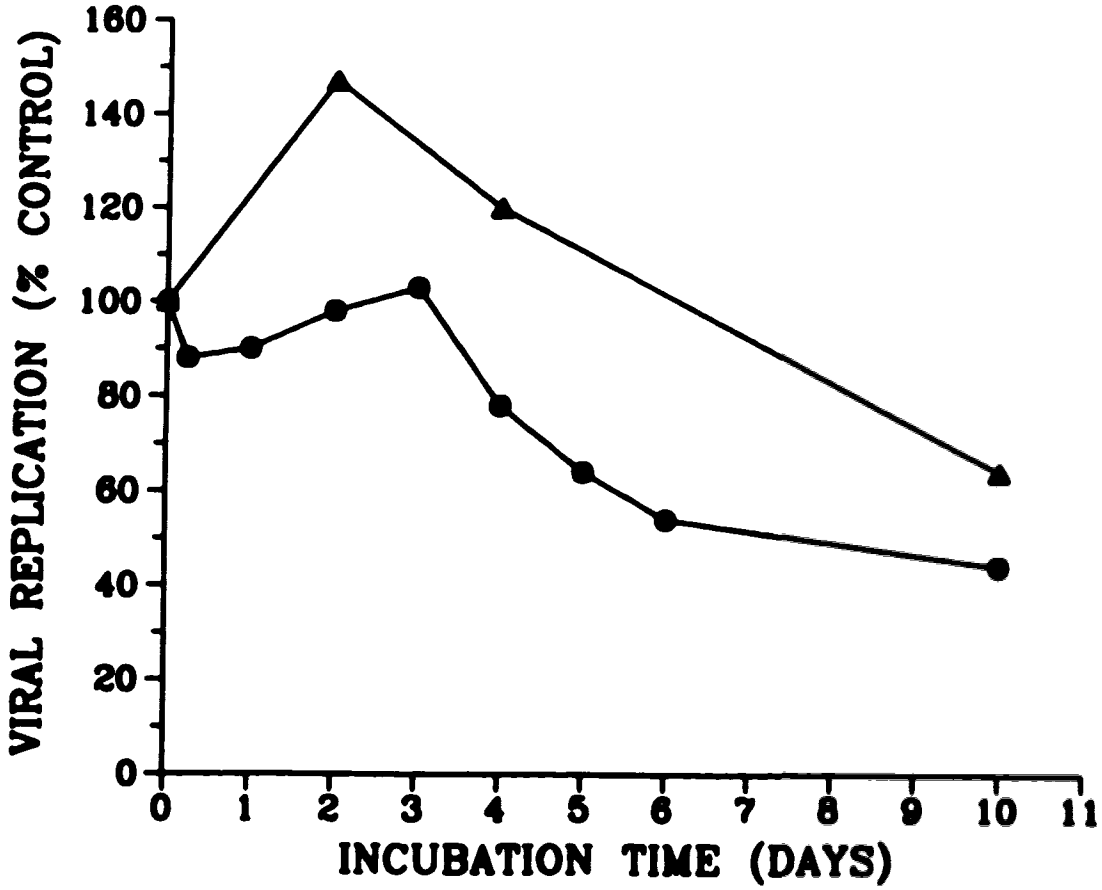

VIRAL REPLICATION (% CONTROL)
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
11
INCUBATION TIME (DAYS)

Figure 4.7 - The effect of ddG and ddC on intracellular levels of cytoplasmic replicating core HBV DNA in 2.2.15 cells. Cells were plated at an initial density of 1.25 x 10^6 cells/60-mm dish and were incubated at 37°C in an humidified environment containing 5% CO_2. The media was replenished on the third day following plating, and on the fifth day post-plating, the cells were given media containing 4 μM ddG, 4 μM ddC, or no drug (control). Fresh media was provided every 2 days thereafter. After 0 to 10 days of treatment, cell samples were harvested and the total cytoplasmic replicating core HBV DNA contained in each sample was isolated according to the procedure outlined in section II.D.2. of this chapter. Sample volumes containing DNA extract derived from one-half of each monolayer sample were electrophoresed in a 0.8% agarose gel. Following Southern transfer of the resolved samples to a nylon membrane and ^{32}P-labeled HBV DNA probe hybridization of the membrane-bound HBV DNA samples, an autoradiographic image was obtained. A) untreated 2.2.15 cells (+ control); B) 2.2.15 cells treated with 4 μM ddG; C) 2.2.15 cells treated with 4 μM ddC; and D) untreated HepG2 cells (- control). The cells were incubated for: a) 0 time; b) 1 day; c) 2 days; d) 4 days; e) 6 days; f) 8 days; and g) 10 days. Lane h contains 780 pg of pAM 6 (HBV DNA-containing plasmid) that has been digested with BamHI, thereby generating 3 DNA fragments of 4.4, 1.9, and 1.3 kilobase pairs (kbp) in size.

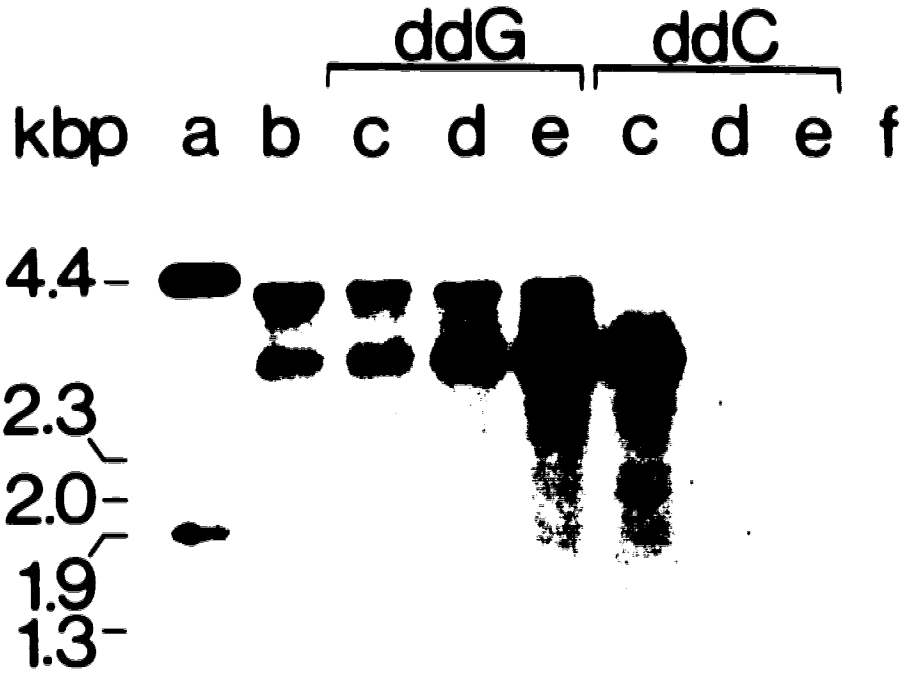
A
B
C
D
a b c d e g b c d e f g d g a e h kbp
4.4
1.9
1.3

the cell population. Similar increases in cell number over time were recorded for ddG-treated (panel B) and ddC-treated (panel C) samples: 1) the ddG-treated samples increased from 5.8 x 10^6 cells/sample to 2.1 x 10^7 cells/sample on day 4; and 2) the ddC-treated samples expanded from from 5.8 x 10^6 cells/sample to 1.6 x 10^7 cells/sample on day 4, and 1.8 x 10^7 cells/sample on day 10. Increased virus production is reported to occur in 2.2.15 cell cultures that have reached confluence (Sells et al., 1988). The time-dependent increase in HBV DNA production by control and ddG-treated 2.2.15 cells is enhanced after the cells reached confluence on day 4 (Figure 4.7, groups A and B).

The two major HBV DNA forms present in replicating core particles, a relaxed circular structure that migrates to a 4.1 kbp region in a 0.8% agarose gel, and a linear form of the genome that is positioned at 3.2 kbp, are visible in the autoradiogram shown in Figure 4.7. When compared to the 10 day control sample (group A, lane g), both the 10 day ddG-treated sample (group B, lane g) and the 10 day ddC-treated sample (group C, lane g) contain a reduced amount of HBV DNA (Figure 4.7). In another experiment, a 10 day exposure to 4, 20, or 50 μM ddC produced a consistent, dose-related decrease in the cytoplasmic HBV DNA content of the 2.2.15 cells. However, of the 4, 20, and 50 μM ddG concentrations tested for 10 days, only the highest drug concentration appeared to induce a slight decrease in the cytoplasmic content of HBV DNA relative to the control. In all cases, the 10-day ddC-treated samples contained predominantly the linear form of the HBV genome, while the control and ddG-treated samples taken at this time point had a more even distribution of the two DNA forms (Figure 4.7).

2.2.15 cells treated for 26 days with 4, 20, or 50 μM ddG did not exhibit a dose-dependent decrease in the cytoplasmic levels of HBV DNA (Figure 4.8). A separate analysis of replicate 26 day control and drug treated samples confirmed the absence of a ddG initiated concentration-dependent decrease in HBV DNA, although the drug treated samples appeared to contain slightly less virus than the controls. A dose-associated antiviral effect was observed for cells treated for 26 days with 4, 20, and 50 μM ddC (Figure 4.8). The antiviral effect of ddC on 2.2.15 cell HBV DNA was verified in a separate analysis of replicate 26 day samples. However, in this repeat analysis, HBV DNA was still detectible in the 50 μM ddC sample. In addition, when compared to the control, the relative level of DNA present in the 4 μM ddC-treated sample shown in Figure 4.8 was higher than the comparative ratio found in the replicate analysis. The HBV DNA present in 26-day ddC-treated samples consisted primarily of the double-stranded, linear form of the virus genome, while ddG and control samples contained both the relaxed, circular and linear arrangements of the DNA.

Figure 4.8 - Dose-dependent inhibition of cytoplasmic HBV DNA in ddG- or ddC-treated 2.2.15 cells . Cells were plated at an initial density of 1.25 x 10^6 cells/60-mm dish and were incubated at 37°C in an humidified environment containing 5% CO_2. The media was replenished on the third day following plating, and on the fifth day post-plating, the cells were given media containing 0, 4, 20, or 50 μM of either ddG or ddC. Fresh media was provided every 2 days thereafter. After 26 days of drug treatment, cell samples were harvested and the cytoplasmic replicating core HBV DNA contained in each sample was isolated according to the procedure outlined in section II.D.2. of this chapter. Each sample lane in a 0.8% agarose gel was loaded with a volume of DNA extract that corresponded to 5.8 x 10^6 cells. After gel electrophoresis, the resolved samples were transferred to a nylon membrane, the membrane was incubated with a ^{32}P-labeled HBV DNA probe, and an autoradiographic image was obtained. The autoradiogram illustrates HBV DNA obtained from cells incubated with b) no drug, c) 4 μM, d) 20 μM, or e) 50 μM, of ddG or ddC. Lane a contains 328 pg of BamHI-digested pAM 6 and lane f represents an HBV-negative control sample prepared from HepG2 cells that were subjected to the same DNA extraction procedure used for the experimental samples. The indicated kilobase pair (kbp) positions were determined by the mobility of the 3 DNA fragments seen in lane a (4.4, 1.9, and 1.3 kbp), and by a Hind III-digested λ DNA standard that was run in the same gel.


ddG
ddC
kbp
a
b
c
d
e
c
d
e
f
4.4
2.3
2.0
1.9
1.3

IV. DISCUSSION

Evidence was presented in Chapter 3 for the intracellular phosphorylation of ddG by endogenous enzymes in duck hepatocyte primary cultures. In the avian cell system, intracellular ddC nucleotide pools accumulated to a lower level than did the corresponding ddG anabolites. When the antiviral activity of ddG, the ddG pro-drug ddDAPR, or ddC was examined in an *in vivo* or *in vitro* avian test system, the purine agents were unique in providing effective inhibition of DHBV (Lee et al., 1989; Suzuki et al., 1988). A link has been established between the ability of animal cells to phosphorylate potential nucleoside analog drugs and the antiviral potency of these select agents (Balzarini et al., 1988; Hao et al., 1988; de Miranda and Good, 1992). In this chapter, the intracellular metabolism and anti-HBV effects of ddG and ddC were explored in the human hepatoblastoma cell lines HepG2 and 2.2.15.

A. Dideoxynucleoside Metabolism in HepG2 and 2.2.15 Cells

The predominant fate of ddG in drug-treated HepG2 or 2.2.15 cells was cleavage of the nucleoside to yield free guanine and sugar moieties. This initial metabolic step did not occur in cells treated with the PNP inhibitor 8-aminoguanosine, suggesting that phosphorolytic cleavage of ddG is catalyzed by PNP. In both cell lines, reutilization of the ddG-derived guanine moiety lead to the accumulation of guanosine nucleotides and a small amount of phosphorylated adenosine. Although a very low amount of ddG nucleotides was detected in HepG2 cells treated with ddG and 8-NH_2Guo, accumulation of these metabolites in cells treated with ddG alone was not readily apparent due to the very low levels of ddGTP produced and the potential masking of ddGMP and ddGDP by co-elution with other metabolites. For example, under the HPLC conditions employed, ddGMP and AMP standards had overlapping elution times. Indeed, a small amount of ATP was detected in HepG2 cells treated for 24 hr with ddG, suggesting that the radiolabeled product eluting between 13 and 14 min was AMP. Detection of low metabolite levels such as that of ATP in the 24-hr HepG2 cell sample, but not the 26-hr 2.2.15 cell sample was probably due to the higher ratio of [^{3}H]ddG to unlabeled ddG (759 cpm/pmole versus 315 cpm/pmole) employed in the HepG2 cell experiment. Both HepG2 and 2.2.15 cells incubated with ddG produced a metabolite that had a similar retention time to ddGTP. However, this radiolabeled metabolite was not assigned to ddGTP: 1) rather than coincident elution, the [^{3}H]-labeled metabolite eluted immediately prior to an internal ddGTP standard; and 2) if this metabolite was ddGTP, there would be significantly more radiolabeled product in the regions associated with ddGDP and ddGMP elution.

Conversion of ddG to ddG nucleotides has been reported to occur in 2.2.15 cells

(Aoki-Sei et al., 1991). These researchers treated an estimated 2×10^7 cells with 5 μM cold ddG + 5 mCi [^{3}H]ddG/ml for 5 hr. In comparison, in the experiments presented in this chapter, 3×10^6 - 14×10^6 cells were treated with 4 μM ddG containing 0.002 to 0.003 mCi [^{3}H]ddG/ml. Ahluwalia and co-workers (1990) reported the detection of low levels of ddG nucleotides in extracts from the human T-cell line Molt-4 treated for 5 hr with 5 μM ddG (0.005 mCi/ml; 5×10^6 cell equivalents analyzed). However, no ddGTP was detected in the HIV-infected human T-cell line H-9 following a 24-hr incubation with 1 μM ddG (0.01 mCi/ml; 3×10^6 cell equivalents analyzed) (Busso et al., 1990). Similar to the results presented in Chapter 4, the major nucleotide products found in 2.2.15 cells and Molt-4 cells treated with ddG were guanosine nucleotides (Ahluwalia et al., 1990; Aoki-Sei et al., 1991). In addition to guanosine nucleotides, Molt-4 and H-9 cells contained substantial amounts of radiolabeled adenosine nucleotides following a 5- or 24-hr exposure to [^{3}H]ddG (Ahluwalia et al., 1990; Busso et al., 1990). Very low levels of adenosine nucleotides were detected in the [^{3}H]ddG-treated HepG2 and 2.2.15 cells utilized in this chapter.

Intracellular ddG metabolism was reported to differ in two 2.2.15 cell populations obtained from independent sources (Aoki-Sei et al., 1991). Although ddG nucleotides were detected in both cell populations, ddG catabolism may have been a more prominent reaction in 2.2.15 (GA) cells (obtained from G. Acs, Mount Sinai School of Medicine, New York) than it was in 2.2.15 (PR) cells (originally provided by P. Roingeard, Harvard University School of Public Health, Boston), despite confirmation that both cell lines were of the same origin. The experiments presented in Chapter 4 employed 2.2.15 cells that were obtained from M.A. Sells (Mount Sinai School of Medicine, New York), a colleague of G. Acs. Variations in drug metabolism by identical cell lines maintained at different facilities have been reported previously (Furman et al., 1981).

Metabolism of ddC in HepG2 cells lead to the formation of ddCMP, ddCDP, ddCTP, and an unknown metabolite. The human hepatoblastoma cells produced 5 times the amount of total ddC nucleotides (ddCMP + ddCDP + ddCTP) as did the duck hepatocyte cultures. The cytidine metabolite cytidine 5'-diphosphocholine (CDP choline) has an elution time of 3.7 min when analyzed under the HPLC conditions employed in these studies, which suggests that the unknown compound formed by ddC-treated HepG2 cells could be a dideoxy-derivative of CDP choline (ddCDP choline). HIV-infected human T-lymphoblasts convert ddC to ddC nucleotides and to a metabolite identified as ddCDP choline (Cooney et al., 1986). Deamination of ddC would yield ddU, another candidate for the unknown metabolite produced by 2.2.15 cells. No cytidine deaminase activity was associated with the serum supplement NSIV. Although a standard for ddU

was not run, a 2'-deoxyuridine standard was found to elute after 2'-deoxycytidine, indicating that ddU would be retained on the column longer than ddC, unlike the unidentified metabolite which eluted earlier than ddC.

Cell cultures maintained in the presence of ddC exhibited a lower cell population than the controls, whereas ddG-treated populations were comparable to that of the untreated cultures. Ample production of ddC anabolites by 2.2.15 cells may lead to biochemical utilization of this unnatural nucleoside. Dideoxycytidine-induced toxicity manifested in the form of growth retardation could stem from incorporation of the phosphorylated analog into cellular DNA. Cytotoxicity in Molt-4F cells treated with ddC has been correlated with decreased cellular mitochondrial DNA and a reduced rate of glycolysis in the treated cells (Chen and Cheng, 1989).

B. Antiviral Effectiveness of ddG and ddC in 2.2.15 Cells

Dot hybridization of whole cell nucleic acid extracts with an HBV-specific probe indicated that both ddG and ddC treatment of 2.2.15 cells decreased the cellular content of HBV relative to the control. The ddG-treated samples appeared to reach a plateau level of viral replication equal to 40 - 60% of the control value after 6 - 10 days of treatment. However, virus detection by the dot hybridization method does not differentiate between the presumably stable population of integrated HBV DNA in the cell and the non-integrated viral DNA produced during replication.

Agarose gel analysis of whole cell nucleic acid extracts from 2.2.15 cells indicated that the cells contain both episomal and integrated HBV DNA sequences. Treatment of the integrated HBV DNA with EcoRI produced a 3.6 kbp fragment suggesting that a complete copy of the viral genome had been excised from the cellular DNA. Sells and co-workers (1988) reported that EcoRI digestion of 2.2.15 cell nuclear DNA produced a major 3,182 base pair, HBV-specific DNA species, indicating that 2.2.15 cells contain full-length, HBV DNA genome sequences integrated into the cellular chromosomes. No cccDNA was detected in the whole cell DNA preparations analyzed in Chapter 4, although the 2.2.15 cell nucleus is reported to contain this form of the virus (Sells et al., 1988).

A concentration-associated decrease in the amount of cytoplasmically-located HBV DNA occurred in 2.2.15 cells treated with ddC. However, cells exposed to 4 - 50 μM ddG did not produce a consistent, dose-related decrease in the amount of HBV DNA present in replicating core particles isolated from the cell cytoplasm. When both the metabolism and antiviral results obtained from ddG-treated 2.2.15 cells are considered, the poor antiviral activity can be directly related to the inability of the cells to produce detectible levels of ddG nucleotides. Although ddC metabolism was only explored in the

parent HepG2 cell line, the HBV-infected, derivative cell line 2.2.15 may present a comparable metabolic profile for this nucleoside analog. The production of micromolar quantities of ddC nucleotides by HepG2 cells treated with 4 μM ddC correlates well with the dose-associated decrease in HBV DNA detected in 2.2.15 cells treated with 4, 20, and 50 μM ddC.

Aoki-Sei et al. (1991) found that 2.2.15 cells obtained from different sources produced disparate antiviral results when treated with ddG. In 2.2.15 (GA) cells treated for 22 days with 50, 100, or 200 μM ddG, HBV DNA synthesis was reduced by only 31, 43, and 37%, respectively (Aoki-Sei et al., 1991). In contrast, 2.2.15 (PR) cells treated with 5, 20, and 50 μM ddG for the same exposure period produced, respectively, a 60, 74, and 92% reduction in extrachromosomal HBV DNA (Aoki-Sei et al., 1991). Although ddG nucleotides were detected in samples from both cell populations, the 2.2.15(GA) cells appeared to produce a larger amount of ddG cleavage products and metabolites stemming from subsequent reutilization of the guanine moiety (Aoki-Sei et al., 1991). The results from Ahluwalia et al. (1990) provide a positive correlation between increased ddG nucleotide formation and enhanced antiviral activity in the human T-cell lines Molt-4 and CEM. Although ddGTP was not detected in HIV-infected H-9 cells, a ddG concentration of 0.1 - 1 μM reduced viral replication in these cells by 50% (Busso et al., 1990). In this case, a low intracellular level of ddGTP could be responsible for the potent antiviral activity attributed to ddG.

The experiments performed in Chapter 4 indicated that ddG phosphorylation products were not detectible in the ddG-treated 2.2.15 cells used in these studies. The lack of ddG anabolism combined with its ineffective anti-HBV activity in this population of cells supports the idea that anabolic activation of ddG is of primary importance for the antiviral activity of this agent. As seen in the results from Chapter 3 and 4, drug metabolism varies depending on the host tissue involved. Other researchers have presented evidence that cells of the same origin can evolve in culture to produce vastly different drug metabolism and antiviral results (Aoki-Sei et al., 1991). As yet, the metabolic fate of ddG in normal human liver tissue is not known. The ultimate effectiveness of ddG as an anti-HBV therapeutic agent will depend on its intracellular metabolism in this tissue.

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CHAPTER 5
OVERALL SUMMARY

I. GENERAL DISCUSSION AND CONCLUSIONS

The series of studies presented in this body of work were undertaken to characterize the intracellular metabolism of the purine dideoxynucleoside analogs ddDAPR and ddG in hepatocyte cultures and to correlate the drug metabolism results with the antiviral effect of these compounds on hepadnavirus replication.

Previous work established that the purine nucleoside analog ddDAPR functions as an effective inhibitor of DHBV replication in both an *in vitro* and *in vivo* avian test system (Lee et al., 1989; Suzuki et al., 1988). A similar antiviral effect was achieved *in vitro* when ddG was used to treat DHBV-infected duck hepatocyte primary cultures (Lee et al., 1989). In contrast, pyrimidine nucleoside analogs are less effective inhibitors of the virus in duck hepatocytes (Lee et al., 1989; Yokota et al., 1990).

The comparable antiviral activities of ddDAPR and its deamination product ddG led to the investigation of ddDAPR metabolism in blood, one of the initial biological tissues encountered *in vivo* by an agent that is administered by injection. The work presented in Chapter 2 clearly indicates that ddDAPR is rapidly converted to ddG by duck blood. Conversion of ddDAPR to ddG also occurs in human blood samples. In this milieu, deamination of ddDAPR yielded a more biologically stable product, suggesting that the reported *in vivo* antihepadnaviral effect of ddDAPR in Pekin ducks actually derives from ddG or a metabolite thereof (Lee et al., 1989; Suzuki et al., 1988).

Metabolism of the pro-drug ddDAPR and its subsequent deamination product ddG was first explored in duck hepatocyte primary cultures, one of the few cell culture systems capable of supporting hepadnavirus replication. The restricted natural host range of hepadnaviruses combined with the inability to propagate these viruses in conventional tissue culture models has led to utilization of this cell culture system in the screening of potential antihepadnaviral agents (Civitico et al., 1990; Lee et al., 1989; Yokota et al., 1990).

Similar to the results obtained from whole blood, the apparent conversion of ddDAPR to ddG occurred in duck hepatocyte primary cultures. Further drug metabolism studies in this cell culture model focused on ddG, with metabolism results obtained from use of the ineffective anti-DHBV agent ddC provided as a comparison (Lee et al., 1989). The work presented in Chapter 3 illustrates that metabolism of the effective anti-DHBV agent ddG in duck hepatocytes leads to the formation of intracellular ddG nucleotide pools that represent a combined total concentration of 2 - 4 μM. Furthermore, the apparent inability of these cells to catabolize ddG resulted in the maintenance of an

intracellular level of ddG that reflected the initial nucleoside analog concentration. The metabolism data clearly indicates that phosphorylation of ddG is the major pathway of ddG utilization in these cells. Duck hepatocytes also produced ddC nucleotide pools in a concentration of 1.4 μM for ddCMP + ddCDP + ddCTP.

The potent antiviral effect exhibited in ddG-treated duck hepatocytes may arise from the ability of these cells to maintain relatively high ddG nucleotide pool sizes, leading to dideoxynucleotide incorporation into, and subsequent chain termination of viral DNA. Alternatively, chemotherapeutic intervention of a ddG derivative with a unique aspect of the virus life cycle could translate into a selective antiviral effect. One such candidate target for a ddG derivative, proposed by Suzuki and co-workers (1988), involves the initial deoxyguanosine nucleotide linkage to the virus primer protein, a polypeptide that is necessary for the initiation of viral DNA (-)-strand synthesis (Bartenschlager and Schaller, 1988; Wang and Seeger, 1992). Incorporation of a dideoxyguanosine nucleotide residue at this point, combined with an inability of the protein-bound, unnatural residue to be excised by a proofreading 3' → 5' exonuclease activity, could selectively and irreversibly block replication of the virus (Suzuki et al., 1988). In comparison to ddG, the antiviral potential of ddC may rely on a different and less sensitive mechanism of action. The poor anti-DHBV activity observed in ddC-treated ducks and duck hepatocytes could result from the formation of insufficient quantities of ddC nucleotides to produce an antiviral effect. In this case, competition for ddC nucleotides by viral and cellular enzymes would compound the problem of drug availability for an antiviral interaction. However, the intracellular total ddC nucleotide level was 74% of the total ddG nucleotide pools following a 24 to 26 hr treatment with the corresponding dideoxynucleosides (5.4 pmoles/10^6 cells of ddC nucleotides vs 7.3 pmoles/10^6 cells of ddG nucleotides). It is questionable whether this relatively minor disparity in nucleotide pool sizes could account for the observed marked differences in the antiviral effects of ddG and ddC on DHBV replication both *in vivo* and *in vitro* (Lee et al., 1989; Suzuki et al., 1988). It is possible that the differing antiviral activities result from the intracellular production of a ddG metabolite capable of exerting a more selective and potent antiviral effect than is produced by the corresponding ddC metabolite.

It is not known whether the duck hepatocyte culture system is a relevant vehicle for the screening of potential anti-HBV pharmacological agents. Results presented in Chapter 3 indicate that the outcome of ddG metabolism is highly dependent on the cell type. A survey of ddG metabolism in duck hepatocytes, rat hepatocytes, and a human T-lymphoblastoid cell line provided evidence for both qualitative and quantitative

differences in ddG utilization and subsequent metabolite formation by these cells. As a result, drug treatment studies were initiated in an alternative, HBV-permissive tissue culture model in order to compare the drug metabolism and antiviral activities of potential antiviral agents with the results obtained from duck hepatocytes.

Dideoxyguanosine phosphorylation was not detectible in the ddG-treated 2.2.15 cells utilized in Chapter 4. The predominant fate of ddG in drug-treated HepG2 or 2.2.15 cells is catabolism of the nucleoside to yield the nucleobase and sugar moieties. In these cells, reutilization of the ddG-derived nucleobase produces substantial amounts of radiolabeled guanosine nucleotides. The lack of ddG anabolism to ddG nucleotides combined with its ineffective anti-HBV activity in this population of 2.2.15 cells supports the idea that anabolic phosphorylation of ddG is of primary importance for the antiviral activity of this agent. Dose-associated inhibition of HBV in ddC-treated 2.2.15 cells correlated with the ability of the parent cell line HepG2 to produce ddC nucleotides at a concentration level of approximately 5 μM. Cell culture growth retardation was associated with ddC treatment.

The results presented in Chapters 3 and 4 indicate that phosphorylation of the nucleoside analog ddG occurs in duck hepatocyte primary cultures but not in the HBV-infected, continuous cell line 2.2.15. It is generally believed that intracellular phosphorylation of nucleoside-type agents is a prerequisite to their antiviral activity (De Clercq, 1993). In the present study, ddG phosphorylation in duck hepatocytes correlates with the reported potent antiviral effect of this agent in the avian system, while in 2.2.15 cells, the absence of ddG anabolism can be related to a lack of antiviral activity (Lee et al., 1989). Although ddG had no apparent effect on HBV replication in the 2.2.15 cells employed in the experiments presented in Chapter 4, there is some *in vivo* evidence that ddG can induce an antiviral effect in HBV-infected chimpanzees (Unpublished results, Dr. D.L.J. Tyrrell). Dot hybridization analysis of serum samples obtained from chimpanzees treated twice daily for 7 to 10 days with 10 mg/kg of the ddG pro-drug ddDAPR indicated that the serum HBV DNA levels decreased after 3 days of treatment (Unpublished results, Dr. D.L.J. Tyrrell). The antiviral effect in these animals suggests that the *in vivo* chimpanzee liver tissue is capable of ddG activation, a result that would be more similar to the antiviral and metabolism data obtained from the *in vitro* duck hepatocyte primary culture system than the 2.2.15 cell model used in this thesis. If *in vitro* cell culture systems are to be successfully employed in the screening of potential anti-HBV agents, it is manditory that the chosen culture system mimic the biochemical environment of the *in vivo* human liver cell.

Transformed cells maintained in culture frequently exhibit genetic and metabolic abnormalities (Kletzien and Perdue, 1974; Knowles et al., 1980; Thorens et al., 1990). Furthermore, phenotypic or genetic changes affecting the metabolism and/or antiviral activity of nucleoside analogs have been identified in continuous cell cultures that share the same origin, but were maintained in separate research facilities (Aoki-Sei et al., 1991; Furman et al., 1981). For example, 2.2.15 cells obtained from two independent sources exhibited disparate antiviral and metabolic results following treatment with ddG (Aoki-Sei et al., 1991). Periodic cloning of immortalized cell cultures is important for preservation of an homogeneous cell preparation. The possibility that phenotypic and genetic alterations have or may occur in immortalized cell lines maintained in culture brings into question the validity of their use as a representative model for *in vivo* liver tissue.

Primary cultures of hepatocytes provide an alternative cell culture system for the screening of potential antiviral drugs. Although primary cultures are nonproliferating and can be cultivated for a limited time, they have the potential to provide a realistic approximation of chemotherapeutic drug interaction with the virus in a normal liver cell environment. Successful preparation of hepadnavirus-infected primary cultures of duck, woodchuck, and human hepatocytes have been reported (Gripon et al., 1988; Shimizo et el., 1986; Thézé et al., 1987; Tuttleman et al., 1986).

Increased culture viability and maintenance of cell differentiation occurs in hepatocyte primary cultures grown in co-culture with another cell type (Clement et al., 1984; Guguen-Guillouzo et al., 1983; Reid and Jefferson, 1984). Viral replication was maintained for 2 months in DHBV-infected duck hepatocyte primary cultures co-cultivated with rat liver epithelial cells, allowing extended antiviral treatment studies to be conducted in an hepatocyte primary culture cell system (Fourel et al., 1989). Although not included in this thesis, nucleoside drug metabolism was examined in a preparation of human hepatocytes maintained in co-culture with rat liver epithelial cells (RLEC). The human hepatocyte primary cultures were originally established by Dr. Eve Roberts (University of Toronto) using liver biopsies obtained from young children and were grown in the presence of an RLEC "feeder" layer. The co-cultures were eventually passaged and maintained in continuous culture. Drug metabolism studies were conducted using the co-culture cells as well as RLEC cell samples alone. However, conclusive metabolism results could not be obtained from the co-cultivation cell system.

The differing antiviral activities induced by ddG and ddC in duck hepatocytes as compared to 2.2.15 cells could also stem from actual differences in the two hepadnaviruses. The possibility of a differential drug interaction occurring with two

separate viruses from the *Hepadnaviridae* family could be addressed through a series of drug treatment experiments utilizing avian hepatocytes transfected with the human virus and human liver tissue-derived cells (i.e. - HepG2) transfected with the duck virus. Cross-transfection of these viruses into the corresponding foreign host tissues has not been reported.

As yet, the metabolic fate and antiviral activity of ddG in *in vivo* human liver tissue is not known. The ultimate effectiveness of ddG as an anti-HBV therapeutic agent will depend on its intracellular metabolism in this tissue.

II. BIBLIOGRAPHY

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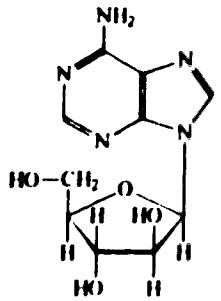
Appendix I

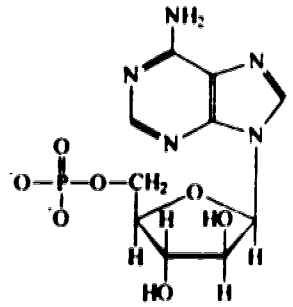
Chemical Structure of Compounds Tested for Anti-Hepadnavirus Activity

1) Arabinosyl Nucleosides

a) Purines

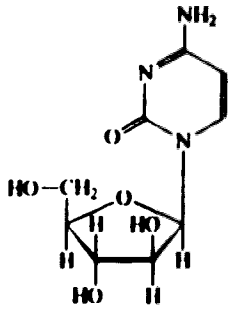


1) ara-A
9-β-D-arabinofuranosyladenine

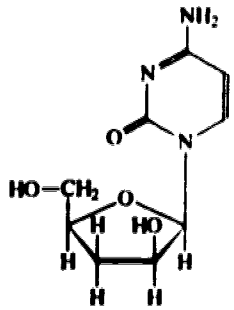


2) ara-AMP
9-β-D-arabinofuranosyladenine monophosphate

b) Pyrimidines

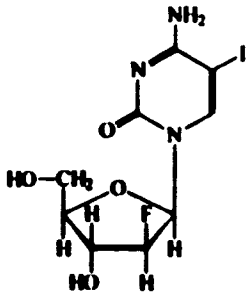


1) ara-C
1-β-D-arabinofuranosylcytosine

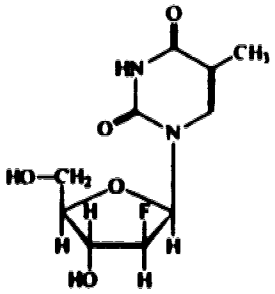


2) D-ara-C
1-β-D-3'-deoxyarabinofuranosylcytosine

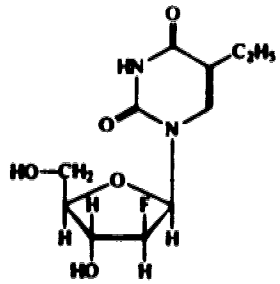
2) Subsituted Arabinosyl Nucleosides



1) FIAC
1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine



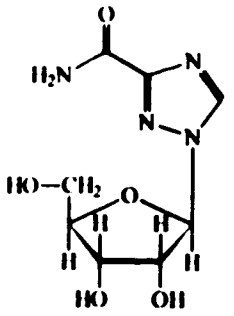
2) FMAU
1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil



3) FEAU
1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil

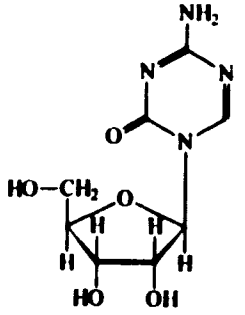
3. Base-Modified Nucleosides

a) Purines

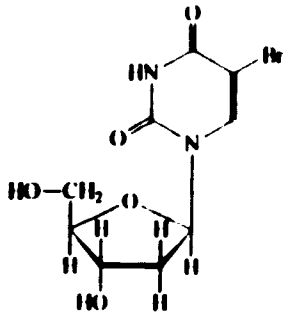


1) ribavirin
1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide

b) Pyrimidines



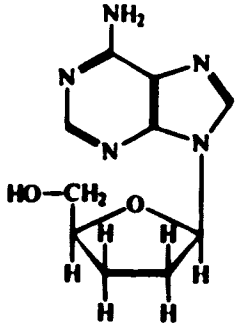
1) 5-aza-Cyd
5-azacytidine



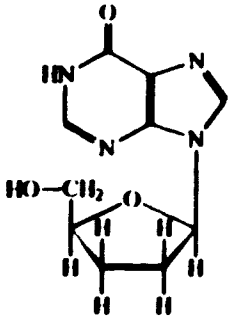
2) BDU
5-bromo-2'-deoxyuridine

4. Dideoxynucleosides

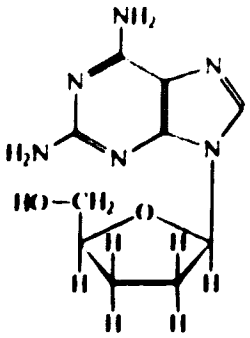
a) Purines



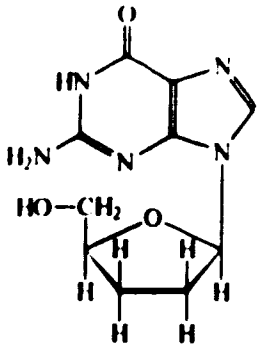
1) ddA
2',3'-dideoxyadenosine



2) ddI
2',3'-dideoxyinosine

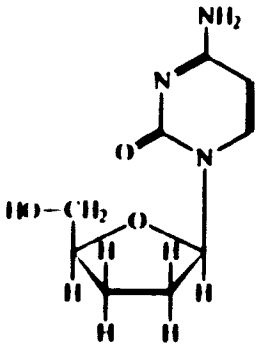


3) ddDAPR
2,6-diaminopurine 2',3'-dideoxyriboside

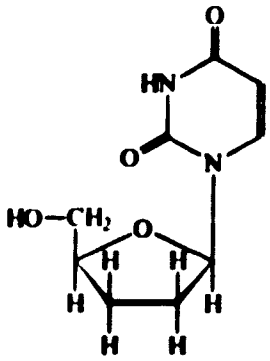


4) ddG
2',3'-dideoxyguanosine

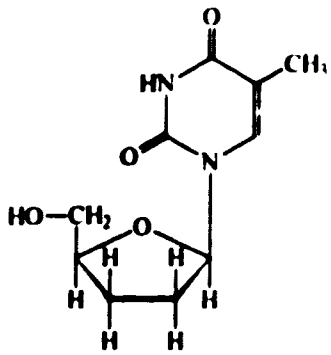
b) Pyrimidines



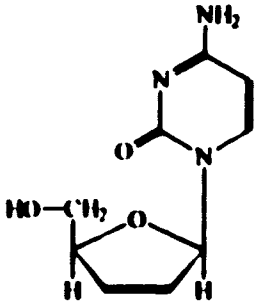
1) ddC
2',3'-dideoxycytidine



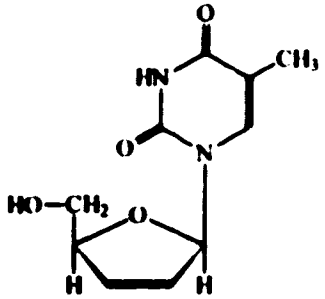
2) ddU
2',3'-dideoxyuridine



3) ddT
2',3'-dideoxythymidine

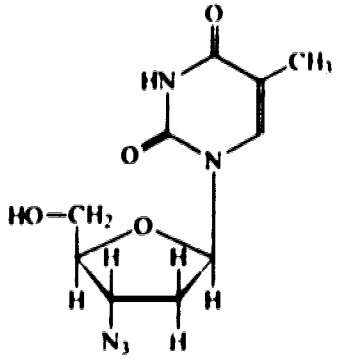


4) D4C
2',3'-didehydro-2',3'-dideoxycytidine

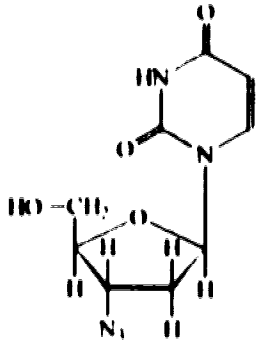


5) d4T
2',3'-didehydro-2',3'-dideoxythymidine

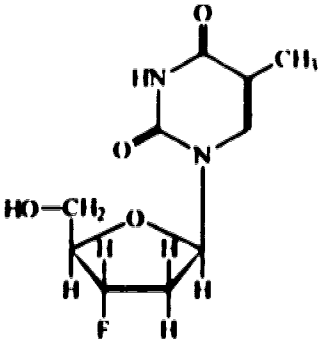
5. Substituted Dideoxynucleosides



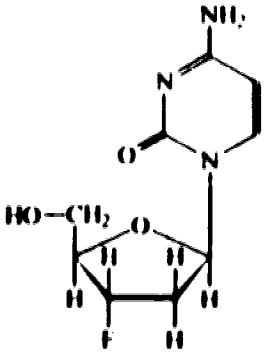
1) AZT
3'-azido-2',3'-dideoxythymidine



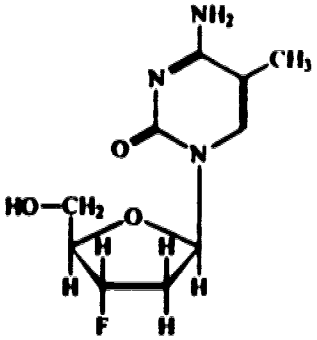
2) AZU
3'-azido-2',3'-dideoxyuridine



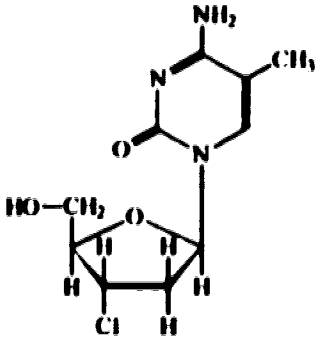
3) 3'-FddT
2',3'-dideoxy-3'-fluorothymidine



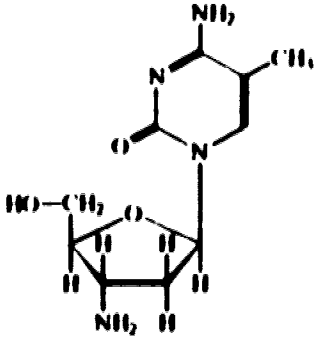
4) 3'-FddC
2',3'-dideoxy-3'-fluorocytidine



5) FddMeC
2',3'-dideoxy-3'-fluoro-
5-methylcytidine

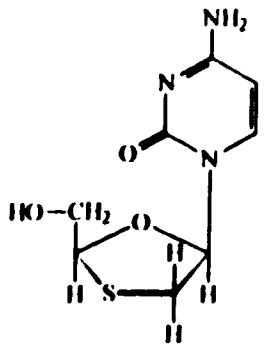


6) ClddMeC
3'-chloro-2',3'-dideoxy-
5-methylcytidine

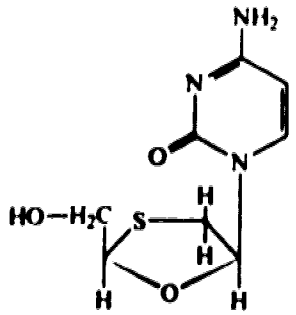


7) AddMeC
3'-amino-2',3'-dideoxy-
5-methylcytidine

6. Heterocyclic Dideoxynucleosides

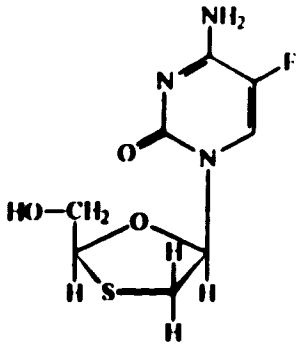


1) (+)BCH-189
2',3'-dideoxy-3'-thiacytidine

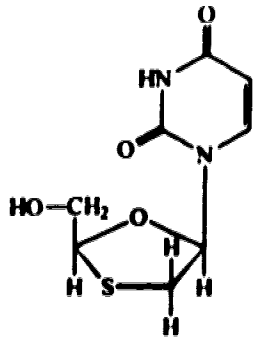


2) (-)BCH-189; 3TC
1-β-L-2',3'-dideoxy-3'-thiaribofuranosylcytosine

(NOTE: a racemic mixture of 1) and 2) is (±)SddC)

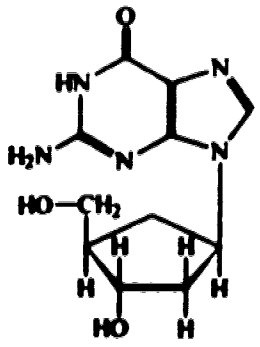


3) FTC
5-fluoro-2',3'-dideoxy-3'-thiacytidine



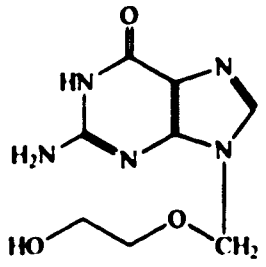
4) SddU
2',3'-dideoxy-3'-thiauridine

7. Carbocyclic Nucleosides

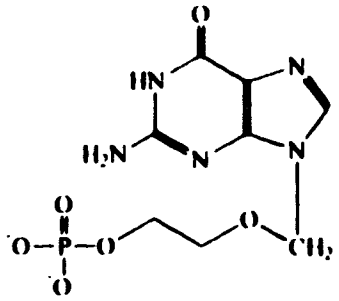


1) 2'-CDG
9-(1(R)-3(S)-hydroxy-4(S)-hydroxymethylcyclopentyl)-guanine

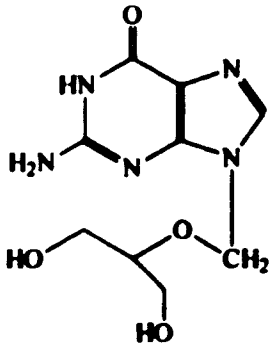
8. Acyclic Nucleosides
   a) Purines



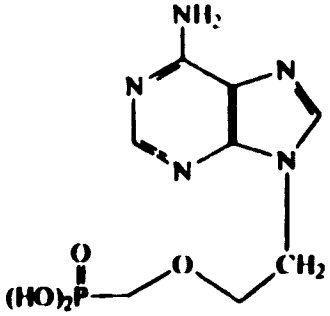
1) acyclovir
9-(2-hydroxyethoxymethyl)guanine



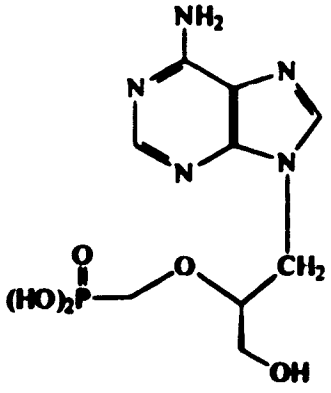
2) acyclovir monophosphate



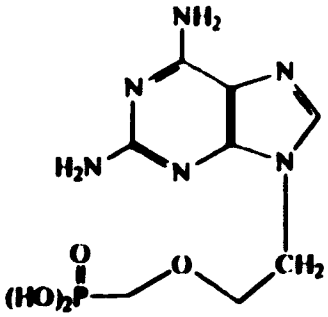
3) ganciclovir; DHPG
9-(1,3-dihydoxy-2-propoxymethyl)guanine



4) PMEA
9-((2-phosphonylmethoxy)ethyl)adenine

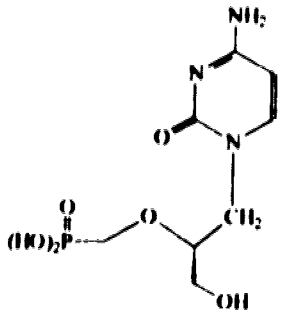


5) HPMPA
(S)-9-((3-hydroxy-2-phosphonylmethoxy)-propyl)adenine



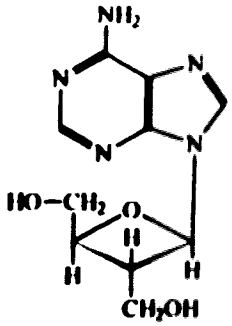
6) PMEDAP
9-((2-phosphonylmethoxy)ethyl)-2,6-diaminopurine

b) Pyrimidines

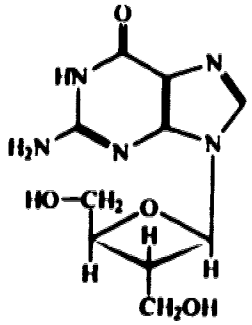


1) HPMPC
(S)-1-((3-hydroxy-2-phosphonylmethoxy)propyl)cytosine

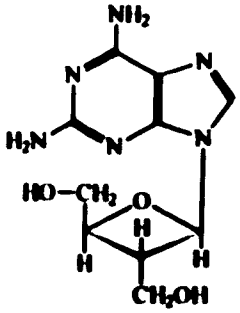
9. Oxetanosyl-N-glycosides
a) Purines



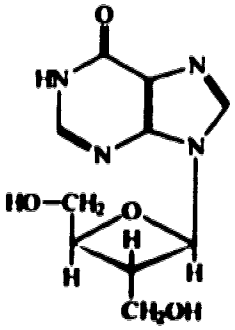
1) OXT-A; oxetanocin-A
9-(2-deoxy-2-hydroxymethyl-β-D-*erythro*-oxetanocyl)adenine



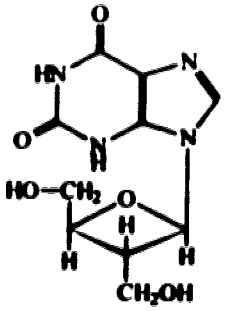
2) OXT-G
9-(2-deoxy-2-hydroxymethyl-β-D-*erythro*-oxetanocyl)guanine



3) 2-amino-OXT-A
9-(2-deoxy-2-hydroxy-methyl-β-D-*erythro*-oxetanocyl)2,6-diaminopurine



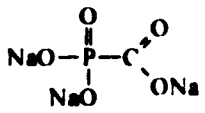
4) OXT-H
9-(2-deoxy-2-hydroxy-methyl-β-D-*erythro*-oxetanocyl)hypoxanthine



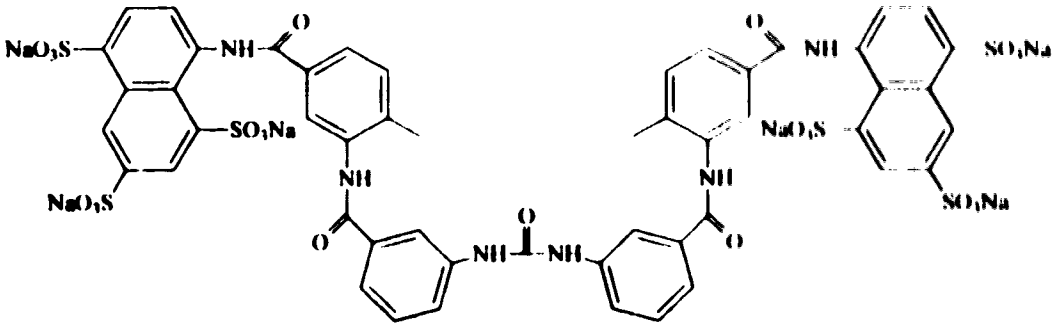
5) OXT-X
9-(2-deoxy-2-hydroxy-methyl-β-D-*erythro*-oxetanocyl)xanthine

10. Non-Nucleosides

a) Phosphonoformate; Foscarnet; PFA

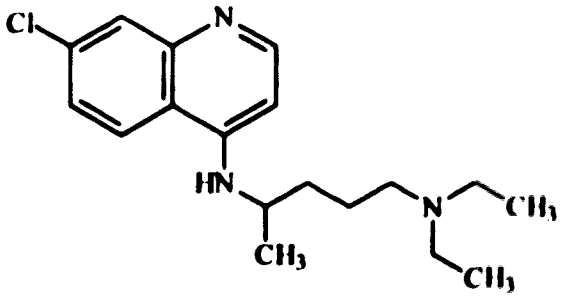


b) Suramin (hexasodium salt)



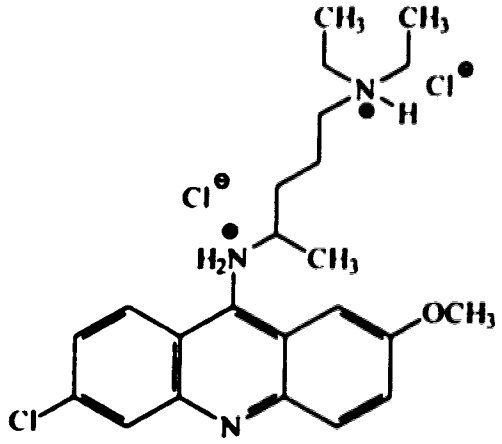
8,8'-{carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]}bis-1,3,5-naphthalenetrisulfonic acid

c) Chloroquine



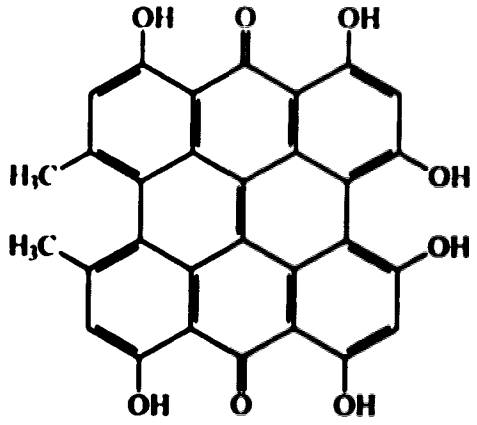
7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline

d) Quinacrine Hydrochloride.



6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-
2-methoxyacridine dihydrochloride

e) Hypericin



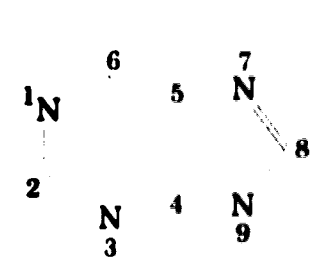
1,3,4,6,8,13-hexahydroxy-10,11-dimethylphenanthro-
[1,10,9,8-opqra]perylene-7,14-dione

Appendix II

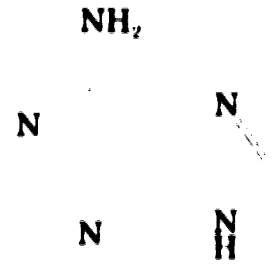
Physiological Purines and Pyrimidines: Chemical Structures and Numbering Systems

1. Bases

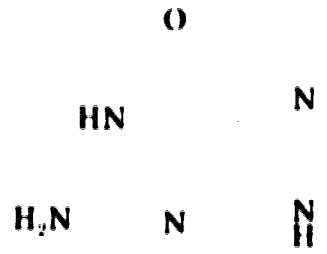
a) Purines



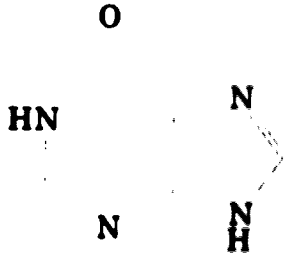
1) Numbering System



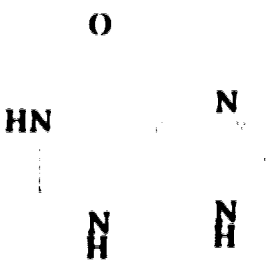
2) Adenine



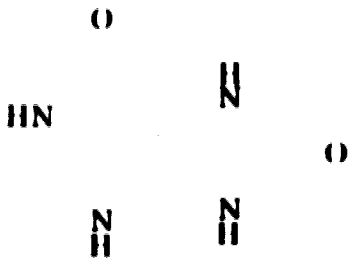
3) Guanine



4) Hypoxanthine

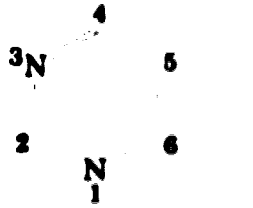


5) Xanthine

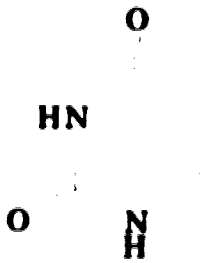


6) Uric Acid

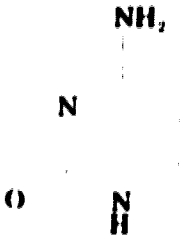
b) Pyrimidines



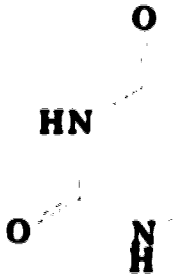
1) Numbering System



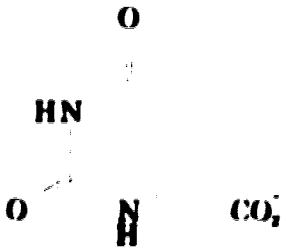
2) Uracil



3) Cytosine



4) Thymine

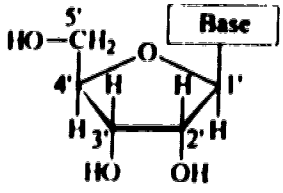


5) Orotate

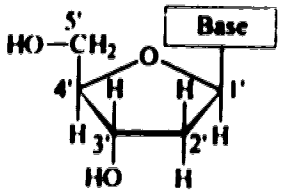
Appendix II, continued

2. Nucleosides

a) Numbering System

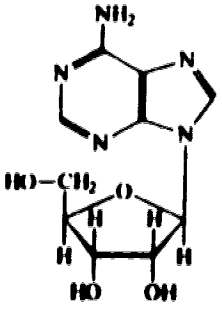


Ribonucleoside

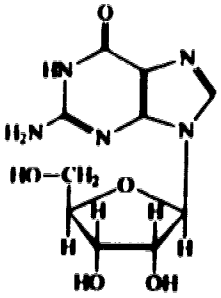


Deoxyribonucleoside

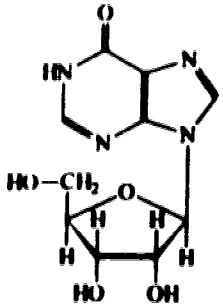
b) Purine Nucleosides



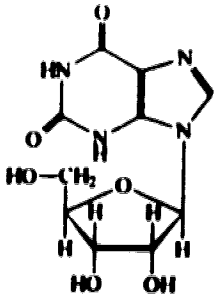
1) Adenosine



2) Guanosine

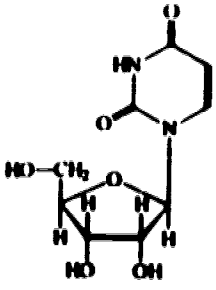


3) Inosine

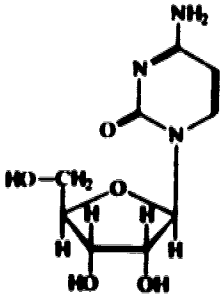


4) Xanthosine

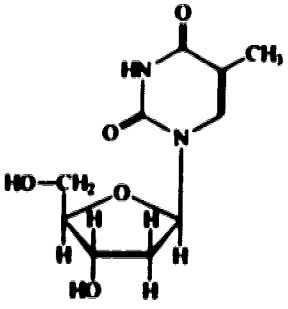
c) Pyrimidine Nucleosides



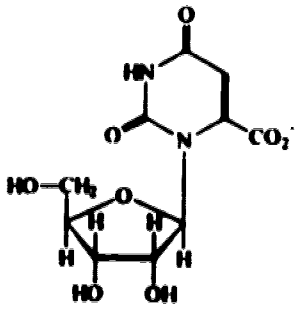
1) Uridine



2) Cytidine



3) Thymidine



4) Orotidine

APPENDIX III
Composition of Reagents Described in Materials and Methods

1. 0.5 M Alamine/freon
   3.1 ml Alamine 336
   9.4 ml Freon TF

2. Denhardt's reagent (50X stock)
   0.01 g/ml Ficoll (Type 400, Pharmacia)
   0.01 g/ml polyvinylpyrrolidone
   0.01 g/ml BSA (Pentex Fraction V)
   Filter and store small aliquots at -20°C

3. Elution buffer
   0.33 M LiCl
   0.167 N NaOH
   0.33M Hepes

4. 6X Gel-loading buffer
   0.25% bromophenol blue
   0.25% xylene cyanol FF
   30% glycerol
   Store at 4°C

5. Lysis solution, pH 7.5
   0.01 M Tris/HCl
   0.001 M EDTA
   0.05 M NaCl
   8% sucrose
   0.25% Nonidet P-40
   Store at 4°C

6. Neutralizing solution, pH 8.0
   1.0 M Tris/HCl (pH 8.0)
   1.5 M NaCl
   Sterilize by autoclaving; store at RT

7. Nick-translation buffer (10X)
   0.5 M Tris/HCl (pH 7.5)
   0.1 M $MgSO_4$
   0.001 M dithiothreitol
   500 µg/ml BSA (Fraction V; Sigma)
   Store in small aliquots at -20°C

8. PBS and dPBS (phosphate-buffered saline without and with divalent cations)
   0.137 M NaCl
   2.68 mM KCl
   10.6 mM Na_2HPO_4
   1.47 mM KH_2PO_4
   Adjust to pH 7.4 at RT
   For dPBS, the above recipe is supplemented with:
   0.492 mM $MgCl_2$
   0.901 mM $CaCl_2$

9. Prehybridization solution
   25% formamide
   5X SSPE
   5X Denhardt's reagent
   0.1% SDS
   100 µg/ml denatured, fragmented salmon sperm DNA
   This solution is prepared fresh

10. RIPA buffer
   0.15 M NaCl
   1% sodium deoxycholate
   1% Triton X 100
   0.1% SDS
   0.01 M Tris/HCl, pH 7.4
   Store at $4^{o}C$

11. Silicon 550/paraffin oil
   425 ml Silicon 550
   75 ml paraffin oil
   Specific gravity = 1.03 g/ml (measured with a Specific Gravity/Baume Hydrometer)

12. Sodium buffer, pH 7.4
   0.120 M NaCl
   0.020 M Tris
   0.003 M K_2HPO_4
   0.001 M $MgCl_2$
   1.80 mM $CaCl_2$
   to pH 7.4 with HCl

13. 20X SSC, pH 7.0
   3.0 M NaCl
   0.3 M sodium citrate
   to pH 7.0 with NaOH or citric acid
   Sterilize by autoclaving

14. SSE solvent
    4 parts ethyl acetate
    1 part isopropanol
    2 parts H_2O
    Combine by several cycles of vigorous shaking followed by settling
    Allow the layers to separate overnight
    Collect the top (organic) layer; store at RT

15. 20X SSPE, pH 7.4
    3.0 M NaCl
    0.20 M NaH_2PO_4
    0.02 M EDTA
    to pH 7.4 with NaOH
    Sterilize by autoclaving

16. 5X TBE buffer
    0.45 M Tris/borate
    0.01 M EDTA
    Store at RT; discard if a precipitate forms

17. TE buffer, pH 8.0
    0.01 M Tris/HCl, pH 8.0
    0.001M EDTA (pH 8.0)
    Sterilize by autoclaving; store at RT

18. TE-Sarkosyl buffer, pH 8.0
    0.01 M Tris/HCl (pH 8.0)
    0.01 M EDTA
    1% Sarkosyl (N-lauroyl sarcosine)
    Store at $4^{o}C$

19. Tris-saturated phenol
    liquified phenol
    0.1% 8-hydroxyquinoline
    Extract several times with an equal volume of 0.5 M Tris/HCl, pH 8.0
    Extract several times with an equal volume of TE buffer, pH 8.0
    Store under TE buffer at $4^{o}C$ in a brown bottle

20. 0.4% Trypan blue stock solution
    0.4g trypan blue
    0.81g NaCl
    0.06g KH_2PO_4
    95 ml H_2O
    Heat to boiling to dissolve, then cool
    Adjust to pH 7.2 - 7.3 with NaOH
    Adjust volume to 100 ml

21. 0.04% Trypan blue stain solution
    1 part 0.4% Trypan blue stock solution
    9 parts PBS

22. Tritosol
    52g PPO
    3.5g POPOP
    10 L xylene
    609 ml ethylene glycol
    2.44 L 98% ethanol
    4.35 L Triton X-100

23. Trypsin solution, pH 7.4-7.5
    8 g/l KCl
    0.4 g/l NaCl
    1 g/l $NaHCO_3$
    0.58 g/l dextrose
    0.5 g/l trypsin
    0.2 g/l disodium EDTA
    Adjust to pH 7.4 - 7.5
    Filter-sterilize; store at $4^{o}C$

END

28-08-96

FIN