INVESTIGATION OF WOODCHUCK HEPATITIS VIRUS-CELL INTERACTIONS

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by

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ABSTRACT

Hepatitis B virus (HBV) is a highly hepatotropic DNA virus, which causes several life threatening liver diseases. These diseases are a major public health problem of worldwide proportions. Initial binding of HBV to host cells is poorly understood. However, virus attachment is an event of utmost importance because it determines viral species specificity and cell tropism and, in consequence, viral pathogenicity. Among hepadnaviruses, the woodchuck hepatitis virus (WHV) displays the highest degree of molecular and biological similarity to HBV. Pending the availability of applicable cell cultures, a woodchuck model of hepatitis B offers a valuable *in vivo* system for the study of hepadnaviral attachment.

The aim of this thesis was to recognize the properties of WHV binding to the host cell surface. The studies revealed that the WHV envelope (WHsAg) binds to woodchuck hepatocyte plasma membranes (HPMs) with kinetics that suggest specific ligand-receptor interaction. Further, they also demonstrated that a 330-kD HPM molecule is involved in the virus attachment. Plasma membranes isolated from woodchuck splenocytes and kidney cells also bound WHsAg, however, to a lesser extent, when compared to HPMs. These non-hepatic membranes exhibited a similar 330-kD WHV binding molecule. Results obtained through virus and lectin affinity chromatography and enzymatic digestions revealed that the binding of the WHV envelope displayed by the 330-kD molecule is mediated both by N-linked polymannose and O-

linked heparan sulphate, but not by the protein core which links these carbohydrates. This suggests that the 330-kD receptor is a proteoglycan and that its virus binding site is constituted both by heparan sulphate and by polymannose. Preincubation of the WHV envelope with an exogenous glycosaminoglycan, heparin, inhibited the binding of WHV envelope to the 330-kD receptor, as well as to host intact HPMs. This suggests that the WHV attachment may be blocked by heparin-like substances *in vivo*.

The nature of the WHV envelope interaction with host cells was further investigated when a peptide homologous to the N-terminal sequence of the virus envelope preS1 protein, predicted to be exposed at the virion surface, was synthesized and used to test its specificity towards species, cells and subcellular organelles. The peptide demonstrated specific binding to an intracellular component exclusively expressed in woodchuck hepatocytes and lymphoid cells, suggesting that an intracellular receptor molecule(s) may play a role in determining virus tropism.

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AGU	=	Acetyl-β-D-glucosamine
AIDS	=	Acquired immune deficiency syndrome
anti-HBc	=	antibodies to hepatitis B virus core antigen
anti-HBe	=	antibodies to hepatitis B virus e antigen
anti-HBs	=	antibodies to hepatitis B virus surface antigen
anti-HBx	=	antibodies to hepatitis B virus x antigen
anti-WHs	=	antibodies to woodchuck hepatitis surface antigen
anti-WHc	=	antibodies to woodchuck hepatitis core antigen
Asn	=	Asparagine
Asp	=	Aspartic acid
cccDNA	=	Covalently, closed, circular DNA
cDNA	=	Complementary deoxyribonucleic acid
C-ORF	=	Open reading frame of HBV or WHV genome encoding nucleocapsid proteins
cpm	=	Count per minutes
Cys	=	Cysteine
DHBV	=	Duck hepatitis B virus
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid

DR1	=	Direct repeat 1
DR2	=	Direct repeat 2
EGF	=	Epidermal growth factor
ER	=	Endoplasmic reticulum
gpHSA	=	Glutaraldehyde polymerized human serum albumin
gpSA	=	Glutaraldehyde polymerized serum albumin
gpWSA	=	Glutaraldehyde polymerized woodchuck serum albumin
Gal	=	Galactose
Gal NAc	=	N-acetyl galactose
Glc A	=	D-glucoronic acid
Glc NAc	=	N-acetyl glucosamine
Gly	=	Glycine
GSHV	=	Ground squirrel hepatitis virus
HA	=	Haemagglutinin
HAV	=	Hepatitis A virus
HBcAg	=	Hepatitis B core antigen
HBeAg	=	Hepatitis B e antigen
HBsAg	=	Hepatitis B surface antigen
HBV	=	Hepatitis B virus
HCC	=	Hepatocellular carcinoma

HCV	=	Hepatitis C virus
HDV	=	Hepatitis D virus
HEV	=	Hepatitis E virus
HHBV	=	Heron hepatitis B virus
HIV-1	=	Human immunodeficiency virus type-1
HPLC	=	High pressure liquid chromatography
HPM	=	Hepatocyte plasma membranes
HRV	=	Human rhinovirus
HSA	=	Human serum albumin
ICAM-1	=	Intercellular adhesion molecule-1
Ido A	=	Idouronic acid
Ig	=	Immunoglobulin
IL-6	=	Interleukin 6
Ile	=	Isoleucine
JP1	=	Oligopeptide sequence encompassing the N-terminus of the WHV surface antigen
kbp	=	Kilo base pairs
kD	=	Kilo Daltons
KPM	=	Kidney plasma membranes
LFA-1	=	Lymphocyte function associated-antigen-1
Lys	=	Lysine

Met	=	Methionine
MMP	=	Methyl-α-D-mannopyranoside
mRNA	=	Messenger ribonucleic acid
NHS	=	Normal human serum
paS	=	Periodic acid Schiff
PBS	=	phosphate-buffered saline
Phe	=	Phenylalanine
pHSA	=	Polymerized-human serum albumin
P-ORF	=	Open reading frame of HBV or WHV genome encoding polymerase
Pro	=	Proline
RNA	=	Ribonucleic acid
SDS-PAGE	=	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH	=	Sulfhydryl
S-ORF	=	Open reading frame of HBV or WHV genome encoding envelope proteins (*e.g.*, preS1, preS2, S proteins)
SPM	=	Spleen or splenocyte plasma membranes
Thr	=	Threonine
TSH	=	Thyroid stimulating hormone
TX-100	=	Triton X-100
Val	=	Valine

VAP	=	Virus attachment protein
VOPBA	=	Virus overlay protein blot assay
WGA	=	Wheat germ agglutinin
WHsAg	=	Woodchuck hepatitis virus surface antigen
WHV	=	Woodchuck hepatitis virus
X-ORF	=	Open reading frame of HBV or WHV genome encoding X protein

CHAPTER 1

1. INTRODUCTION

1.1 Viruses and their Pathogenicity

Viruses are infective agents which depend on living host cells for their replication (Lycke and Norrby, 1976). The study of viruses began when Dmitrii Iwanowski reported his observation in 1892. He observed that the agent which caused the mosaic disease in tobacco plants passes freely through "bacterial filters". However, it was not until 1898 that Martinus Willem Beijerinck was able to clarify Iwanowski's finding. Beijerinck was confident that the filterable agent which caused the tobacco mosaic disease did not contain bacteria and referred to it as "contagious living fluid" or "virus" (Knight, 1974). Investigations over the past decades led to the discovery of numerous viruses which are pathogenic to humans, animals, insects, plants and bacteria (Murphy and Kingsbury, 1990). In the beginning, the classification of viruses was carried out by several independent groups of investigators. This approach, however, led to confusion because conflicting schemes of classification were used (Murphy and Kingsbury, 1990). In order to resolve this problem, the International Committee on Nomenclature of Viruses recommended that these agents become divided into families, genera and species (Murphy and Kinsbury, 1990). Today, viruses are classified according to the nucleic acid species and morphological structure. Thus, they can be referred to as DNA and RNA viruses depending on the type of nucleic acid constituting their genomes.

Furthermore, in regard to their morphological structure, some virus families consist of naked, whereas others consist of enveloped viruses. Naked viruses possess a protein coat, called capsid or nucleocapsid, which encompasses viral genomic material. In contrast, enveloped viruses possess an additional lipoprotein structure of cellular origin, termed envelope (Kucera and Myrivik, 1985; Rapp, 1983).

Important human infectious diseases such as rabies, small pox, poliomyelitis, haemorrhagic fever, encephalitis, acquired immune deficiency syndrome (AIDS), and inflammatory liver diseases (hepatitis) are caused by viruses (Joklik, 1988). Among viral agents which produce infectious hepatitis, five distinctive types of viruses have been identified to date. These are: hepatitis A virus (non-enveloped, 27-32 nm in diameter RNA virus belonging to picornavirus group; HAV) (Hollinger and Ticehurst, 1990); hepatitis B virus (enveloped, 42 nm DNA virus, a prototype of the hepadnavirus group; HBV) (Robinson, 1990); hepatitis C virus (enveloped, 30-60 nm RNA virus sharing many characteristics with the flaviviruses and togaviruses; HCV) (Hollinger, 1990a); hepatitis D virus (enveloped, 28-39 nm RNA virus, known to share similarities with viroid-like agents and adopting the HBV envelope lipoprotein as its own envelope; HDV) (Wang, *et al.*, 1986; Purcell and Gerin, 1990), and hepatitis E virus (32 nm viral particles carrying RNA detected in stools of patients with enterically transmitted non-A non-B hepatitis; HEV) (Asher *et al.*,1990). In addition, some other viruses, such as the cytomegalovirus, Epstein-Barr virus, yellow fever virus and Rift Valley fever virus, may occasionally cause hepatitis (Patrick and McGee, 1980; Murphy and Kingsbury, 1990).

Nevertheless, among primary hepatopathic viruses, HBV has the biggest epidemiological and clinical significance. Worldwide, HBV is the major cause of life threatening liver diseases, including fulminant hepatitis, chronic hepatitis, post-necrotic cirrhosis and hepatocellular carcinoma (HCC; Robinson, 1990).

Most of the known viruses display a restricted species range and predisposition to replicate within defined host cells. The life cycle of a virus, regardless of its morphological structure and nucleic acid type, can be divided into six distinct yet interdependent stages: attachment, penetration, uncoating, biosynthesis of structural constituents, assembly or maturation and release of virions (Kucera and Myrivik, 1985).

Attachment is the first event in the multiplication cycle of a virus. This step appears to be the most important in determining both host range and tissue tropism. It can be defined as the binding of a virus attachment protein (VAP) to receptor molecules present on the cell surface (Lonberg-Holm and Crowell, 1986). Viral attachment proteins are polypeptides which constitute a part of the envelope in enveloped viruses or an element of the capsid in naked viruses. On the other hand, virus receptors are structures comprising or associated with the plasma membranes of host cells (Lentz, 1990).

1.2 Structure of the Cell Membrane

Biological membranes are organized sheet-like structures consisting mainly of proteins and lipids. They also contain carbohydrates, which are covalently linked to

proteins (termed glycoproteins) and lipids (termed glycolipids) (Stryer, 1988). The accepted model for the overall organization of biological membranes was first proposed by Singer and Nicolson in 1972. This model, called the "fluid mosaic model" exhibits inner and outer surfaces, both structurally and functionally asymmetric. The model possesses three main features: (1) phospholipid (the most abundant lipid in the membranes) and glycolipid molecules are arranged in a bilayer so that only ionic and polar hydrophillic head groups of these lipids are in contact with water, (2) membrane proteins are allowed to diffuse laterally, but not transversely regardless of whether they span the lipid bilayer (integral membrane proteins) or they are loosely associated with the surface of the bilayer (peripheral membrane proteins), and (3) biologic activity of some membrane proteins depends on a proportion of membrane lipids which specifically interact with them (Stryer, 1988).

1.3 Properties of Cellular Receptors for Viruses

Receptors for viruses identified thus far have been biochemically characterized and classified as proteins, lipids or carbohydrates (Lentz, 1990). A number of proteins located on the surface of cells have been found to express binding properties for viruses. Examples of viruses which utilize proteins as cellular receptors are: reovirus type 1 (Maratos-Flier *et al.*, 1988), visna virus (Dalziel *et al.*, 1988), and Sindbis virus (Massen and Terhorst, 1981). Alternatively, viruses may utilize lipid molecules of plasma membranes as their receptors. The vesicular stomatitis virus is an example of such a

virus, because its binding to red blood cells was found to be mediated by plasma membrane phosphatidylserine, phosphatidylinositol and GM3 ganglioside (Mastromarino *et al.*, 1987). Nevertheless, for most viruses, carbohydrate residues associated with the plasma membrane proteins, called glycoproteins, have been identified as receptors. An example of such a carbohydrate residue is sialic acid, which is known to play a crucial role in the attachment of Sendai virus, encephalomyocarditis virus and influenza viruses to targeted cells (Lentz, 1990; March and Helenius, 1989). Another example of a glycoprotein serving as cellular receptor for a virus is the CD4 molecule, which was identified as the major receptor for human immunodeficiency virus type 1 (HIV-1) (Sattentau and Weiss, 1988). This molecule is a member of the immunoglobulin (Ig) superfamily and exists on the surface of thymus-derived lymphocytes and cells of monocyte/ macrophage lineage (Sattentau and Weiss, 1988). In the attachment of some other viruses to host cells, carbohydrates exhibiting an elaborate biochemical structure were found to play a role. For example, proteoglycans which contain high molecular weight carbohydrate moieties, such as heparan sulphate and heparin-like glycosamino-glycans, have been shown to mediate the attachment of herpes simplex virus and pseudorabies, respectively, to susceptible cells (WuDunn and Spear, 1989; Mettenleiter *et al.*, 1990).

Since receptors for viruses are natural constituents of the cell surface, it is possible that they also mediate important physiological functions, such as the binding of antigens, hormones or neurotransmitters (Lentz, 1990). Assigning these functions to

cellular components exhibiting receptor activity for viruses has proved to be a very difficult task. Nevertheless, to date it has been reported that Sindbis virus, rabies virus, reovirus and vaccinia virus recognize the catecholinergic (Tignor *et al.*, 1984), acetylcholine (Lentz *et al.*, 1982), β-adrenergic (Co *et al.*, 1985) and epidermal growth factor (Eppstein *et al.*, 1985) receptors, respectively.

1.4 Virus-Plasma Membrane Interactions

Interactions between virus and host plasma membrane can either be direct or indirect (*i.e.*, mediated by an intermediate molecule). Both direct and indirect interactions can be specific or nonspecific (Lonberg-Holm and Crowell, 1986; Lentz, 1990). Specific virus bindings are usually saturable, dependent on time, temperature, pH, ions and their concentration. In addition, these bindings can be inhibited by an excess of unlabelled virus and by antibodies directed towards viral receptors or viral attachment proteins. However, it should be pointed out that cells which bear virus specific receptors, may not necessarily become infected by a particular virus, despite its specific attachment. For example, when CD4-negative mouse cells were transfected with a cDNA clone encoding the HIV-1 receptor (*i.e.*, CD4 molecule), the virus bound to the transfected cells, but it was still unable to infect them (Maddon *et al.*, 1986). Reasons which may explain why these cells are not susceptible to HIV-1 infection could lie in the fact that perhaps another cell-type specific molecules (*e.g.*, penetration factors), in addition to the CD4 molecule, are required for the virus attachment.

It has also been found that different receptor systems can be involved in the specific binding of a virus to different cell types of the same host. For example, HIV infects cells of the central nervous system *via* a CD4 independent mechanism. Moreover, some cell lines originating from human rhabdomyosarcoma, osteosarcoma, bowel and foreskin fibroblasts also appear to be susceptible to HIV-1 infection but they do not express CD4 molecules (Clapham *et al.*, 1989; Castro *et al.*, 1989; Tateno *et al.*, 1989).

There are also examples of viruses which recognize specific receptors on the surface of a variety of host cells, but only some of these cells can be infected by the virus. For instance, human rhinovirus (HRV) specifically recognizes a molecule of an apparent molecular weight of 90 to 95 kD that possesses 5 extracellular Ig-like domains, named intercellular adhesion molecule-1 (ICAM-1). This molecule is encoded by a gene present on human chromosome 19 and is well known for its widespread distribution on the surface of cells derived from skin, kidneys, liver, thymus, tonsil, lymph nodes and intestine (Dustin *et al.*, 1986). Like the CD4 molecule, ICAM-1 is a member of the immunoglobulin super-family (Dustin *et al.*, 1986; Dustin and Springer, 1988). This molecule is also a ligand for the lymphocyte function associated-antigen-1 (LFA-1) (Elliott *et al.*, 1990) and it is thought that its interaction with LFA-1 plays a role in the adhesion of leukocytes to T cells. It is, necessary to emphasize, that despite the fact that ICAM-1 exists on a variety of cells as mentioned above, only non-ciliated cells of the adenoid and ciliated epithelial cells in nasal polyps support HRV replication (Arruda *et*

al., 1991).

Poliovirus, another member of the picornavirus family, recognizes a cellular receptor which also maps to chromosome 19 (Shepley *et al.*, 1988). In this case, however, the receptor has been identified as a 100-kD integral membrane protein consisting of 3 extracellular immunoglobulin (Ig)-like domains. Although, it has been previously reported that the poliovirus receptor is only detectable on cells that support virus replication, (*i.e.*, oropharyngeal and intestinal epithelium, Peyer's patches of the ileum, and motor neurons within the central nervous system) (Holland, 1961; Shepley *et al.*, 1988), in a recent study, a 3.3-kbp receptor transcript was detected not only in those tissues, but also in kidney, a tissue which does not express any poliovirus binding activity or ability to support the virus replication (Mendelsohn *et al.*, 1989). This finding may suggest that detection of the mRNA for the virus receptor does not mean that a functional receptor is indeed expressed on the cell surface. Conversely, detection of binding sites for virus on these cells does not necessarily mean that the cells are susceptible to infection. This last prediction is supported by the observation that L cells, non-primate cells originating from mouse connective tissue, exhibit binding sites for poliovirus, yet they are not susceptible to virus infection (Barnert *et al.*, 1992).

Virus specific attachment need not necessarily occur through its direct binding to cellular receptors. Some viruses may bind to intermediate molecules, which in turn bind to cell surfaces endowed in specific receptors for these molecules. For example, it has been observed that the infectivity of dengue virus (Halstead and O'Rourke, 1977;

Halstead, 1988) and West Nile virus (Peiris *et al.*, 1981) can be enhanced by coating virions with the specific anti-virus antibodies. In this case, the Fc portion of the Ig molecule interacts with the cell surface receptors for Fc facilitating the uptake of the virus-antibody complex and, in turn, infection of the cells (Lentz, 1990). In another example, the formation of a complex between the West Nile virus and the anti-virus antibodies of the IgM class is followed by the interaction of the complexes with the C3b component of complement present on macrophages. This binding enhances viral infectivity (Cardosa *et al.*, 1986). Similarly, it has been observed that anti-virus antibodies isolated either from infected individuals or from animals inoculated with virus are able to enhance binding of HIV to cells, which bear the Fc receptor. In addition, the attachment of HBV and cytomegalovirus to susceptible human cells has been found to be mediated by intermediate molecules such as polymerized human serum albumin (pHSA; Machida *et al.*, 1984) and β-2-microglobulin (Grundy *et al.*, 1987), respectively. Finally, viruses already attached to their host receptor may act as a bridge for the entry of other viruses. In this regard, it has been demonstrated that infected cells which bear the influenza virus HA molecule(s) not only facilitate the entry of the influenza virus, but also the entry of vesicular stomatitis and Semliki Forest viruses (Fuller *et al.*, 1985).

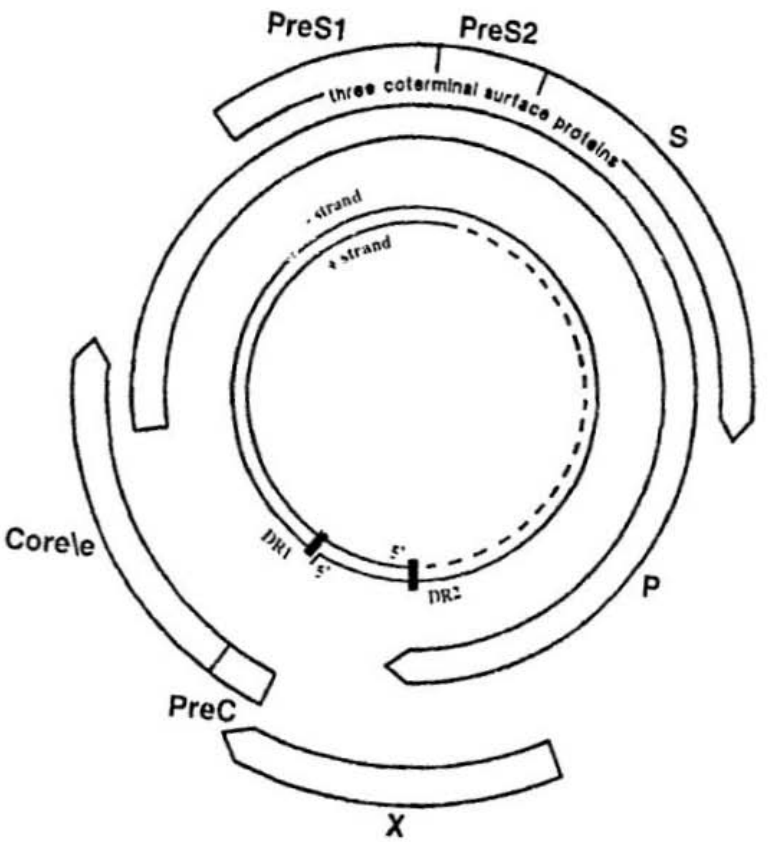
Unlike specific virus interactions, nonspecific virus binding to cells usually results in a non-productive infection. This type of interaction is characterized by a lack of saturability in the presence of excess virus. Nevertheless, it has been reported that some viruses may infect cells as a result of such binding. In this regard, it has been shown

that the non-saturable binding of vesicular stomatitis virus to baby hamster kidney cells leads to productive infection (Lenard, 1986).

1.5 Structure, Genomic Organization and Replication Cycle of Hepatitis B Virus

Hepatitis B virus (HBV) is a prototype of the hepadnavirus family and its structure and molecular organization have been extensively studied. Despite this progress, the nature of initial interactions between the virus and host cells is not yet established. HBV also known as Dane particle (Dane *et al.*, 1970), is generally characterized as a complex DNA virus. The virion has a diameter of approximately 42 nm and consists of lipoprotein envelope about 7 nm thick, which in turn surrounds an electron dense icosahedral nucleocapsid, possessing a diameter of 27 nm (Christie, 1987; Gust *et al.*, 1986). The genomic organization of the HBV has been elucidated as a result of DNA cloning and nucleotide sequencing (Figure 1). As a result of these studies, the DNA of the HBV has been found to be partially double-stranded and consisting of a long, negative strand of approximately 3.2-kbp and a short, positive strand ranging from 1.7 to 2.8-kbp. Furthermore, as a result of a nick on the DNA minus strand, which is located approximately 225 base pairs from the 5'-end of the positive strand, the negative strand is not a closed circle (Robinson, 1990). The circular structure of the HBV DNA is maintained through the cohesive overlap of both strands, which is achieved by positioning the 5'-end of the plus strand 200-300 nucleotides downstream from the 5'-end

Figure 1. Structure and genomic organization of hepatitis B virus. The structural and functional features of the virus genome, include minus(-) and plus(+) strands of DNA, direct repeat (DR) sequences (filled boxes), and the single stranded gap filled by DNA polymerase (dashed line). Viral open reading frames, indicated with arrowheads, reveal the location, direction of transcription and products of translation.


PreS1
PreS2
three coterminal surface proteins
S
- strand
+ strand
Corele
DR1
5'
5'
DR2
P
PreC
X

of the minus strand. Within the cohesive overlap region, there are two 11 base pair direct repeats denoted DR1 and DR2 which are found to be complementary to the respective 3-' and 5-'ends of the adjacent minus strand. These direct repeats, identified as conserved cis-acting elements, represent not only the primary sites for replication of both DNA strands, but also they serve as sites of HBV DNA integration into host genome (Blum *et al.*, 1989). Features which are also characteristic of the HBV genome organization include: (a) the presence of DNA polymerase (DNA-dependent RNA reverse transcriptase) which fills the gap between the 3-' and 5'-ends of the DNA plus strand and is essential for viral gene replication; (b) a protein covalently linked to the 5'-end of the minus strand that probably serves as its transcriptional primer, and (c) an oligoribonucleotide primer covalently attached to the 5'-end of the plus strand whose major role is to initiate the synthesis of the strand (Blum *et al.*, 1989).

Analysis of HBV DNA genome revealed that there are at least four open reading frames (ORF), named S,C,P, and X. All of the ORFs are located on the DNA minus strand. A unique feature of the HBV genome, as well as genomes of other hepadnaviruses, is that the ORFs are overlapped. Therefore, the virus produces substantially more protein per genome unit, when compared to other viruses, and as such is recognized as an unprecedented example of viral genome economy.

The S-ORF includes the preS1, preS2 and S regions, which are delineated by three in frame ATG codons. Translation products of these regions renders proteins of the virion envelope referred to as major (small) or S protein, middle or preS2 protein,

and large or preS1 protein (Robinson, 1990). Characteristically, all of them share the same carboxy-terminus, but differ in their respective amino-terminal sequence. The major protein is 226 amino acids long and it exists both in the glycosylated form, where a complex N-linked glycan is bound to asparagine 146 (GP27), and in the nonglycosylated form (P24). The middle protein is 281 residues long and occurs in two glycosylation forms, as GP33 and GP36, depending on the presence of 1 or 2 glycans. Also, the large protein, either exists in a form which lacks glycosylation (P39) or in a glycosylated form (GP42). Finally, unlike the major and middle proteins, the length of the large protein varies according to HBV subtype. For example, the length of subtype ay is restricted to 389 amino acids, whereas the length of subtype ad is increased by 11 amino acids for a total of 400 amino acids (Tiollais *et al.*, 1985; Blum *et al.*, 1989). These small, medium and large envelope proteins collectively constitute the virus envelope and carry immunological specificity defined as the hepatitis B virus surface antigen (HBsAg). HBsAg is usually produced in a large excess by the virus infected hepatocytes from which it is secreted into the serum, where it exists in the free form as spherical or filamentous particles. These particles have an average diameter of about 22 nm. The length of the filamentous particles could be up to 230 nm (Ginsberg, 1988). Interestingly, the antigen may reach concentrations of 10^{12} to 10^{14} particles per ml of serum (McCollum and Zuckermann, 1981).

The HBsAg contains a number of antigenic determinants. A major group determinant denoted as "a" is shared by all subtypes of HBV. In contrast, two pairs of

subgroup determinants "d or y" and "w or r" are found to be mutually exclusive. In addition, antigenic heterogeneity of the w, q, x and g determinants has also been identified (Robinson, 1990). All of these subtype specific domains are encoded by the preS1 region of the HBV S-ORF. Nevertheless, five determinants, which produce the three major subtypes designated as adw, adr, ayw, are the most frequently observed. Interestingly, each of these subtypes has a tendency to occur in a particular geographical distribution (Blum *et al.*, 1989).

The second ORF, designated as the C-ORF or C gene, contains two in phase start codons, that define the preC and C regions, which in turn code for two overlapping nucleotide polypeptides (Shafritz, 1987). Core protein, also called hepatitis B core antigen (HBcAg), is the shorter of the two peptides. This peptide has a molecular weight of 22 kD (P22) and its multiple repeats form the nucleocapsid of the virus. The core protein can be detected as a structural constituent both in virions and in naked nucleocapsid particles derived from HBV-infected hepatocytes (Robinson, 1990; Blum *et al.*, 1989). It has been found that this protein binds the viral DNA via the 34 amino acids located at its carboxyl-terminus during the assembly of virions. On the other hand, the product encoded by the whole C-ORF, designated as precore protein, displays e antigen specificity, which is referred to as hepatitis B e antigen (HBeAg). The variable size of this protein (*i.e.*, from 16 to 25 kD) results from the posttranslational clipping and modifications (Uy *et al.*, 1986; Robinson, 1990). Due to the cleavage of amino acids encoded by the preC region, as well as the 34 residues encoded by the 3'-end of

the C region (Robinson, 1990), serum-HBeAg displays a molecular weight of 16 kD (P16). It is thought that the formation of the P16 results from the cleavage of the amino-end of the phosphorylated P22 core protein by a signal peptidase and the cleavage of the carboxy-end of the core protein by a trypsin-like protease (Uy *et al.*, 1986; Robinson, 1990). It has been shown that separate messenger RNAs for e and core proteins do not exist and that the amino acid sequences of these proteins are largely identical (Schlicht *et al.*, 1987; Garcia *et al.*, 1988). Despite the above, several important properties distinguish e protein from the core protein. Thus, e protein (HBeAg) is immunologically distinct from the core protein (HBcAg) at the level of B cell response, leading to production of specific anti-HBe and anti-HBc antibodies (Salfeld *et al.*, 1989). Further, HBeAg can be detected as a nonstructural protein circulating in the blood or complexed with anti-HBe, whereas free core particles, as mentioned above, apparently occur only within infected hepatocytes or virions. Finally, while the core protein plays an important role in the replication and infectivity of the virus, e protein is apparently not required for virion replication.

The P-ORF is the largest among the ORFs and covers three-fourths of the HBV genome. It overlaps the C terminus of the C-ORF gene, the entire S-ORF and the N-terminus of the X-ORF (Robinson, 1990). The translation product derived from this gene is a basic polypeptide whose molecular weight approximates 90 kD. Synthesis of this protein is crucial to the successful replication of the HBV genome. It has been demonstrated that the P-ORF product not only carries the virion-associated DNA

polymerase activity, but also the protein primer responsible for initiating synthesis of the DNA negative-strand (Robinson, 1990; Blum *et al.*, 1989).

Finally, the smallest ORF designated as the X-ORF, encodes a transcriptional transactivating protein consisting of 154 amino acids. This protein is a non-specific activator of several viral (Twu and Robinson, 1989), as well as, cellular genes (Twu and Schloemer, 1987). Furthermore, recent evidence suggests that the X gene product could be a novel protein kinase (Wu *et al.*, 1990). It has been proposed that upregulated expression of the X gene product may be involved in the pathogenesis of HCC. Anti-HBx antibodies can be detected in the sera of HBV-infected patients, particularly in individuals with the virus-induced HCC (Pfaff *et al.*, 1987).

Replication of the HBV can be divided into several steps: (1) conversion of the asymmetric circular DNA to covalently closed circular DNA (ccc DNA) within the nucleus due to the action of a DNA polymerase and a ligase; (2) transcription of the circular DNA to RNA template (pregenome); (3) transportation of the pregenomes to cytoplasm and their translation into various viral proteins; (4) the encapsidation of the pre genomes into immature core particles; (5) synthesis of the minus strand of DNA from the pregenomic RNA by reverse transcription; (6) synthesis of the plus strand of DNA from the first DNA strand; (7) formation of relaxed circular double-stranded DNA within core particles; (8) packing of the cores into viral envelope proteins, and (9) secretion from the cells.

Overall, the HBV replication mechanism is fairly well understood. However, it

can not be overemphasized that much less is known about the initial stages of the virus life cycle, particularly, about the virus attachment to host cells and the mode of cellular entry.

1.6 Biology of Hepatitis B Virus Infection

When non-immune individuals are exposed to HBV, the infection may result in the development of clinically, serologically and biochemically evident or silent liver pathology. It is observed that primary infection with the virus (*i.e.*, acute or subclinical hepatitis B) in 90% of adult cases is self-limited and leads to the development of virus neutralizing antibodies directed to HBsAg (anti-HBs; Krugman *et al.*, 1979). It is thought that such cases become permanently immune to HBV re-infection. However, in the remaining 10% of hepatitis cases the replication of HBV is not terminated and the development of chronic hepatitis B is observed. It is generally accepted that the diagnosis of chronic HBV infection is valid when serologic markers of the virus infection persist for more than six months.

In chronic hepatitis, several immunomorphologically distinct forms of the disease can be recognized. The most common forms are: chronic active or aggressive hepatitis, chronic persistent hepatitis, and chronic HBsAg carrier state. Chronic active and persistent hepatitis are the result of permissive HBV infection (Lieberman and Shafritz, 1986). In chronic active hepatitis, the liver usually displays a picture of severe necro-inflammation and hepatocytes show evidence of active virus replication. Thus, HBsAg,

HBcAg and HBeAg can be detected by immunohistochemical staining of individual cells randomly distributed throughout the liver parenchyma. Also, free virions and low molecular weight replication forms of HBV DNA are present in the infected hepatocytes (Lieberman and Shafritz, 1986; Shafritz, 1987). On the other hand, HBsAg, HBeAg, virus specific DNA polymerase, HBV DNA and virions are usually detectable in the serum. Patients in this stage of the disease are considered to be highly infectious (Shafritz, 1987; Grist *et al.*, 1987). In chronic persistent hepatitis, all the above markers of HBV infection and liver injury may also occur, but they are usually transient. The overall picture of this disease is one which is mild and self-limited in most cases.

The majority of clinically unsymptomatic HBV chronic carriers are the result of non-permissive infections (Maynard *et al.*, 1988). In fact, many of these carriers are referred to as "healthy carriers" because they do not express clinical and biochemical evidence of liver disease (Iwarson, 1985). In these cases, HBsAg, HBV DNA and anti-HBe are usually detectable in the serum, but HBeAg is absent (Lieberman and Shafritz, 1986; Iwarson, 1985). Regarding the liver morphology, one may not observe any histological evidence of hepatocyte injury (Iwarson, 1985) or minimal inflammatory reaction even though production of HBsAg is continued and integration of HBV DNA into the liver genome is detectable (Shafritz, 1987).

The relationship between hepatitis B virus carrier state and the development of HCC has been well established. For instance, epidemiological studies which examined the geographic distribution of HCC demonstrated that the sub-Saharan Africa and eastern

Asia are the areas of the world, where both chronic HBV infection and HCC occur at the highest frequencies (Robinson, 1990). In a prospective study where 22,707 Taiwanese male government workers were tested for the presence of HBsAg, the calculated risk of the development of HCC in chronic HBsAg carriers was found to be over 200 times higher than that in healthy individuals (Beasly *et al.*, 1981). Furthermore, among HBsAg carriers, over 50% of deaths are caused by liver cirrhosis or HCC compared to only 2% among the control population (Tiollais *et al.*, 1985). The molecular mechanisms of the HBV-induced HCC are not known. However, two features are common to all cases: chronic HBV infection and the random integration of HBV DNA into genome of the host hepatocytes.

Although HBV is considered to be a hepatotropic virus, its replication has also been accounted for in cells of the lymphatic system (Lieberman *et al.*, 1987; Yoffe *et al.*, 1986; Pontisso *et al.*, 1984). It is also well known that HBV infection may lead to the development of life-threatening extrahepatic diseases namely, serum sickness-like syndrome, polyarteritis nodosa (Grocke, 1975; Michalak, 1978) and glomerulonephritis (Combes *et al.*, 1971; Brzosko *et al.*, 1974). These diseases result from the deposits of virus antigen-antibody complexes in the walls of blood vessels.

1.7 The Hepadnavirus Group

HBV is the prototype for the hepadnavirus family which consists of several related viruses that share similar genomic organization, virion morphology, and prominent

hepatotropism (Melnick, 1982; Feitelson and London, 1990). Among the animal viruses related to HBV, woodchuck hepatitis virus (WHV) was the first to be discovered in a *Marmota monax* colony established in the Philadelphia Zoologic Garden (Summers *et al.*, 1978). Then, other hepadnaviruses were found in the following species: ground squirrel hepatitis virus (GSHV) in *Spermophilus beecheyi* (Marion *et al.*, 1980); duck hepatitis B virus (DHBV) in domestic Pekin ducks (Summers *et al.*, unpublished data) and in *Anas domesticus* (Mason *et al.*, 1980), tree squirrels hepatitis virus (TSHV) in *Scurus carolinensis pennsylvanicus* (Feitelson *et al.*, 1986), heron hepatitis B virus (HHBV) in *Ardea cinerea* (Sprengel *et al.*, 1988), and chipmunk hepatitis virus in *Tamias sibiricus asiaticus* (Yoo and Park 1988; Yoo and Kim 1987). Nevertheless, of all the HBV related animal viruses WHV displays the closest genetic, immunological and pathological similarities to HBV, when judged by nucleic acid sequence homology (*i.e.*, approximately 70%), viral antigen cross-reactivity, and the immunovirological and morphological profiles of induced liver diseases. Similar to HBV infection in humans, an episode of acute WHV hepatitis in woodchucks may progress to persistent infection and the development of HCC. Interestingly, it has been shown that approximately 70% of woodchucks inoculated with WHV shortly after birth develop the chronic virus carrier state leading to hepatoma (Popper *et al.*, 1987). This situation mimics the progression of chronic HBV infection to HCC in individuals who acquired the infection in the neonatal period from mothers chronically infected with the virus (Popper *et al.*, 1987).

1.8 Interactions between Hepatitis B Virus and the Cell Surface

The binding of HBV to host hepatocytes is thought to take place as a result of the interaction between the domains of the virus envelope (virus attachment proteins) and the binding sites (receptors) present on the cell surface.

Research on the nature of HBV attachment to hepatocytes was initiated by studies where the interaction between gpHSA and isolated hepatocytes was tested. Imai and colleagues (1979) were the first to demonstrate that HBsAg particles specifically bound human or chimpanzee albumin artificially cross-linked with glutaraldehyde, but not similarly treated albumins purified from other species. On this basis, it was hypothesized that the attachment of HBV to hepatocytes could be mediated by a naturally occurring analogue of polymerized host albumin. This hypothesis gained significant support when a receptor for gpHSA was identified in the preS2 region of the HBV envelope (Machida *et al.*, 1984).

To add support to this concept, other investigators have demonstrated that the binding of gpHSA to isolated hepatocytes (Lenkei *et al.*, 1977; Trevisan *et al.*, 1982; Wright *et al.*, 1987) and purified liver plasma membranes (Michalak, 1986) is consistent with specific receptor-ligand interactions. Further, it has also been shown that the interaction of glutaraldehyde-polymerized woodchuck serum albumin (gpWSA) and highly purified woodchuck hepatocyte plasma membranes (HPMs) was ligand specific, rapid and expressed saturable kinetics (Michalak and Bolger, 1989). In addition, the binding was found to be sensitive to temperature, pH and ionic strength. Examination

of the binding kinetics exhibited by HPM for gpWSA revealed the presence of two distinct classes of membrane-associated binding sites. One class includes sites which mediate high affinity, low capacity binding for gpWSA, whereas the second one consists of sites which display low affinity and high capacity for the ligand. Furthermore, solubilization of HPMs with the non-ionic detergent Triton X-100, as well as enzymatic digestions revealed that high affinity, low capacity binding sites are integral HPM proteins containing a carbohydrate moiety. In contrast, the binding expressed by low affinity, high capacity gpSA binding sites is dependent on membrane lipids (Michalak and Bolger, 1989). In addition, it has been shown that HepG2 cells also exhibit two classes of binding sites for gpHSA (Dash *et al.*, 1991). Moreover, in another relevant study, evidence has been obtained indicating that human liver plasma membranes also display high affinity for gpHSA (Pontisso *et al.*, 1989a,b, 1990).

It is necessary to emphasize that, in theory, the functional significance of the interaction between the envelope middle preS2 protein of HBV and gpHSA lies not in the binding of gpHSA *per se*, but in the binding of a naturally occurring molecule behaving as gpHSA. The nature of such molecule(s) is not yet well recognized. Nevertheless, it has been demonstrated that the binding of specific monoclonal antibody (Q 19/10) to the peptide encoded by the preS2 region of HBV genome can be inhibited by a constituent present in normal human serum (NHS). Since the size of this serum constituent(s) ranges from 40 kD to 100 kD, it has been expected that this inhibitor might be a naturally modified form of human albumin (Heermannn *et al.*, 1988).

Regarding the possible participation of a monomeric form of HSA in HBV attachment, published studies have shown somewhat conflicting results. For example, experiments performed by Yu and colleagues (1985) and Pontisso and co-workers (1989a), indicated that monomeric HSA does not play a role in the binding of HBV to hepatocytes. In contrast, more recent studies presented by Krone and colleagues (1990) have shown that serum-derived HBsAg particles and recombinant HBsAg spheres containing middle envelope protein exhibit the ability to bind both monomeric HSA as well as gpHSA. Moreover, the binding of HSA or gpHSA to HBsAg was drastically reduced when the preS2 sequence was removed from the serum-derived HBsAg particles by trypsin digestion or when recombinant HBsAg spheres containing preS2-encoded epitope were pre-incubated with normal human serum. These results suggest that native HSA is able to compete for gpHSA sites carried by the preS2-encoded sequence. Furthermore, when the polypeptide composition of serum-derived HBsAg was analyzed, monomeric HSA was found to be associated with the antigen particles (Krone *et al.*, 1990). These results ascertained by Krone and colleagues (1990), are in agreement with the previous findings made by Michalak and co-workers (1980), which demonstrated that the incorporation of HSA into HBsAg occurs intracellularly and most likely within the endoplasmic reticulum of HBV-infected hepatocytes. In addition, it was also shown that when intracytoplasmic deposits of HBsAg were treated with 2-mercaptoethanol, their ability to bind specific anti-HSA antibodies significantly decreased, whereas their potential to bind exogenous gpHSA evidently increased. These data suggested that

disulphide bonds play a role in the incorporation of albumin into HBsAg structure and that this association already occurs at the intracellular level (Michalak *et al.*, 1980). Similarly, it has been found that anti-HSA antibodies bind to serum-derived HBsAg particles. However, such binding is very low when the disulphide bonds of HBsAg are broken through treatment with 2-mercaptoethanol, (Krone *et al.*, 1990). Thus it can be concluded that HBsAg in the circulation possesses not only covalently bound HSA, but also that a portion of HSA is reversibly associated with the HBV envelope (Krone *et al.*, 1990). Reversibility of HSA binding to HBV was also observed by Lu and coworkers (1988), who demonstrated that a prolonged storage of virions results in the dissociation of HSA from HBV particles.

Interestingly, when the middle and the large proteins of the HBV envelope were separated by gel electrophoresis, electroblotted onto nitrocellulose and incubated with native HSA or different forms of gpHSA and then identified with labelled anti-HSA antibodies, it was found that naturally occurring HSA did not recognize the viral proteins, whereas all tested forms of gpHSA were able to bind to both envelope middle and large proteins. Thus, it appears that the observed binding of glutaraldehyde modified albumin to HBsAg could be due to a glutaraldehyde-induced change in the HSA conformation or charge (Krone *et al.*, 1990). Consequently, these results prompted other researchers to search for alternate methods for HSA modification, which would produce albumin molecules capable of binding HBsAg *in vivo*. Among others Thung and co-workers (1989) demonstrated that a polymer obtained through HSA cross-linkage with

guinea pig liver transglutaminase (pHSA-T) binds serum-derived HBsAg. This binding can be blocked by monoclonal antibodies to the envelope middle protein as well as by polyclonal anti-HBs antibodies, but not by antibodies that react with the large envelope protein sequence encoded by the preS1 region of the HBV S-ORF. Overall, the collected evidence clearly demonstrates that the preS2-encoded sequence of the HBV envelope is involved in the recognition of the host hepatocytes *via* a modified HSA as an intermediate molecule. They also suggest that specific receptors, which mediate the binding of serum albumin modified by glutaraldehyde treatment are present on the hepatocyte surface.

Epitopes other than those located within the preS2-encoded sequence of the HBV envelope also seem to be involved in the attachment of the virus to host hepatocytes. Neurath and colleagues (1985) have found specific binding of HBsAg to HepG2 cells. The inhibitory effect of overlapping synthetic peptides homologous to the preS1 and preS2 amino acid sequences on this binding was tested (Neurath *et al.*, 1986). The results revealed that residues 21-47 of the envelope large (preS1) protein, constitute the site mediating binding to these cells. Also, antibodies to the 21-47 peptide were able to inhibit the binding. In addition, antibodies raised against a "partially purified receptor fraction" from HepG2 cells inhibited the attachment of serum-derived HBsAg and a peptide analogous to the N-terminus of the preS1 protein to HepG2 cells. The above studies demonstrated that the amino acid sequence, encoded by the preS1 region of the HBV S-ORF, carries the attachment site involved in the virus binding to the tested cells. However, one must take into consideration the fact that HepG2 cells are tumorigenic

cells derived from human hepatoma, not normal human hepatocytes. Consequently, binding studies involving these cells may not reflect, in its entirety, the binding nature and characteristics expected for normal human hepatocytes.

In the recent studies, it has been shown that the envelope large protein of the HBV not only displays affinity for HepG2 cells or human hepatocyte membranes (see below), but it also recognizes cultured cells of non-hepatic origin (*e.g.*, amnion, Wish, neuroblastoma and SK-N-SH cells, and B lymphocytes; Neurath *et al.*, 1990). Furthermore, antibodies produced against a crude HepG2 lysate seem to be able to inhibit not only the interaction of serum-derived HBsAg with HepG2 cells, but also its binding to those cells (Neurath *et al.*, 1986, 1990). These findings raise the possibility that the binding of HBsAg to HepG2 cells is in fact, not cell specific and that other cultured cell lines could express the same type of receptor for HBV as HepG2 cells. The postulated receptor on these cells was primarily identified as a 66 kD protein and was recognized by antibodies raised against the previously mentioned "partially purified receptor" from HepG2 cells (Neurath *et al.*, 1990). In addition, Dash and Gerber (1992) demonstrated that the PreS1 (21-47) bound not only to HepG2 cells, non-hepatic cells (*i.e.*, kidney cells and fibroblasts), but also to a 68-kD binding protein identified in the HepG2 cell lysate. It should be emphasized, however, that the functional significance of the observed interactions can not be evaluated because there is no evidence that these cells, with the exception of HepG2 cells (Bchini *et al.*, 1990), are able to support HBV replication. There are also studies, however, which demonstrate that the preS1-encoded

sequence binding to normal human hepatocytes is cell specific. For example, it has been shown that yeast-derived large HBsAg protein displays the ability to bind plasma membranes purified from human liver (Pontisso *et al.*, 1989 a,b). This interaction was shown to be saturable and could be prevented by competition with unlabelled recombinant HBsAg particles containing the large envelope protein or by monoclonal antibodies raised against a synthetic peptide homologous to residues 27-49 of the preS1 protein. In another study, serum-derived subviral HBsAg particles and complete virions were also shown to bind human liver plasma membranes (Pontisso *et al.*, 1990). In addition this interaction was found to be dependent on the presence of preS1 proteins and inhibited by a monoclonal antibody which recognizes the preS1 (27-49) epitope (Pontisso *et al.*, 1989 b).

As a result of the efforts made to find receptors for HBV, which at the same time recognize physiologically important molecules, it was found that the amino acid sequences of the preS1 (21-47) epitope and the α-1-chain C-terminal sequence of the human immunoglobulin A (IgA) display a significant degree of homology. Also, some homology between the preS2-region encoded sequence and the amino acid sequence of IgA was observed. Consequently, the possibility that HBV may use a hepatocyte receptor for polymeric IgA as its receptor was raised (Neurath *et al.*, 1986). Interestingly, Neurath and Strick (1990) demonstrated that immunological cross-reactivity between IgA and preS (21-47) region of the HBV envelope protein exists, however, their experimental findings do not support the view that the receptor for polymeric IgA

functions as a binding site for HBV. Nevertheless, three lines of experimental evidence have been put forward in an attempt to explain the postulated role of the IgA receptor in HBV binding to host hepatocytes (Pontisso *et al.*, 1990). First, the specific binding of a labelled preS1 (21-32) oligopeptide to human liver plasma membranes was not only inhibited by addition of cold preS1 (21-32) peptide, but also by a human IgA. Secondly, the binding of radiolabelled IgA to these membranes was inhibited by addition of cold IgA or serum-derived HBV particles. Finally, it was observed that when membrane protein blots were independently incubated with either IgA, preS1 (21-32) peptide or viral particles, the same three bands of molecular weight 19, 34, 38 kD were consistently detected. In a recent study, Neurath and coworkers (1992) showed that the preS(21-47) also binds to interleukin 6 (IL-6), a cytokine produced by a variety of human cells *in vivo* as well as by various cell lines (*i.e.*, U266, H9, FS-4, HepG2, Cos-1, Namalwa, SH-N-SH, Cates 1B cells) or when cells are stimulated with mitogens such as Concanavalin A.

In order to determine the involvement of the third constituent protein of the HBV envelope, the major S protein, in the attachment of HBV to cells, several cell lines originating from liver (human and chimpanzee), kidney (human, monkey, owl, canine, feline and hamster), lymphoid tissues (ape and human), as well as from several other tissues of different species were tested for their ability to bind recombinant HBsAg (rHBsAg) particles containing only the major S protein (Peeples *et al.*, 1987). It was observed that among the cells that were examined only the Vero and CV-1 cell lines, derived from the kidney of the African green monkey, were able to bind rHBsAg. In

addition, 22-nm serum-derived HBsAg particles were also able to bind Vero and CV-1 cells. The specificity of the observed binding of rHBsAg to Vero cells was confirmed by the following criteria: (1) the binding of ^{125}I-rHBsAg was inhibited by unlabelled rHBsAg particles and (2) preincubation of rHBsAg with antiserum raised against these recombinant particles blocked the rHBsAg binding to the cells. In addition, it was found that each Vero cell possesses a finite number of binding sites for rHBsAg, which is approximately 2.4×10^5 sites per cell and that the binding is mediated by a single class of high affinity receptors (Peeples *et al.*, 1987), which display properties of a protein endowed with sialic acid (Komai *et al.*, 1987). Thus, if one looks at the range of experiments which tested the direct binding of HBV envelope major S protein to cellular surfaces, it is apparent that this protein displays affinity for cells maintained in culture that originate from organs other than human liver. Although the HBV major S protein does not bind to human hepatocytes *in vitro*, it is still possible that the protein may participate in the recognition of binding sites on human cells other than hepatocytes.

Overall, the status of our present understanding in regards to the participation of HBV envelope proteins in host and cell specific recognition can be summarized as follows: (1) it is most likely that amino acid sequences encoded both by the preS1 and preS2 regions of the HBV S-ORF contain domains involved in the virus attachment to human hepatocytes. Nevertheless, it appears, that the preS1-encoded sequence possesses a domain which directly binds HPMs, whereas the sequence encoded by the preS2 region attaches to HPM *via* a hypothetical analogue of gpHSA that may naturally occur in the

circulation. At present, the involvement of the major S protein in HBV recognition of human cells is strictly hypothetical; (2) in contrast to the relatively well recognized character of the HBV attachment proteins, the nature of hepatocyte receptors for HBV requires further extensive investigation. Preliminary data suggests that the receptor involved in the direct binding of the preS1-encoded sequence could be the hepatocyte receptor for IgA and/or IL-6. The hepatocyte ability to bind gpSA is better recognized and it has been found that two distinct classes of HPM-associated receptors, with high and low affinity for gpHSA are involved in this binding; (3) host and cell specificity of the interactions mediated by the HBV envelope proteins is only vaguely established and there is lack of convincing evidence demonstrating that HBV cell tropism is in fact determined at the level of the initial viral attachment; (4) because HBV is also capable of replicating in host cells other than hepatocytes, the ability of the virus envelope proteins to specifically interact with the surface of other human cells, particularly with lymphoid cells, have to be examined before conclusions can be made regarding the involvement of these proteins in the determination of the virus host range and tissue tropism.

1.9 Interactions between other Members of the *Hepadnaviridae* Family and Host Cells

It is necessary to point out that interactions between other hepadnaviruses and the surfaces of respective host cells have not for the most part been studied. However,

because of the postulated participation of a specifically modified human serum albumin in the attachment of HBV to human HPMs, the possibility that WHV envelope proteins (WHsAg) exhibit binding sites for glutaraldehyde-treated woodchuck serum albumin (gpWSA) has been tested. In a study reported by Pohl and coworkers (1986), it was determined that such binding sites do not exist on the intact WHV virions, 22-nm subviral WHsAg particles nor on WHsAg polypeptides. Furthermore, when a gpWSA affinity column was prepared and ^{125}I-labelled WHsAg particles were passed through the column, specific binding of WHsAg to this column did not take place (Michalak, unpublished). However, it is necessary to mention that when highly purified serum-derived WHsAg particles were subjected to Western blot analysis using antibodies raised against woodchuck serum albumin, it was evident that these preparations contained albumin monomers with apparent molecular weight of 67-kD (Lin and Michalak, unpublished). These data indicate that the WHV is unlikely to possess a receptor for gpWHV; however, albumin seems to be incorporated into the virus envelope, similarly as is also the case for HBV. The fact that HBV, but not WHV, exhibits binding activity for gpSA should not be viewed as a surprise because, unlike the major S proteins of hepadnaviruses, the amino acid sequences encoded by the preS1 and preS2 regions of the S-ORFs of HBV and WHV display only a limited homology (Snisky *et al.*, 1984). In fact, it is expected that the narrow species specificity displayed by hepadnaviruses could be related to the specific recognition of cellular receptors by epitopes present on their preS sequences. In a recent study, Kuroki and Ganem (1992) reported that the envelope

proteins encoded by the preS regions of DHBV and HHBV, unlike the HBV and WHV preS envelope proteins, bound with high affinity the same 180 kD glycoproteins of duck hepatocyte extracts.

1.10 Attempts to Establish Hepadnavirus Infection in Cultured Cells

At the present time, there are no cell lines which efficiently support HBV or WHV replication after exposure to infectious virions. However, it was observed that primary normal adult human hepatocytes cultured in the presence of DMSO apparently can be susceptible to limited HBV infection (Grippon *et al.*, 1988). The mechanism leading to the establishment of HBV infection in these cells remains unknown. It is possible, that DMSO may artificially favor the expression of cell surface receptors, which can be recognized by the virus and which in turn may facilitate the virus penetration into the cell. In another study, the *in vitro* infection of primary human fetal hepatocytes with HBV was attempted (Takahiro *et al.*, 1989); the authors claimed that these cells can be infected by virions, that they support the production of all viral constituents, that they accumulate HBV DNA in a covalently closed circle form, and that they release infectious virions. However, there is no data supporting these observations. Infection of HepG2 cells with HBV virions has also been successful (Bchini *et al.*, 1990). It appears, however, that the observed infectivity is highly dependent upon pre-treatment of the cells with dexamethasone and insulin, as well as on the continuous presence of these hormones in the culture medium. This suggests that the HepG2 susceptibility for

HBV infection may require stimulation of intracytoplasmic factors, which in turn may facilitate virus attachment, penetration or targeting of virions to a proper subcellular compartment.

On the other hand, there are a number of human and non-human cell lines, which can support HBV replication after transfection with HBV DNA (*i.e.*, HepG2, Huh 6-C15 cells) (Sells *et al.*, 1987; Sureau *et al.*, 1986; Tsurimoto *et al.*, 1987).

Primary hepatocyte lines derived from livers of woodchucks chronically infected with WHV are capable of supporting active virus replication for up to 3 months (Theze *et al.*, 1987). To date, normal woodchuck hepatocytes in culture have not been shown to be infected after exposure to WHV virions. In contrast, infection of primary duck hepatocytes with DHBV has been accomplished (Tuttleman, 1986). However, since this avian virus is phylogenetically distant from mammalian hepadnaviruses, it is unlikely that this model mimics interactions between HBV and surfaces of human cells. Also, the limited infectibility of duck hepatocytes by DHBV (*i.e.*, about 10% of the cells exposed to the virus), loss of albumin synthesis soon after plating of the cells, and change in morphology of hepatocytes from typical polygonal to a fibroblast-like shape after the plating, makes the studies on the initial virus-cell interaction in this *in vitro* model difficult to perform and evaluate. Consequently, the hepatocellular receptor for the DHBV has not yet been identified.

In conclusion, unlike the pathway of hepadnaviral replication, the mechanism of virion attachment to targeted cells remains an ill understood event in hepadnaviral

infection, despite the variety of efforts reported to date. It is hoped that a better understanding of the nature of the initial interactions between these viruses and host cells will result in effective means and strategies of treating HBV-induced diseases. In an attempt to broaden the scope of knowledge pertaining to the recognition and structure of cellular receptors for HBV, the studies described herein were undertaken using a woodchuck model of hepatitis B.

1.11 Experimental Strategy

Among the hepadnaviruses WHV displays the closest molecular, structural, and biological characteristics to those displayed by HBV (section 1.7). It is hoped that identification of host cellular constituents responsible for the binding of WHV would, by analogy, allow the identification of HBV receptor(s). Hence, the use of a woodchuck model should not only overcome the difficulties in obtaining virus and cell materials for the study, but may also provide a ready-to-use *in vivo* system for testing of therapeutic agents, resulting from the characterization of these viral receptors. Since envelope proteins of WHV, in contrast to HBV, do not recognize gpSA (Pohl *et al.*, 1986), the likelihood that an analogue of glutaraldehyde-polymerized woodchuck serum albumin may participate as a bridging molecule in virus binding to hepatocyte is unlikely. Consequently, the investigations presented in this thesis encompass the characterization of the direct binding of WHV envelope particles (WHV surface antigen - WHsAg) constituted by all three envelope proteins of the virus, *i.e.*, large, middle and major

proteins, to purified woodchuck HPMs and to plasma membranes of other woodchuck cells, and the recognition of the biochemical nature of the cell receptor molecules. In addition, since it is highly probable that epitopes exposed at the surface of the virion are more likely to interact with cellular receptors, an oligopeptide homologous to the extreme N-terminal amino acid sequence of the preS1 protein of WHV envelope, predicted to be located at the virion surface, was synthesized and used to test the possible involvement of the preS1 region of the virus in the determination of its host and cell specificity.

Accordingly, the specific aims of these studies were as follows:

1) To develop or adopt assays which allow the identification and analysis of WHV envelope-receptor interactions.

2) To determine WHV receptor activity in highly purified plasma membrane preparations derived from woodchuck hepatocytes, splenocytes and kidney cells using the assays developed in (1) above.

3) To prepare a soluble fraction of purified woodchuck hepatocyte plasma membranes displaying receptor activity for WHV and to compare the kinetics of the WHV envelope binding to this fraction with those displayed by native hepatocyte plasma membranes.

4) To purify hepatocyte plasma membrane constituents enriched in WHV receptor activity using virus affinity chromatography.

5) To characterize the biochemical properties of molecules expressing WHV envelope binding, through the employment of lectin affinity chromatography and

enzymatic digestions.

6) To test whether an exogenous analogue of a carbohydrate moiety, which was identified as a WHV binding constituent in the course of the study, can inhibit the attachment of WHV envelope to that receptor molecule and to native woodchuck hepatocyte plasma membranes.

7) To test the binding of an oligopeptide homologous with the sequence of the WHV envelope preS1 protein for woodchuck tissues and subcellular fractions.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Animals

Woodchucks *(Marmota monax)* with an approximate weight of 4 kg, were kept as a colony at the Faculty of Medicine, Memorial University of Newfoundland. Healthy and WHV-infected animals were held separately, under environmental, dietary and biosafety conditions suitable for this species. Blood samples were collected after administration of *i.v.* anaesthesia, using ketamine hydrochloride (100 mg/ml) in benzethonium chloride (0.1%) and xylazine hydrochloride (100 mg/ml) in a ratio of 0.87 to 0.13 for an approximate total volume of 1.5 ml. Liver perfusion was performed under a deep general anaesthesia induced by an overdose of ketamine and xylazine hydrochloride mixture.

2.2 Purification of WHV Envelope Particles and Evaluation of their Purity

WHV envelope particles were isolated from a pool of WHsAg-positive serum samples collected from a single woodchuck chronic carrier of WHV, essentially by the procedure previously described by Gerlich and coworkers (1980).

2.2.1 Buffers and chemicals

1. 0.01 M Tris [hydroxymethyl] amino methane-hydrochloric acid (Tris-HCl), pH 7.4, containing 0.1 M NaCl and 5 mM ethylenediamine-tetraacetic acid (EDTA) (TBES).
2. 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (phosphate-buffered saline, PBS).
3. CsCl crystals (Pharmacia Fine Chemicals, Uppsala, Sweden).
4. Agarose beads (Bio-Gel A-5m, 100-200 mesh; Bio-Rad Laboratories, Richmond, CA).

2.2.2 Methods

2.2.2.1 Preparation of a Bio-Gel A-5m column

Agarose beads were washed with PBS according to the procedure advised by the manufacturer to remove any preservatives and de-gassed under a negative pressure. Then, the beads were packed on a 80 x 1 cm column and equilibrated with TBES.

2.2.2.2 Column chromatography of WHsAg-positive serum samples

A serum sample of 3 ml was applied to the Bio-Gel A-5m column and fractionated into 2 ml aliquots at a flow rate of 0.5 ml/min using TBES. The protein content of the resulting fractions was determined through the use of a Gilford instrument spectrophotometer (Gilford Instruments Laboratory Inc., Oberlin, OH) set at a wavelength of 280 nm. Fractions of the first protein peak, containing the partially purified WHV envelope particles (*i.e.*, crude WHsAg preparation), were combined and

immediately subjected to further purification.

2.2.2.3 Purification of WHV envelope particles from crude WHsAg by CsCl gradient centrifugation

A two-ml aliquot of crude WHsAg preparation was made up to a density of 1.30 g/cm^3 by the addition of CsCl crystals and then layered on top of a 2 ml CsCl solution (1.35 g/cm^3) contained in a Beckman ultra clear tube (14 x 98 mm). Subsequently, 2 ml volumes of CsCl solutions with densities of 1.27, 1.24 and 1.18 g/cm^3 were added. The gradients were centrifuged at 86,000 x g in a SW40 rotor (Beckman Instruments Inc., Palo Alto, CA) for 40 hr at 10^{0}C. Then, 0.5 ml fractions were collected beginning at the top of each gradient. The presence of WHsAg in the fractions was determined by taking advantage of the cross-reactivity displayed by the commercially available radioimmunoassay for HBsAg (Ausria II; Abbott Laboratories, North Chicago, Il). The assay was performed according to the procedure proposed by the supplier. It was observed that WHsAg banded between 1.18 and 1.19 g/cm^3 of CsCl (*i.e.*, fractions 6-10). The respective fractions were combined and concentrated by negative pressure dialysis against PBS using a Micro-ProDiCon apparatus (Bio-Molecular Dynamics, Beaverton, Ore).

2.2.2.4 Assessment of the purity of WHV envelope particles

The purity of the WHV envelope preparation was assessed by analysis of its protein composition in a sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), using a 5-16% linear gradient and conditions described in section 2.20. Furthermore,

morphological homogeneity of the envelope particles was established through ultrastructural examination. For this purpose, the preparation was spotted on 100-mesh copper grids coated with formvar and then with carbon, and negatively stained with 1% phosphotungstic acid. The grids were examined in a JEM-1200 EX electron microscope (Japan Electro-Optical Laboratory, Japan) operated by the Electron Microscopy Unit at the Faculty of Medicine, Memorial University of Newfoundland.

2.3 Synthesis of the JP1 Sequence

In order to recognize the possible involvement of the WHV preS1 sequence in regards to specific cellular interactions, a synthetic peptide homologous to a portion of the extreme amino terminal sequence of the preS1 protein was prepared. The peptide (designated JP1) encompassing amino acids MGNNIKVTFNPDK (in a single-letter amino acid code) was synthesized on a p-alkoxybenzyl ester type resin by the Merrifield solid-phase method (Stewart and Young, 1984), using a semi-automatic peptide synthesizer (Labortec SP 640 Bubendorf, Switzerland). Cysteine residue was added to the carboxyl-terminal of the peptide to facilitate the possibility of coupling to carriers and labelling. Cysteine was used as a derivative of the resin (Omni Biochemicals, National City, CA). The α-amino group of each amino acid was protected with 9-fluorenylmethyloxycarbonyl (Fmoc). The side chain, of lysine was protected with tert-butylcarbonly (BOC), whereas those of aspartic acid, threonine and cysteine were protected with tert-butyl. Prior to each coupling, Fmoc protecting group was cleaved

with piperidine in dimethyl formamide (DMF) (1:1) and presence of the free α-amino group was assessed by the Kaiser test (Stewart, 1983). Asparagine was coupled as its p-nitrophenyl ester in the presence of 1-hydroxybenzotriazole (HOBt) in DMF. Both 1,3-dicyclohexyl carbodiimide (DCC) and HOBt in DMF were used to couple the other amino acids. Completeness of coupling was monitored by the Kaiser test. Cleavage of the synthesized peptide from the resin and removal of side chain protecting groups, namely BOC and tert-butyl (except for cysteine), was achieved by treatment with 50% trifluoroacetic acid in dichloromethane, containing anisole and 2-mercaptoethanol under an atmosphere of N_2 gas at 25^{0}C for 2 hr and 30 min. To remove the cysteine side chain protecting group, the peptide was dissolved in water and adjusted to pH 4.0 by the addition of 10% ammonium hydroxide. Mercuric II acetate was then added and the solution was stirred at pH 4.0 for 5 hr (Chang *et al.*, 1980; Felix *et al.*, 1978). Hydrogen sulphide was passed through the above solution for 15-30 min to precipitate mercury as mercuric sulphide (HgS). The HgS precipitate was filtered off and the filtrate was lyophilized. Then, in order to convert any methionine oxide that may have been formed to methionine and to reduce disulphide bridges of cysteine, the peptide was stirred in the presence of a solution consisting of 2 N acetic acid with a few drops of 2-mercaptoethanol at room temperature under an atmosphere of N_2 gas for 16 hr. The solutions were then lyophilized to recover the peptide.

The purity of the peptide was checked by thin layer chromatography, paper chromatography, and reverse phase high pressure liquid chromatography (HPLC) at

25^{0}C. HPLC was performed by employing a Waters C-18 column measuring 300 x 4 mm where a 0-50% linear gradient of acetonitrile in 0.1% trifluoroacetic acid over 60 min at a flow rate of 1.0 ml/min was used. The detection of the peptide was accomplished with UV rays set at a wavelength of 260 nm.

The synthesis of JP1 peptide and evaluation of its purity was done with help from Drs. N.S. Rangaraju and V.S. Ananthanarayanan, formerly from the Department of Biochemistry, Memorial University of Newfoundland.

2.4 Heparin

Based on the results of experiments performed during the preparation of this thesis, it became important to test the behaviour of an exogenous sulphated glycosaminoglycan as a WHV-binding substance. Heparin was chosen because of its well characterized chemistry, biological action, hydrophilicity, and accessibility. Sodium heparin from Becton Dickinson (Rutherford, NJ) was used.

2.5 Radiolabelling Procedure

Purified serum-derived WHV envelope particles, JP1 sequence and heparin were labelled with Na^{125}I by the lactoperoxidase/glucose oxidase method according to the procedures recommended by the supplier for the commercially available labelling kit (Bio-Rad Enzymobead radioiodination reagent, Bio-Rad Laboratories). This method of radiolabelling is based on studies reported by Marchalonis (1969), Morrison and Bayse

(1970), Phillips and Morrison (1970) and Morrison and colleagues (1971).

2.5.1 Reagents and buffers

1. 0.5 mCi of a carrier free $Na^{125}I$ (Amersham Corporation, Arlington Heights, IL)
2. 2% dextrose solution in distilled water mutarotated overnight.
3. Enzymobeads consisting of lacto-peroxidase and glucose oxidase immobilized on beads (Bio-Rad Laboratories). The beads were rehydrated in 0.5 ml of distilled water and left to stand for 2 hr at 25°C prior to radiolabelling.
4. A column (0.5 cm x 20 cm) of Sephadex G-25 (Pharmacia Fine Chemicals).
5. 0.2 M sodium phosphate buffer, pH 7.2.
6. PBS

2.5.2 Method

1. Fifty μl of sodium phosphate buffer, 25 μl of WHV envelope preparation (100 μg protein) or JP1 (100 μg of weight) or heparin (100 μg of weight), 50 μl of Enzymobeads, and 0.5 mCi $Na^{125}I$ (10μl) were mixed together at room temperature.
2. The radiolabelling reaction was initiated by addition of 25 μl of dextrose solution. Then, the reaction components were mixed and left to stand for 30 min at room temperature.
3. The mixture was applied on a Sephadex G-25 column in order to quench the

reaction and to separate the radiolabelled material from the unbound $Na^{125}I$. The column was washed with PBS and the radioactivity of each 0.5-ml fraction was determined in a Beckman Gamma 300 Counting System (Beckman Instruments, Inc., Irving, CA). The fractions constituting the first peak of radioactivity were combined and used further as a probe.

2.6 Preparation of Woodchuck, Human and Mouse Tissue Homogenates

Woodchuck liver, spleen and kidney tissues were obtained from a healthy WHV-negative animal. Fragments of human liver and spleen were obtained during a routine autopsy of an individual without clinical and histological evidence of known liver disease. Liver and spleen tissues were also obtained from a healthy mouse. All tissues were cut into small pieces, washed extensively with TBS until blood was completely removed, and homogenized with a Brinkmann homogenizer (Brinkmann Instruments Co., Westbury, NY). The resulting homogenates were then filtered through 4 layers of surgical gauze to remove connective tissue, frozen in liquid nitrogen and stored at -70°C until use. Protein content was determined by the method of Lowry and co-workers (1951), using bovine serum albumin (BSA; Sigma) as a standard.

2.7 Liver Perfusion

To isolate hepatocytes, woodchuck livers were perfused with a collagenase solution using the two-step perfusion technique as described by Seglen (1976).

2.7.1 Buffers

1. 9.22 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid] (HEPES; Sigma Chemical Company), pH 7.4, containing 142.1 mM NaCl and 6.71 mM KCl (Ca^{2+}- and Mg^{2+}-free buffer).
2. 0.05% collagenase (type IV; > 125 units/mg; Sigma) in 9.22 mM HEPES, pH 7.6, containing 66.7 mM NaCl, 6.71 mM KCl and 4.77 mM $CaCl_2$ (collagenase buffer).
3. 27.7 mM HEPES, 30.1 mM N-Tris[hydroxymethyl]methyl-2-amino ethanesulfonic acid mixed with 2-([2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino) ethanesulfonic acid (TES; Sigma), 36.3 mM N-Tris[hydroxymethyl]methyl glycine (Triscine; Sigma), pH 7.6, 68.46 mM NaCl, 5.37 mM KCl, 1.23 mM $CaCl_2$, 0.64 mM $MgCl_2$, 1.1 mM KH_2PO_4 and 0.83 mM Na_2SO_4 (Ca^{2+}- and Mg^{2+}-containing buffer).

2.7.2 Methods

1. Approximately 0.75 ml of heparin (Hepalean; 1000 USP units/ml; Organon Canada Ltd., Toronto, Ont.) was *i.v.* injected prior to the administration of the anaesthesia.
2. Once the animal was found to be in a deep anaesthesia and was unable to

respond to pain, laparotomy was performed. As the first, the portal vein was exposed and sutures were loosely placed around the vessel. Subsequently, the vein was cannulated and the canulae was tightly secured with the already placed sutures.

3. Following attachment of the perfusion tubing to the cannulae, the peristaltic pump (Sorvall peristaltic pump, Newtown, CT) was turned on and the liver was perfused with 250 ml of Ca^{2+}- and Mg^{2+}-free buffer, preheated at 37^{0}C and saturated with a mixture of 95% O_2 and 5% CO_2. The intrahepatic pressure was vented by opening the *vena cava* shortly after the beginning of the perfusion. The preperfusion continued for about 10-15 min. at a flow rate of 2 ml/min.

4. The liver was then excised and perfused outside the body with 200 ml of recirculating, oxygenated collagenase buffer at a flow rate of 2 ml/min at 37^{0}C for 20-25 min.

5. Subsequently, the liver was disconnected and the tissue gently minced with the fingers. The suspension was then incubated with 4 volumes of collagenase buffer for an additional 10 min. at 37^{0}C, while being slowly gassed with a mixture of 95% O_2 and 5% CO_2.

2.8 Isolation of Hepatocytes and Evaluation of their Purity

A suspension of the collagenase-treated liver cells was used for isolation of

hepatocytes.

2.8.1 Buffers

1. Ca^{2+}- and Mg^{2+}-containing buffer described above.

2. 0.25 M sucrose in 5 mM Tris-HCl buffer, pH 7.4, supplemented with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) (0.25 M sucrose-EDTA-PMSF buffer, pH 7.4).

2.8.2 Methods

1. Liver cells were filtered through 4 layers of surgical gauze to remove cell aggregates and debris, and the filtrate was centrifuged at 200 x g using a JA 20 Beckman rotor (Beckman Instruments, Inc., Palo Alto, CA) for 5 min at 10^0C.

2. The resulting pellet was washed by diluting it with 5 volumes of Ca^{2+}- and Mg^{2+}-containing buffer and centrifuged as before. Washings and centrifugations were repeated for a total of 5 times. The progress in the purification of hepatocytes was monitored by examination of the hepatocyte pellets using a phase contrast microscope.

3. The final pellet of the hepatocytes was resuspended in 0.25 M sucrose-EDTA-PMSF, pH 7.4 buffer and allowed to equilibrate for 30 min on ice. Phase contrast microscopy and ultrastructural examination demonstrated that more than 95% of the isolated liver cells were hepatocytes.

2.9 Preparation of Hepatocyte Plasma Membranes (HPMs)

Plasma membranes were prepared from isolated woodchuck hepatocytes according to the procedure described by Touster and coworkers (1970).

2.9.1 Buffers

1. 5 mM Tris-HCl buffer, pH 8.0 (Tris buffer).
2. 0.25 M sucrose in 5 mM Tris-HCl buffer, pH 8.0 (0.25 M sucrose buffer).
3. 34% (w/w) sucrose in 5 mM Tris-HCl buffer, pH 8.0 (34% sucrose buffer).
4. 57% (w/w) sucrose in 5 mM Tris-HCl buffer, pH 8.0 (57% sucrose buffer).
5. 0.25 M sucrose, 1 mM EDTA, 1 mM PMSF in 5 mM Tris-HCl buffer pH 8.0 (0.25 M sucrose-EDTA-PMSF buffer, pH 8.0).

2.9.2 Methods

1. To the suspension of hepatocytes, 3 volumes of cold 0.25 M sucrose buffer were added. The cells were homogenized in a Potter-Elvehjem homogenizer mounted in a drill press at 1000-1200 rpm for 5 passes of the teflon pestle.
2. The homogenate was centrifuged at 1000 x g using a JA 20 rotor (Beckman Instruments) for 10 min to separate nuclei and cellular debris. The resulting supernatant was saved and kept on ice.
3. The pellet was re-suspended in the same initial volume of 0.25 M sucrose and rehomogenized with 3 passes of the pestle and then centrifuged at 1000 x g for 10 min as described above. The supernatant was saved and kept on

ice.

4. Re-homogenization and re-centrifugation of the pellet resulting from step 2 was repeated a third time to increase the yield of plasma membranes. The final pellet, designated as the nuclear fraction, was immediately frozen and kept at -70^{0}C until further use.

5. All three supernatants resulting from steps 2-4 were combined and centrifuged at 33,000 x g for 7.5 min using a SW 27 rotor (Beckman Instruments). The rotor was first accelerated to 33,000 x g at maximum acceleration then the speed control was turned off. The time elapsed between the beginning of acceleration, and the beginning of braking was approximately 7.5 min.

6. The supernatant and the "fluffy pink" layer packed on top of the resulting pellet, was carefully aspirated and kept at 4^{0}C. Subsequently, the pellet was suspended by gentle rubbing with a plastic rod in 3 vol of 0.25 M sucrose buffer and homogenized with one pass of the pestle.

7. The mixture was centrifuged as in step 5. and the resulting supernatant was removed. The pellet, termed mitochondrial fraction, was saved and kept at -70^{0}C until further use.

8. The supernatants recovered in steps 6 and 7 were combined and centrifuged at 100,000 x g for 100 min using a 50.2 Ti Beckman rotor. The resulting supernatant, including the congealed fat floating on its surface, was

carefully aspirated leaving behind a whitish layer which was loosely packed on the top of the pellet.

9. In order to further purify plasma membranes, the pellet was homogenized together with 1.5 volumes of 57% sucrose buffer with two passes of the pestle. The pestle was rinsed thrice with 5 ml of 57% sucrose buffer and this aliquot was combined with the homogenate.

10. Subsequently, the homogenate aliquoted into 10 ml amounts was placed at the bottom of Beckmann ultra clear (25 x 98 mm) tubes to which 20 ml of 34% sucrose buffer was layered on top. Finally, the gradient was completed when 8.5 ml of 0.25 M sucrose buffer was added over the 34% sucrose buffer. The gradients were centrifuged at 78,000 x g for 16 hr in a SW27 Beckman rotor.

11. Plasma membranes (originally designated as P2; Touster *et al.*, 1970) appeared as a thick white band at the interface of the 0.25 M sucrose and the 34% sucrose buffers. Once the uppermost layer containing the congealed fat was aspirated, the membranes were carefully removed with the aid of a pasteur pipette.

12. Finally, in order to remove sucrose from the HPM preparation, the membranes were diluted with Tris buffer and centrifuged 100,000 x g for 1 hr using a 50.2 Ti Beckman rotor. The resulting supernatant was discarded and the plasma membrane pellet was resuspended in 0.25 M

sucrose-EDTA-PMSF buffer, pH 8.0.

13. HPMs were frozen in liquid nitrogen and stored at -70°C until further use. Protein concentration was determined by the method of Lowry and colleagues (1951), using BSA as a standard.

2.10 Preparation of Plasma Membranes from Splenocytes (SPMs)

Isolation of plasma membranes from isolated woodchuck splenocytes was essentially performed as described by Misra and coworkers (1975).

2.10.1 Buffers

1. 5 mM Tris-HCl buffer, pH 7.4.
2. 10 mM Tris-HCl buffer, pH 7.4.
3. 5 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, (TBS).
4. 5 mM Tris-HCl buffer, pH 7.4, containing 1.5 M NaCl (NaCl-Tris buffer).
5. 20% sucrose (w/w) in 10 mM Tris-HCl buffer, pH 7.4 (20% sucrose-Tris buffer).
6. 30% sucrose (w/w) in 10 mM Tris-HCl buffer, pH 7.4 (30% sucrose-Tris buffer).
7. 40% sucrose (w/w) in 10 mM Tris-HCl buffer, pH 7.4 (40% sucrose-Tris buffer).
8. 10 mM Tris-HCl buffer, pH 8.0 containing 1 mM EDTA and 1 mM PMSF (Tris-EDTA-PMSF buffer).

2.10.2 Reagents

1. Histopaque 1119 (Sigma).
2. Ethidium bromide (Sigma).
3. 3,6- bis [Dimethylamine] acridine (acrinidine orange; Sigma).

2.10.3 Methods

1. Spleens removed from four healthy woodchucks were extensively washed in cold TBS and then allowed to stand for 10 min on ice.
2. The organs were gently minced with the fingers and pressed against a coarse stainless steel mesh with the drop-wise addition of TBS. The cell suspension was then washed in TBS by sedimentation until the red coloration in the supernatant was no longer present.
3. The resulting cell suspension was layered on Histopaque 1119 in such a way that the volume ratio of the cell to Histopaque was 2:1. The gradient was centrifuged at 700 x g for 20 min using a IEC swing out 253 rotor (International IEC, Inc., Needham Heights, Mass).
4. The splenocyte fraction located between the Histopaque 1119 and TBS was removed, washed in TBS by centrifuging twice at 500 x g for 7.5 min using a IEC swing out 253 rotor, and re-suspended in 10 ml of TBS. Macrophages were depleted by adsorption to plastic for 1 hr at 37^0C under 95% O_2 and 5% CO_2 atmosphere.
5. Examination of the macrophage-free cell isolate under phase-contrast

microscopy revealed that the cells displayed morphological features resembling those of human medium- and small-size lymphocytes. A slight contamination with erythrocytes, which amounted to less than 2% of the total number of cells, was removed by addition of 900 μl 3% acetic acid and centrifugation. Staining of the splenocytes with ethidium bromide-acridine orange solution followed by examination under a fluorescent microscope showed that viable mononuclear cells constituted more than 95% of the isolated cells.

6. The splenocytes were suspended in NaCl-Tris buffer and gently homogenized in a Potter-Elvehjem homogenizer mounted in a drill at 1000-1200 rpm. 7. The homogenate was centrifuged at 300 x g for 15 min using a JA 20 Beckman rotor to remove nuclei and cellular debris. The supernatant was saved and kept on ice.

8. The pellet was resuspended in TBS, gently homogenized with another 5 passes of the pestle and centrifuged as indicated above. Then the supernatant was carefully collected and the pellet, designated as a nuclear fraction, was saved and stored at -70^{0}C.

9. The combined supernatants were centrifuged at 4000 x g for 20 min using a Beckman SW 27 rotor to remove mitochondria contamination. The resulting supernatant was saved and the pellet was re-suspended in the same initial volume of TBS, and centrifuged at 4000 x g in a SW 27 Beckman

rotor for 30 min. The supernatant was saved and used in the next step. The sediment, termed mitochondrial fraction, was saved and kept at -70°C until further use.

10. The resulting supernatants from step 9 were combined and centrifuged at 20,000 x g for 1 hr using a Beckman 50.2 Ti rotor. The pellet containing crude plasma membranes was suspended in 40 ml of 20% sucrose-Tris buffer, homogenized with 5 gentle passes of the pestle, and divided into 10 ml aliquots.

11. Each aliquot was then layered on 12 ml of 30% sucrose-Tris buffer, which in turn was layered on top of 12 ml of 40% sucrose-Tris buffer. The gradients were prepared in Beckman ultra clear (25 x 98 mm) tubes and then centrifuged at 33,000 x g for 16 hr using a Beckman SW 27 rotor.

12. The purified plasma membranes, which appeared as a light brown colour band at the 30-40% sucrose interface, were collected and diluted with 10 volumes of 10 mM Tris-HCl buffer, and then centrifuged at 100,000 x g using a 50.2 Ti Beckman rotor for 1 hr in order to remove the sucrose.

13. The membrane pellets were resuspended in Tris-EDTA-PMSF buffer, immediately frozen in liquid nitrogen, and stored at -70°C until use. Protein concentration was measured by the method of Lowry and colleagues (1951).

2.11 Preparation of Kidney Cell Plasma Membranes (KPMs)

Once the liver perfusion was completed, the kidneys were removed from the animals. Kidney tissue was cut in small pieces, washed in TBS, and homogenized with a Brinkmann homogenizer. The resulting homogenate was filtered through four layers of surgical gauze to remove connective tissue. Subsequently, plasma membranes were purified following the previously described procedure for the isolation of HPMs.

2.12 Evaluation of the Purity of Plasma Membrane Preparations

The purity of the plasma membranes was assessed by measuring the activity of marker enzymes, reported to be specific for defined subcellular constituents. Thus, 5'-nucleotidase and (Na^+,K^+)-ATPase were used as enzymatic indicators of plasma membrane purity, whereas glucose-6-phosphatase and succinate cytochrome C reductase were assayed to measure the respective microsomal and mitochondrial contamination. The assessment of the plasma membrane purity was determined by calculating the ratio (enrichment) between the activity displayed by each of the above enzymes in the membrane fraction and that found in the initial cell or organ homogenates used for the membrane isolation.

2.12.1 5'-Nucleotidase assay

This enzyme was assayed according to a colorimetric procedure originally described for the determination of the enzyme activity in serum samples. The assay was performed using a kit commercially available from the Sigma Chemical Company, which

is based on the method described by Dixon and Purdom (1954).

2.12.1.1 Reagents and solutions

1. 2.38 mM adenosine 5'-monophosphate, pH 7.5 (adenosine 5'-monophosphate substrate).
2. 1.52 mM sodium β-glycerophosphate, pH 7.5 (glycerophosphate substrate).
3. 30% trichloroacetic acid (TCA) (v/v) in distilled water.
4. Reagents for the determination of free inorganic phosphate (see section 2.12.3).

2.12.1.2 Method

1. To tubes containing 4.8 ml of adenosine 5'-monophosphate substrate (tube 1) and 4.8 ml of glycerophosphate substrate (tube 4), 50 μg plasma membrane protein or organ homogenate protein in 0.2 ml of Tris-EDTA-PMSF, pH 8.0 buffer was added.
2. Similarly, as a control, 0.2 ml of Tris-EDTA-PMSF, pH 8.0 buffer was added to tubes containing 4.8 ml of adenosine 5'-monophosphate substrate (tube 2) and 4.8 ml of glycerophosphate substrate (tube 3).
3. Tubes containing the assayed preparations and the controls were incubated in a shaking water bath for 15 min at 37°C. Then, the tubes were placed in an ice bath and 1.0 ml aliquots of cold TCA (30%) were added to each tube to stop the enzymatic reaction.
4. Fifty μg of protein in 200 μl of Tris-EDTA-PMSF buffer was added to

controls and allowed to stand for 10 min on ice. Any precipitate formed was removed by centrifugation for 5 min. using a IEC clinical centrifuge. Four-ml aliquots of the clear supernatant were used to determine the content of inorganic phosphate utilizing the procedure outlined below.

5. The 5'-nucleotidase activity was calculated by subtracting the net absorbance of the non-specific phosphatase activity (tubes 3 and 4) from the net absorbance found in tubes 1 and 2. Enzymatic activity was expressed as μmole phosphate /min/mg protein.

2.12.2 Determination of (Na^+, K^+)-ATPase

This enzyme, being a marker for the sinusoidal portion of the hepatocyte plasma membranes (Sztul *et al.*, 1987), was assayed in the presence and absence of a cardiac glucoside inhibitor, ouabain by modifying the methodology originally described by Sulakhe and co-workers (1971).

2.12.2.1 Reagents and solutions

1. 58.2 mM Tris-HCl buffer, pH 7.2, containing 0.465 mM ouabain (Sigma), 0.12 M NaCl, 23.5 mM KCl, 5.88 mM $MgCl_2$, and 1.17 mM EDTA (incubation medium I).
2. 58.2 mM Tris-HCl buffer, pH 7.2, containing 0.12 M NaCl, 23.5 mM KCl, 5.88 mM $MgCl_2$, and 1.17 mM EDTA (incubation medium II).
3. 0.724 mM sodium phosphate monobasic buffer, pH 7.5.
4. 12% TCA (v/v) in distilled water.

5. Reagents for the determination of free inorganic phosphate (see section 2.12.5).

6. 40 mM adenosine triphosphate (ATP) in distilled water (freshly prepared and kept on ice until use.)

2.12.2.2 Method

1. To tubes 1 and 2 containing 850 μl of incubation medium I and incubation medium II, respectively, 50 μg of tested protein in a volume of 0.05 ml of Tris-EDTA-PMSF buffer, pH 8.0 was added and equilibrated for 5 min in a shaking water bath at 37^{0}C.

2. As controls, two other tubes containing 0.85 ml incubation medium II (tube 3 and 4) were prepared. Subsequently, 0.15 ml and 0.05 ml of distilled water was added to tubes 3 and 4,respectively. One ml aliquot of a 0.724 mM sodium phosphate buffer was poured into a fifth tube.

3. To initiate the enzymatic reaction, 100 μl of 0.04 M ATP was added to tubes 1, 2, and 4. The reaction was allowed to take place in a shaking water bath for 15 min at 37^{0}C. Reaction was terminated by adding 2.0 ml of cold TCA to each of the 5 tubes. The tubes were then kept on ice for 5 min.

4. All tubes were centrifuged for 5 min using a IEC clinical centrifuge. After centrifugation, 2.0 ml aliquots of the supernatants were pipetted out and placed in new tubes. Then, 2.0 ml of distilled water was added to each

tube.

5. The amount of inorganic phosphate released into the medium was estimated as described below (see section 2.12.5). The values obtained were corrected for the non-enzymatic hydrolysis of ATP. According to the principles of the assay, the difference between the amount of detected inorganic phosphate in the absence and presence of ouabain results from the activity of the sinusoidal plasma membrane marker, (Na^+,K^+)-ATPase, whereas the values obtained in the presence of ouabain are assumed to be due to the presence of Mg^{2+}-dependent ATPase. The activity of the enzyme was expressed as μmole phosphate/min/mg protein.

2.12.3 Determination of glucose-6-phosphatase activity

Glucose-6-phosphatase was measured by determining the rate of inorganic phosphate release from glucose-6-phosphate as described by Aronson and Touster (1974).

2.12.3.1 Solutions

1. 100 mM sodium-glucose-6-phosphate, adjusted to pH 6.5 with 3 M HCl.
2. 35 mM histidine, adjusted to pH 6.5 with 3 M HCl.
3. 10 mM EDTA, adjusted to pH 7.0 with 3 *M* HCl.
4. 0.724 mM sodium phosphate monobasic buffer, pH 7.5.
5. 8% TCA in distilled water (v/v).
6. Reagents for the determination of free inorganic phosphate (see section 2.12.5).

2.12.3.2 Methods

1. A reaction mixture was prepared by combining sodium-glucose-6-phosphate, histidine, EDTA, and distilled water in a ratio 2:5:1:1. The enzymatic reaction began when 0.05 ml of Tris-EDTA-PMSF buffer containing 100 μg of protein was added to a tube (tube 1) containing a 640 μl aliquot of the above reaction mixture.

2. In a volume of 0.69 ml, the following control tubes were also carried throughout the assay: distilled water (tube 2), 100 μg of assayed protein (tube 3), 640 μl of assay mixture (tube 4), 0.69 ml of 0.724 mM sodium phosphate buffer containing 0.5 μmole of sodium phosphate (tube 5). Tubes 3 and 4 were supplemented with distilled water to a total volume of 0.69 ml. Subsequently, all tubes were capped and immersed for 30 min in a shaking water bath maintained at 37^{0}C.

3. The reaction was stopped by cooling the tubes in a ice bath, then, 2.31 ml of cold TCA was added in each tube.

4. Finally, all tubes were centrifuged in a IEC clinical centrifuge and 2.0 ml aliquots of the supernatants were pippeted out and diluted to a total volume of 4.0 ml with distilled water. Inorganic phosphate was determined by the procedure described below. The glucose-6-phosphatase activity was corrected for any inorganic phosphate not enzymatically released from glucose-6-phosphate. The activity of the enzyme was defined as μmoles

phosphate/min/mg protein.

2.12.4 Evaluation of succinate-cytochrome C reductase activity

The assessment of this mitochondrial enzyme was based on a modified version of the assay described by Green and colleagues (1955).

2.12.4.1 Buffers

1. 0.1 mM cytochrome C in 50 mM sodium phosphate buffer, pH 7.5, containing 0.3 mM KCN (phosphate-cytochrome C buffer).
2. 50 mM succinate in distilled water.

2.12.4.2 Method

1. To each of two cuvettes containing 3.0 ml aliquots of phosphate cytochrome C buffer, 50 μl of protein in 100 μl of Tris-EDTA-PMSF buffer was added and mixed well.
2. Changes in absorbance at 550 nm were then monitored for 5 min on both cuvettes at 15 second intervals using the Gilford spectrophotometer 240 attached to a Gilford 6050 chart recorder (Instrument Laboratories Inc., Oberlin, OH).
3. The reaction began by the addition of 50 μl of 50 mM succinate to one of the cuvettes. The change in absorbance in both cuvettes was recorded for 5 min. Enzyme activity was expressed in μmole/ min/mg. The extinction coefficient used was 18.5 mM/cm.

2.12.5 Inorganic phosphate assay

Inorganic phosphate determination was based on the method reported by Fiske and Subbarow (1925).

2.12.5.1 Solutions

1. 0.724 mM sodium phosphate buffer, pH 7.5.
2. Acid molybdate stock solution in sulphuric acid (Sigma).
3. Fiske and Subbarow solution (Sigma). The solution was filtered through a Whatman No. 3 filter paper and the resulting colourless to light-yellow solution was tightly capped and stored in the dark at room temperature.

2.12.5.2 Method

1. To tubes containing 0.1, 0.2, 0.4, 0.6, 0.8 or 1.0 ml aliquots of a sodium phosphate buffer, distilled water was added to a final volume of 4.0 ml. Subsequently, 1.0 ml of molybdate solution and 0.25 ml of Fiske and Subbarow solution was added to each tube.
2. Tubes were mixed and colour was allowed to develop for 10 min. Absorbance of each sample was then measured at 660 nm.

2.13 Enzymatic Digestions of Plasma Membranes

Plasma membranes were digested with several types of enzymes in order to determine the biochemical nature of the constituents responsible for recognition of WHV envelope.

2.13.1 Enzymes, reagents and buffers

1. Heparinase, also called Heparin lyase II or heparinase II from *Flavobacterium heparinum* (100-300 international units (IU)/mg solid; Sigma).
2. Heparitinase I also called heparinase III from *Flavobacterium heparinum* (200-600 IU/mg solid; Sigma).
3. Chondroitinase ABC from *Proteus vulgaris* (0.2-1.0 IU/mg solid; Sigma).
4. Endoglycosidase F/N-Glycosidase F from *Flavobacterium meningosepticum* (600 IU/mg protein; Boehringer Mannheim Biochemica, Germany).
5. Neuraminidase from *Clostridium perfringens* attached to beaded agarose (40 IU/gm agarose; Sigma).
6. Pronase from *Streptomyces griseus* attached to beaded agarose (500-700 IU/gm agarose; Sigma).
7. 150 mM Tris-HCl buffer, pH 7.2, containing 10 mM $CaCl_2$ and 20 mM EDTA (Tris-$CaCl_2$-EDTA buffer).
8. 1% (w/v) n-octyl β-d-glucopyranoside (OGP;Sigma) in 100 mM sodium phosphate buffer, pH 7.0, containing 50 mM EDTA, (phosphate-OGP-EDTA buffer).
9. PBS containing 10 mM EDTA (PBS-EDTA buffer).
10. 45% sucrose in PBS.
11. 1% SDS (w/v) containing 1% 2-mercaptoethanol (SDS-mercaptoethanol).
12. 2 M HCl.

13. BSA (Sigma).

2.13.2 Methods

2.13.2.1 Plasma membrane digestion with heparinase, heparitinase or chondroitinase ABC.

Purified plasma membranes (250 μg protein/sample) were digested for 24 hr with 1 IU of heparinase at 30^{0}C or heparitinase at 42^{0}C or chondroitinase ABC at 37^{0}C in 100 μl of Tris-$CaCl_2$-EDTA buffer. Following these digestions, the samples were boiled for 2 min to destroy the enzymes. As a control, the same amounts of the enzymes were suspended in Tris-$CaCl_2$-EDTA buffer and heated under the same conditions in absence of plasma membrane proteins.

2.13.2.2 Plasma membrane treatment with endoglycosidase F/N-glycosidase F.

In the first step, plasma membranes (250 μg of protein) suspended in 25 μl of Tris-$CaCl_2$-EDTA buffer were denatured by the addition of 25 μl of SDS-mercaptoethanol solution followed by 2 min boiling period. The denatured membranes were then supplemented with 150 μl of phosphate-OGP-EDTA buffer, boiled again for 2 min and cooled to 22^{0}C. Subsequently, the membranes were digested with 1 IU of endoglycosidase F/N-glycosidase F for 24 hr at 37^{0}C and then boiled for 2 min to inactivate the enzyme.

2.13.2.3 Plasma membrane digestion with pronase or neuraminidase.

One mg protein of plasma membranes in PBS-EDTA buffer was digested with 3 IU of pronase immobilized on beaded agarose for 2, 30 or 120 min at 37^{0}C.

Similarly, one mg protein sample of the membranes in PBS-EDTA buffer, previously adjusted to pH 5.0 with 2 M HCl, was digested with 0.6 IU of neuraminidase attached to beaded agarose for 60 min at 37^{0}C. The digested samples were then carefully layered on 400 μl of 45% sucrose in a 1.5 ml Eppendorf tube, and centrifuged in a Savant microfuge (Savant Instruments, Inc., Hicksville, NY) at 1000 x g for 10 min to separate the digested proteins from the enzyme attached to the beads. Protein determination on digested and undigested (control) plasma membrane samples was performed by the Lowry method (1951) using BSA as a standard.

2.14 Determination of WHV Envelope Binding to Hepatocyte Plasma Membranes in a Sedimentation Assay

The principle of this assay was based on our observation that free, radiolabelled WHV envelope particles and purified plasma membranes migrate separately in the 5-45% sucrose gradient, whereas WHsAg radioactivity bound to the membranes co-sediments with the peak of the membrane proteins in the middle of the gradient (Michalak, 1986).

2.14.1 Buffers

1. 5% sucrose (w/w) in 5 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 1 mM PMSF (5% sucrose buffer).
2. 45% sucrose (w/w) in 5 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 1 mM PMSF (45% sucrose buffer).

3. 60% sucrose (w/w) in 5 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 1 mM PMSF (60% sucrose buffer).

2.14.2 Method

1. A linear gradient created by mixing 1.5 ml of the 5% sucrose buffer with an equal volume of 45% sucrose buffer was placed on the top of 0.5 ml of a 60% sucrose cushion in a Beckmann ultra clear (9 x 52 mm) tube.
2. Plasma membranes (1 mg protein/ml), pre-incubated with 450 ng of ^{125}I-WHsAg protein for 1 hr at 22^{0}C, were placed at the top of the gradient and centrifuged at 200,000 x g for 2 hr using a SW 50.1 Beckman rotor.
3. 250 μl fractions were collected from the top of the gradient and their radioactivity content was measured in a gamma counter.
4. In control experiments, HPMs alone, HPMs from chronic carriers incubated with ^{125}I-WHsAg, and ^{125}I-WHsAg alone were fractionated in separate gradients under the same conditions.

2.15 Solubilization of Plasma Membranes with Triton X-114

To produce a water soluble fraction of plasma membranes, the purified membranes were treated with the non-ionic detergent Triton X-114 as described by Bordier (1981).

2.15.1 Solutions

1. 1% Triton X-114 in 10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M

NaCl (1% Triton X-114).

2. 2% Triton X-114 in 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl (2% Triton X-114).

3. 0.06% Triton X-114 in 10 mM Tris-HCl buffer, pH 7.4, containing 6% (w/w) sucrose and 150 mM NaCl (sucrose-Triton X-114).

2.15.2 Methods

1. 3 mg of membrane protein suspended in 1% Triton X-114 was overlayed on 300 μl of a sucrose-Triton X-114 solution in a 1.5 ml Eppendorf tube.

2. The tube was then incubated at 30^{0}C for 3 min. and centrifuged at 300 x g for 3 min at 25^{0}C in a IEC clinical centrifuge.

3. The upper phase was removed from the tube and diluted with an equal volume of 2% Triton X-114 solution, again overlayed on the sucrose cushion mentioned in point 1, incubated at 30^{0}C for 3 min. and centrifuged as previously described.

4. Finally, the upper aqueous phase was collected and its protein content determined.

2.16 Purification of Plasma Membrane Constituents by Affinity Chromatography on a WHsAg#SH-Cellulose Column

Purified serum-derived WHV envelope particles were covalently bound to sulfhydryl (SH) cellulose beads. The resulting beads were used as an affinity matrix to

prepare the column (WHsAg#SH-cellulose affinity column) on which water soluble plasma membrane constituents expressing WHV binding activity were purified.

2.16.1 Materials

1. Aqueous phase of HPMs and KPMs obtained after treatment of the membranes with Triton X-114 as described in the section 2.15.
2. WHV envelope particles isolated from serum of a chronic carrier of WHV according to the procedure described in section 2.2.

2.16.2 Chemicals and solutions

1. Sulfhydryl cellulose beads (SH-cellulose; Sigma).
2. 0.1 M sodium acetate buffer, pH 5.0 (acetate buffer).
3. 0.25 M N-N'-p phenylenedimaleimide prepared by dissolving N-N'-p phenylenedimaleimide in dimethylformamide (phenylenedimaleimide solution).
4. 0.1 M phosphate buffer, pH 7.0, containing 10 mM EDTA (phosphate-EDTA buffer).
5. 10 mM Tris-HCl buffer, pH 7.0, containing 0.14 M NaCl, and 3 mM NaN_3 (Tris buffer).
6. 0.1 M sodium phosphate buffer, pH 7.2 containing 0.14 M NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (washing buffer).
7. 4 M $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.2 (4 M $MgCl_2$ buffer).

8. 1% Triton X-100 in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.14 M NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (1% TX-100 buffer or regeneration buffer).

9. PBS.

10. PBS containing 0.02% NaN_3 (preservation buffer).

2.16.3 Methods

2.16.3.1 Preparation of WHsAg#SH-cellulose column.

1. To 0.5 g of SH-cellulose suspended in 5.0 ml of the acetate buffer was added 2.5 ml of phenylenedimaleimide solution. The suspension was incubated for 1 hr at 30°C. Subsequently, the mixture was supplemented with 20 ml of the phosphate-EDTA buffer and centrifuged at 300 x g using an IEC clinical centrifuge. The resulting pellet was washed five times with the phosphate buffer and finally resuspended in 9.0 ml. of the same buffer.

2. To 3.0 ml of the activated SH-cellulose, 1.3 mg protein of serum-derived WHV envelope particles suspended in 3.5 ml of PBS was added and allowed to react for 20 hr at 25°C while shaking.

3. The suspension was centrifuged at 300 x g using a IEC clinical centrifuge and the supernatant pipetted out and saved.

4. The beads were washed 3 times in Tris buffer and then incubated once again with the supernatant recovered in step 3. for 24 hr at 25°C.

5. Subsequently, the bead suspension was centrifuged at 300 x g for 10 min.

The resulting supernatant was saved and tested for protein content according to the method described by Lowry and colleagues (1951). The pellet (WHsAg#SH-cellulose) was suspended in 5.0 ml of Tris buffer and packed into a 4.0 x 0.5 cm glass column.

2.16.3.2 Chromatography of soluble plasma membranes on a WIIsAg#SII-cellulose column.

1. The column was first equilibrated with 10 ml of washing buffer. Then a 0.4 ml aliquot containing 1 mg of soluble membrane proteins, was applied to the top of the column and allowed to run through it until all of the aliquot had interacted with the matrix. The column was then stopped for 20 min to provide sufficient time for the binding to take place. The material not absorbed was removed from the column by washing with washing buffer, which in turn was applied at an approximate rate of 0.6 ml/min. The wash was reapplied onto the column and allowed to interact with the matrix for an additional 20 min as described above. After the second incubation, the column was washed extensively with the washing buffer until the recorded protein absorbance at 280 nm reached zero.

2. Subsequently, protein bound to the column was eluted with 4 M $MgCl_2$ applied to the column at a flow rate of 0.4 ml/min. One-ml fractions were collected by hand until the absorbance at 280 nm approached zero.

3. The column was then washed with washing buffer and the absorbance of the

washed material was monitored at 280 nm until it approached zero.

4. The fractions eluted with $MgCl_2$ found to have an absorbance at 280 nm were pooled, dialysed against PBS and concentrated using a Micro-ProDi-Con apparatus. Their protein content was determined by the procedure reported by Lowry and co-workers (1951).

5. The binding capacity of the column was regenerated by applying 10 ml of 1% Triton X-100 buffer followed by 20 ml of preservation buffer and kept at 4^0C until further use.

2.17 Purification of Plasma Membrane Constituents by Affinity Chromatography on a Concanavalin A#Sepharose 4B Column

Concanavalin A is known for its affinity to a variety of carbohydrates which include N-linked-mannose rich moieties and glycosaminoglycans. In order to determine if these moieties serve as WHsAg-binding elements, Concanavalin A linked to Sepharose 4B (Concanavalin A#Sepharose 4B affinity column) was used and the aqueous phases of plasma membranes were fractionated on this column.

2.17.1 Material

Aqueous phase of hepatocyte or kidney cell plasma membranes prepared as described in section 2.15.

2.17.2 Chemicals and buffers

1. Concanavalin A attached to CNBr-activated Sepharose 4B beads (Sigma)

2. 0.1 M Tris-HCl buffer, pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$ (wash buffer).

3. 0.3 M methyl-α-D-mannopyranoside in 0.1 M Tris-HCl buffer, pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$ (0.3 M MMP buffer).

4. 1.5 M NaCl in 0.1 M Tris-HCl buffer, pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$ (1.5 M NaCl buffer or regeneration buffer).

5. PBS.

2.17.3 Methods

2.17.3.1 Preparation of a Concanavalin A#Sepharose 4B affinity column.

Concanavalin A attached to CNBr-activated Sepharose 4B beads was packed in a 6.0 x 0.5 cm column. The column was extensively washed with PBS to remove any preservatives and then washed with 20 ml of regeneration buffer. The column was kept at 4^0C until use.

2.17.3.2 Chromatography of plasma membrane soluble fraction on Concanavalin A#Sepharose 4B column

1. Solubilized plasma membranes (1 mg protein/ml) were applied to the column and incubated in a manner similar to that conducted for WHsAg#SH-cellulose column.

2. The column was washed with wash buffer until the absorbance at 280 nm approached zero. Elution of the constituents specifically bound to Concanavalin A was accomplished by applying 0.3 M MMP buffer as 1.0-

ml fractions were collected and their protein content monitored at 280 nm.

3. Fractions containing eluted material were pooled, dialysed and concentrated using a Micro-ProDicon apparatus. The protein content was evaluated by the Lowry *et al.*, (1951) method and the polypeptide profile by SDS-PAGE (section 2.20.). The binding activity for WHsAg was tested with the dot-blot radioligand binding assay described in section 2.19 and in the legend of Figure 16.

4. The column was regenerated through extensive washing with regeneration buffer.

2.18 Purification of Plasma Membrane Constituents by Affinity Chromatography on a Wheat Germ Agglutinin Column

To determine whether terminal sialic acid residues of tested plasma membranes displayed binding activity for WHV envelope, the solubilized membrane proteins were fractionated on a wheat germ agglutinin column (WGA#Sepharose 6MB affinity column).

2.18.1 Material

Aqueous phase of hepatocyte or kidney plasma membrane prepared as described in section 2.15.

2.18.2 Chemicals and buffers

1. Wheat germ agglutinin attached to Sepharose 6MB beads (Sigma).

2. 0.1 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (wash buffer).

3. 0.3 M N acetyl-β-D-glucosamine in 0.1 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (0.3 M AGU buffer).

4. PBS.

2.18.3 Methods

2.18.3.1 Preparation of wheat germ agglutinin#Sepharose 6MB affinity column.

The column was prepared by packing the wheat germ agglutinin attached to Sepharose 6MB beads in a bed volume of 6.0 x 0.5 cm. Then, the matrix was washed extensively with PBS to remove any preservative and kept at 4^0C until it was needed.

2.18.3.2 Chromatography of plasma membranes on wheat germ agglutinin# Sepharose 6MB column

1. The column was equilibrated with wash buffer and an aqueous fraction of Triton X-114 solubilized membranes (1mg protein/ml) was applied to the column. The column was incubated under conditions previously described for the WHsAg#SH-cellulose column.

2. The unbound material was washed out from the column with washing buffer until the absorbance at 280 nm reached zero, whereas the constituents specifically bound to the matrix were eluted with 0.3 M AGU buffer.

3. Protein content in the 1.0 ml fractions collected was monitored at 280 nm.

Fractions demonstrating the highest protein readings were pooled and dialysed.

4. The column was regenerated by an extensive washing with PBS.

2.19 Assessment of WHV Envelope or JP1 Sequence Binding to Plasma Membranes or Heparin in a Nitrocellulose Dot-Blot Radioligand Binding Assay

This assay was developed to test the binding potential exhibited by tissue homogenates, native plasma membrane, plasma membrane fractions resulting from the purification of their water soluble constituents on various affinity columns, and in some cases, subcellular fractions for either WHV envelope or the JP1 sequence. In addition, this assay was used to determine whether heparin, an extracellular sulphated glycosaminoglycan, exhibits binding activity for WHV envelope.

2.19.1 Materials and buffers

1. Nitrocellulose membrane (45 micron pore size; Bio-Rad Laboratories).
2. 96-well microfiltration apparatus (Bio-Dot apparatus; Bio-Rad Laboratories).
3. PBS.
4. 10% fetal calf serum (FCS) in PBS (v/v).

2.19.2 Methods

1. An 8 x 12 cm nitrocellulose sheet was soaked in PBS and assembled in a

microfiltration apparatus.

2. Samples containing the desired amount of protein or heparin were supplemented with PBS for a total volume of 100 μl and placed in the wells of the apparatus. The samples were allowed to filter through the membrane by gravity for 30 min at 22^{0}C. Subsequently, vacuum was applied to the apparatus for a few seconds to dry out the wells.

3. The nitrocellulose sheet was cut into 0.5 cm strips, in such a way that each strip encompassed several dots. The strips were then incubated with 10% FCS for 1 hr at 22^{0}C to block any non-specific binding. Blocking solution was decanted and strips were washed thrice with PBS, for a total of 45 min.

4. The strips were incubated (unless otherwise indicated), either with approximately 3 μg of ^{125}I-labelled WHV envelope protein, 3 μg of ^{125}I-JP1 peptide or 3 μg of ^{125}I-heparin in 1.0 ml of PBS for 1 hr at 22^{0}C, followed by 16 hr at 4^{0}C.

5. The strips were then washed three times for 15 min with PBS, air-dried and exposed to Kodak X-Omat RT for 12 hr.

6. After autoradiography each spot was cut out and the associated radioactivity quantified using a gamma counter. The background radioactivity measured in control dot-blots, which lacked protein or heparin was subtracted from each determination. Alternatively, quantification of the binding activity was determined using serial two-fold dilutions of a given substrate and by

finding the spot with the least amount of protein where binding was still observed on an autoradiogram.

2.20 Polyacrylamide Gel Electrophoresis and Gel Stainings

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was essentially performed according to the procedure of Laemmli (1970).

2.20.1 Reagents and solutions

1. 30% acrylamide (w/v), 0.8% methylene bisacrylamide (w/v) in distilled water (acrylamide-Bis).
2. 0.4% SDS (solubilized in distilled water; w/v) in 1.5 M Tris-HCl buffer, pH 8.8 (lower Tris).
3. 0.4% SDS (solubilized in distilled water; w/v) in 0.5 M Tris-HCl buffer, pH 6.8 (upper Tris).
4. 0.1% SDS (solubilized in distilled water; w/v) in 95.91 mM glycine and 12.39 mM Tris buffer (reservoir buffer).
5. 12.5 % upper Tris (v/v) in distilled water, containing 10% glycerol (v/v), 5% 2-mercaptoethanol (v/v), 30% SDS (v/v) (sample buffer).
6. 75% glycerol in PBS (v/v).
7. 3-5 mg ammonium persulfate in 5 ml of distilled water (ammonium persulfate solution).
8. N,N,N'N'-Tetramethylethylenediamine (Temed; Bio-Rad Laboratories)

9. 0.1% 3'5'5'5'-Tetrabromophenol sulfonylphthalein in PBS (bromophenol blue tracking dye; Sigma).

2.20.2 Materials

1. Gradient maker (Pharmacia Fine Chemicals).
2. Protean II slab cell apparatus (Bio-Rad Laboratories).
3. Mini-Protean II dual slab cell apparatus (Bio-Rad Laboratories).
4. Gel destainer (Bio-Rad Model 556, Bio-Rad Laboratories).
5. Slab gel dryer (Hoefer Scientific Instruments., San Francisco, CA).
6. SDS-PAGE molecular weight standards-high range (Pharmacia Fine Chemicals).
7. SDS-PAGE molecular weight standards-low range (Bio-Rad Laboratories).

2.20.3 Methods

2.20.3.1 Preparation of continuous 5-16% SDS-polyacrylamide gels.

1. Two gel solutions 5% and 16% were prepared. The 5% gel solution was made using, 4.5 ml of acrylamide, 3.0 ml of distilled water, 28 μl ammonium persulphate and 9 μl of Temed. The 16% gel solution was prepared by addition of 3.9 ml of 75% glycerol, 28 μl of ammonium persulphate and 9 μl of Temed to 4.5 ml of acrylamide.
2. A 5-16% acrylamide gradient was made by mixing the 5% and 16% gel solutions in the gradient maker and pouring the mixture into the gel sandwich of a Protean II slab cell apparatus at a rate of 0.31 ml/min.

3. Once the 16 x 20 cm gel was polymerized the stacking gel solution was prepared by mixing 1.0 ml of acrylamide, 2.5 ml of upper Tris, 6.4 ml of distilled water, 150 μl of ammonium persulphate and 10 μl of Temed.

4. Immediately after the stacking gel solution was poured onto the polymerized separating gel, a comb was inserted into this solution.

5. After polymerization (about 20 min), the comb was removed by pulling straight up to avoid disturbing the well dividers.

6. Protein samples predestined for loading, (30-50 μg protein) were diluted with an equal volume of sample buffer to a total volume of 100 μl and boiled for 2 min. In addition, 25 μl aliquots of low and high range molecular weights standards diluted in PBS (1:20 v/v) and supplemented with an equal volume of sample buffer were run on each gel. Ten μl aliquots of tracking dye was added to each sample before loading on the gel.

7. Samples were run at a constant current of 25 mA, until the tracking dye reached the top of the separating gel, then the current was increased to 40 mA.

2.20.3.2 Preparation of 8% SDS-polyacrylamide gel.

1. The separating gel solution was prepared by mixing 2.66 ml of 30% acrylamide, 2.5 ml of lower Tris, 4.69 ml of distilled water, 150 μl of ammonium persulphate, and 5 μl of Temed. The mixture was then poured

into the assembled gel sandwich of a Mini-Protean II dual slab cell, overlayed with distilled water, and allowed to set for 30 min. Following polymerization of the gel, filter paper was used to removed the excess of distilled water. The stacking gel solution was then prepared as described above.

2. The stacking solution was prepared and wells were formed following the procedure described above.

3. Ten μg protein samples were diluted with an equal volume of sample buffer for a total volume of 20 μl and boiled for 2 min. High molecular weight standards were prepared by adding a 5 μl aliquot of a stock solution diluted 1:20 (v/v) in PBS to an equal volume of the sample buffer. Once the samples were cooled, 5 μl of tracking dye was added.

4. Proteins were electrophoretically separated at a constant voltage of 200 mV until the tracking dye reached the bottom of the separating gel.

2.20.3.3 Preparation of 10% SDS-polyacrylamide gel.

This gel was prepared using the procedure described for the 8% SDS polyacrylamide gel, except that the separating solution consisted of 3.33 ml of acrylalmide, 2.5 ml lower Tris, 4.02 of distilled water, 150 μl of ammonium persulphate and 5 μl of Temed.

2.20.3.4 Coomassie brillant blue staining of polyacrylamide gels.

Staining of gels for protein content was performed through the use of

Coomassie brillant blue solution as described below.

2.20.3.4.1 Reagents and solutions

1. 25% 2-propanol (v/v) in 10% acetic acid (fixative).
2. 0.05% Coomassie brilliant blue R-250 (w/v) in 25% 2-propanol (v/v) and 10% acetic acid (staining solution).
3. 10% methanol (v/v) in 7.5% acetic acid (destainer solution).

2.20.3.4.2 Method

1. Following electrophoresis, the gels were soaked with staining solution for 30 min at 22°C.
2. After staining, gels were decolorized in the presence of an excess of destaining solution in a gel destainer apparatus.

2.20.3.5 Periodic acid-Schiff staining of SDS-polyacrylamide gels.

Staining of gels for carbohydrate content was done using periodic acid-Schiff staining method as described by Fairbanks and colleagues (1971).

2.20.3.5.1 Reagents and solutions

1. 25% 2-propanol (v/v) and 10% acetic acid (v/v) in water (fixative).
2. 0.5% periodic acid (w/v) in water.
3. 0.5% sodium arsenate (w/v) in 5% acetic acid.
4. 0.1% sodium arsenate (w/v) in 5% acetic acid.
5. 5% acetic acid.
6. 0.1% sodium metabisulphite (w/v) in 0.01 M HCl (metabisulphite/HCl).

7. 200 ml of Schiff reagent (Fisher Scientific Company, Fair Lawn, NJ), decolorized with 2 g of activated charcoal, stirred and filtered.

2.20.3.5.2 Method

1. The gel was submersed in the fixative solution for 2 hr at 22^{0}C. The fixative was decanted and the gel was incubated with 0.5 % periodic acid for a further 2 hr, while shaking.
2. The gel was then washed using the following cycle:
   a) 0.5% sodium arsenite-acetic acid for 60 minutes (twice)
   b) 0.1% sodium arsenite-acetic acid for 20 minutes (twice)
   c) 5% acetic acid for 10-20 min.
3. Following this washing the gel was transferred to a tray containing 20 ml of Schiff's solution and left to stand overnight at 4^{0}C.
4. The staining reagent was decanted and the gel was washed with metabisulphite/HCl, until addition of formaldehyde to the washing solution did not turn it pink.

2.21 Virus Overlay Protein Blot Assay (VOPBA)

Proteins separated by SDS-PAGE were transferred onto nitrocellulose using the Trans-Blot apparatus (Bio-Rad Laboratories) under the conditions recommended by the supplier.

2.21.1 Buffers

1. 25 mM Tris-HCl buffer, pH 8.3, containing 192 mM glycine and 20% methanol (v/v; Tris-Glycine).

2. PBS.

3. 10% fetal calf serum (FCS) in PBS (v/v).

2.21.2 Materials

1. Nitrocellulose membrane (45 micron pore size; Bio-Rad Laboratories).

2. Bio-Rad Trans-Blot cell apparatus (Bio-Rad Laboratories).

2.21.3 Method

1. The gel (5 x 8 cm) and a sheet of nitrocellulose (8 x 10 cm) were first equilibrated with Tris-glycine transfer buffer for 30 min at 22^{0}C.

2. Subsequently, the nitrocellulose membrane was laid over the gel and sandwiched between filter paper, porous pads and plastic supports of the apparatus. The nitrocellulose-gel sandwich was then assembled in a Trans-Blot cell apparatus.

3. The proteins were electrophoretically transferred from the gel onto nitrocellulose at a constant current of 0.40 A for 8 hr at 4^{0}C.

4. The resulting blot was air-dried and then incubated with 1% FCS in PBS for 15 min at 22^{0}C and placed in a plastic bag containing fresh 1% FCS in PBS.

5. Subsequently, a working dilution of the radiolabelled ligand (*i.e.*, 3 μg of radiolabelled WHV envelope, ^{125}I-heparin or ^{125}I-WHsAg protein

preincubated with heparin) suspended in a total volume of 3 ml of 1% FCS in PBS was added to the bag. The bag was sealed and incubated for 1 hr at 22^{0}C and then 16 hr at 4^{0}C.

6. The nitrocellulose membrane was removed from the bag, washed with PBS three times for 15 min and air-dried. Autoradiography was performed by exposing the membrane to a Kodak X Omat RT film for 12 hr.

2.22 Evaluation of the Inhibitory Activity of Heparin on the WHV Envelope Binding to Plasma Membranes

To assess the ability of heparin to block the interaction between WHV envelope subviral particles and plasma membranes, ^{125}I-WHsAg (3 μg protein/100 μl) aliquots were preincubated with 1 or 5 mg heparin/sample for 1 hr at 22^{0}C and 4 hr on ice, followed by extensive dialysis against PBS to remove excess heparin. Then, the dialysed mixtures were separately incubated with nitrocellulose strips containing dots of HPM proteins for 1 hr at 22^{0}C, followed by 16 hr at 4^{0}C, washed and exposed to autoradiography for 12 hr as described in section 2.19. Likewise, nitrocellulose blots of plasma membrane proteins transferred from 10% SDS-polyacrylamide gels were incubated with a mixture of ^{125}I-WHsAg preincubated with cold heparin. This mixture was obtained by incubating ^{125}I-labelled WHsAg (3 μg protein/sample) with cold heparin (1 mg/sample) for 1 hr at 22^{0}C and then 4 hr at 4^{0}C. Finally, the mixture was adjusted to 3.0 ml with PBS and extensively dialysed against PBS. The protein blots were

incubated for 1 hr at 22^{0}C and 16 hr at 4^{0}C with the mixture, washed and exposed to autoradiography for 12 hr as described in section 2.21.

CHAPTER 3

3. RESULTS

3.1 Purity of the Isolated Plasma Membranes

To assess the purity of the isolated woodchuck hepatocyte, spleen and kidney plasma membranes, characterization of the activities of four marker enzymes was performed. The activities of 5'-nucleotidase and Na^+-K^+-ATPase were tested because these enzymes have been found to be reliable indicators of plasma membrane purity, whereas glucose-6-phosphatase and succinate cytochrome C reductase activities were evaluated as markers of endoplasmic reticulum (ER) and mitochondria, respectively. These two last enzymes are commonly used as indicators of organelle contamination in plasma membrane preparations (Touster and coworkers, 1970). The results of these assays are presented in Tables 1, 2 and 3. As expected, the yield and relative enrichment for enzymes specific to plasma membranes, were significantly higher in the purified membrane preparations than those detected in the cell and tissue homogenates, originally used for the membrane isolations. In contrast, the presence of glucose-6-phosphatase and succinate cytochrome C reductase in the purified plasma membranes was found to be only marginal and non-existent, respectively. In other words, the results of these enzymatic tests revealed that the isolated membranes were essentially free of organelle contamination stemming from the endoplasmic reticulum and mitochondria. Thus, these membranes showed a high degree of purity according to the generally

Table 1. Activity of marker enzymes in woodchuck hepatocyte plasma membrane preparations.

Enzyme	Enzyme yield[a,b] (%)	Relative enrichment[a,c]
5′ Nucleotidase	10.79 ± 4.10	17.40 ± 3.32
Na^+-K^+-ATPase	8.96 ± 1.84	8.05 ± 1.84
Glucose-6-phosphatase	1.53 ± 0.51	1.55 ± 0.40
Succinate cytochrome C reductase	n.d.[d]	n.d.[d]

[a] Statistics refer to the means ± the standard deviation of three determinations.

[b] The yield of each enzyme marker in the final membrane preparations is given as a % of its total activity, as detected in the initial hepatocyte homogenate.

[c] Relative enrichment of a given enzyme is defined as the ratio of its specific activity in plasma membranes to its specific activity detected in hepatocyte homogenate, the latter being normalized to 1.

[d] n.d., not detected, whereas in the hepatocyte homogenate there was 3.11×10^{-2} μmol.min^{-1}.mg protein^{-1}.

Table 2. Activity of marker enzymes in woodchuck spleen plasma membrane preparation.

Enzyme	Enzyme yield[a,b] (%)	Relative enrichment[a,c]
5′ Nucleotidase	10.25	12.10
Na^+-K^+-ATPase	12.10	11.40
Glucose-6-phosphatase	1.31	1.35
Succinate cytochrome C reductase	n.d.[d]	n.d.[d]

[a] The results are from a single determination of enzyme activity in the isolated membranes.
[b] The yield of each enzyme marker in the final membrane preparation is given as a % of its total activity, as detected in the initial spleen homogenate.
[c] Relative enrichment of a given enzyme is defined as the ratio of its specific activity in the plasma membrane preparation to its specific activity detected in the spleen homogenate, the latter being normalized to 1.
[d] n.d., not detected, whereas in the spleen homogenate there was 1.78×10^{-2} $\mu mol.min^{-1}.mg\ protein^{-1}$.

Table 3. Activity of marker enzymes in woodchuck kidney plasma membrane preparation.

Enzyme	Enzyme yield[a,b] (%)	Relative enrichment[a,c]
5′ Nucleotidase	9.12	15.30
Na^+-K^+-ATPase	7.25	11.40
Glucose-6-phosphatase	1.20	1.25
Succinate cytochrome C reductase	n.d.[d]	n.d.[d]

[a] The results are from a single determination of enzyme activity in the isolated membranes.
[b] The yield of each enzyme marker in the final membrane preparation is given as a % of its total activity, as detected in the initial kidney homogenate.
[c] Relative enrichment of a given enzyme is defined as the ratio of its specific activity in plasma membrane to its specific activity detected in the kidneyy homogenate, the latter being normalized to 1.
[d] n.d., not detected, whereas in the kidney homogenate there was 2.43×10^{-2} μmol.min^{-1}.mg protein^{-1}.

accepted biochemical criteria.

3.2 Purity of the WHV Envelope Preparation

Serum-derived WHsAg particles purified by column chromatography and isopycnic centrifugation in cesium chloride gradients were examined by SDS-PAGE and electron microscopy in order to assess their polypeptide composition, overall purity and morphological homogeneity. Analysis of the SDS-polyacrylamide gels revealed an array of bands, which indicated that all constituent proteins of the WHV envelope were present in the purified preparation. In Figure 2, the proteins with molecular weight of 24 and 27-kD (native and a glycosylated form of the major or S protein), 33-kD (middle or preS2 protein) and the 39 and 42-kD (native and glycosylated form of large or preS1 protein) are clearly shown. In addition, the 52- and 58-kD, and 67- and 74-kD band pairs are also visible. These proteins likely represent dimers of 24- and 27-kD and 34- and 36-kD proteins, respectively. The 36-kD protein was not detected in a monomeric form in this particular WHsAg preparation. Fractions 6 to 10 resulting from the fractionation of WHsAg on a CsCl gradient were combined and analysed by SDS-PAGE (Figure 2, lane C). These fractions demonstrated the presence of all constituents polypeptides of the WHV and they were subsequently used as a ligand in the binding experiments described in this thesis. Results of ultrastructural examination, shown in Figure 3, revealed that the WHsAg preparation contained mainly spherical particles measuring 22 nm in diameter and some filaments of the same diameter approximating

Figure 2. SDS-polyacrylamide gel of purified serum-derived WHV envelope particles. Fractions 1 to 5 (lane B), 6 to 10 (lane C), 11 to 16 (lane D) collected from the top of a CsCl gradient were subjected to electrophoresis in a 5-16% SDS-polyacrylamide gel as described in section 2.20.3.1. Protein bands were stained with 0.05% Coomassie brilliant blue. Numbers on the left side of the panel indicate molecular weight standards in kD (lane A), whereas those on the right side indicate molecular size of the peptides detected in the WHV envelope preparation.

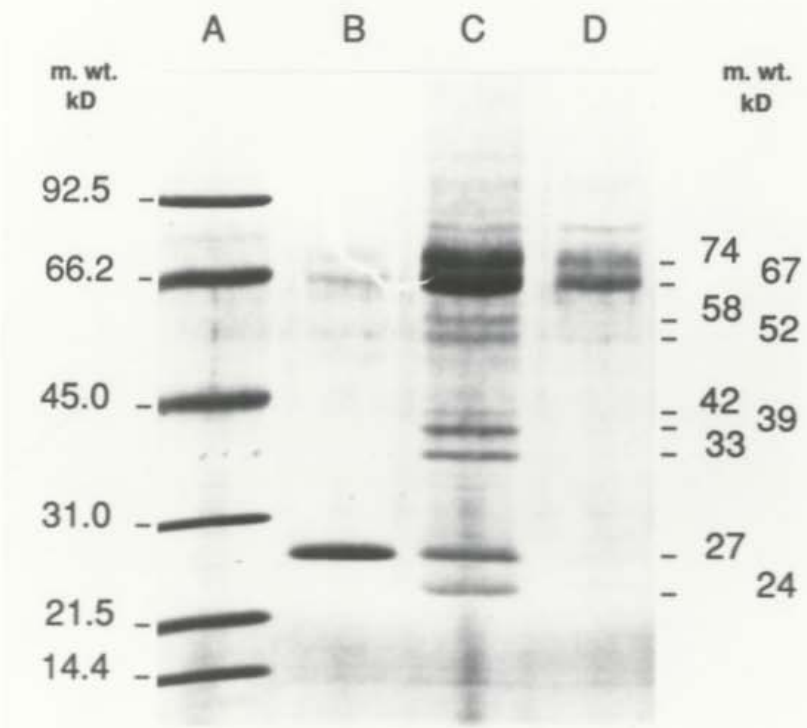
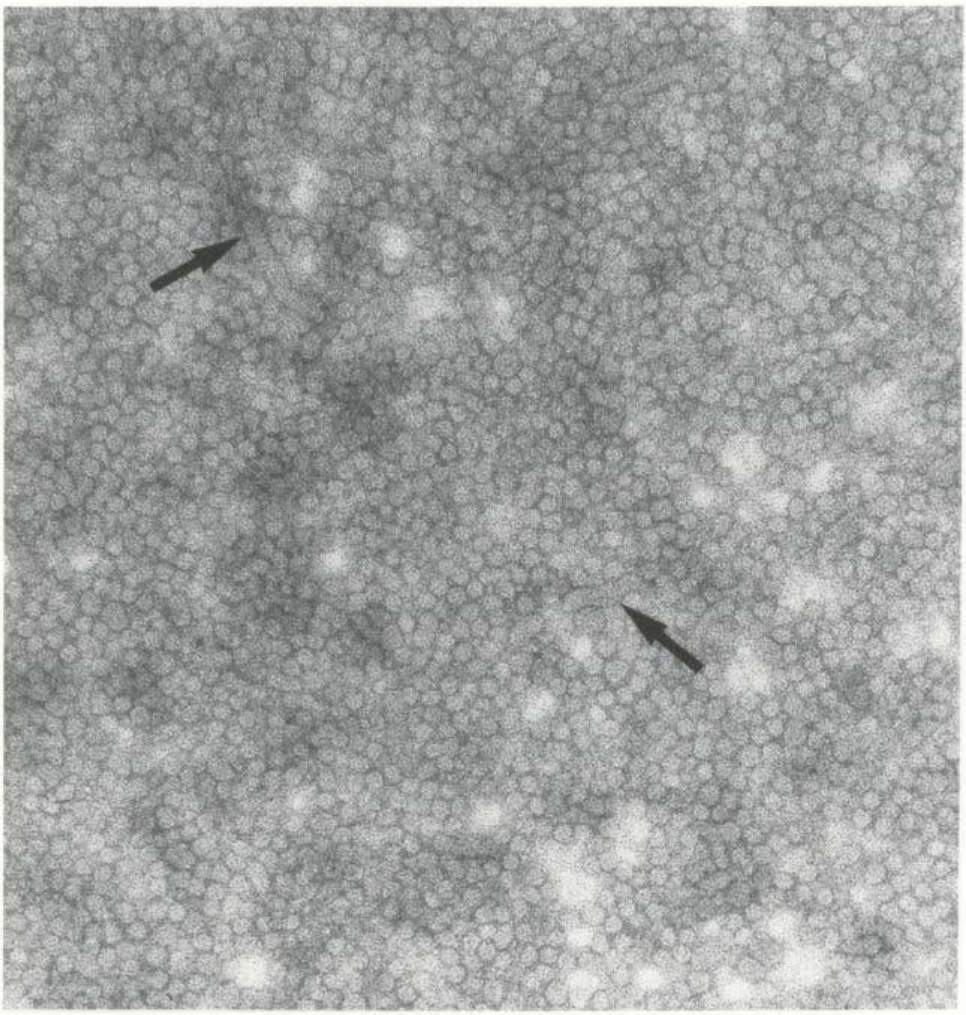

A
B
C
D
m. wt.
kD
92.5
66.2
45.0
31.0
21.5
14.4
m. wt.
kD
74
67
58
52
42
39
33
27
24

Figure 3. Electron micrograph of purified serum-derived WHV envelope particles. The micrograph demonstrates the presence of numerous spherical and few filamentous particles (arrows) recovered from fractions 6 to 10 following centrifugation on a CsCl gradient (see Figure 2). The preparation was negatively stained with 1% phosphotungstic acid. x 34,700.



60 to 250 nm in length.

3.3 WHV Envelope Binding to Normal and WHV-Infected Hepatocyte Plasma Membranes

The WHV envelope binding ability to normal woodchuck HPMs and HPMs isolated from an animal chronically infected with WHV was first tested using radiolabelled WHsAg and a sedimentation assay described in section 2.14. Prior to this experiment, in order to determine the most appropriate incubation time for the binding, the ^{125}I-labelled envelope particles were incubated with the membranes at different time periods at room temperature and then fractionated across a 5-45% sucrose gradient (Figure 4). The experiment demonstrated that an incubation time of 60 or 90 min resulted in approximately the same degree of binding of WHsAg to HPMs, and the cpm values obtained were clearly higher than that observed after an incubation time of 30 min. Thus, incubations of ^{125}I-WHsAg with plasma membranes in subsequent experiments were performed for 60 min at room temperature.

The extent of WHsAg binding to normal and infected woodchuck HPMs was investigated by fractionation of plasma membranes incubated with ^{125}I-WHsAg across a 5-45% sucrose gradient. Free ^{125}I-WHsAg, not incubated with HPMs, remained at the top of the gradient and was detectable in fractions from 1 to 9 (Figure 5). Analysis of the ^{125}I-WHsAg distribution after the incubation of the labelled envelope particles with normal HPMs revealed that some WHsAg radioactivity sedimented in the middle of the

Figure 4. Time dependence of WHV envelope binding to normal woodchuck hepatocyte plasma membranes. One mg of membrane protein was incubated with a constant amount of ^{125}I-WHsAg (450 ng protein) for the indicated time periods and the mixture was then fractionated on a 5-45 % sucrose gradient. WHsAg radioactivity bound to HPM proteins recovered in fractions 10-14 of the gradient was determined as described in "Materials and Methods".

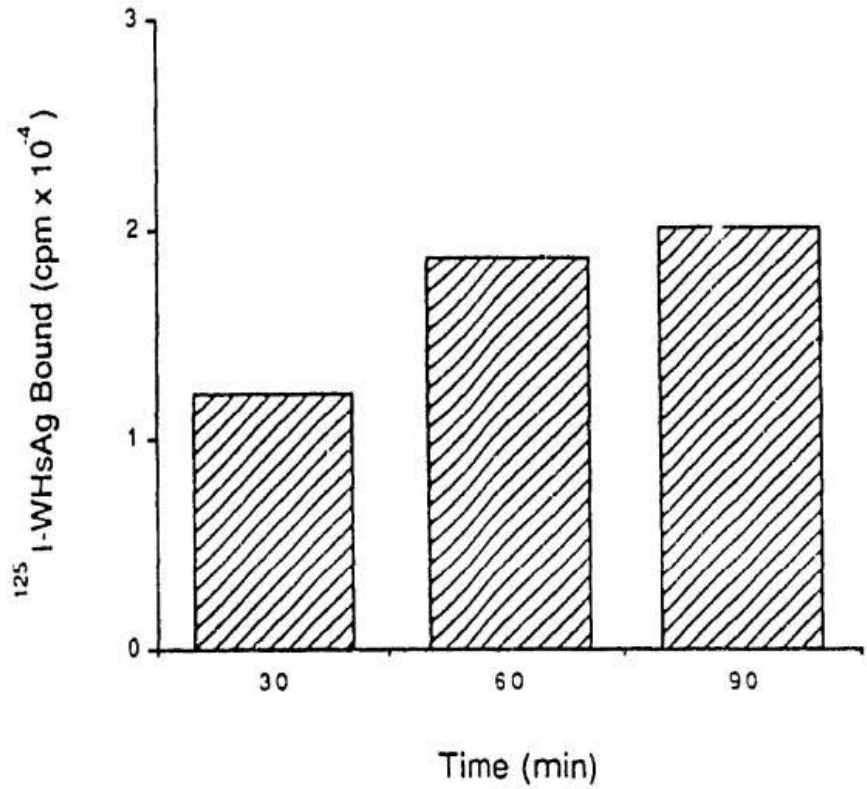
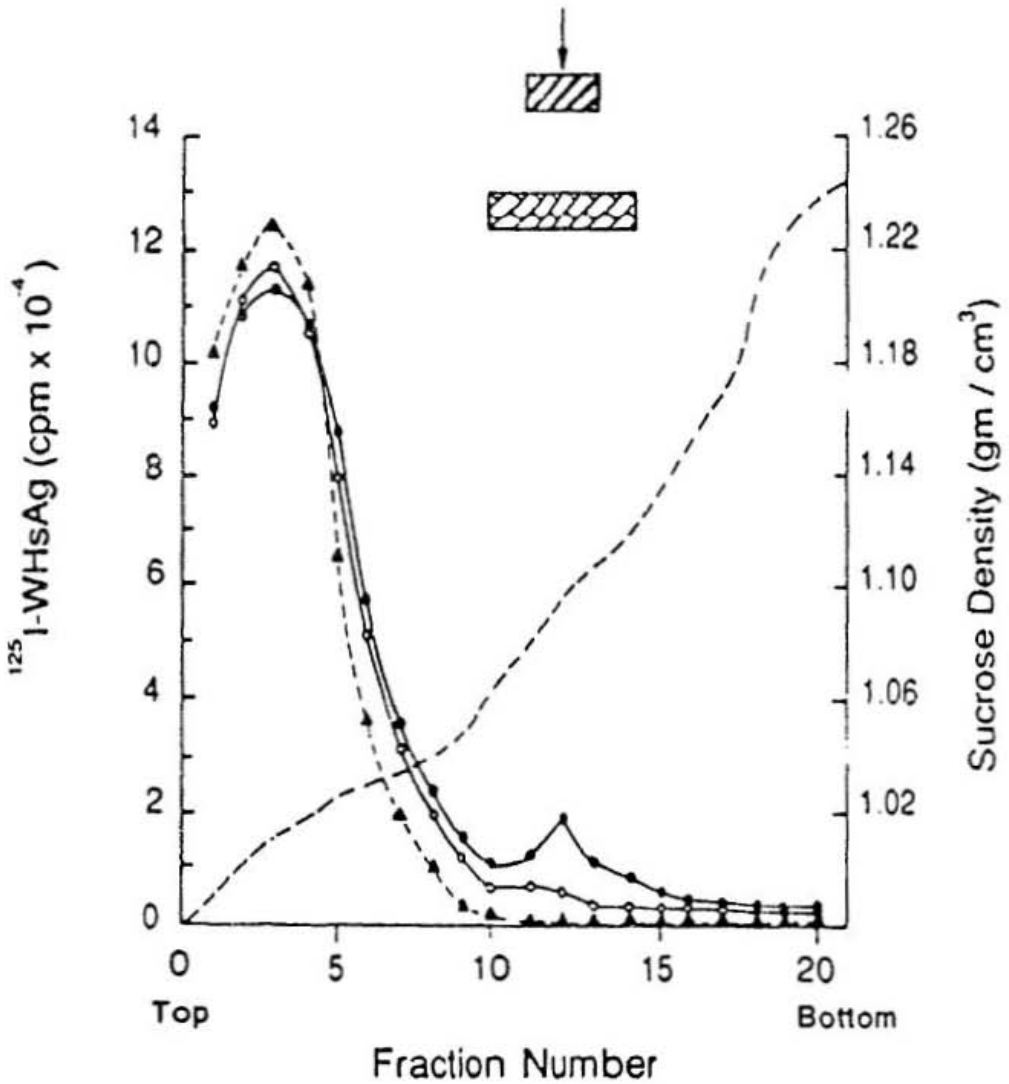

3
2
1
0
^{125}I-WHsAg Bound (cpm x 10^{-4})
30
60
90
Time (min)

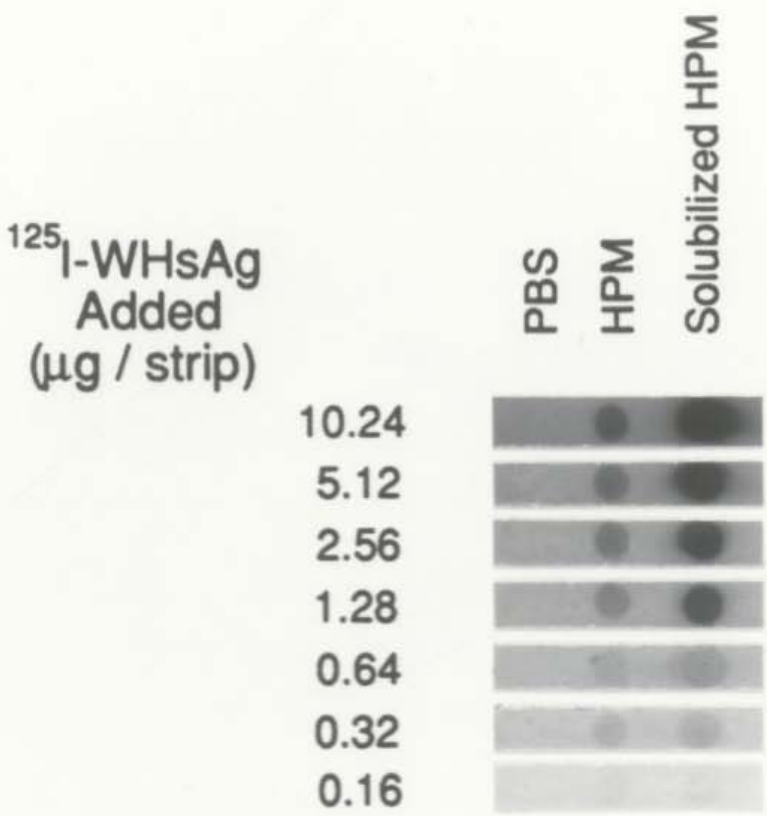
Figure 5. Distribution of free WHsAg and WHsAg incubated with purified woodchuck hepatocyte plasma membranes in a 5-45% sucrose gradient. Normal and WHV-infected hepatocyte plasma membranes were incubated with ^{125}I-labelled WHsAg and fractionated in a sucrose gradient as described in "Materials and Methods". The symbols represent radioactivity distribution of free ^{125}I-WHsAg (triangles), ^{125}I-WHsAg incubated with normal membranes (filled circles), and ^{125}I-WHsAg incubated with infected membranes (empty circles). Tile-shaded box represents the fractions which encompass the plasma membrane protein peak. Striped-shaded box represents the plasma membrane fractions positive for WHsAg, whereas the arrow indicates the fraction where both the highest membrane protein content and WHsAg activity were detected.


^{125}I-WHsAg (cpm x 10^{-4})
Sucrose Density (gm / cm^3)
Fraction Number
Top
Bottom
0
2
4
6
8
10
12
14
1.02
1.06
1.10
1.14
1.18
1.22
1.26
0
5
10
15
20

gradient (fractions 11-13; stripe-shaded box; Figure 5). Moreover, when all fractions were assayed for protein content, only those recovered from the middle of the gradient exhibited high levels of protein (tile-shaded box; Figure 5). This suggested that WHV envelope particles bind to normal HPMs. In contrast, when HPMs derived from a woodchuck chronic carrier of WHV were incubated with the labelled WHV envelope, binding of ^{125}I-WHsAg was not detected (Figure 5). This result remains in agreement with the finding previously described in this laboratory (Michalak, 1988). It was postulated in the latter study that receptors for WHV present on hepatocyte plasma membranes of animals persistently infected with the virus may already be occupied by the virus envelope proteins or be modified due to virus protein insertion into the membrane, therefore, they would be unable to interact with exogenous WHV envelope particles. The behaviour demonstrated by infected HPMs has been investigated and described in detail in other studies (Michalak and Churchill, 1988; Michalak *et al.*, 1989; Michalak *et al.*, 1990; Lin and Michalak, 1991). These studies led to characteri-zation of the interactions between the WHV proteins and structural constituents of the hepatocyte plasma membrane in sequential stages of WHV-induced hepatitis and in hepatocellular carcinoma.

Based on the preliminary findings obtained by employing the sedimentation assay, a nitrocellulose dot-blot radioligand binding assay was used to determine the manner in which WHsAg-HPM binding is accomplished. In Figure 6, the binding of ^{125}I-WHsAg to native HPMs, derived from a healthy woodchuck, and to water-soluble proteins,

Figure 6. Binding of increasing concentrations of WHV envelope proteins to native and solubilized hepatocyte plasma membranes in a nitrocellulose dot-blot radioligand binding assay. Five μg protein samples of native (untreated) membranes (HPM) and water-soluble HPMs resulting from Triton X-114 extraction (solubilized HPM) were spotted onto nitrocellulose strips and incubated with the indicated amounts of ^{125}I-WHsAg. Background was visualized using PBS instead of plasma membranes.


^{125}I-WHsAg Added (µg / strip)
PBS
HPM
Solubilized HPM
10.24
5.12
2.56
1.28
0.64
0.32
0.16

resulting from the treatment of these HPMs with Triton X-114, increases as the concentration of labelled WHV envelope is raised.

Measurements of the WHsAg radioactivity bound to dot-blots of native HPMs and water-soluble HPM proteins demonstrated that both HPM preparations displayed saturable binding for ^{125}I-WHsAg (Figure 7). Furthermore, these data also demonstrate that solubilized HPMs evidently bind more WHsAg than do intact HPMs at comparable protein concentrations.

Preincubation of native HPMs with 0.5 to 2.5 μg protein from unlabelled WHV envelope particles partially inhibited the binding of ^{125}I-WHsAg (Figure 8). However, when HPMs were preincubated with concentrations higher than 5 μg of unlabelled WHV envelope particles, the block was not observed, instead the binding increased. This finding is consistent with the possibility that WHsAg particles could form self-aggregates when high concentrations of WHsAg were used. This notion was supported by the observation that cold WHsAg alone at concentrations higher than 3μg was able to bind ^{125}I-WHsAg in a nitrocellulose dot-blot radioligand binding assay (data not shown). For this reason, when ^{125}I-WHsAg was employed as a ligand in binding experiments its concentration did not exceed 3 μg.

The interaction between WHV envelope particles and native HPMs appear to be ruled by binding kinetics which could be biphasic in nature (Figure 9). Hence, for HPM protein concentrations below 2.5 μg a concentration-dependent curvilinear pattern of binding was observed. On the other hand, when higher membrane protein concentrations

Figure 7. Binding kinetics of increasing concentrations of WHV envelope proteins to constant amounts of native and water-soluble hepatocyte plasma membrane proteins. Five μg protein samples of intact HPMs and HPMs solubilized with Triton X-114 were spotted on nitrocellulose and incubated with increasing amounts of ^{125}I-WHsAg (specific activity 2.66 x 10^7 cpm/μg protein) as described in "Materials and Methods". The symbols represent the binding of ^{125}I-WHsAg to native HPMs (full circles) and solubilized HPMs (empty circles). Control dots incubated with PBS were used as negative controls to determine background binding. The points are the values obtained after subtraction of background binding.

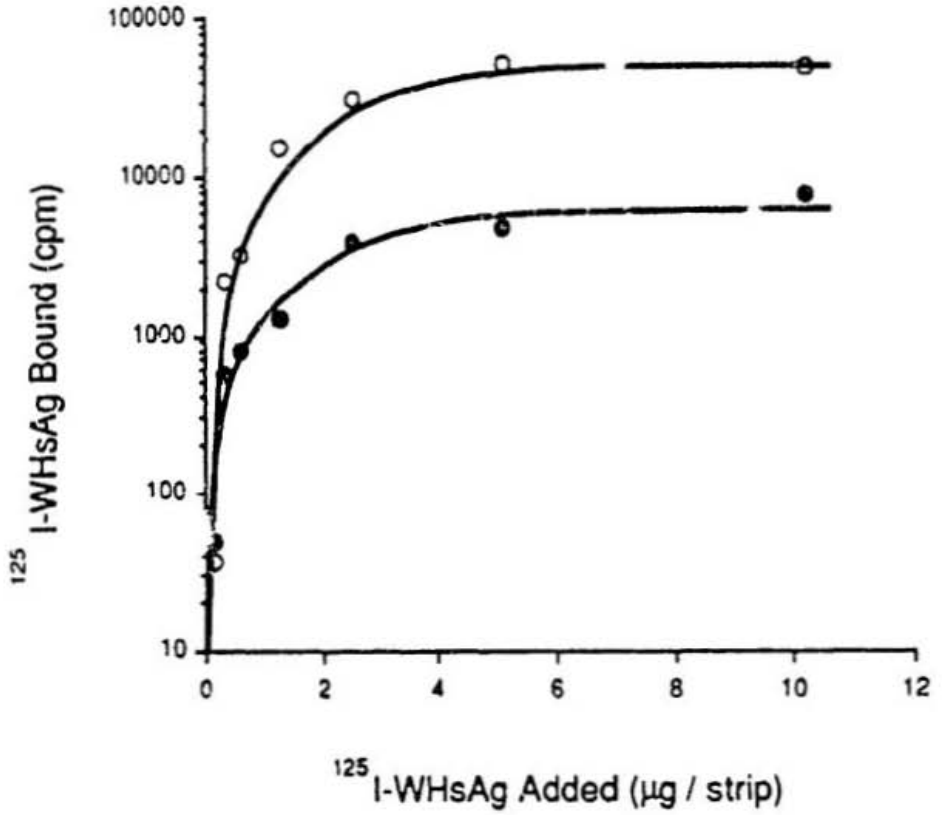

100000
10000
1000
100
10
0
2
4
6
8
10
12
125 I-WHsAg Bound (cpm)
125 I-WHsAg Added (µg / strip)

Figure 8. Effect of preincubation of hepatocyte plasma membranes with unlabelled WHV envelope particles on the binding of ^{125}I-WHsAg. Five μg of HPM proteins were preincubated with the indicated amounts of cold WHsAg proteins. The mixtures were spotted onto nitrocellulose, incubated with a constant amount of ^{125}I-WHsAg (3 μg protein), washed and exposed for autoradiography. Blots incubated with PBS instead of the membrane-WHsAg mixtures were included as negative controls to determine background binding. The relative WHsAg binding was calculated by dividing the net cpm (*i.e.*, after subtraction of background binding) of the ^{125}I-WHsAg bound to the HPM preincubated with cold WHsAg by the net cpm of the ^{125}I-WHsAg bound to HPM in the absence of cold WHsAg. The final relative binding was obtained from the ratio (R) by calculating 1-R. Negative values indicate that cold WHsAg prevented ^{125}I-WHsAg binding, whereas positive values suggest that self-aggregation of WHsAg particles took place.

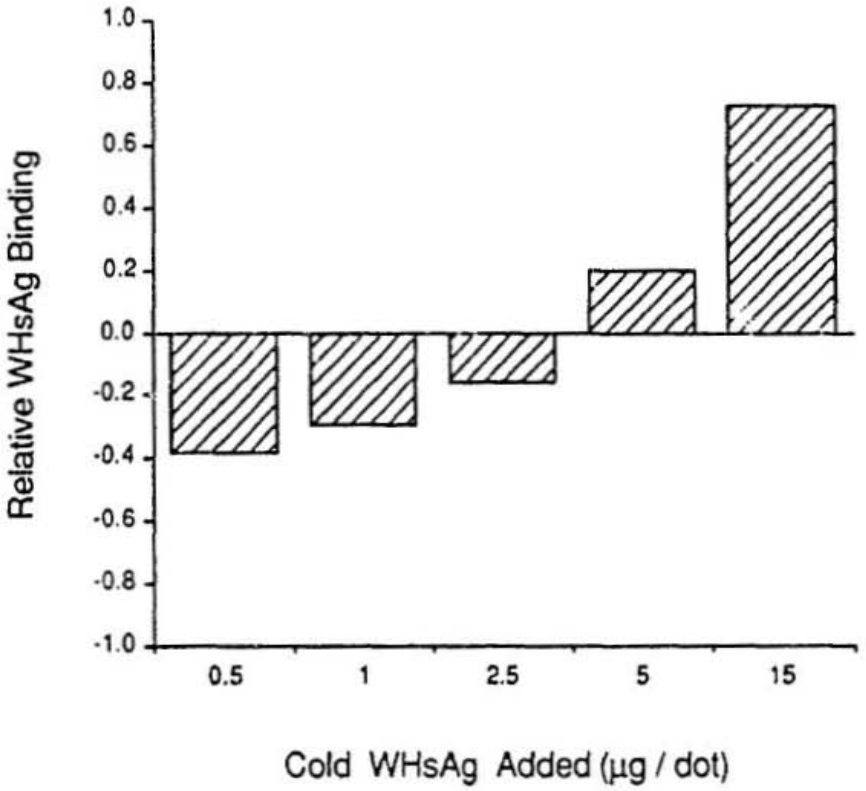
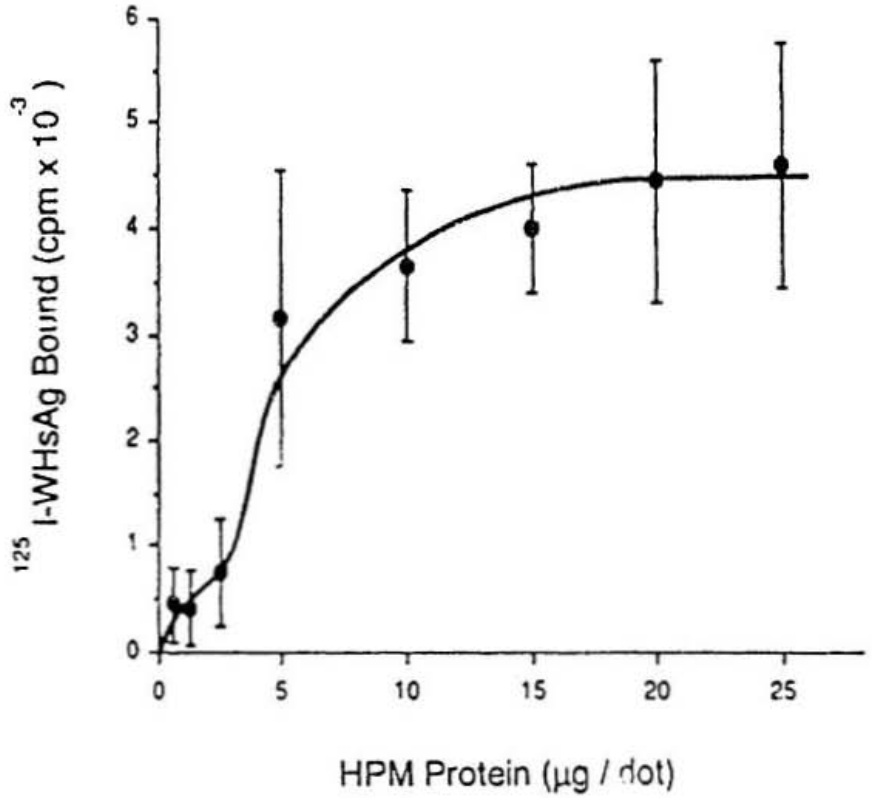

Relative WHsAg Binding
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
0.5
1
2.5
5
15
Cold WHsAg Added (µg / dot)

Figure 9. Binding kinetics of constant amounts of WHV envelope proteins to increasing concentrations of hepatocyte plasma membrane proteins. Samples of purified woodchuck HPMs containing the indicated amounts of protein were spotted onto nitrocellulose and incubated with ^{125}I-labelled WHsAg (3 μg protein) as described in "Materials and Methods". Control dots incubated with PBS were used as negative controls to determine background binding. The points are the mean values of cpm from three separate experiments after subtraction of background binding. Bars represent standard deviations of the means.


^{125}I-WHsAg Bound (cpm x 10^{-3})
0
1
2
3
4
5
6
0
5
10
15
20
25
HPM Protein (µg / dot)

were used (above 2.5 μg) a second component of the binding occurred. This pattern of binding kinetics suggests that the interaction was mediated by two classes of saturable binding sites with different affinities for WHsAg. However, since the binding kinetics were evaluated using a nitrocellulose dot-blot radioligand binding assay it was not technically possible to determine the amounts of unbound ^{125}I-WHsAg. Thus, the Scatchard analysis of the data could not be performed. It is necessary to mention that an effort to assess quantities of unbound ^{125}I-WHsAg using the sedimentation assay was also not successful.

3.4 WHV Envelope Binding to Water-Soluble HPM Constituents

The study of receptors is best accomplished when the molecules of interest can be released from their cellular structures in a water-soluble form so that they can be purified to homogeneity. Ionic and non-ionic detergents are among the most commonly used agents for this purpose. In this study, Triton X-114 was chosen primarily because solubilization of plasma membranes with this mild non-ionic detergent results in the reproducible separation of membrane proteins into a detergent-rich phase and an aqueous phase containing hydrophobic and hydrophillic proteins, respectively (Bordier, 1981). The detergent phase contains proteins spanning and tightly (*e.g.*, covalently) bound to the membrane lipid bilayer, called integral membrane proteins. In contrast, the aqueous phase predominantly contains peripheral proteins of hydrophillic nature (water-soluble proteins), loosely associated with the membrane lipid core. Since it has been documented

that the aqueous phase of cellular membranes carries receptors of physiological importance, for example quisqualate receptor (Chang *et al.*, 1991) and the urokinase plasminogen activator receptor (Behrendt *et al.*, 1991), it was reasonable to expect that the HPM aqueous phase should also express binding activity for the WHV envelope. A preliminary observation demonstrating that water-soluble proteins of HPMs not only bind WHsAg, but also that the binding is more efficient than for intact (untreated) HPMs (Figure 6), supported this possibility. Further, when increasing concentrations of intact and Triton X-114-solubilized HPMs were incubated with a constant amount of ^{125}I-WHsAg (Figure 10), it was clearly observed that the water-soluble HPM proteins bind significantly more ^{125}I-WHsAg than intact membranes. Quantitation of the WHV envelope binding to the soluble HPM proteins showed between 1.6 and 3.4 times greater binding than that calculated for native membranes tested at comparable protein concentrations (Figure 11). These results convincingly demonstrated that hydrophillic proteins of woodchuck HPMs were enriched for WHsAg-binding activity over intact HPMs. Furthermore, incubation of increasing concentrations of Triton X-114-solubilized membranes with a constant amount of ^{125}I-WHsAg suggested that the solubilized HPMs may exhibit a single class of saturable binding sites for the WHV envelope (Figure 12). Therefore, it was concluded that the Triton-114-solubilized woodchuck hepatocyte plasma membranes represent a convenient source of molecules which may serve as receptors for WHV.

Figure 10. Binding of WHV envelope to increasing concentrations of intact and solubilized HPM proteins in a nitrocellulose dot-blot radioligand binding assay. Samples of intact HPMs (top strip) and water-soluble HPM proteins resulting from Triton X-114 treatment (bottom strip) were spotted on nitrocellulose at the indicated protein concentrations and incubated with a constant amount of ^{125}I-WHsAg (3 μg protein). Background radioactivity was visualized using PBS instead of plasma membranes.

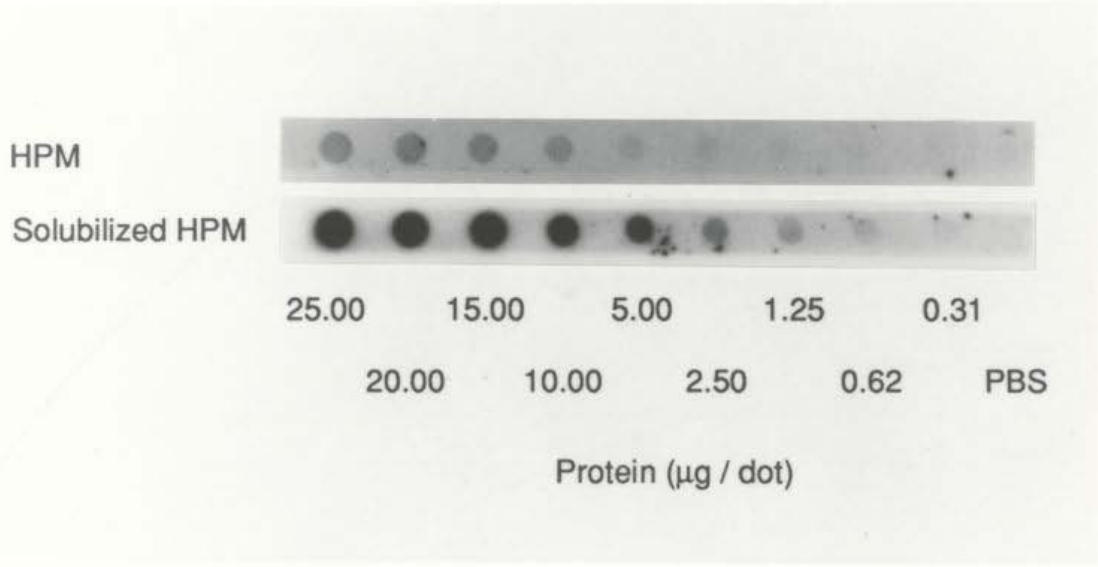

HPM
Solubilized HPM
25.00
15.00
5.00
1.25
0.31
20.00
10.00
2.50
0.62
PBS
Protein (µg / dot)

Figure 11. Comparison of WHV envelope binding to intact and water-soluble woodchuck hepatocyte plasma membrane proteins. Native (untreated) and Triton X-114-solubilized HPMs were spotted onto nitrocellulose at the indicated concentrations and were incubated with a constant amount of ^{125}I-WHsAg (3 μg protein) as described in "Materials and Methods". Control dots containing PBS were used to determine background radioactivity. The WHsAg binding ratio was calculated by dividing the net cpm (*i.e.*, after the subtraction of background binding) of the ^{125}I-WHsAg bound to solubilized HPM by the net cpm of the WHsAg bound to the intact membranes at the same membrane protein concentration. The data represent the average of four experiments each with three membrane protein concentrations and the bars indicate the standard deviations of the means.

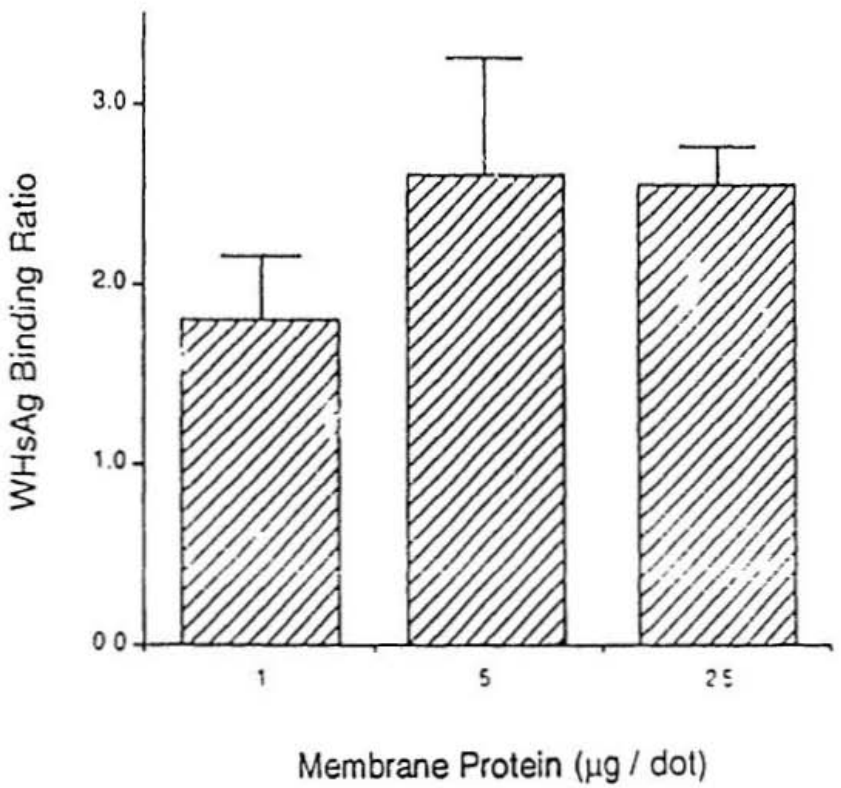
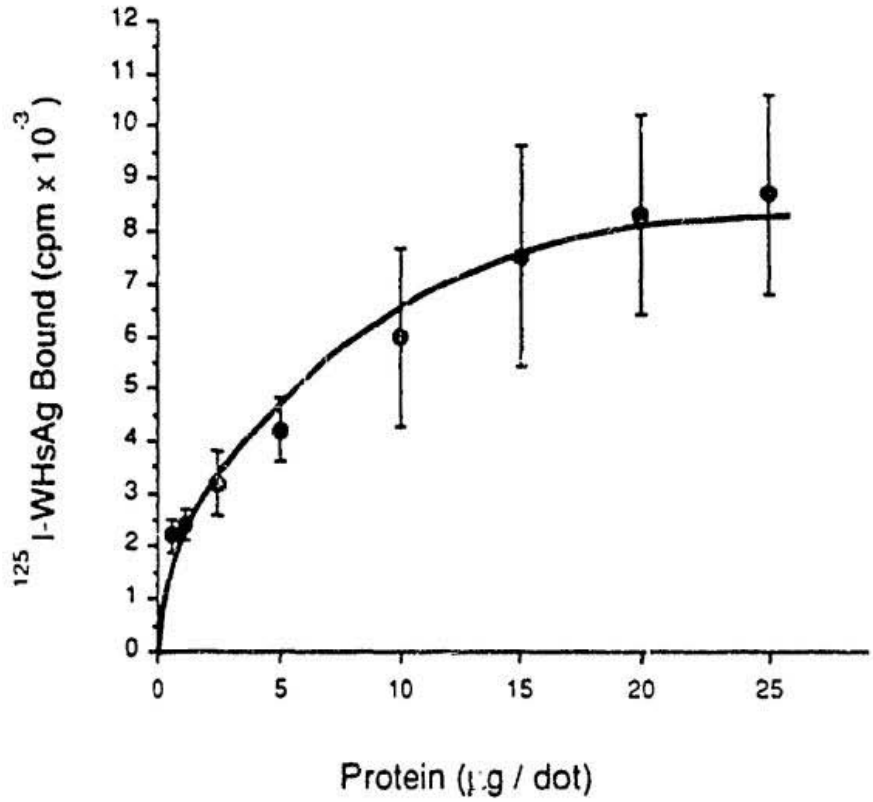

WHsAg Binding Ratio
3.0
2.0
1.0
0.0
1
5
25
Membrane Protein (µg / dot)

Figure 12. Kinetics of WHV envelope binding to increasing concentrations of solubilized proteins of woodchuck hepatocyte plasma membranes. Samples containing the indicated amounts of water-soluble proteins resulting from treatment of HPMs with Triton X-114 were spotted onto nitrocellulose by microfiltration at the indicated protein concentrations and incubated with ^{125}I-labelled WHsAg (3 μg protein) as described in "Materials and Methods". Control dots incubated with PBS were used as negative controls to determine background binding. The points are the mean values of cpm from three separate experiments after subtraction of background binding. Bars represent the standard deviations of the means.


^{125}I-WHsAg Bound (cpm x 10^{-3})
0
1
2
3
4
5
6
7
8
9
10
11
12
0
5
10
15
20
25
Protein (µg / dot)

3.5 WHV Envelope Interaction with Plasma Membranes of Non-Hepatic Origin

Although WHV is regarded as a primarily hepatotropic virus, in order to test whether receptors for the virus are present on cells other than hepatocytes, plasma membranes derived from woodchuck splenocytes and kidney cells were used as substrates. Prior to these experiments HPM, SPM and KPM preparations were subjected to SDS-PAGE analysis in an attempt to see if common polypeptide and carbohydrate bands occur. Stainings of the SDS-polyacrylamide gels with Coomassie brilliant blue or periodic acid Schiff reagent revealed that the protein and carbohydrate patterns of the membranes were distinct overall; however, some common bands (*e.g.*, 330-kD protein) were also observed (Figure 13). Since the majority of bands were stained by both Coomassie brilliant blue and periodic acid Schiff reagent, it is likely that they represent polypeptides complexed with carbohydrate moieties. Furthermore, no significant differences in the protein or carbohydrate patterns were observed between native membranes and their respective aqueous phases resulting from the treatment with Triton X-114 (data not shown).

Results shown in Figure 14 demonstrate that plasma membranes derived from woodchuck splenocytes and kidney cells evidently bind less WHV envelope protein than HPMs, when tested in a nitrocellulose dot-blot binding assay. In fact, assuming that HPMs express the maximum number of binding sites for the WHV envelope and that all these sites became saturated with ^{125}I-WHsAg under the present assay conditions (*i.e.*,

Figure 13. Protein and carbohydrate patterns of woodchuck hepatocyte, splenocyte and kidney cell plasma membranes determined by SDS-PAGE. Plasma membrane proteins (10 μg/sample) derived from hepatocyte (HPM), splenocyte (SPM), and kidney cells (KPM) were separated by electrophoresis in a 8% SDS-polyacrylamide gel and stained with either 0.05% Coomassie brilliant blue (left panel) or periodic acid Schiff reagent (right panel). Numbers indicate the positions of the molecular weight protein standards expressed in kD values.

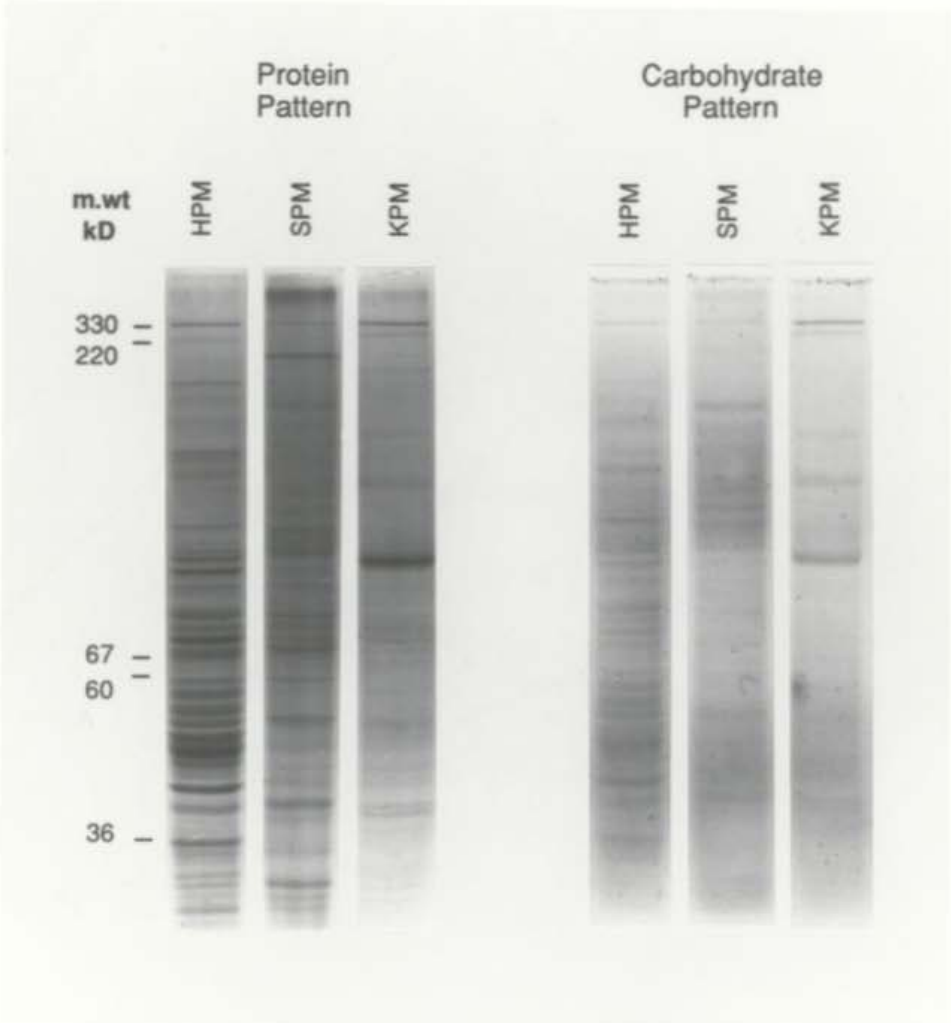
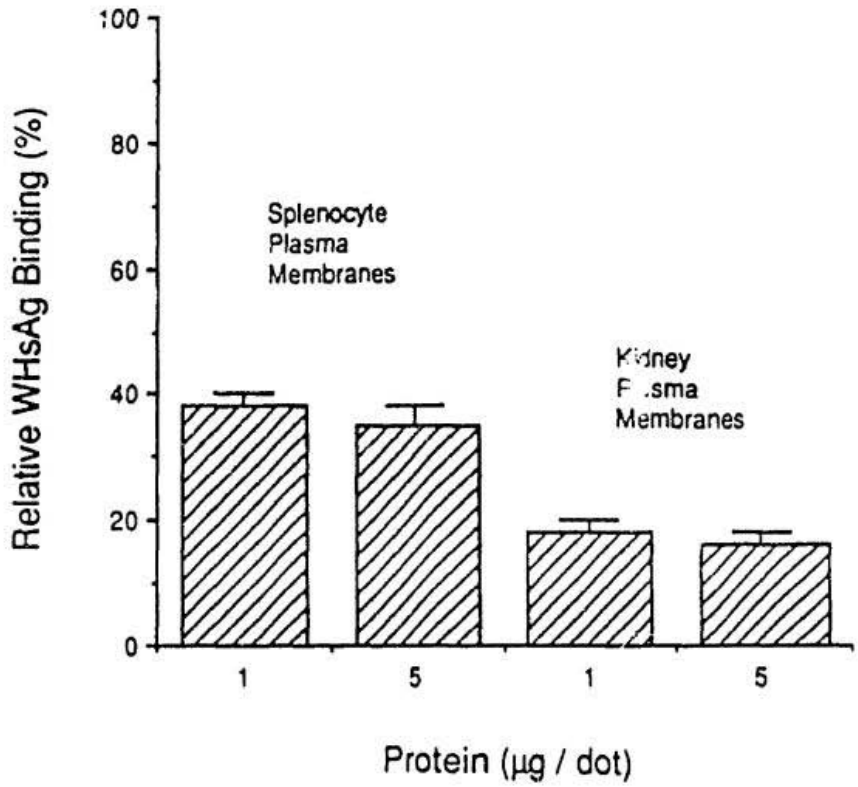

Protein
Pattern
Carbohydrate
Pattern
m.wt
kD
HPM
SPM
KPM
HPM
SPM
KPM
330
220
67
60
36

Figure 14. Relative binding of WHV envelope particles to plasma membranes derived from different woodchuck organs. Membrane proteins originating from hepatocytes, splenocytes and kidney cells were immobilized on nitrocellulose at the indicated concentrations and incubated with a constant amount of ^{125}I-WHsAg (3 μg protein) as described in "Materials and Methods". The relative WHsAg binding was calculated by dividing the net cpm of ^{125}I-WHsAg bound to splenocyte or kidney cell plasma membranes by the net cpm of the radioactivity bound to hepatocyte plasma membranes, the latter being normalized to 100%. The mean cpm values were obtained from six separate experiments after subtraction of background binding. Bars represent the standard deviations of the means.


Relative WHsAg Binding (%)
100
80
60
40
20
0
Splenocyte
Plasma
Membranes
Kidney
Plasma
Membranes
1
5
1
5
Protein (µg / dot)

100 % binding), SPMs and KPMs displayed a WHsAg binding activity quantified as being approximately 40% and 18%, respectively, of that expressed by HPMs. However, it is necessary to take into consideration that HPMs were used in a highly purified form and were derived from an isolated, homogeneous population of liver parenchymal cells, whereas SPMs were isolated from a population of splenic lymphoid cells containing mainly lymphoid cells, and KPMs from all cells of the kidney.

Overall, the results stemming from these experiments suggest that woodchuck lymphoid cells may display receptors for WHV, whereas the existence of these receptors on KPM is less likely. In addition, differences in the ability of the plasma membranes to interact with WHV envelope proteins could be related to the different composition of the membranes visualized by SDS-PAGE analysis.

3.6 Strategy for Purification of Plasma Membranes Receptors for WHV by Affinity Chromatography on WHsAg and Concanavalin A Affinity Columns

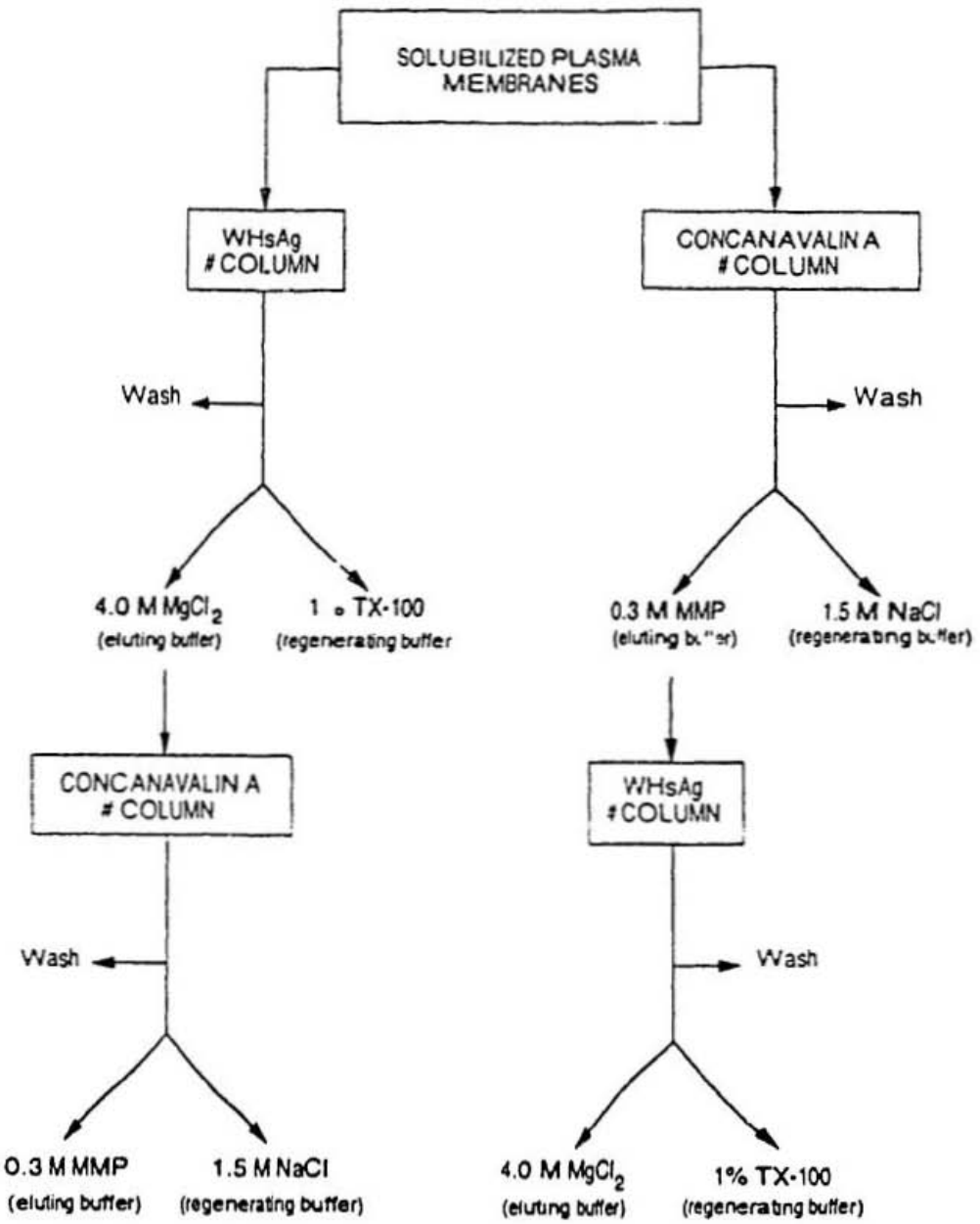
To identify plasma membrane constituents which exhibit binding for WHV envelope, woodchuck HPMs and KPMs, representing membranes with high and low WHsAg binding activity, respectively, were further investigated.

Based on the observation that HPM water-soluble constituents efficiently bind the WHV envelope and on the prediction that carbohydrate moieties of the plasma membranes may play a role in this binding, two affinity matrices, one carrying WHsAg

and the other Concanavalin A (Con A), were used. The WHsAg affinity column was chosen because it was likely that this approach would result in the isolation of constituents exhibiting specificity for the WHV envelope. This expectation was based on the previous successful isolations of cellular receptor for viruses using a virus affinity chromatography (*e.g.*, Tomassini and Colono, 1986; Allaway and Burness, 1987). On the other hand, the Concanavalin A affinity column was used because it was of interest to test whether cell surface proteins, which commonly exhibit carbohydrate moieties, display binding activity for WHV envelope and because preliminary data on HPM digestions with neuraminidase suggested that sialic acid residues are unlikely to participate in the WHV envelope binding.

A WHsAg#SH-cellulose column was employed for the purification of the WHV envelope binding constituents after a series of preliminary experiments in which optimal conditions for the elution of these constituents were established. Among the tested eluting buffers, such as 3 M NaCl buffer (pH 7.2), 0.2 M glycine-HCl buffer (pH 2.2) and 4 M $MgCl_2$ buffer (pH 7.2), the latter was found to be the most efficient. Furthermore, it was determined that treatment of the column with 1% Triton X-100 in 0.1 M sodium phosphate buffer (pH 7.2), containing 0.14 M NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, improved recovery of the specifically bound HPM elements in subsequent absorption-elution cycles (data not shown). Consequently, 1% Triton X-100 was employed to regenerate the virus affinity column. Following, preliminary evaluation of the WHV-binding activity expressed by the eluates recovered from the WHsAg and

Figure 15. Flow chart depicting the strategy of affinity chromatography studies aimed at purifying plasma membrane constituents capable of WHV envelope binding and determining the role of carbohydrate moieties in the WHV binding.


SOLUBILIZED PLASMA MEMBRANES
WHsAg # COLUMN
CONCANAVALIN A # COLUMN
Wash
Wash
4.0 M $MgCl_2$ (eluting buffer)
1 % TX-100 (regenerating buffer)
0.3 M MMP (eluting buffer)
1.5 M NaCl (regenerating buffer)
CONCANAVALIN A # COLUMN
WHsAg # COLUMN
Wash
Wash
0.3 M MMP (eluting buffer)
1.5 M NaCl (regenerating buffer)
4.0 M $MgCl_2$ (eluting buffer)
1% TX-100 (regenerating buffer)

Concanavalin A columns, a scheme shown in Figure 15 was employed to purify plasma membrane constituents enriched in the WHV receptor activity and to determine participation of the carbohydrate moieties in the virus binding.

3.7 WHV Envelope Binding Activity of the Plasma Membrane Constituents Purified on WHsAg and Concanavalin A Affinity Columns

In order to determine the WHV envelope binding exhibited by plasma membrane constituents recovered from affinity columns, a semi-quantitative dot-blot radioligand binding assay was used (Figure 16). Quantitation of the ^{125}I-WHsAg binding activity displayed by the aqueous phases of HPMs and KPMs prior to chromatography on a WHsAg affinity column gave values of 7+ and 5+, respectively (Table 4). This difference became even more pronounced when proteins of HPMs and KPMs specifically bound to the WHsAg#SH-cellulose column and recovered in the $MgCl_2$ eluate were tested for WHsAg binding. Thus, while HPM proteins exhibited maximum binding activity for WHsAg (*i.e.*, 7+), the KPM proteins had only a value of 2+ (Table 4). In contrast, the washes containing HPM and KPM constituents not bound to the column, expressed WHsAg binding values of 3+ and 2+, respectively (Table 4). It is likely, that the activity exhibited by the HPM wash could be due to overload of the column.

In Figure 17, comparison of the SDS-PAGE profiles of the HPM proteins prior to chromatography on a WHsAg#SH column and recovered in the wash and $MgCl_2$ eluate

Figure 16. Diagram of the scoring used in the semi-quantitative assessment of WHV envelope binding to plasma membrane proteins in a nitrocellulose dot-blot radioligand binding assay. Two-fold serial dilutions of membrane proteins were spotted onto nitrocellulose by microfiltration; the filter was then incubated with ^{125}I-WHsAg, washed, and exposed for autoradiography. Background radioactivity was assessed using PBS instead of plasma membrane proteins. Quantitation of the WHsAg binding was determined by finding the spots on the autoradiograms corresponding to the dots containing the least amount of membrane protein to which binding of WHsAg was still observed (presented in the diagram as black-shaded circles). The extent of WHsAg binding was scored according to the numbering system shown on the left side of the panel.

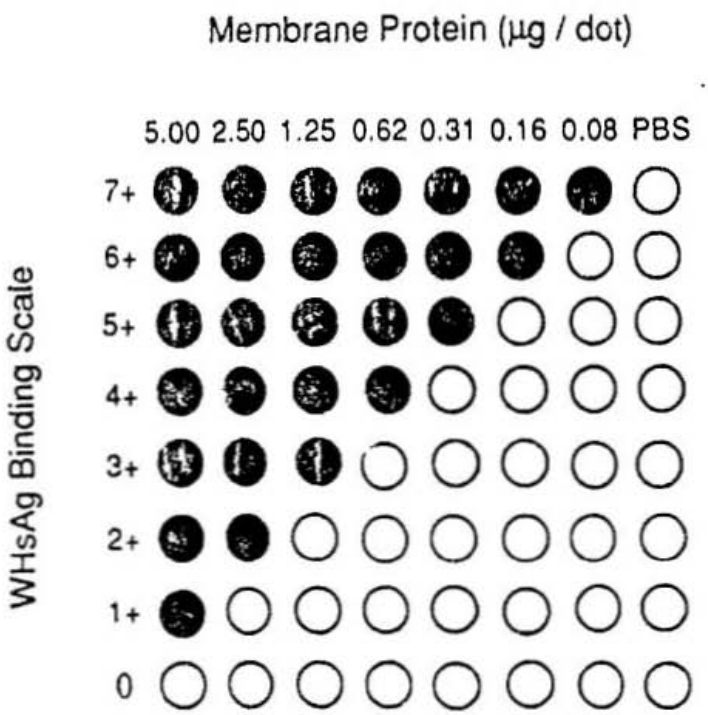
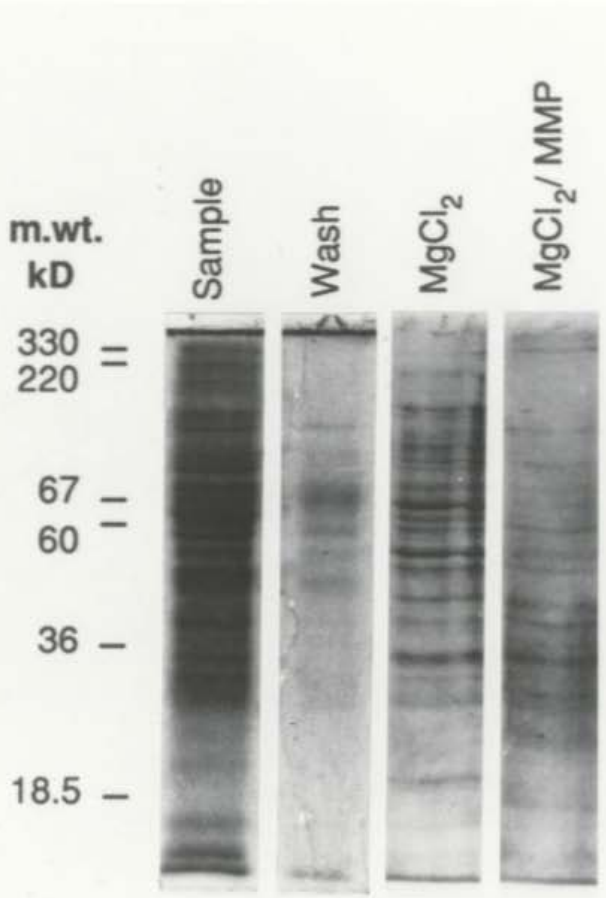

Membrane Protein (µg / dot)
5.00 2.50 1.25 0.62 0.31 0.16 0.08 PBS
WHsAg Binding Scale
7+
6+
5+
4+
3+
2+
1+
0

Table 4. Recovery of WHV envelope binding activity in fractions resulting from the chromatographic separation of solubilized woodchuck hepatocyte and kidney cell plasma membrane on WHsAg and Concanavalin A affinity columns.

Origin of membranes[a]	WHsAg binding[b]				
	Prior to column	WHsAg # column		Concanavalin A # column	
		Wash[c]	$MgCl_2$ eluate[d]	Wash[c]	MMP eluate[f]
Hepatocyte plasma membranes	7+[g]	3+	7+	4+	7+
Kidney cell plasma membranes	5+	2+	2+	2+	3+

[a] Aqueous phases of the Triton X-114 extracted plasma membranes.
[b] Quantified by the nitrocellulose dot-blot ^{125}I-WHsAg binding assay performed as described in "Materials and Methods".
[c] 0.1 M sodium phosphate buffer, pH 7.2, containing 0.14 NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$.
[d] 4 M $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.2.
[e] 0.1 M Tris-HCl buffer, pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$.
[f] 0.3 M methyl-α-D-mannopyranoside in 0.1 M Tris-HCl buffer, pH 7.2, containing 1 mM $CaCl_2$ and $MnCl_2$.
[g] The numbers refer to the binding activity assessed according to the WHV envelope binding scale presented in figure 15.

Figure 17. Protein profiles of water-soluble hepatocyte plasma membranes before and after their fractionation on WHsAg and Concanavalin A columns determined by SDS-PAGE. Membrane proteins (10μg protein/lane) separated by electrophoresis in a 10% SDS-polyacrylamide gel were stained with 0.05% Coomassie brilliant blue. Water-soluble membrane proteins (lane sample) were applied to the WHsAg#SH-cellulose column, unbound elements washed out (lane wash) and the specifically bound constituents eluted with 4 M $MgCl_2$ (lane $MgCl_2$). Subsequently, the $MgCl_2$ eluate was applied onto a Concanavalin A column, the column was washed, and the specifically bound constituents were eluted with 0.3 M MMP (lane $MgCl_2$/MMP) (see also Figure 18). Numbers indicate the electrophoretic mobility of the molecular weight protein markers.

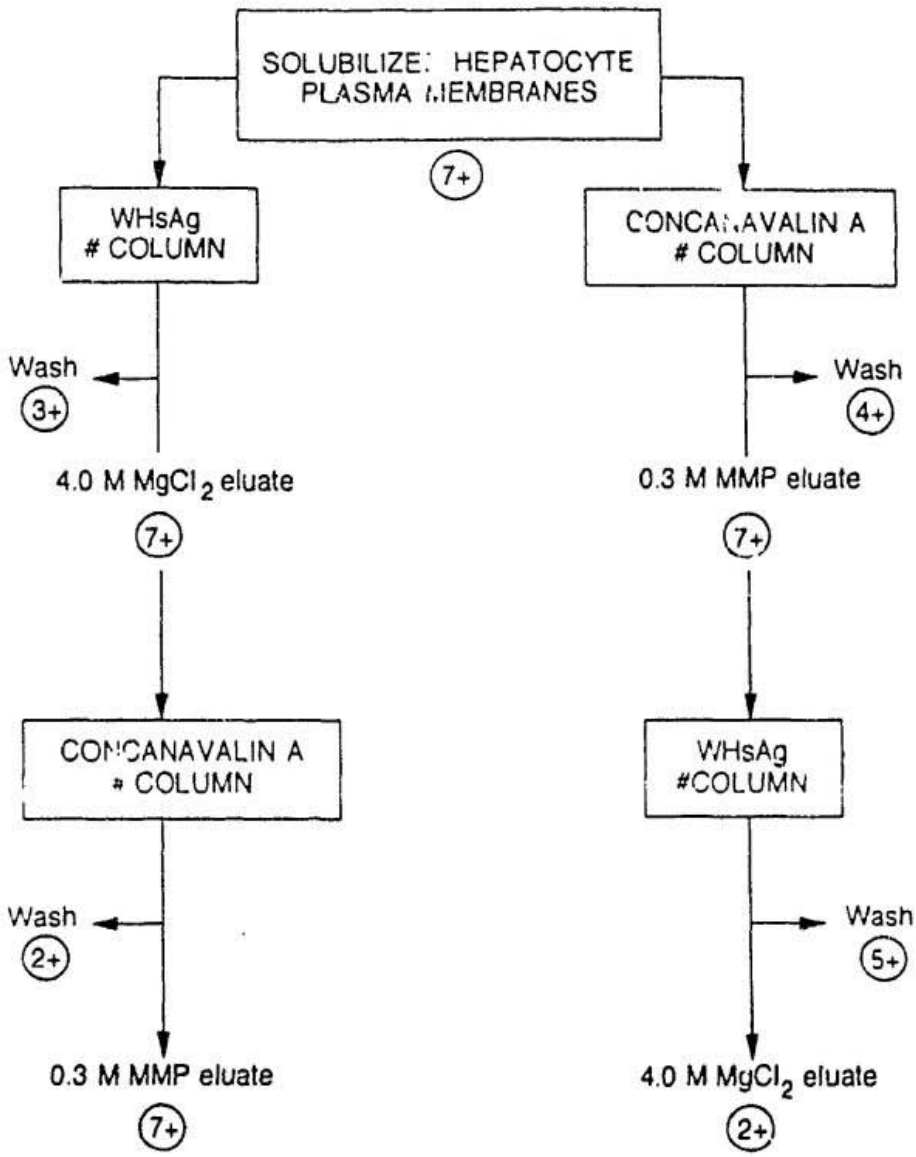

m.wt.
kD
330
220
67
60
36
18.5
Sample
Wash
MgCl2
MgCl2/ MMP

demonstrate that some of the bands observed in the eluate were also present in the wash. This observation reinforces the possibility that column overloading may have taken place. However, in the case of KPMs, the observed WHV envelope binding activity for the wash does not appear to be due to overloading of the WHsAg column since both the wash and $MgCl_2$ eluate displayed a similar WHsAg binding potential (*i.e.*, 2+; Table 4).

The binding exhibited by fractions resulting from the chromatography of solubilized HPMs and KPMs on a Concanavalin A affinity column revealed that HPM glycoconjugates specifically eluted with 0.3 M MMP were able to bind the virus envelope (value of 7+; Table 4). In parallel experiments where solubilized KPMs were employed, a WHsAg binding activity of 3+ was found in the MMP eluate. In contrast, the washes showed binding values of 4+ and 2+ for solubilized HPM and KPM proteins, respectively (Table 4).

The results obtained from WHsAg and Concanavalin A affinity columns provide important information about the biochemical composition of the plasma membrane proteins capable of recognizing the WHV envelope. Thus, due to the oligosaccharide specificity exhibited by Concanavalin A, it is most likely that proteins bound to this affinity matrix exhibited either N-linked polymannose, N-linked bi-antennary complex (Baenziger and Fiete, 1979), O-linked heparin-like glycosaminoglycans (Monges *et al.*, 1989) or a combination of the above. On the other hand, the wash containing constituents not bound to this column should be void of the above mentioned type of carbohydrates, unless the column was overloaded.

Figure 18. Summary of results on the WHV envelope binding activity recovered from the alternate sequence in which water-soluble HPM proteins were chromatographed on WHsAg and Concanavalin A affinity columns. The WHsAg binding was quantified using nitrocellulose dot-blot ^{125}I-WHsAg binding assay as described in "Materials and Methods". Numbers contained within the circles correspond to the values found on the WHsAg binding scale shown in Figure 16.


SOLUBILIZE: HEPATOCYTE PLASMA MEMBRANES
7+
WHsAg # COLUMN
CONCANAVALIN A # COLUMN
Wash
3+
Wash
4+
4.0 M MgCl2 eluate
0.3 M MMP eluate
7+
7+
CONCANAVALIN A # COLUMN
WHsAg #COLUMN
Wash
2+
Wash
5+
0.3 M MMP eluate
4.0 M MgCl2 eluate
7+
2+

On the contrary, the WHsAg binding activity exhibited by the KPM wash, was more likely due to carbohydrate moieties which lack the above mentioned N- or O-linkage.

To directly determine the nature of HPM oligosaccharides eluted with $MgCl_2$ from WHsAg affinity column, the 4M $MgCl_2$ eluate was re-chromatographed on a Concanavalin A#Sepharose 4B column (Figure 15). Quantification of the WHsAg binding activity specifically eluted from a Concanavalin A column with 0.3 M MMP in the nitrocellulose dot-blot ^{125}I-WHsAg binding assay, revealed that the activity exhibited by the $MgCl_2$ eluate (*i.e.*, 7+) was recovered in the MMP eluate (*i.e.*, $MgCl_2$/MMP eluate 7+; Figure 18). Furthermore, when these two eluates were subjected to SDS-PAGE, the analysis revealed that the observed protein profiles appeared to have the same predominant polypeptide bands (Figure 17). This may suggest that most of the proteins specifically retained by the WHsAg#SH-cellulose column contained carbohydrates enriched in either N-linked polymannose, N-linked bi-antennary complex, O-linked heparin-like glycosaminoglycans or a combination of these.

In parallel experiments, Triton X-114-solubilized HPMs were first applied to a Concanavalin A affinity column, and the resulting MMP eluate was chromatographed on the WHsAg affinity column (Figure 15). Analysis of the WHsAg binding activity by nitrocellulose dot-blot assay revealed that the WHsAg binding observed in the MMP eluate (*i.e.*, 7+; Figure 18) became partitioned between the wash (*i.e.*, 5+; Figure 18) and the $MgCl_2$ eluate (*i.e.*, 2+; Figure 18) after chromatography on the WHsAg#SH-cellulose column. This result indicates that only a fraction of the HPM proteins whose

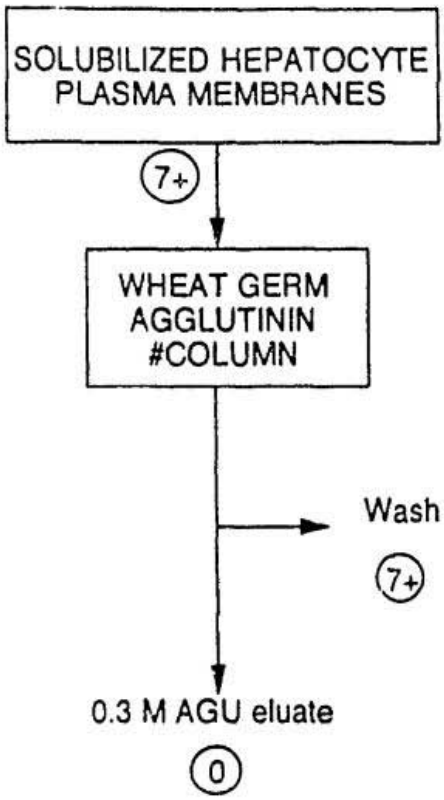
glycosidic residues are recognized by Concanavalin A specifically interacts with the WHV envelope. Furthermore, since these interactions are dissociated by 4 M $MgCl_2$ this implicates that the WHsAg binding is likely mediated by charged glycosidic residues.

Overall, alternating the sequence by which affinity chromatography was performed indicates that while most, if not all, of the WHsAg binding activity present in the $MgCl_2$ eluate can be subsequently recovered from a Concanavalin A column with 0.3 M MMP, only a portion of the activity eluted with MMP was then eluted with 4 M $MgCl_2$ from a WHsAg column. This suggests that constituent(s) which mediate binding of the WHV envelope to the hydrophillic phase of woodchuck HPMs is endowed with glycosidic residue(s) and that the interaction has an ionic character.

3.8 Determination of the Role of Sialic Acid Residues in WHsAg-HPM Binding by Affinity Chromatography on Wheat Germ Agglutinin Column

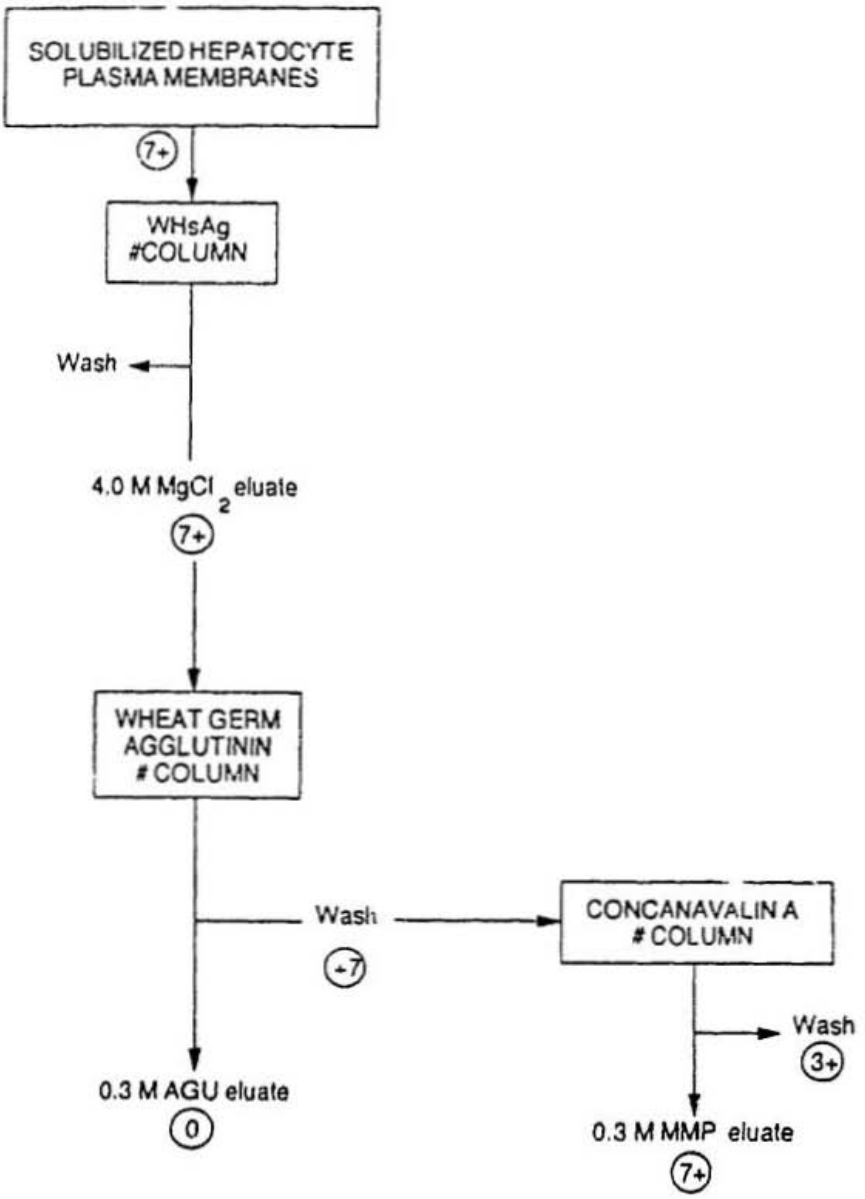
It has been demonstrated that the plasma membrane sialic acid plays a crucial role in the attachment of several viruses such as polyoma virus (Fried *et al.*, 1981), encephalomyocarditis virus (Burness and Pardoe, 1981), influenza A virus (Paulson *et al.*, 1979), influenza C virus (Rogers *et al.*, 1986) and Sendai virus (Paulson *et al.*, 1979) to their target cells. It is also well known that wheat germ agglutinin interacts specifically with sialic acid residues (Montsigny *et al.*, 1980). To determine whether sialic acid participates in the binding of WHV to hepatocyte plasma membrane, the

Figure 19. Schematic diagram showing the procedure and the results obtained on the quantitation of WHV envelope binding activity following the fractionation of water-soluble HPMs on a wheat germ agglutinin affinity column. The WHV envelope binding was evaluated using the nitrocellulose dot-blot binding assay as described in "Materials and Methods". The numbers contained within the circles correspond to the values on the WHsAg binding scale shown in Figure 16.


SOLUBILIZED HEPATOCYTE PLASMA MEMBRANES
7+
WHEAT GERM AGGLUTININ #COLUMN
Wash
7+
0.3 M AGU eluate
0

aqueous phase of HPMs was chromatographed on a wheat germ agglutinin (WGA)affinity column and the resulting washes and AGU eluates were tested for WHsAg binding using the nitrocellulose dot-blot radioligand binding assay (Figure 19). Interestingly, the results demonstrated that HPM proteins bound to the WGA affinity column and eluted with AGU buffer did not express binding activity for the WHV envelope (Figure. 19). In contrast, proteins which were incapable of binding to WGA and, thus, detected in the wash retained the WHsAg binding potential (*i.e.*, 7+; Figure 19). This result strongly suggests that HPM proteins containing sialic acid (*i.e.*, N-linked tri-, bi-antennary complex type and/or O-linked oligosaccharides) are not involved in the recognition of the WHV envelope. The above finding was confirmed when the $MgCl_2$ eluate obtained through the chromatography of solubilized HPM proteins on the WHsAg affinity column was subsequently chromatographed on the wheat germ affinity column (Figure 20). As expected, the envelope binding activity was recovered in the wash of the WGA column (*i.e.*, 7+), whereas the AGU eluate did not demonstrate any binding potential for the WHV particles (*i.e.*, 0; Figure 20). Furthermore, in order to further test if the wash from a WGA column contained proteins exhibiting the same type of carbohydrate moieties as those interacting with Concanavalin A (see Figure 18), the wash was re-chromatographed on a Concanavalin A column (Figure 20). As expected, the three-step column chromatography resulted in a MMP eluate which exhibited a high level of WHV envelope binding activity (*i.e.*, 7+; Figure 20). Therefore, the data indicate that N-linked oligosaccharides, such as N-linked polymannose and/or a O-linked heparin-like

Figure 20. Schematic diagram showing the procedure and results obtained on the quantitation of WHV envelope binding activity following fractionation of water-soluble HPM proteins through three consecutive affinity columns. Membrane proteins eluted from a WHsAg affinity column with 4 M $MgCl_2$ were re-chromatographed on a wheat germ agglutinin column and the resulting wash was re-chromatographed on a Concanavalin A column. The WHsAg binding activity expressed by the eluates and washes was determined using the nitrocellulose dot-blot binding assay as described in "Materials and Methods". The numbers contained within the circles correspond to the values on the WHsAg binding scale shown in Figure 16.


SOLUBILIZED HEPATOCYTE PLASMA MEMBRANES
7+
WHsAg #COLUMN
Wash
4.0 M MgCl2 eluate
7+
WHEAT GERM AGGLUTININ # COLUMN
Wash
+7
CONCANAVALIN A # COLUMN
Wash
3+
0.3 M AGU eluate
0
0.3 M MMP eluate
7+

glycosaminoglycan, but not O-and/or N-linked tri-, bi-antennary, complex type, oligo-saccharides possessing sialic acid, mediate the interaction between WHV envelope and HPMs.

3.9 WHV Envelope Binding to a Hepatocyte Plasma Membrane Associated 330-kD Receptor Molecule

To further investigate the nature of the HPM binding activity for WHsAg, a virus overlay protein binding assay (VOPBA) was performed. In this assay, plasma membrane proteins separated on an SDS-polyacrylamide gel were electroblotted onto nitrocellulose and probed with ^{125}I-WHsAg. As shown in Figure 21, WHsAg recognized a single protein band of an approximate molecular weight of 330-kD. Interestingly, re-examination of the SDS-PAGE profiles revealed that the 330-kD polypeptide band was consistently detected in native HPM (Figure 13) as well as in the $MgCl_2$ eluates from a WHsAg#SH-cellulose column (Figure 17) or in the MMP eluates after chromatography of the HPM aqueous phase on a Concanavalin A column (data not shown).

3.10 Effects of Enzyme Treatments on the Binding Activity of the 330-kD Receptor for the WHV Envelope

In view of the fact that the 330-kD polypeptide was a protein specifically eluted from the Concanavalin A column, and not bound by WGA, it was expected to be compo

Figure 21. Binding of WHV envelope to hepatocyte plasma membrane in a virus overlay protein blot assay. Purified woodchuck HPMs (10μg protein) were separated by electrophoresis in a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose sheet. The blot was incubated with ^{125}I-WHsAg, washed, air-dried and the bound radioactivity visualized by autoradiography as described in "Materials and Methods". The number on the left side of the figure indicates the molecular weight of the protein band which exhibits WHsAg binding activity. The top and the bottom of the transfer are indicated on the right side.

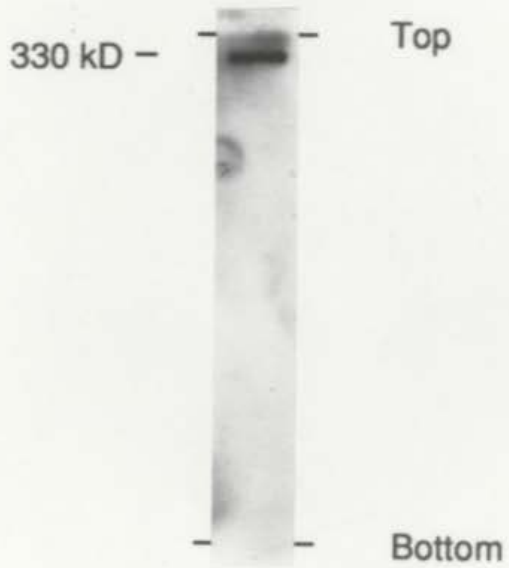
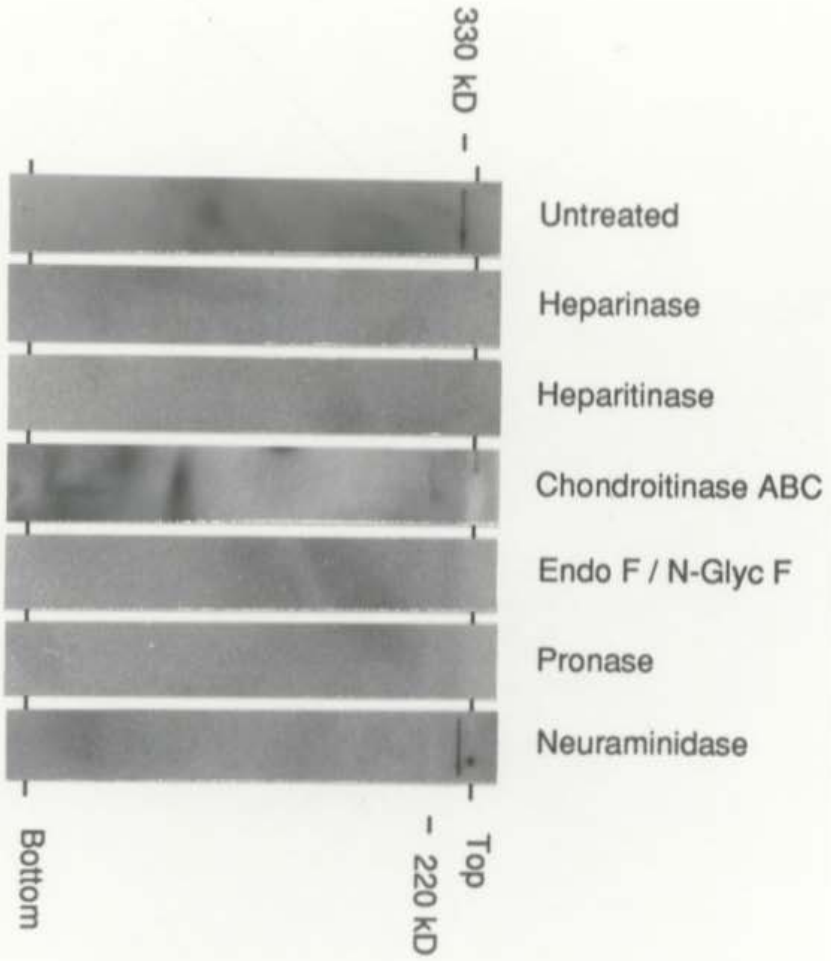

330 kD
Top
Bottom

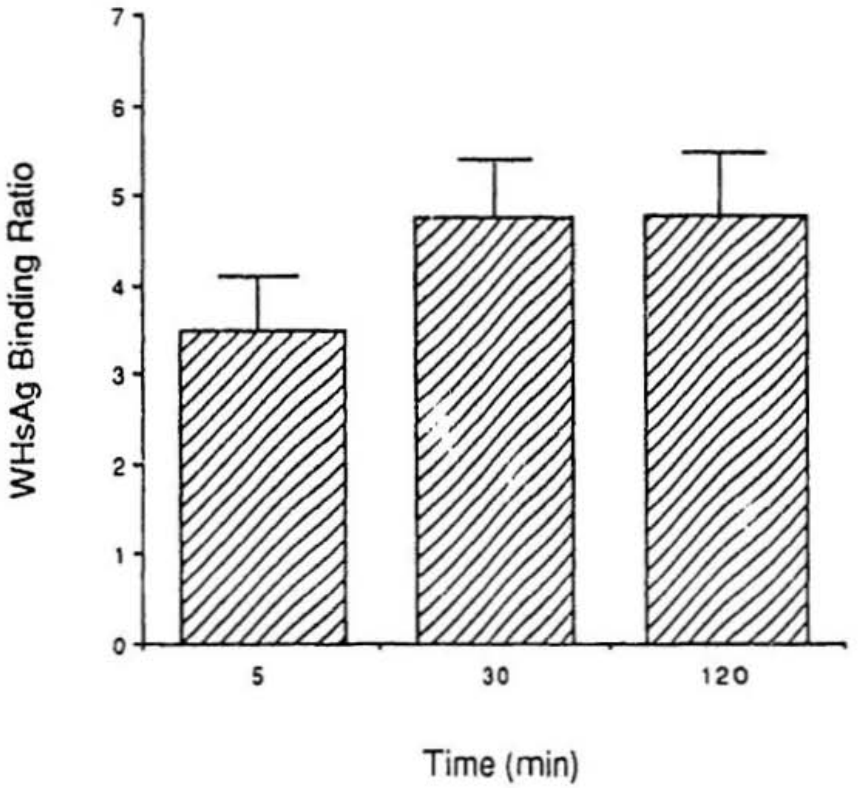
Figure 22. Effects of enzymatic digestions on the hepatocyte plasma membrane 330-kD WHV envelope-binding protein. Ten μg protein of native HPMs (lane: untreated), membranes digested with heparinase, heparitinase, chondroitinase ABC, endoglycosidase F/N-glycosidase F (lane: EndoF/N-GlycF), pronase or neuraminidase were separated by electrophoresis in a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose as described in "Materials and Methods". Blots were incubated with ^{125}I-WHsAg, washed and the bound radioactivity visualized by autoradiography. The electrophoretic mobility of the 330-kD and 220-kD proteins specifically recognized by the virus envelope, relative to the top and the bottom of the transfer are shown.


330 kD
Untreated
Heparinase
Heparitinase
Chondroitinase ABC
Endo F / N-Glyc F
Pronase
Neuraminidase
Bottom
Top
220 kD

sed of a protein core with N-linked polymannose and/or an O-linked glycosaminoglycan moiety, such as heparin or heparan sulphate (Doyle and Kan 1972; Monges *et al.*, 1989). To provide more detail on the biochemical properties of the 330-kD molecule, the membranes were treated with enzymes capable of depolymerizing oligosaccharides by cleaving specific saccharide structures. For this purpose, HPM proteins digested with either heparinase, heparitinase, chondroitinase ABC, endoglycosidase F/N-glycosidase F or neuraminidase were separated by SDS-PAGE, transferred onto nitrocellulose, and incubated with ^{125}I-WHsAg. Autoradiograms showed that digestions of HPMs with heparinase, heparitinase or endoglycosidase F/N-glycosidase F, but not with neuraminidase abolish WHsAg binding to the identified 330-kD band (Figure 22). In addition, digestion of HPMs with chondroitinase ABC produced a 220-kD protein band, instead of a 330-kD protein, capable of WHsAg binding. Since both heparinase and heparitinase exhibit substrate specificities for heparan sulphate, the absence of WHV envelope binding following treatment of HPMs with these enzymes strongly suggests that the 330-kD band reflects the existence of a macromolecule which is constituted by protein and heparan sulphate. This finding is consistent with the results of experiments employing Concanavalin A and WGA affinity columns, which indicated that an O-linked heparin-like glycosaminoglycan or N-linked polymannose, but not O- and N-linked tri-, bi- antennary complex, oligosaccharides possessing sialic acid or a combination of these, are responsible for the WHV observed envelope binding activity. However, since the treatments of HPMs with endoglycosidase F/N-glycosidase F also resulted in the

elimination of the 330-kD band, we are led to conclude that a N-linked polymannose carbohydrate moiety may also constitute part of the 330-kD molecule and contribute to the binding of the WHV envelope. Further, the 330-kD macromolecule may also contain carbohydrate bonds resembling those occurring in chondroitin sulphate since chondroitinase ABC digestion of HPMs rendered a 220-kD polypeptide. Concomitant analysis of the protein patterns resulting from the above enzymatic digestions by SDS-PAGE, revealed that the loss of the WHsAg binding due to digestions with heparinase, heparitinase and endoglycosidase F/N-glycosidase F was not accompanied by the elimination of the 330-kD protein band when stained with Coomassie brilliant blue (data not shown). This result implies that the band reflects the existence of a high-molecular weight protein-carbohydrate complex, whose interaction with WHV is mediated by its carbohydrate content, yet its electrophoretic mobility likely depends on the protein core. The results of digestion of HPMs with pronase supported this possibility by demonstration that the 330-kD band was no longer detectable when HPM proteins were treated with the enzyme and analysed by VOPBA (Figure 22). On the other hand, when nitrocellulose dot-blots containing immobilized pronase-treated HPMs (*i.e.*, 2 hr at 37°C) were incubated with ^{125}I-WHsAg, it was observed that the treatment not only did not abolish the WHsAg binding but the binding was increased (Figure 23). This further supported the view that carbohydrates, but not the protein core of the 330-kD macromolecule, mediate the binding of the WHV envelope. Analysis of the WHsAg binding exhibited by HPM treated with pronase for 5, 30 and 120 min and then incubated

Figure 23. Comparison of the WHV envelope binding displayed by native and pronase digested hepatocyte plasma membranes. Five μg protein samples of native (untreated) and HPM treated with pronase for 5, 30 and 120 min were spotted on nitrocellulose and incubated with a constant amount of ^{125}I-labelled WHsAg (3 μg protein) as described in "Materials and Methods". Control dots incubated with PBS were used as negative controls to determine background binding. The WHsAg binding ratio was calculated by dividing the net cpm (*i.e.*, after subtraction of background binding) of the ^{125}I-WHsAg bound to pronase treated HPMs by the net cpm of the ^{125}I-WHsAg bound to native membranes, at the same protein concentration. The data represent the average of three experiments. Bars indicate the standard deviations of the means.


WHsAg Binding Ratio
0
1
2
3
4
5
6
7
5
30
120
Time (min)

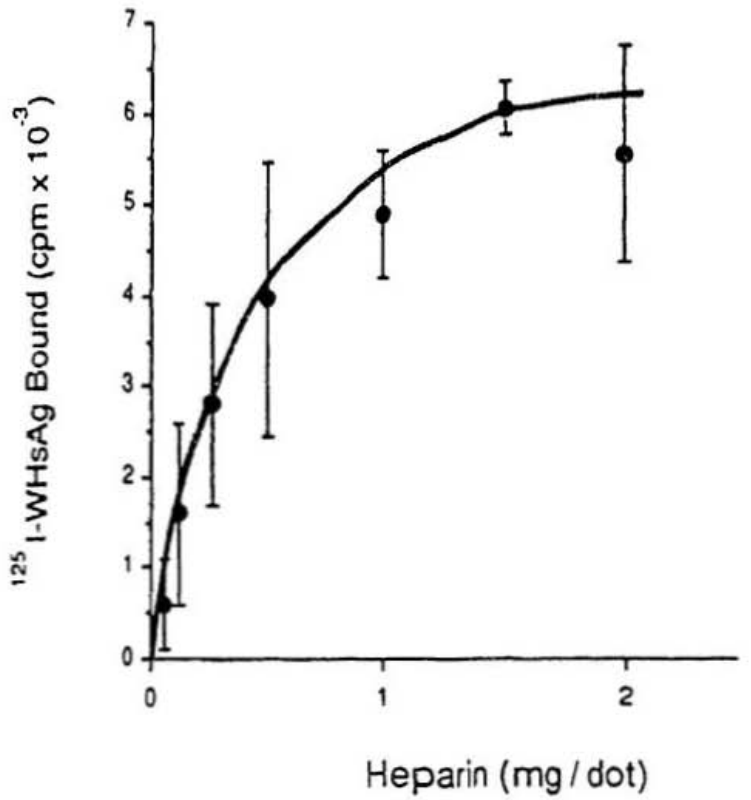
with the virus envelope particles demonstrated that the binding activity was evidently higher when compared to untreated HPMs. Moreover, the binding ability displayed by HPMs after digestion with pronase for 5 min appeared to be somewhat lower than that observed after 30 or 120 min, suggesting that the progressive proteolytic digestion may lead to the exposure of WHV binding sites.

3.11 WHV Envelope Binding to Heparin

Because it was found that a heparan sulphate-like moiety is essential to the binding of the WHV envelope to the 330-kD receptor, it became important to test whether an extracellular heparan sulphate-like compound could bind the envelope particles and inhibit the ability of the WHV envelope to recognize binding sites on HPMs. Among the glycosaminoglycans available, the sodium salt of heparin was chosen because it displays significant biochemical similarities to heparan sulphate and is commonly available in a highly purified form (Kjellen and Lindahl, 1991).

Binding of the WHV envelope to heparin was evaluated using the nitrocellulose dot-blot ^{125}I-WHsAg binding assay. The incubation of nitrocellulose strips containing increasing amounts of immobilized heparin with a constant amount of ^{125}I-WHsAg revealed that the envelope binds to heparin with apparent saturable kinetics (Figure 24).

Figure 24. Kinetics of WHV envelope binding to increasing concentrations of heparin. Varying amounts of heparin immobilized onto nitrocellulose were incubated with a constant amount of ^{125}I-labelled WHsAg (3 μg protein), washed and the bound radioactivity determined as described in "Materials and Methods". Control dots containing PBS were used to assess background radioactivity. The points are mean values of cpm from three separate experiments after subtraction of background binding. Bars represent the standard deviations of the means.


^{125}I-WHsAg Bound (cpm x 10^{-3})
0
1
2
3
4
5
6
7
0
1
2
Heparin (mg / dot)

3.12 Heparin as an Inhibitor of WHV Envelope Binding to Hepatocyte Plasma Membranes

Exposure of extensively dialysed ^{125}I-WHsAg preincubated with different amounts of cold heparin to nitrocellulose dot-blot strips each containing 5 μg of immobilized HPM protein, revealed that the binding of the WHV envelope to HPM is inversely proportional to the concentration of heparin added to WHsAg (Figure 25). In fact, when 3 μg of WHV envelope protein was preincubated with 5000 μg of heparin and then the mixture extensively dialysed to remove excess of heparin, binding of the particles to HPMs was nearly eliminated (Figure 25).

3.13 Binding of Heparin to Hepatocyte Plasma Membranes

To exclude the possibility that an inhibitory effect displayed by heparin on the WHsAg binding to the 330-kD macromolecule is due to direct heparin binding to the same macromolecule and, at the same time, to determine which polypeptides of woodchuck HPMs are able to recognize heparin, HPM proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and incubated with ^{125}I-labelled heparin. Predominant bands with an approximate molecular weight of 115, 78, 50-58, 45 and 42 kD, but not 330-kD band, were visualized on the autoradiogram (Figure 26). In contrast, when a similar HPM protein blot was incubated with ^{125}I-WHsAg pre-incubated with cold heparin, no bands were detected (Figure 26). Therefore, these results indicate that: (1) free heparin does not bind to the 330-kD band; however heparin complexed

Figure 25. Effect of heparin on the binding of WHV envelope to woodchuck hepatocyte plasma membranes. HPMs immobilized on nitrocellulose at 5 μg protein per dot were incubated with ^{125}I-WHsAg (3 μg protein), which was pre-incubated with the indicated amounts of heparin and dialysed; PBS similarly treated was used as the control. The data represent the mean cpm $\pm$ SD of three experiments after subtraction of background binding.

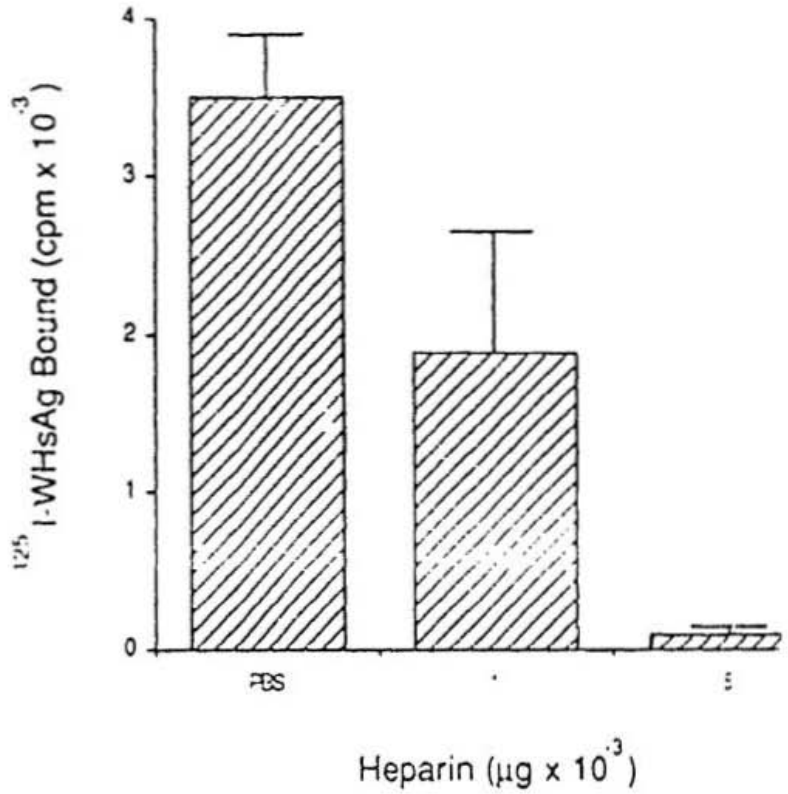
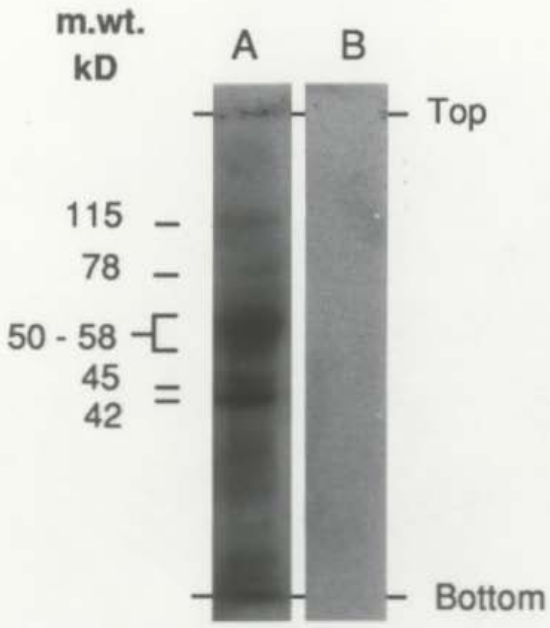

^{125}I-WHsAg Bound (cpm x 10^{-3})
4
3
2
1
0
PBS
Heparin (µg x 10^{-3})

Figure 26. Identification of heparin binding proteins in woodchuck hepatocyte plasma membranes by ligand overlay protein binding assay. Membrane proteins were separated by electrophoresis in a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose as described in "Materials and Methods". The nitrocellulose blots were incubated with 3 μg of ^{125}I-heparin (lane A) or with 3 μg of ^{125}I-WHsAg protein pre-incubated with 1000 μg of heparin and dialysed (lane B). Bound radioactivity was visualized by autoradiography. Numbers on the left side of the figure indicate the apparent molecular weights of the most predominant bands which exhibit heparin binding activity. ^{125}I-WHsAg preincubated with heparin does not produce any visible HPM bands (lane B).

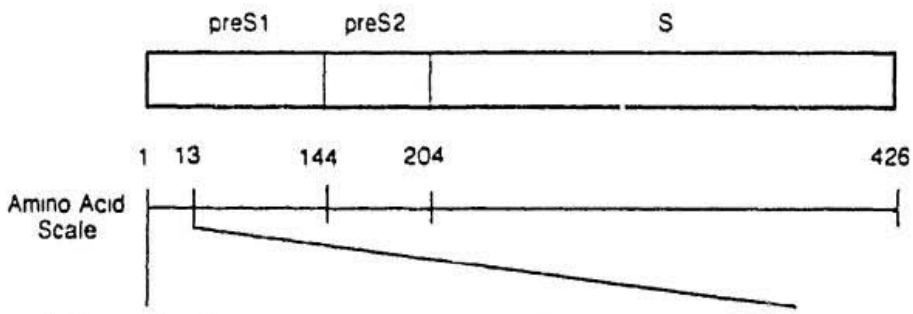

m.wt.
kD
A
B
Top
115
78
50 - 58
45
42
Bottom

with ^{125}I-WHsAg inhibits the binding of the WHV envelope to this HPM molecule, (2) free heparin, but not heparin complexed with the WHV envelope particles, recognizes HPM proteins other than 330-kD protein band, and (3) it is highly unlikely that heparin represents a bridge molecule for WHV binding to woodchuck hepatocyte plasma membrane.

3.14 Selection and Purity of the Synthesized JP1 Sequence

Computer assisted analysis performed by other investigators has suggested that the amino acid sequences encoded by the preS1 region of the S (envelope) gene of hepadnaviruses have the most peripheral location in the virion (Schaeffer *et al.*, 1986; Neurath *et al.*, 1986). In addition, the analysis performed in this laboratory with the use of the PC Gene software (Intelligenetics Inc., Mountain View, CA) predicted that the tetrapeptide Asn-Pro-Asp-Lys, occurring at position 10-13 of the WHV preS1 protein (Figure 27), is hydrophillic and is likely to adopt a β-turn configuration (Figure 28). Thus, since it is generally accepted that the above features are indicative of an amino acid sequence which can be exposed on the outer surface of the protein, it was thought that this viral sequence could be involved in important functions of the virus such as mediating interactions with cellular constituents. To test whether the binding activity expressed by the woodchuck hepatocyte and splenocyte plasma membranes for the WHV envelope, including that displayed by the 330-kD proteoglycan, is mediated by the N-terminal amino acid sequence of the WHV preS1 protein, a peptide named JP1, carrying

Figure 27. Sequence of the first 13 amino acids at the N-terminus of the WHV preS1 envelope protein and the synthesized sequence of the oligopeptide JP1.

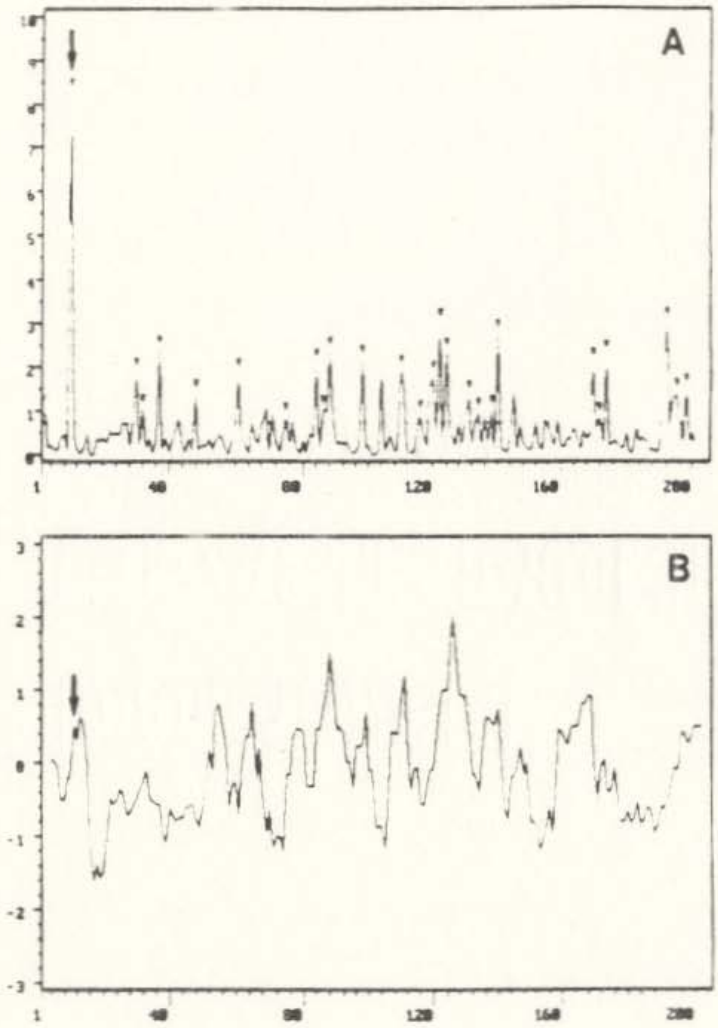


(NH_2) - Met Gly A .n Asn 'le Lys Val Thr Phe Asn Pro Asp Lys - (COOH)

(NH_2) - Met Gly Asn Asn Ile Lys Val Thr Phe Asn Pro Asp Lys Cys - (COOH)

JP1 Sequence

Figure 28. Hydrophilicity and β-turn plots predicted for the amino acid sequence of the preS regions of the WHV envelope proteins. Arrows indicate the predicted hydrophillic peak (panel A) and the corresponding location of the β turn (panel B).



Amino Acid Scale

the predicted Asn-Pro-Asp-Lys site, was chemically synthesized. The JP1 peptide sequence shown in Figure 27 was synthesized according to the solid phase method and purified to homogeneity using HPLC. The absorption spectra shown in Figure 29 revealed a single peak, demonstrating that the oligopeptide preparation was in excess of 99% purity.

3.15 Binding of the JP1 Peptide to Host and Non-Host Specific Tissue Homogenates

In an attempt to recognize whether the JP1 peptide is able to bind to woodchuck and non-woodchuck tissue constituents, the radiolabelled peptide was incubated with homogenates originating from livers and spleens of a healthy woodchuck, human and mouse. Results demonstrate that woodchuck spleen and liver tissue homogenates clearly exhibited binding for the ^{125}I-labelled JP1 peptide. In contrast, neither the woodchuck kidney homogenate nor the non-host specific tissue homogenates derived from human or mouse expressed binding activity for the peptide (Figure 30). Furthermore, when the amount of JP1 radioactivity bound to woodchuck spleen and liver homogenates was quantified, the former displayed at least a 10-fold higher binding potential (data not shown).

Figure 29. HPLC profile of the synthesized oligopeptide (JP1). The peptide was eluted from a non-polar C-18 matrix column over the time period of 60 min as described in "Materials and Methods". The peak corresponds to the absorption reading of the peptide at 260 nm.

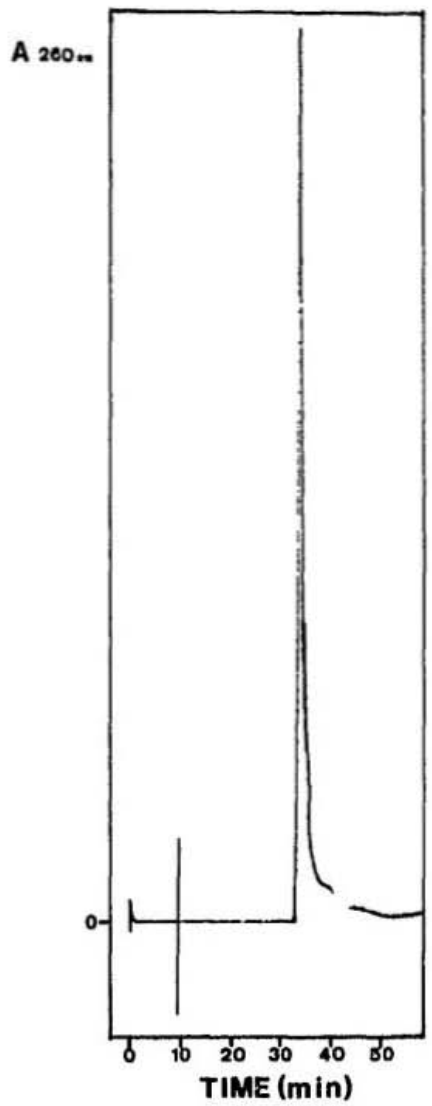
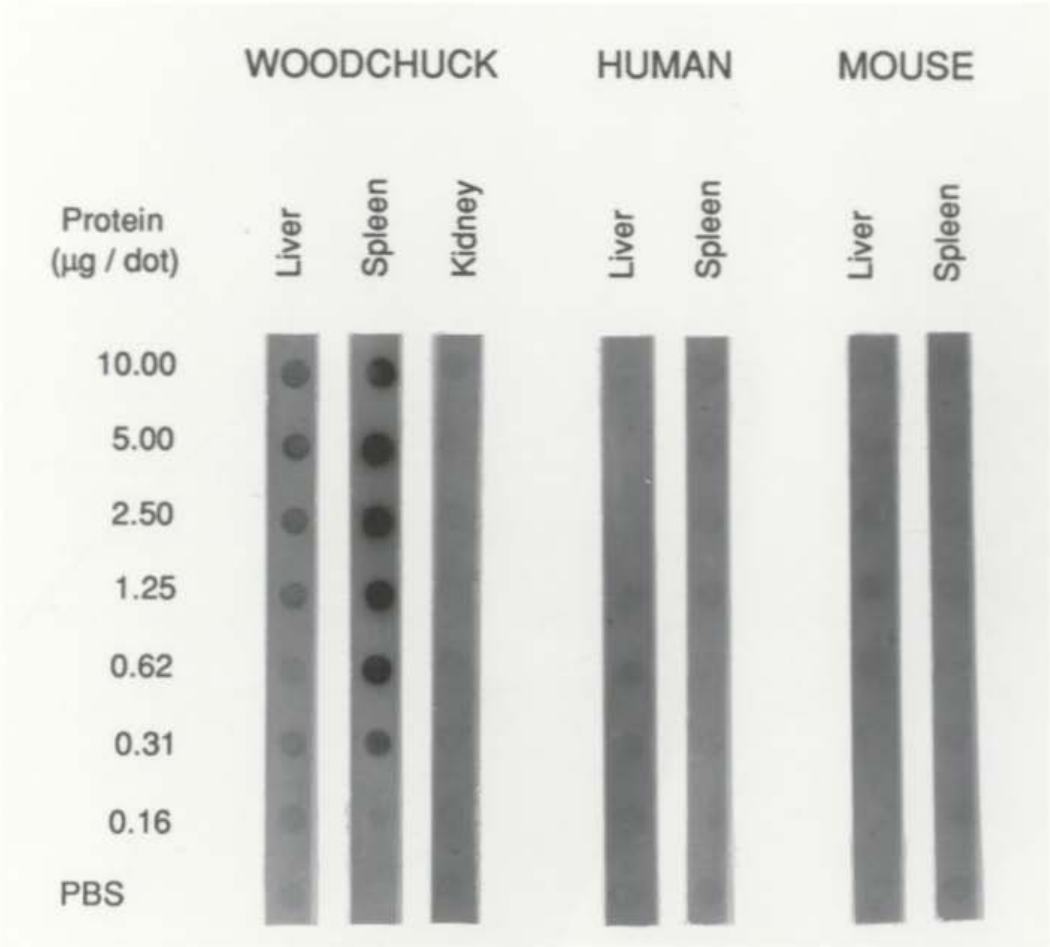

A 260 nm
0
0
10
20
30
40
50
TIME (min)

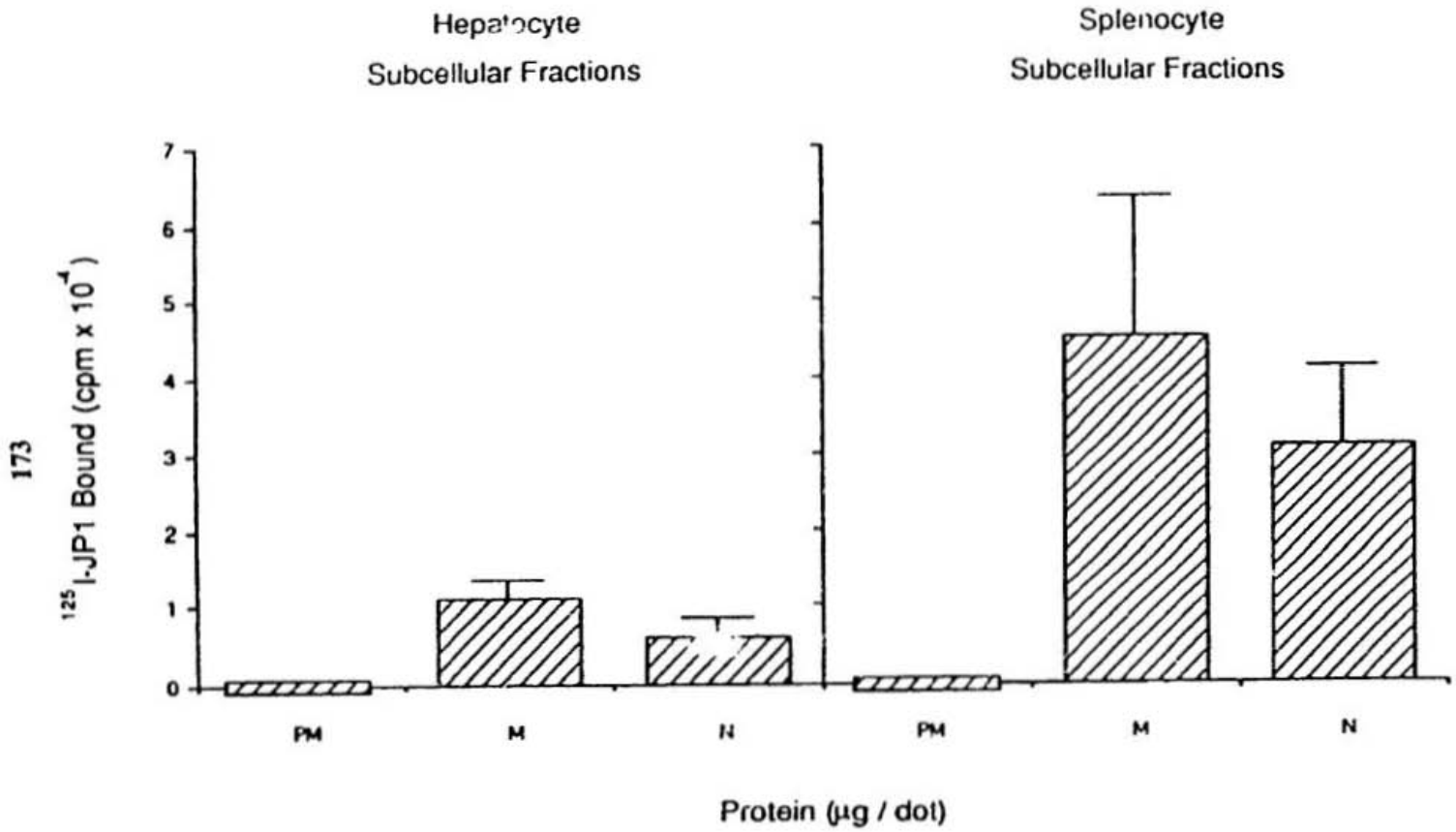
Figure 30. Binding of the synthetic JP1 oligopeptide to woodchuck, human, and mouse tissue homogenates. Serial two-fold dilutions of protein samples from the various tissue homogenates were spotted onto nitrocellulose, incubated with ^{125}I-JP1, washed, air-dried, and exposed for autoradiography as described in "Materials and Methods". Background radioactivity was visualized using PBS instead of protein samples.


WOODCHUCK
HUMAN
MOUSE
Protein
(µg / dot)
Liver
Spleen
Kidney
Liver
Spleen
Liver
Spleen
10.00
5.00
2.50
1.25
0.62
0.31
0.16
PBS

3.16 Binding of the JP1 Sequence to Subcellular Fractions Derived from Woodchuck Hepatocytes and Splenocytes

To test whether the binding activity observed by woodchuck spleen and liver homogenates was restricted to plasma membranes or to constituents present in other subcellular fractions, fractions enriched in mitochondria and nuclei were purified from isolated hepatocytes and splenocytes and tested for ^{125}I-JP1 binding using a nitrocellulose dot-blot assay. Surprisingly, it was found that the JP1 sequence bound to the mitochondrial and nuclear-enriched fractions of both cell types, but not to the plasma membranes of these cells (Figure 31). This observation excluded the possibility that the JP1 sequence recognizes the 330-kD molecule identified as the WHV receptor in the course of this study. Since the splenocyte mitochondrial and nuclear fractions exhibited a 3 to 5-fold higher potential to bind the oligopeptide than the corresponding hepatocyte fractions (Figure 31), this may suggest that a binding site specifically recognizing the preS1 sequence of WHV may exist at the intracellular level and be expressed in woodchuck lymphoid cells to a much higher extent than in hepatocytes. Identification of the postulated intracellular virus attachment molecule for WHV exceeds the aims of this thesis and will be continued in further studies by this laboratory.

Figure 31. Binding of the JP1 sequence to purified woodchuck hepatocyte and splenocyte subcellular fractions. ^{125}I-labelled JP1 was incubated with dots containing 25 μg protein of plasma membranes (PM), mitochondria (M), or nuclei (N), the dots were cut out and the bound radioactivity determined as described in "Materials and Methods". Control dots incubated with PBS were used as negative controls to determine background binding. The data represent the mean of three independent experiments after subtraction of background binding. Bars represent the standard deviations of the means.


Hepatocyte
Subcellular Fractions
Splenocyte
Subcellular Fractions
^{125}I-JP1 Bound (cpm x 10^{-4})
0
1
2
3
4
5
6
7
PM
M
N
PM
M
N
Protein (µg / dot)

CHAPTER 4

4. DISCUSSION

The studies described in this thesis were aimed at assessing the character of the initial interaction between the WHV envelope (WHsAg) and host cells and to recognize the properties of cellular molecules mediating this binding. In this respect, plasma membranes and other subcellular fractions isolated from woodchuck hepatocytes, splenocytes and kidney cells, as well as WHV envelope particles were purified and used as reactants. The biochemical properties of the plasma membrane constituents mediating the observed binding were studied using virus and lectin affinity chromatography and enzymatic digestions. In the course of the study, a 330-kD proteoglycan containing heparan sulphate and polymannose-like activity was identified as a host plasma membrane receptor contributing to the binding of WHV. In addition, an oligopeptide homologous with the N-terminal amino acid sequence of the WHV envelope preS1 protein, containing a structure predicted to be exposed on the virion's surface, was synthesized and used to explore its predisposition toward host and cell interactions. The results revealed that the peptide did not interact with the 330-kD receptor, however, it appeared to recognize an intracellular element specific for woodchuck hepatocytes and lymphoid cells.

To date, among the methods used for receptor purification, affinity column chromatography appears to be the most efficient and one of the most common. Several cellular receptors have been identified and purified to homogeneity employing this

technique. For instance, the receptors for the thyroid stimulating hormone (TSH; Tate *et al.*, 1975; Fenzi *et al.*, 1978), and epidermal growth factor (EGF; Cohen *et al.*, 1980), as well as ,α adrenergic (Graham *et al.*, 1982; Regan *et al.*, 1982), β adrenergic (Vauquelin *et al.*, 1977; Vauquelin *et al.*, 1979), and muscarinic acetylcholine (Andre *et al.*, 1983) were characterized using affinity chromatography. Furthermore, affinity chromatography has been successfully used for the isolation and characterization of receptors for the reovirus 3 and encephalomyocarditis virus (Co *et al.*, 1985; Pardoe and Burness 1980). Virus specific affinity chromatography was also used in preliminary studies, which attempted the isolation of HBV receptors from HepG2 cells (Neurath *et al.*, 1986). Promising results from the above study encouraged the use of a similar approach for the purification of cellular components expressing WHV binding activity. In addition, a virus overlay protein blot assay was used in this study to identify and characterize a plasma membrane-associated WHV receptor. This technique has been used to characterize putative cell receptors for a number of viruses, including Sendai virus (Gershoni *et al.*, 1986), human cytomegalovirus (Adlish *et al.*, 1990), reovirus (Verdin *et al.*, 1989), mouse hepatitis virus (Boyle *et al.*, 1987), lymphocytic choriomeningitis virus (Borrow and Oldstone, 1992), and, recently, encephalomyocarditis virus (Jin *et al.*, in preparation). Since identification and biochemical characterization of cell surface receptors for WHV were the main objectives of this study, steps were taken to ensure that the woodchuck plasma membrane and WHV envelope preparations used in the experiments were of the highest possible purity. Characterization of the receptor

molecules was also preceded with experiments that assessed whether the plasma membranes derived from woodchuck cells indeed expressed binding activity for the WHV envelope.

4.1 Biochemical Characterization of Woodchuck Plasma Membrane Preparations

The surface of the hepatocyte has three faces: a blood-sinusoidal, a bile-canicular and a contiguous face. The receptors for ligands circulating in blood (*e.g.*, hormones; Wisher and Evans, 1975) are mainly located on the plasma membrane of the blood-sinusoidal face. Since hepadnaviruses spread throughout the blood, it is expected that their receptors should exist on the blood-sinusoidal face of the hepatocyte surface. To date, a variety of methods have been reported for the isolation of HPMs. The method published by Touster and coworkers (1970) is of particular interest to this thesis because the membrane fraction, originally designated as P2, was found to be enriched in molecules which are characteristic for the blood-sinusoidal face of hepatocytes. For example, receptors for galactose-terminated glycoproteins and insulin (Doyle and colleagues 1979), as well as, the enzyme Na^+-K^+-ATPase have been identified on these membranes.

The results of the enzymatic assays shown in Table 1 are indicative that the isolated woodchuck HPMs by the method described by Touster and colleagues (1970), were essentially free from mitochondrial and microsomal contamination, which represent

the most commonly detectable impurities in plasma membrane preparations. Nevertheless, when these results are compared to the published data, a few discrepancies are noticed. For example, although the yield of 5'-nucleotidase activity is lower in the purified woodchuck HPMs (10.79%±4.10; Table 1) compared to that shown by Touster and colleagues (1970) in rat liver plasma membranes (*i.e.*, 24.1%±4.0), it is still significantly higher than that reported by Enrich and colleagues (1986) in rat hepatocytes (*i.e.*, 4.8%±0.19). On the other hand, no significant difference is observed when the relative enrichment for the enzyme, that is the activity of the enzyme in the plasma membranes divided by the enzyme activity detected in the initial homogenate used for the membrane purification, the latter normalized to 1 (*i.e.*, 17.40±3.32; Table 1), is compared to that reported by others (Touster *et al.*, 1970, Cook *et al.*, 1983 and Enrich *et al.*, 1986). In regards to the yield of Na^+-K^+-ATPase (*i.e.*, 8.96%±1.84; Table 1) and its relative enrichment (*i.e.*, 8.05 ± 1.84; Table 1) in woodchuck HPMs, it was found that although the former is significantly higher than that shown by Enrich and colleagues (1986) (*i.e.*, 2.88%±0.64), the latter is comparable to the results reported by these authors (*i.e.*, 10.08±2.83). Furthermore, woodchuck HPMs contained only a marginal level of glucose-6-phosphatase activity which is an enzyme occurring mainly in microsomes. Such a low level of this enzyme activity is in keeping with that found by Touster and colleagues (1970) and Enrich and coworkers (1986). It should be pointed out, however, that the relative enrichment of glucose-6-phosphatase, as reported by Enrich and coworkers (1986), in rat hepatocyte plasma membranes is 10-fold lower (*i.e.*,

0.11±0.02), than that published by Touster and coworkers (1970) (*i.e.*, 1.1±0.1). An explanation for such discrepancy has been put forward by Touster and coworkers (1970). They suggest that HPM preparations that demonstrate a very low glucose-6-phosphatase activity can in fact be contaminated with constituents other than endoplasmic reticulum, such as nuclear envelopes and mitochondria outer membranes. Finally, succinate cytochrome C reductase activity, a marker for mitochondria, was not detected in the isolated HPMs. A low level of activity for this enzyme was detected by Cook and coworkers (1983) in rat HPM preparations, in spite of the fact that the procedure used to isolate their membranes was the one reported by Touster and colleagues (1970).

The yield and relative enrichment for 5'-nucleotidase exhibited by plasma membranes purified from woodchuck spleen (SPMs) are comparable to the results for rat spleen plasma membranes, (*i.e.*, yield of 14.3% and relative enrichment of 14.1) published by Misra and colleagues (1975). On the other hand, values obtained for the relative enrichment of glucose-6-phosphatase tabulated in Table 2 was higher (*i.e.*, 1.35) than that found in rat spleen plasma membranes (i.e., 0.52; Misra *et al.*, 1975). Such a result, however, does not necessarily mean that the woodchuck SPM preparation contained non-desirable levels of endoplasmic reticulum contamination, since the relative enrichment for this enzyme in this preparation is in total agreement with that obtained for woodchuck HPMs (i.e., 1.55±0.40; Table 1). Finally, like HPMs, woodchuck SPMs were also free of any mitochondrial contamination, as indicated by the absence of succinate cytochrome C reductase activity.

The purity of woodchuck kidney plasma membranes was also evaluated using the above mentioned enzyme markers. The yields for the plasma membrane 5'-nucleotidase (*i.e.*, 9.12%; Table 3) and Na^+-K^+-ATPase (*i.e.*, 7.25%; Table 3) were lower than that reported by Hazel and Landrey (1988) (*i.e.*, 34.4% and 24.5%, respectively). In contrast, the respective relative enrichments obtained by these authors (*i.e.*, 17.0 for 5'-nucleotidase and 13.0 for Na^+-K^+-ATPase) were comparable to those obtained in the present study. Furthermore, when woodchuck KPMs were assayed for endoplasmic reticulum contamination only low levels of glucose-6-phosphatase activity (*i.e.*, yield of 1.20% and relative enrichment of 1.25; Table 3) were detected. This result is in contrast to data published by Hazel and Landrey (1988) who reported a significantly higher yield for this enzyme (*i.e.*, 10-15% and a relative enrichment of 2.86). This discrepancy indicates that the woodchuck KPM preparation was far less contaminated with endoplasmic reticulum than the trout kidney plasma membranes obtained by the above mentioned investigators. On the other hand, succinate cytochrome C reductase activity in both woodchuck KPMs (Table 3) and trout kidney plasma membranes (Hazel and Landrey, 1988) was not detectable. The absence of activity for this enzymatic marker strongly suggests that KPMs did not contain mitochondrial contamination.

Overall, the woodchuck hepatocyte, spleen and kidney cell plasma membranes were found to be highly pure according to accepted biochemical criteria.

4.2 Polypeptide Composition and Structural Characterization of Woodchuck Virus Envelope Particles

WHV envelope particles were purified from the serum of a woodchuck WHV carrier according to the procedure described by Gerlich and colleagues (1980). This procedure involved purification of the particles by gel filtration and cesium chloride fractionation through an isopycnic density gradient. The assessment of WHsAg presence in fractions generated by centrifugation in cesium chloride, revealed that the fractions corresponding to the isopycnic density ranging from 1.18 to 1.19 g/cm^3 contained most of the WHsAg activity. Ultrastructural examination of these fractions demonstrated the presence of abundant spherical and a few filamentous WHV envelope particles. Both the isopycnic density at which the WHV envelope particles were recovered and their morphology are in keeping with previously reported results. For example, Summers and colleagues (1978) showed that WHV envelope particles sediment to an approximate density between 1.192 and 1.204 g/cm^3 and that WHV spherical particles are more abundant in these fractions when compared to their filamentous counterparts.

Examination of the isolated envelope particles by SDS-PAGE demonstrated that their polypeptide composition was analogous to a pattern reported by Pohl and colleagues (1986). A minor exception was, however, noticed when the 36-kD band, that corresponds to one of two glycosylated forms of the middle protein, was absent in the majority of our preparations despite the fact that in all WHsAg preparations, molecular weight equivalents for the major, middle and large WHV envelope proteins were present.

However, the 74-kD band, which most likely represents a dimer of the 36-kD polypeptide, was always detectable. Furthermore, unlike the WHsAg preparation presented by Pohl and coworkers (1986), two sets of pairs, namely the 52 and 58-kD and 67 and 74-kD, thought to be dimers for the 24 and 27-kD and 34 and 36-kD, respectively, were observed. Overall, it is certain that the WHV envelope preparation used in the experiments described in this thesis was pure and that the above mentioned exceptions were most likely attributed to formation of peptide dimers.

4.3 Binding of WHsAg to Native Hepatocyte Plasma Membranes

The results presented in Figures 4, 6 and 7 demonstrate that woodchuck HPMs undoubtedly express ability to bind WHsAg. In Figure 5, distribution analysis of the radiolabelled WHsAg pre-incubated with HPMs on the 5-45% sucrose gradient demonstrated that, unlike plasma membranes derived from a woodchuck chronic WHV carrier, normal hepatocyte plasma membranes display binding for exogenous ^{125}I-WHsAg. This affinity, viewed as a peak of the WHsAg radioactivity co-sedimenting with membrane proteins in the middle of the gradient, corresponds to the membrane-^{125}I-WHsAg complex, not to the free ^{125}I-WHsAg, which was found to sediment within the top fractions of this sucrose gradient. In contrast, as shown in the same figure, exogenous ^{125}I-WHsAg was unable to form a complex with infected HPMs. Failure to localize the peak of WHsAg radioactivity sedimenting together with the peak of WHV-infected HPM proteins is consistent with the previous observation made in this laboratory

(Michalak, 1988). In that study, when woodchuck HPMs were fractionated across a 5-45% sucrose gradient only one protein peak, localized in the middle of the gradient, was observed. Furthermore, only this peak, but not fractions before or after it, retained WHsAg reactivity. It has been proposed that the inability of the infected plasma membranes to bind exogenous ^{125}I-WHsAg may be due to the saturation of HPM receptors for WHV by WHsAg. A similar phenomenon is apparently observed on the surface of target cells in the course of a variety of chronic virus infections (Longberg-Holm and Philipson, 1974). It is also possible that resistance of infected HPMs in binding WHsAg is due to the insertion of the virus envelope polypeptides or other viral proteins into the HPM, leading to a restructuring of the membrane in such a way that the WHV receptors are modified or even abrogated. Indeed, most of the WHsAg incorporated into HPMs in woodchucks infected with WHV behaves as an integral membrane protein, suggesting that the incorporation is irreversible (Michalak and Churchill 1988; Michalak *et al.*, 1989). Furthermore, Western immunoblotting studies performed with the use of HPMs, isolated from different immunomorphological stages of WHV hepatitis, proved that the WHV envelope major S and preS proteins, as well as core polypeptides of the virus are incorporated into infected HPMs as integral membrane proteins (Lin and Michalak, 1991).

Incubation of woodchuck HPM proteins with WHV envelope particles for different periods of time, demonstrated that the WHsAg-hepatocyte plasma membrane interaction is time dependent (Figure 4). Moreover, analysis of the data generated by

incubating constant amounts of native or Triton X-114 solubilized woodchuck HPMs (receptor) with increasing amounts of WHV envelope (ligand; Figure 7) and vice-versa (Figure 9 and 12), revealed that in both cases the binding is saturable. Therefore, since saturability of the receptor is generally accepted as an indicator of ligand specificity, it can be concluded that the binding displayed by woodchuck HPMs for WHV envelope is likely to be mediated by specific receptor molecules. However, despite the saturable kinetics displayed by the WHV envelope binding to both water-soluble and native HPMs (Figure 7, 9 and 12), it is clear that they differ in the manner in which they achieve the saturation plateau. For instance the shape of the curve depicting the binding kinetics of native HPMs for the virus envelope appears to be biphasic suggesting that probably two classes of receptors exhibiting unequal WHsAg binding affinities participate in the binding (Figure 9). On the other hand, the shape of the binding kinetics for the water-soluble HPMs is monophasic, indicating that most likely only one class of receptor molecule mediates the binding (Figure 12). Furthermore, preincubation of HPMs with cold WHsAg partially inhibited the binding of the radiolabelled WHV envelope particles to membranes (Figure 8). This finding supports the conclusion that the observed binding displayed properties of specific ligand-receptor interaction. It was observed, however, that ^{125}I-labelled WHsAg is capable of binding to cold WHsAg and that the extent of this self-aggregation increases with the concentration of virus protein. Conversely, this phenomenon was not observed, when concentrations of labelled or cold envelope subviral particles were below 3μg protein per assay. Therefore, due to the behaviour of the

WHsAg, determination of the dissociation constant, as well as values of maximal binding was not possible. In spite of the above, the data provides clear evidence that highly purified woodchuck HPMs and the corresponding soluble fraction obtained by treatment with Triton X-114, exhibit binding sites for WHV envelope. This finding remains in agreement with those made by Pontisso and coworkers (1989b), who showed that a much more crude preparation of human liver plasma membranes displayed binding for the HBV envelope.

4.4 Binding of WHsAg to Woodchuck Spleen and Kidney Plasma Membranes

Although the liver is the major target of hepadnaviral invasion, HBV DNA replicative intermediates and virus specific mRNA have also been found in non-hepatic tissues such as spleen (Lieberman *et al.*, 1987) and in peripheral blood mononuclear cells (Yoffe *et al.*, 1986; Pontisso *et al.*, 1984; Michalak *et al.*, in press). In the case of woodchuck hepatitis virus, large quantities of the virus DNA replicative intermediates have also been detected in the liver, spleen (Korba *et al.*, 1987,1988b, 1989a) and, in smaller amounts, in peripheral blood mononuclear cells (Korba *et al.*, 1986). In addition, in woodchucks chronically infected with WHV, trace quantities of WHV DNA have been detected in kidneys, pancreas, ovary and testis (Korba *et al.*, 1990). In a duck model of hepatitis B, evidence of active replication of DHBV has been found in liver and in extrahepatic tissues such as pancreas, kidney, spleen, brain, lung, heart and intestine

(Halpern *et al.*, 1983; Jilbert *et al.*, 1987a,b; Tagawa *et al.*, 1985; Hosoda *et al.*, 1990). Certainly, one should keep in mind that studies involving the detection of viral nucleic acids refers very often not only to replicative DNA intermediates, virus specific DNA transcripts and viral mRNA, but also to non-replicative forms of viral DNA. The presence of viral DNA in the replicative form and of viral specific RNA, suggests that the organs or cells, from which these nucleic acids were extracted, are capable of supporting virus multiplication. However, detection of non-replicative forms of hepadnavirus DNA alone in cells can not exclude the possibility that their presence is a result of DNA contamination originating from blood, where freely circulating virions commonly exist. Thus, the identification of replicative forms of viral nucleic acid is certainly more meaningful in terms of the viral pathogenesis than the sole presence of non-replicating viral DNA. Further, identification of replicative forms of viral genome in a given cell type indicates that surface cellular receptors must have been present to allow virus entry and replication.

There is evidence, in addition to the identification of viral DNA replicative intermediates and virus specific mRNA, which supports the view that receptors for hepadnaviruses may exist on non-hepatic cells. Neurath and coworkers (1990) have reported that human peripheral blood B lymphocytes, some haematopoietic cell lines of the B lineage, amnion, neuroblastoma, and embryonic carcinoma cells display binding activity for HBsAg. In fact, these authors claim that these receptors are the same as those found on the surface of HepG2 cells, reported in one of their earlier studies

(Neurath *et al.*, 1986). However, one should keep in mind that cells which express surface receptor for the virus may not be able to support virus replication conversely cells which support virus proliferation may lack the continuous expression of surface receptors for the virus. In this respect, Neurath and colleagues (1992) recently demonstrated that Concanavalin A seems to be capable of inducing HBV receptors on human cells which do not normally express them, such as the Hut-78 and Molt-3 T cell lines. Similarly, HBV receptors on the surface of peripheral blood mononuclear cells and U 937 cells were apparently induced by liposaccharide (LPS). Perhaps what is more striking in these studies are the following three findings: (a) several cell lines cultured in single cell suspension (*e.g.*, Hep G2 [liver hepatoma] and Cos-1 [SV-40-transformed African Green monkey kidney cells]) bound to the affinity column containing HBV preS1 (21-47) peptide, but the binding was inhibited by preincubation of the cells with cytokine interleukin-6 (IL-6); (b) polyclonal antibodies to IL-6 inhibited the binding of HBV preS1 (21-47) oligopeptide to these cell lines from 45% to almost 100%, and (c) release of most, if not all, IL-6 from the surface of the cell lines by pretreating the cells at pH 4.0, abrogated their attachment to HBV preS1 (21-47) oligopeptide. Thus, it seems to be clear from these studies that HBV receptors exist both on hepatocytes and on many cells of non-hepatic origin.

Data obtained in the course of the present study indicate that WHsAg binding activity was exhibited by plasma membranes derived from cells of two extrahepatic woodchuck tissues, namely the spleen and, to a much lesser extent, kidney (Figure 14).

These results suggest that lymphoid cells may be a particularly good target for WHV invasion. In this regard it is well documented that woodchuck spleen cells are capable of supporting active WHV replication and that they contain large amounts of WHV DNA, in animals with chronic WHV hepatitis (Korba *et al.*, 1987, 1988 a,b). As mentioned above, WHV DNA replicative intermediates can also be found in kidneys of chronically infected woodchucks (Korba *et al.*, 1990), although at a much lower copy number when compared to liver or spleen. Thus, results from the present study remain apparently in good agreement with the identification of HBV DNA at the molecular level in the respective organs. Interestingly, woodchuck SPMs showed approximately 40% of WHsAg binding activity when compared to HPMs, whereas, in the case of KPMs, it did not exceed 20% (Figure 14).

In an attempt to analyse further the discrepancy in the extent of WHV envelope binding to plasma membranes derived from various cell types, we chose plasma membranes which displayed high (HPMs) and low (KPMs) WHsAg binding activity for further investigation.

4.5 One-Step Purification of WHsAg-Binding Constituents from Woodchuck Hepatocyte and Kidney Plasma Membranes by Affinity Chromatography

As mentioned earlier, virus affinity chromatography was chosen in order to characterize cell surface receptors for the WHV envelope proteins. Since binding to

affinity matrices is best performed when water-soluble substrates are used, woodchuck HPMs and KPMs were subjected to Triton X-114 treatment. This treatment resulted in the production of two phases *e.g.* hydrophobic and hydrophillic, however only the latter was used in subsequent experiments. Solubilization of HPMs with Triton X-114 proved to be a good approach because the resulting aqueous phase proteins demonstrated an enhanced WHsAg binding activity compared to the native HPMs, as shown in Figures 10 and 11. In the initial steps of the WHV receptor isolation, a few significant improvements were made to the purification strategy. For instance, highly purified woodchuck plasma membranes essentially free from cellular contamination were used instead of a crude homogenate of cells; this may represent an improvement over the methods used by others (*e.g.*, Neurath *et al.*, 1986). In addition, a soluble fraction of HPMs was used to ensure that efficient recovery of the WHsAg-binding molecules from affinity matrices took place. According to the general principles of affinity column chromatography, when solubilized plasma membranes were chromatographed on the WHsAg-affinity column, the wash should contain only those membrane proteins which do not have receptor activity for WHsAg. However, the dot-blot radioligand binding assay used for the semi-quantitative evaluation of the WHsAg binding revealed that the wash from HPMs exhibited a residual WHsAg binding activity (Table 4; Figure 18). It is pertinent to this study to explain why the above wash displayed such WHsAg binding activity. One possibility is that some membrane proteins which recognize WHV envelope did not interact with WHsAg because the latter was covalently bound to

cellulose through the same surface structures that are also recognized by cellular proteins. The most likely possibility, however, is that not all HPM proteins, which expressed WHV envelope binding potential, were able to bind to WHsAg#SH-cellulose because the column binding capacity was exceeded. In fact, since the $MgCl_2$ eluates of HPMs from the column demonstrated maximum WHsAg binding activity in the nitrocellulose dot-blot ^{125}I-WHsAg binding assay (*i.e.*, value of 7+), this suggests that all binding sites on the column were occupied before the specifically bound proteins were eluted. Consequently, an excess of the membrane proteins which would otherwise bind the WHsAg#SH-cellulose column were unable to do so, thus, appearing as a part of the wash. It was further observed that when the amounts of soluble HPM proteins not exceeding 0.5 mg/ml were applied on the WHsAg column, the WHV envelope binding activity was not detectable in the wash (data not shown). This indicates that the column under routinely used conditions (*i.e.*, 1 mg HPM protein loaded in a volume of 1 ml) was overloaded with proteins capable of recognizing virus envelope. These conditions, however, ensured maximum recovery of WHV envelope binding proteins in the $MgCl_2$ eluate.

SDS-PAGE analysis of hepatocyte plasma membrane proteins recovered in the $MgCl_2$ eluates showed a multiband pattern, suggesting that virus may bind to more than one plasma membrane protein (Figure 17). This finding supports the notion that the virus envelope may assume characteristics of a multivalent ligand. Otherwise, it may suggest that the observed multiband pattern in the $MgCl_2$ eluates reflects presence of both specific receptor(s) and proteins that were structurally associated with them. These

complexes were resistant to dissociation with Triton X-114 but they were dissociated under reducing conditions of SDS-PAGE. The phenomenon of virus direct binding to multiple plasma membrane proteins has been observed in other viruses, *e.g.*, reovirus binding to mouse T cells (Choi *et al.*, 1990). In addition, analysis of HPM proteins recovered in the washes from the WHsAg#SH-cellulose column revealed that, although the washes did not display all the proteins present in the $MgCl_2$ eluates, they did have some common proteins (namely those ranging in molecular weight from 140-kD to 28-kD; data not shown). This further supports the view that the WHsAg#SH-cellulose column was overloaded with HPM constituents that exhibited binding for the WHV envelope.

In eukaryotic cells, the carbohydrate content of the plasma membranes ranges from 2 to 10% by weight (Alberts *et al.*, 1983). Hepatocytes maintained in cultures have also been shown to have a variety of externally oriented glycoproteins on the plasma membrane (Le and Doyle, 1984). As mentioned in the introduction (section 1.3), several studies have demonstrated that glycosylated proteins serve as receptors for physiologically important ligands and, at the same time, may serve as receptors for viruses. For example, the acetylcholine and the epidermal growth factor receptors function as receptors for the rabies (Lentz *et al.*, 1982) and vaccinia viruses (Eppstein *et al.*, 1985), respectively. Since carbohydrate moieties associated with the plasma membrane proteins appear to be the cell surface constituents most often involved in virus specific recognition, it became important to test whether carbohydrate moieties of the

woodchuck plasma membranes participate in the recognition of WHV envelope. Preliminary assessment of the carbohydrate composition of woodchuck HPMs, SPMs and KPMs using separation by SDS-PAGE and periodic acid-Schiff staining demonstrated that most, if not all, of the protein bands stained also for carbohydrate (Figure 13). This result suggests that most of the proteins present in the plasma membranes were covalently associated with some type of carbohydrate moiety. It is important to point out that several different types of carbohydrate moiety can be bound to plasma membrane proteins.

In general, protein glycosylation is achieved through the covalent linkage of a carbohydrate to a polypeptide chain. Among the known types of linkage, the most common are those where either the reducing end of N-acetylglucosamine (GlcNAc) is bound to the β-amide group of asparagine (N-glycosidic bond) or the reducing end of N-acetylgalactose (GalNAc) is bound to the hydroxyl group of serine or threonine (O-glycosidic bond; Le and Doyle, 1984; Lotan and Nicolson, 1979). The known structures stemming from oligosaccharides linked by N-glycosylation can be classified into two types: (a) polymannose, which contains more than three mannose residues peripheral to the mannose-$(GlcNAc)_2$ core and (b) the complex type, in which sialic acid occupies non-reducing terminal positions on both the main and branch oligosaccharide chains and are linked *via* galactose-GlcNAc sequences to internal mannose residues. On the other hand, O-glycosidically-linked oligosaccharides usually have a core structure such as galactose-β-(1,3)-GalNAc to which either fucose is linked to galactose by α-(1,2) or sialic acid is

linked to galactose or to GalNAc by either α-(2,3) or α-(2,6) (Cummings *et al.*, 1989). Nevertheless, there is yet another type of O-glycosidic link, which has been found to be characteristic of protein-carbohydrate complexes, called proteoglycans. In this case, the glycosidic linkage is between the reducing group of xylose and the hydroxyl group of serine, as it is the case in the charged glycosaminoglycans, chondroitin A, B or C, heparin or heparan sulphate. The xylose residue, in turn, is linked to two galactoses resulting in a trisaccharide link to which one of the following can be covalently bound: chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate, heparan sulphate or heparin (Alberts and colleagues 1983; Roden 1980). Corneal keratan sulphate, however, is N-linked to asparagine of a protein *via* mannose and GlcNAc residues. Alternatively, skeletal keratan sulphate is O-linked to either serine or threonine units *via* a GalNAc residue (Kjellen and Lindahl, 1991; Roden, 1980).

In terms of the basic glycan structure of heparin or heparan sulphate, it has been determined that it is constituted by [GlcA β1,4 linked to GlcNAc α1,4]$_n$ linkages. Thus, in general, these polysaccharide structures are quite similar to each other (Kjellen and Lindahl, 1991). However, heparin usually contains more N- and O- sulphate groups and a higher proportion of Idouronic acid (IdoA) units. The galactosaminoglycans, such as chondroitin-4-sulphate, chondroitin-6-sulphate and dermatan sulphate are by in large generated by the structure [GlcA β1,3 linked to GalNAc β1,4]$_n$. The heterogeneity of these carbohydrate structures is owed to the presence of GlcA and IdoA units and the location of sulphate groups. The keratan sulphate glycosaminoglycan is generated by the

[Gal β1,4 linked to GlcNAc β1,3]$_n$. The sulphate group may be located on either or both Gal and GalNAc units (Kjellen and Lindahl, 1991). Finally hyaluronic acid consists of repeating disaccharide units of GlcA β1,3 linked to GlcNAc (Roden, 1980).

The development of lectin affinity chromatography and enzymatic digestions set the stage for a more detailed characterization of glycoproteins. Lectins are a group of proteins known for their specific recognition and binding to a single glycosyl residue (Goldstein and Hayes, 1978). This residue is usually found to be at the non-reducing terminal group of a carbohydrate chain. However, the specificity of the residue interaction with the lectin can be influenced by the nature of the glycosidic linkage, steric factors or even by non-specific interactions (Hedo, 1984). In view of the fact that lectins express a remarkable specificity to defined carbohydrate residues, these proteins have been commonly used as probes for the identification of cellular glycoproteins. An example of such a probe is Concanavalin A, well known for its α-D-mannose specificity (Hedo, 1984). This lectin has been widely used to isolate and purify various forms of N-linked oligosaccharides such as polymannose and bi-antennary complexes (Baenziger and Fiete, 1979; Krusius *et al.*, 1976). In addition, Concanavalin A also expresses affinity for the O-linked glycosaminoglycans, such as heparin (Doyle and Kan, 1972; Monges *et al.*, 1989) and most likely heparan sulphate, since it is chemically very similar to heparin (Kjellen and Lindahl, 1991).

In view of the fact that N-linked glycosylated proteins may perform a pivotal role as cellular receptors for viruses, an attempt was made to determine whether some of

these molecules serve as receptors for the WHV envelope. To test this possibility, the water-soluble phase of woodchuck HPMs, as well as the $MgCl_2$ eluates of the membrane constituents obtained from the WHsAg#SH-cellulose column, were subjected to chromatography on a Concanavalin A column. Specific elution of carbohydrate moieties from the Concanavalin A column with 0.3 M MMP revealed that the recovered moieties express a high binding activity for the WHV envelope particles (Table 4, and Figure 18). This suggested that N-linked oligosaccharides and/or heparin-like glycosaminoglycans, which are known to interact with Concanavalin A, are involved in the WHV binding to the hepatocyte surface.

The results on the WHV envelope binding to the water-soluble constituents of HPMs recovered in the eluates after one-step chromatography on the WHsAg#SH-cellulose or Concanavalin A columns suggested that at least two types of interactions existing between WHsAg and the membranes can be distinguished on the basis of their susceptibility for dissociation with a high molar $MgCl_2$ and MMP. Thus, the recovery of the WHV envelope binding constituents after elution with 4M $MgCl_2$ indicates that an ion-dependent bond mediates the interaction, whereas, the recovery of the binding activity in the MMP eluate from a Concanavalin A column implies the existence of a carbohydrate-dependent bond mediated by N-linked polymannose and/or O-linked heparin-like glycosaminoglycan. The involvement of an N-linked bi-antenary complex is not as likely because this type of molecule exhibits a very low affinity for Concanavalin A (Baenziger and Fiete, 1979).

The presence of carbohydrates on the surface of hepatocytes have been well demonstrated. For instance, it has been shown that the rat liver plasma membrane dipeptidyl peptidase IV exhibits N-linked polymannose and N-linked complex carbohydrates (Hartel-Schenk *et al.*, 1991). In addition, the presence of heparan sulphate on the surface of rat liver cells has also been reported (Kjellen *et al.*, 1980, 1981; Soroka and Farquhar, 1991; Brandan and Hirschberg, 1989; Oldberg *et al.*, 1979).

To establish whether there are differences in the nature of binding sites exhibited by HPMs and KPMs for the WHV envelope, a water-soluble phase of KPMs was also chromatographed on a WHsAg affinity column. In spite of the fact that KPMs were solubilized using the same procedure as that used for HPMs, the membrane constituents eluted with 4M $MgCl_2$ from a WHsAg column rendered a much lower binding activity for the WHV envelope than the constituents derived from HPMs when tested at comparable protein concentrations (Table 4). Similarly, in the same experiment, the corresponding wash displayed limited WHsAg binding (Table 4), suggesting that part of the KPM proteins capable of WHsAg recognition was unable to interact with the WHV envelope when it is bound to a cellulose matrix. In other words, some KPM constituents that expressed a potential for WHsAg binding may have failed to recognize WHV envelope probably because WHsAg bound to the SH-cellulose matrix may have blocked or obstructed the epitopes involved in the binding to the membranes.

Also, as in the case of HPMs, chromatography of a water-soluble fraction of KPMs on a Concanavalin A affinity column was able to establish that some of the

membrane glycosylated proteins express WHsAg binding activity. However, specific elution of KPM carbohydrates from the Concanavalin A column clearly demonstrated that the eluted elements express a significantly lower WHV envelope binding activity when compared to HPM carbohydrates eluted and tested under similar conditions. Analysis of the results obtained through the fractionation of solubilized HPMs and KPMs on WHsAg and Concanavalin A affinity chromatography remain in agreement with the initial observations, indicating that native KPMs bound significantly less WHsAg than HPMs. They also indicate that the same types of carbohydrate moieties are likely involved in the binding of WHsAg to those membranes. These results also appears to be in agreement with the carbohydrate content of the membranes visualized by p.a.S. stain after separation by SDS-PAGE, where KPMs evidently displayed less numerous bands with carbohydrate content than HPMs.

Overall, the results on the one-step affinity chromatography experiments suggest that the WHV envelope binding displayed by the plasma membranes is closely associated with the content of carbohydrate moieties capable of interacting with Concanavalin A.

4.6 Purification of WHV Envelope Binding Constituents from Hepatocyte Plasma Membrane by Multi-Step Affinity Chromatography.

To further characterize the nature of WHV envelope binding and to purify plasma membrane receptors, HPM constituents specifically eluted from a WHsAg affinity

column were re-chromatographed on a Concanavalin A column. This two-step procedure proved to be a very useful approach because it demonstrated that both the 4 M $MgCl_2$ eluate from the WHsAg affinity column and the subsequent MMP eluate from the Concanavalin A column displayed the maximal binding activity for WHV envelope, when this activity was measured by the nitrocellulose dot-blot ^{125}I-WHsAg binding assay (Figure 18). This suggests that most, if not all, membrane proteins recognized by WHV envelope through ion-dependent interactions contain N-linked carbohydrates and/or heparin-like moieties. On the other hand, when the water-soluble fraction of HPM proteins was subjected to a reverse two-step purification procedure, in which the MMP eluate from a Concanavalin A column was subsequently re-chromatographed on a WHsAg affinity column, the resulting $MgCl_2$ eluate expressed a substantially lower WHV envelope binding activity compared to that displayed by the initial MMP eluate. However, under the above conditions, most of the constituents that expressed WHsAg binding activity were washed out from the WHsAg column under weak ionic conditions with washing buffer that contained 0.14 M NaCl. These results indicate that among HPM proteins with N-linked oligosaccharides and/or associated heparin-like glycosaminoglycans that were capable of interacting with the WHV envelope, were those which may recognize the virus through strong ionic interactions dissociable by 4 M $MgCl_2$ and others that can be dissociated under weak ionic conditions.

As mentioned above, there is a remote possibility that oligosaccharides containing sialic acid such as N-linked bi- and tri-antennary complexes and/or O-linked

oligosaccharides, play a role in the binding of the WHV envelope to the hepatocyte surface. It has been well recognized that WGA is capable of specifically binding the terminal sialic acid exhibited by N-linked bi- and tri- antennary complexes and O-linked oligosaccharides (Bhavanandan and Katlic, 1979; Montsigny *et al.*, 1980). Therefore, in order to determine whether these types of HPM oligosaccharides exhibit WHV envelope binding potential, a soluble fraction of HPMs was chromatographed on a WGA affinity column. The results of this experiment clearly demonstrated that sialic acid residues of HPMs lack any ability to interact with the WHV envelope (Figure 19). Thus, in spite of the fact that sialic acid residues have been shown to be crucial for the attachment of other viruses, such as polyoma (Fried *et al.*, 1981), encephalomyocarditis (Burness and Pardoe, 1981), myxovirus (Paulson *et al.*, 1979) and influenza C (Rogers *et al.*, 1986), its participation in the attachment of WHV to the hepatocyte surface can be excluded. To confirm this observation, two-step affinity chromatography was employed by re-chromatographing the $MgCl_2$ eluate of HPM constituents obtained from the WHsAg column on the WGA column (section 3.8). In this case, all the WHsAg binding activity displayed by the $MgCl_2$ eluate was recovered in the wash from the WGA column and none was present in the AGU eluate (Figure 20). To strengthen the conclusion that a N-linked polymannose oligosaccharide or an O-linked heparin-like substance, but not O- or N- linked oligosaccharide moieties containing sialic acid, mediate binding of WHV to HPMs, the wash from the WGA column was re-chromatographed on the Concanavalin A column (Figure 20). This chromatographic

procedure fully confirmed previous results by demonstrating that the MMP eluate from the Concanavalin A column retained all the WHsAg binding activity exhibited by the wash from the WGA column (Figure 20). Therefore, the results obtained through affinity chromatography studies indicate that O-linked oligosaccharides or N-linked bi- and tri- antennary complex type oligosaccharides containing sialic acid residues do not play a role in the binding of WHV to woodchuck HPMs. At the same time, they strongly suggest that HPM heparin-like substances and/or a polymannose participate in the attachment of the virus to the hepatocyte surface.

4.7 Detection and Biochemical Characterization of a 330 kD Receptor Protein for WHV

Employment of virus and lectin affinity chromatography allowed purification of plasma membrane proteins, which are involved in the binding of the WHV envelope. Analysis of these proteins by SDS-PAGE revealed a wide range of molecular weights (Figure 17). This multiband protein pattern of the purified preparation expressing high WHV receptor activity most likely reflects the presence of complexes between WHV envelope receptor protein(s) and other proteins of plasma membranes, which are structurally associated with the receptor proteins and are sensitive to dissociation under the reducing conditions employed in SDS-PAGE but not to the treatment with Triton X-114. In an attempt to distinguish between true receptor proteins and those which could structurally or by other means be associated with the receptor molecules, a VOPBA was

employed. In this assay, plasma membrane proteins were separated by electrophoresis in a SDS-polyacrylamide gel, transferred onto nitrocellulose and incubated with the radiolabelled WHV envelope preparation. When woodchuck native HPMs, soluble proteins of HPMs and affinity purified membrane preparations were subjected to this type of analysis, a single band with an apparent molecular weight of 330-kD was consistently detected as the only one capable of binding the WHV envelope. Retrogressive analysis of the protein profiles and carbohydrate patterns of native and detergent-solubilized plasma membrane preparations, as well as membrane constituents specifically eluted from the WHsAg and Concanavalin A affinity columns, in SDS-polyacrylamide gels, revealed that the carbohydrate-containing 330-kD protein band was conserved through the employed purification procedures. However, it also became evident that the 330-kD band occurs not only in woodchuck hepatocyte plasma membranes, but also in SPMs and KPMs. Analysis of SPMs and KPMs by a virus overlay protein assay (data not shown) supported the conclusion that the 330-kD band exhibits binding activity for the WHV envelope in these plasma membrane preparations.

In order to determine the biochemical nature of the 330-kD receptor molecule, soluble proteins of HPMs were subjected to a variety of enzyme digestions and then analyzed in the ^{125}I-WHsAg overlay protein binding assay. Treatments with heparinase or heparitinase resulted in the abolition of the ability exhibited by the 330-kD protein to recognize the WHV envelope (Figure 22). It is known that heparinase specifically depolymerizes heparin as well as heparan sulphate (*e.g.* Mclean *et al.*, 1985; Mclean,

1987), whereas heparitinase depolymerizes heparan sulphate (Mclean, 1987). Therefore, considering the obtained result it can be concluded that a heparan sulphate moiety is the constituent of the molecule seen as a 330-kD band. This finding is supported by the fact that hepatocytes have been reported to express heparan sulphate, but not heparin, on their surface (Hook, 1984). On the other hand, digestion of HPMs with endoglycosidase F/N-glycosidase F, known to cleave bonds between two N-acetylglucosamine and N-acetylglucosamine and asparagine, also abolished the binding activity exhibited by the 330-kD band. Since results from lectin affinity chromatography indicated that WHsAg binding constituents may exhibit not only an O-linked heparin-like moiety but also N-linked polymannose-rich carbohydrates, where bonds between two acetlyglucosamine and N-acetlyglucosamine and asparagine exist, it was not completely surprising that this enzyme was also able to inactivate WHsAg binding displayed by the 330-kD band.

Thus, since removal of both heparan sulphate and polymannose specificity by carbohydrate-specific digestions completely abolished the 330-kD receptor activity, this strongly suggests that these two specificities participate in the formation of the WHV binding site on the 330-kD molecule. In contrast, digestion of HPM soluble proteins with chondroitinase ABC did not result in the elimination of the WHV envelope binding. However, the treatment resulted in the appearance of a faint WHsAg reactive band at an approximate molecular weight of 220-kD on the virus overlay protein binding assay (Figure 22). This finding may suggest that the observed shift in the molecular weight of the 330-kD molecule can result from the partial dissociation of the 330-kD

macromolecule due to removal of chondroitinase ABC-sensitive bond. At the same time, this result suggests that the dissociation of this bond does not influence the attachment of WHV.

Since digestion with neuraminidase did not affect the WHsAg binding displayed by the 330-kD receptor, this indicates that carbohydrates exhibiting terminal sialic acid do not contribute the binding of the WHV envelope. This finding remains in agreement with the results of affinity chromatography experiments that employed wheat germ agglutinin columns (Figures 19 and 20).

Finally, treatment of intact as well as, water-soluble HPM proteins with pronase led to the disappearance of the 330-kD band and the apparent loss of the WHsAg binding activity when the resulting residues were tested by the VOBPA using ^{125}I-WHsAg. This result demonstrates that protein is an important structural component of the identified receptor molecule. Interestingly, when the digested membranes were tested in a nitrocellulose dot-blot ^{125}I-WHsAg binding assay, prolonged proteolytic digestion (*i.e.*, up to 2 hr) of HPMs was unable to inhibit the WHV envelope binding. This behaviour strongly suggests that WHV envelope binding is not mediated by protein but by carbohydrate constituents associated with the protein core of the 330-kD receptor. The increase of the WHsAg binding after pronase digestion may reflect fragmentation of the receptor molecules, which may lead to uncover additional binding sites originally hidden in the intact membrane. Overall, the results from enzymatic digestions and affinity chromatography experiments indicate that the 330-kD receptor molecule is a protein-

carbohydrate complex, where carbohydrates such as charged heparan sulphate and, likely, N-linked polymannose are bound to the protein back bone. The findings also indicate that carbohydrate components of the receptor molecule, but not the protein core, mediate binding of WHV.

Most proteoglycans consist of one or more glycosaminoglycan chains linked to a protein core. The surface of the hepatocyte has been determined to have two forms of proteoglycan: (1) a hydrophobic (intercalated) proteoglycan that possesses a protein core and is rooted in the lipid bilayer of the plasma membrane (Kjellen *et al.*, 1981) and (2) a hydrophillic (peripheral) proteoglycan, which is apparently retained on the surface of hepatocytes by a receptor that has affinity for the glycosaminoglycan, heparan sulphate. (Kjellen *et al.*, 1980). The molecular weight of the peripheral heparan sulphate proteoglycan on rat hepatocytes has been estimated to be 75-kD (Oldberg *et al.*, 1979), whereas the molecular weight of its protein core and heparan sulphate chains was determined to be 27-kD and 14-kD, respectively (Kjellen and Hook, 1983). Moreover, although the molecular weight of the protein core for the intercalated proteoglycan was higher (*i.e.* 35-kD) than that of peripheral proteoglycan, the size and distribution of the N-sulphate groups along the glycosaminoglycan chains remained the same in both intercalated and peripheral proteoglycans. It appears that the rat hepatocyte is capable of processing hydrophobic heparan sulphate proteoglycan to the corresponding hydrophillic form (Brandan and Hirschberg, 1989). These researchers also suggest that hepatocytes are not likely to exhibit a hydrophobic heparan sulphate proteoglycan that is

directly anchored to the membrane by inositol phospholipids, rather the hydrophobic nature of the protein core serves as the required anchor. The structure of proteoglycans on the hepatocyte surface in other species is not yet established.

In the case of herpes simplex virus (WuDunn and Spear, 1989) and cytomegalovirus (Neyts *et al.*, 1992), it has been proposed that the interaction of virions with cell surface requires the presence of heparan sulphate and that this moiety is required to concentrate virions at the cell surface so that their binding to other, perhaps high affinity surface receptors, be facilitated. In this regard, it is interesting to note that Neurath *et al.*, (1992) had proposed that the participation of IL-6 as a bridging molecule in the binding of human HBV to the surface of the cells may involve a proteoglycan that is attached to the cell membrane by a phosphotidyl inositol anchor. Thus, it could be postulated that the 330-kD proteoglycan described in this thesis may also function as a means to concentrate the virus at the hepatocyte surface or as the cellular receptor that directly mediates the binding and penetration of WHV to the host cell.

In summary, affinity chromatography and enzymatic digestions revealed that the identified 330-kD receptor for WHV expressed on woodchuck HPMs is a high molecular weight, sulfated proteoglycan constituted by an O-linked heparan sulphate and N-linked polymannose moiety, which are bound to a protein core. It is unlikely that the 330-kD macromolecule is the receptor that determines hepatotropism of WHV, since this macromolecule not only exists on hepatocytes but is also expressed on other woodchuck cells. This implies that the virus cell tropism has to be determined by a mechanism

involving another cell surface or intracellular, virus-specific receptor system.

4.8 Inhibition of WHV Envelope Binding to Targeted Membranes by Heparin

Certainly, molecules viewed as being able to prevent the interaction between viruses and their respective cellular surface receptors are very important because they may block the attachment and entry of the virus to the targeted cells, and prevent infection.

It is conceivable that inhibition of virus binding to the surface of the cells may be achieved either through: (1) blocking the cellular receptors for the virus, (2) blocking viral epitopes involved in the recognition of the specific cellular surface receptors, or (3) through a simultaneous blocking of both the viral epitopes and the cellular receptors involved in the viral attachment. The blocking can be specific, when a blocker is acting directly on a virus receptor or on a viral epitope that is involved in the recognition of targeted cells (*e.g.*, specific antibodies, anti-idiotypic antibodies carrying images of viral receptors, soluble forms of viral receptors, chemicals with specific affinity to viral receptors or virus attachment proteins). On the other hand, blocking can also be non-specific. In this case, when the block of the virus binding is achieved through the interaction of an agent, which does not specifically recognize viral or cellular constituents mediating viral attachment, but is able to bind to a variety of molecules on the surface of virions or cells, usually by electrostatic forces. Among non-specifically acting

blockers, the effect of sulphated glycosaminoglycans was tested on the infectivity of several different viruses by monitoring the concentration required to inhibit virus-mediated cytopathogenicity, inhibition of plaque formation or virus adsorption to cells (*i.e.* Baba *et al.*, 1988b; Mettenleiter *et al.*, 1990; WuDunn and Spear, 1989).

Heparin and other sulphated polysaccharides (*i.e.*, dextran sulphate, pentosan polysulfate, fucoidan and carrageenans) have been found to express high inhibitory activities for the herpes simplex virus type 1 and 2 (Baba *et al.*, 1988b; WuDunn and Spear 1989), cytomegalovirus (Baba *et al.*, 1988b; Neyts *et al.*, 1992; Kari and Gehrz, 1992), vesicular stomatitis virus (Baba *et al.*, 1988b), vaccinia virus (Baba *et al.*, 1988b), immunodeficiency virus type 1 (Baba *et al.*, 1990; Schols *et al.*, 1990; Baba *et al.*, 1988a; Nagumo and Hoshino, 1988; Callahan *et al.*, 1991) and pseudorabies virus (Mettenleiter *et al.*, 1990). The inhibitory effect of these sulphated glycosaminoglycans on the viral attachment appears to be independent of any direct effect on cellular receptors specifically recognized by the virus attachment proteins. For example, Callahan *et al.*, (1991) reported that preincubation of CD4-positive Molt-4 cells and H9 cells, both infected with immunodeficiency virus type 1, with dextran sulphate did not affect their ability to bind the recombinant gp120, which constitutes the HIV attachment protein, or soluble CD4 which represents the HIV cellular receptor, respectively. However, during the course of the mentioned studies it was noticed that dextran sulphate inhibited the recognition of the gp120 attachment protein by the anti-gp120 monoclonal antibody. Interestingly, this antibody does not block the gp120-CD4 interaction, but it

apparently neutralizes HIV because it is directed against positively charged amino acids present in the V3 loop region of gp120. The authors propose that in spite of the fact that dextran sulphate does not interfere with the gp120-CD4 binding, it probably disrupts processes critical to HIV infection. They believe that the V3 loop, exhibiting such high positively charge residues, is instrumental in ensuring that the HIV particle effectively interacts with the negatively charged cell surface. Thus, they postulate that the interaction of the negatively charged dextran sulphate with the V3 loop of HIV would not only mask its positive charge but also add an additionally negative potential, thereby electrostatically preventing the interaction between the virion and the cell. Indeed, there is additional support for the view that electrostatic interactions form the basis of the inhibitory effect displayed by sulphated polysaccharides in the attachment of HIV to targeted cells. For instance, Baba *et al.* (1988a) and De Clercq (1989) have demonstrated that lack of sulphation in dextran sulphate reduces its inhibitory effect on HIV attachment. Similarly, Lycke *et al.* (1991) demonstrated that, unlike highly sulphated heparan sulphate, heparan sulphate preparations of low sulphate content failed to show significant interaction with the herpes simplex virus type 1 and 2.

It is interesting to note that the attachment of other envelope viruses such as the herpes simplex virus type 1 and 2 to HEp-2 cells was inhibited by heparin, a readily available proteoglycan with anti-coagulant activity. Thus, it is thought that this glycosaminoglycan may occupy sites on the virions that are necessary for effective attachment (WuDunn and Spear, 1989). These authors do not propose the mechanism

which governs this inhibitory effect. The attachment of another enveloped virus, pseudorabies, to NBDK (Madin-Darby bovine kidney) and RK (rabbit kidney) cells was also inhibited by heparin (Mettenleiter *et al.*, 1990). In this case, the authors do suggest that the arginine-rich (positively charged) region present near the amino terminus of the pseudovirus attachment glycoprotein (gIII) interacts with the polyanionic heparin, leading to the inhibition of the binding of pseudovirus particles to the target cells.

With regard to hepadnaviruses, influence of a sulphated polysaccharide on the interactions between HBsAg or WHsAg and their specific antibodies was investigated (Venkateswaran *et al.*, 1989). In the mentioned study, a sulphated polysaccharide isolated from *Pelvetia fastigiata*, a marine algae, was tested. The polysaccharide was apparently found to be efficient in the inhibition of formation of the antigen-antibody complexes. It was also observed that the extent of the inhibition depends on the structural features displayed by the polysaccharides. For instance, the inhibitory activity exhibited by the 500-kD, 8-kD and 5-kD dextran sulphate was 63.9%, 12.3% and 0%, respectively. This may suggest that the inhibitory activity of the oligosaccharide could be somehow related to its molecular weight. On the other hand, the extent of inhibition of the antigen-antibody interaction in this experimental system also seems to depend on the type of glycosidic residues used, since the 500-kD fucoidan from *Pelvetia fastigiata* and *Fucus disticus* displayed a percent inhibition of 91.1% and 92.9%, whereas the above mentioned 500-kD dextran sulphate displayed a significantly lower percent of inhibition (*i.e.*, 63.9%). Furthermore, the authors report that the presence of sulphate

is essential to the inhibitory activity of fucoidans and their removal abolished fucoidan's capacity to inhibit the formation of the antigen-antibody complexes. Thus, the extent to which sulphated polysaccharides inhibit the interaction between hepadnaviral envelopes and their respective antibodies may depend on the type of glycosidic residues present, molecular weight, and availability of charged sulphate groups.

The effectiveness of the polysaccharides derived from *Pelvetia fastigiata* was also tested *in vivo* using woodchucks chronically infected with WHV (Venkateswaran *et al.*, 1989). Animals were *i.p.* administered with 5 mg of fucoidan from *Pelvetia fastigiata* once a week and bled bi-weekly to determine the WHsAg titre. Treatment continued for 6 months after which liver biopsy was performed. As controls, a different group of woodchucks chronically infected with WHV were subjected to the same protocol but treated with PBS instead of fucoidan. The study revealed that this polysaccharide has no beneficial effect on the status of the disease. The authors suggest that the lack of any observable effect may be due to the fucoidan's inability to reach the site of action because of its polyanionic nature. However, there could be two other possible explanations for the lack of the effect, which were not mentioned by the authors. The first one is based on the possibility that viral epitopes recognized by fucoidan may not be involved in the attachment of the virus to the targeted cells. In this context, the effect of fucoidan on the binding to the WHV envelope to the host hepatocytes has not been evaluated by the authors. Secondly, and most important, it is unlikely that this type of treatment would be effective in chronic WHV carriers because constant replication of the

virus in the majority of hepatocytes and integration of the virus DNA to host genome have already taken place. It is also unlikely, that the polysaccharide influences DNA synthesis, consequently, cessation of virus replication or synthesis of virus epitopes that attract cytotoxic T cells in already infected cells can not take place. However, it is possible that the spread of WHV infection to non-infected hepatocytes might be, in theory, abrogated using this type of treatment. Perhaps, in future studies, this treatment should be re-evaluated at the time when healthy woodchucks are inoculated with the infectious virus.

Since experiments reported in this thesis demonstrated that the attachment of WHV envelope to host hepatocyte plasma membranes is mediated by a receptor which contains a heparan sulphate entity, it was reasonable to test whether preincubation of the virus envelope particles with heparin would inhibit their binding to the 330-kD receptor and to HPMs as a whole. The results of the performed experiments revealed that, preincubation of the WHV envelope with an excess of heparin, followed by extensive dialysis of the treated subviral particles to remove free heparin, lead to complete elimination of the virus binding both to the identified 330-kD receptor and to native woodchuck HPMs. Furthermore, even though several HPM polypeptides were identified as capable of interacting with free heparin, the 330-kD band was not among them. Therefore, it is highly unlikely that the inhibitory effect exhibited by heparin on the WHV envelope binding, resulted from the direct binding of heparin to the 330-kD receptor for WHV. In turn, this indicates that the observed inhibition was in fact the

result of heparin's interaction with WHV envelope. This was further supported by the results of experiments where increasing concentrations of heparin were incubated with WHsAg. They clearly demonstrated that the heparin's effectiveness is dependent on its concentration. Further, since preincubation of the WHV envelope with heparin was followed by an extensive dialysis, it is highly unlikely that free heparin coexisted with the WHV envelope-heparin complexes. These data suggest that the WHV-heparin complexes appear stable even though they are likely sustained by electrostatic forces. The stability of these complexes was confirmed when the preincubation of the same quantities of WHsAg with heparin was performed in different reaction volumes (data not shown). Despite the fact that the reaction volume was increased threefold, the 330-kD band was not visualized on the HPMs blots, indicating that the WHV envelope-heparin complex does not readily dissociate on dilution. This observation seems to remain in agreement with data obtained on the interaction between heparin and herpes simplex virus (Nahmias and Kibrick, 1964). The authors of the above mentioned work demonstrated that when virus-heparin mixture was used undiluted or diluted by a factor of 10, the cytopathic effect on amnion cultures was still not observed, indicating that the complex was stable under the tested conditions.

One of the major concerns arising from the use of compounds acting on the attachment of viruses to host cells is that many of them may cause undesirable side effects. Undoubtedly, heparin belongs to these types of compounds, since it expresses a strong anticoagulant activity. Recently, however chemically treated heparin with a very

low anti-thrombin activity has been produced (Baba *et al.*, 1990). This novel heparin is also capable of inhibiting the attachment of HIV to host cells, thus retaining its antiviral activity (Baba *et al.*, 1990). It would be very interesting to test whether such an analogue of heparin will be able to block hepadnavirus attachment *in vivo* conditions and prevent hepadnaviral infection in a woodchuck model of hepatitis B. The inhibitory effect displayed by heparin on the WHV envelope binding to host cells may open the possibility for further intensive investigations of its anti-hepadnaviral activity.

4.9 Binding of the JP1 Sequence to Woodchuck and Non-Host Specific Cellular Constituents

Since WHV envelope interacted with the 330-kD plasma membrane receptor, of woodchuck cells of both hepatic and non-hepatic origin, it became apparent that it will be unlikely that this interaction could explain the predominant tropism of the WHV to the liver and lymphoid tissues. However, even though experiments performed in this study revealed that both plasma membranes of hepatic and non-hepatic origin exhibit binding activity for the WHV envelope, tissue tropism of WHV could still be determined at the plasma membrane level by a virus receptor other than 330-kD. Experiments performed by Neurath and colleagues (1986, 1990, 1992) revealed that a synthetic peptide homologous to a sequence encompassing residues 21-47 of the HBV envelope preS1 protein has affinity for the surface of HepG2 cells as well as for cytokine IL-6. Also, receptors for the HBV preS1 (21-47) peptide and for IL-6 have been demonstrated

to occur on a variety of human cells of non-hepatic origin. For instance, the percentage of cells of non-hepatic origin (*e.g.* Cates 1B, SH-N-SH, Namalwa, Wish and peripheral blood B lymphocytes) that bound serum-derived HBsAg and preS1 (21-47) peptide was comparable to that observed for HepG2 cells (Neurath *et al.*, 1990). This data indicate that both the HBV envelope and the virus preS1 (21-47) sequence were recognized by hepatic as well as non-hepatic cells. Therefore, the cell surface receptor recognized by virions and the peptide can not be considered to be the sole factor that determines liver tropism for HBV.

It is generally accepted that protein epitopes exposed on the virus surface may mediate important viral functions and, among others, participate in virus binding to host cells. Furthermore, viral envelope amino acid sequences that exhibit high probabilities for β-turn and high hydrophilicity are likely to be exposed on the surface of the virion. Both the JP1 sequence of WHV and the preS1 (21-47) sequences of HBV exhibit a β-turn and, in both cases, the predicted β-turn corresponds to the predicted occurrence of a hydrophillic peak. Since the JP1 sequence is homologous to the N-terminus of the WHV preS1 protein and this portion of the preS1 protein is preferentially localized on the surface of virions (Heermann *et al.*, 1984), it is likely that the JP1 sequence can be exposed on the surface of WHV particles.

Performed experiments clearly demonstrated that JP1 sequence interacted exclusively with woodchuck liver and splenic tissue, but not with homogenates of woodchuck kidney nor with liver and spleen tissues of human and mouse origin. This

may suggest that this viral peptide exhibits the ability to specifically recognize a host and tissue specific component. Also, the results indicate that this component is present in woodchuck tissues which are known to support WHV replication *in vivo* (Korba *et al.*, 1988b). Furthermore, although the peptide does not interact with woodchuck HPMs and SPMs it binds to fractions of these cells where mitochondria and nuclei sediment. Thus, the JP1 sequence, in addition to the exhibited species and tissue specificity, may also be capable of specifically recognizing a subcellular organelle. This very interesting behaviour requires further investigations to determine a role, if any, of the identified sequence in the specific recognition by WHV of a host and cell type specific constituent at the intracellular level (Michalak *et al.*, 1991). Nevertheless, the JP1 oligopeptide clearly does not recognize the detected 330-kD receptor molecule on plasma membranes derived from woodchuck hepatocytes.

Several studies have demonstrated that N-terminal sequences of proteins display covalently linked myristate. Myristoylated proteins have been assigned the role of virion assembly for the (*e.g.*, Moloney murine leukemia virus (Rein *et al.*, 1986), rotavirus (Clark and Desselberger, 1988), poliovirus (Paul *et al.*, 1987), polyoma virus and SV40 (Streuli and Griffin, 1987)). On the other hand, there are other myristoylated proteins whose role includes not only assembly but also entry of virus into cells, as it seems to be the case in poliovirus (Chow *et al.*, 1987). In the case of retrovirus, myristoylation is required for intracellular transport, but not the formation of virus particles (Rhee and Hunter, 1987). It has also been demonstrated that the N-terminus of the HBV, GSHV,

DHBV and WHV preS1 proteins have the potential to become modified by the post-translational addition of myristic acid (Persing *et al.*, 1987). It was shown that the myristoylation of the HBV preS1 protein occurs intracellularly (Persing *et al.*, 1987). Moreover, in regards to the functional role assumed by myristic acid, in HBV infection it has been reported that myristoylation alone was insufficient for membrane insertion or endoplasmic reticulum localization of the virus preS1 protein (Kuroki *et al.*, 1989). Since then Macrae *et al.*, (1991) have demonstrated that the myristoylated DHBV preS1 protein is essential for cell entry (*i.e.*, infectivity), but not for viral assembly. Therefore, the inability exhibited by woodchuck HPMs and SPMs in expressing binding activity for the non-myristoylated JP1 oligopeptide, may suggest that, like the myristoylation of DHBV large protein, a similar protein modification at the N-terminus of the WHV preS1 protein may be crucial to the effective recognition of binding sites on the surface of the targeted cell. On the other hand, even though HBV preS1 (21-47) peptide is somehow similar to the JP1 of WHV, in that both exhibit sequences that are likely to be exposed on the virion surface, such a property does not necessarily mean that those hepadnaviral amino acid sequences are the only ones recognized by cellular receptors. While the HBV preS1 (21-47) is apparently recognized by HepG2 and other cells of non-hepatic origin, the same does not occur with the JP1 sequence. Instead, the latter seems to be recognized by intracellular binding sites expressed only by the host hepatocytes and lymphoid cells. Nevertheless, an epitope present in the WHV JP1 sequence clearly is not involved in the interaction between WHV envelope and the identified 330-kD plasma membranes

receptor for WHV.

4.10 Conclusions

Overall, the findings of this study can be summarized as follows:

1. The envelope of WHV displayed saturable binding to a highly purified woodchuck hepatocyte plasma membrane preparation and to soluble proteins derived from those membranes; both interactions follow the characteristics expected of a specific ligand-receptor association. The water-soluble proteins, exhibited a greater potential for the binding of the WHV envelope than the native membrane at comparable protein concentrations, indicating that peripheral proteins loosely associated with the membrane lipid bilayer are involved in the virus binding.

2. Binding sites for WHV envelope were not restricted to the surface of the host hepatocytes, but were also present on the plasma membranes of woodchuck splenocytes and kidney cells. Membranes of non-hepatic origin displayed a distinctly lower binding capacity for the virus envelope than did the hepatocyte plasma membranes (approximately, 40% and 18% of the binding to hepatocyte membranes for splenocyte and kidney cells, respectively).

3. The search for the WHV receptor in the soluble fraction of woodchuck hepatocyte plasma membrane proteins, using a virus overlay protein blot assay revealed that only a 330-kD molecule was capable of specific recognition of the virus envelope. This virus binding molecule was also expressed on the plasma membranes of woodchuck

splenocytes and kidney cells.

4. Characterization of the 330-kD molecule by lectin affinity chromatography and enzymatic digestions revealed that the WHV envelope binding activity was determined by O-linked heparan sulphate and N-linked polymannose but not by the protein core of the 330-kD macromolecule. This suggests that the 330-kD receptor is a protein-carbohydrate complex and that the virus binding site is formed by the structural cooperation between heparan sulphate and polymannose.

5. The binding of the WHV envelope particles to the 330-kD receptor molecule, as well as to the host native hepatocyte plasma membrane could be inhibited by preincubation of the WHV envelope with an exogenous glycosaminoglycan, heparin. This suggests that heparin or its analogue has the potential to become a therapeutic agent, which could be important in the prevention of hepadnaviral infection by blocking virus attachment.

6. The synthetic amino acid sequence of the WHV envelope preS1 protein, which is likely to be exposed on the virion surface, did not bind to the 330-kD receptor or to plasma membranes, but was capable of host and cell specific recognition of an intracellular component, exclusively present in woodchuck hepatocytes and splenocytes. This suggests that this WHV sequence participates in determination of the virus host and cell tropism at the intracellular level. Further studies should establish the significance of this phenomenon in the life cycle of WHV.

4.11 Future work

Future work may involve the following:

1. Determination of the amino acid sequence of the protein core of the 330-kD molecule which was identified as the receptor for WHV, and comparison of its sequence with those of previously identified cell surface proteoglycans, in an attempt to predict the physiological role of this molecule.

2. Testing whether free carbohydrates constituting the 330-kD macromolecule, heparin or heparin analogues void of anticoagulant activity can prevent hepadnavirus infection in a woodchuck model of hepatitis B.

3. Searching for molecules other than the 330-kD proteoglycan which may serve as receptors for WHV on the surface of woodchuck cells naturally targeted by the virus.

CHAPTER 5

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