INTERACTION OF THE NUCLEAR NUCLEOSIDE TRIPHOSPHATASE WITH NUCLEAR MEMBRANE CHOLESTEROL

by

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BRAM RAMJIAWAN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Statement

The work reported in this thesis was undertaken at the St. Boniface General Hospital Research Centre, in conjunction with the Department of Physiology of the University of Manitoba, Winnipeg Manitoba. Supervision of this work was provided by Dr. Grant N. Pierce. I declare that the work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text; and that this material has not previously been submitted, either in part or in full, for a degree at this or any other university.

August 19, 1996

Bram Ramjiawan

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Abstract

The nucleoside triphosphatase (NTPase) which is located on the inner face of the nuclear membrane appears to regulate the nucleocytoplasmic transport of materials by controlling the opening of the nuclear pore complex. However, little is known about the influence that the nuclear membrane itself may have on NTPase activity. The purpose of these studies was to investigate the influence of changes in nuclear membrane cholesterol may exert upon nuclear NTPase activity. We were able to successfully increase the cholesterol content of nuclear membranes after exposure to cholesterol-enriched liposomes. Nuclear NTPase activity was potently stimulated (~150-200 % of control) by an increase in the nuclear membrane cholesterol content. The Vmax of the NTPase activity in the presence of ATP or GTP was significantly increased after cholesterol enrichment without altering the affinity of the enzyme for these moieties.

The cholesterol content of nuclei isolated from hepatic tissue was relatively low in comparison to that typically exhibited by other membrane fractions. Because of this, it was difficult to further deplete the nuclear membrane of cholesterol. Oxidation of nuclear membrane cholesterol was adopted as an alternative approach to liposomal methods for changing membrane cholesterol. Although oxygen derived free radicals can oxidize membrane cholesterol. Nuclear membrane cholesterol was oxidized in situ with cholesterol oxidase (to selectively oxidize cholesterol) and NTPase activity measured.

HPLC analysis confirmed the formation of cholesterol oxides. The activity of the NTPase was strikingly inhibited by cholesterol oxidase. The Vmax of the NTPase was significantly decreased after cholesterol oxidase treatment but the Km value was unchanged.

These results clearly demonstrate that the cholesterol content of the nuclear membrane can be effectively modified and this has significant effects on the nuclear NTPase activity. These data have important implications for nuclear pore function and the trafficking of compounds into and out of the nucleus in the cell, particularly when the cholesterol content of the cell becomes altered.

2 General Introduction

Advances in biochemistry, cell and molecular biology, have resulted in a huge body of information regarding the cell nucleus. The nucleus is the primary depot of genetic material and information of the cell. It occupies about 10% of the total cell volume and contains DNA and its associated proteins. Nuclear size in the cell can vary depending upon the cell type. Typically, the nucleus of mammalian cells has a diameter of 3 to 10 μm and is separated from the cytoplasm by the nuclear envelope (11). Nuclear proteins are actively and posttranslationally transported across the nuclear envelope. This transport is a highly selective process that can be divided into distinct steps. Receptor binding to the nuclei followed by translocation through the nuclear envelope is the first step (9). Receptor binding is mediated by nuclear localization signals that have been identified in many nuclear proteins. The second step involves translocation through the nuclear envelope. Translocation is an energy-dependent process that is regulated by the nuclear pore complex.

The proper functioning of a cell depends on its ability to communicate with its environment. External stumuli often interact with membrane receptors which in turn induce second messengers that ultimately affect transcription factors. These transcription factors can either repress or activate the expression of certain genes resulting in a specific pattern of proteins in a particular cell. The cytoplasm contributes signals to and from the

nucleus to modulate gene expression. DNA replication, RNA transcription and processing all occur within the nucleus. The precise mechanisms by which the contol systems of the genome operates is an area of intense research. Particularly important is the question of factors that govern gene expression since the content and constitution of DNA is the same in almost all cells, yet each cell type differentiates in a unique manner.

a. The Nuclear Envelope

The nuclear membrane and its associated structures are collectively termed the nuclear envelope. The nuclear envelope provides a separation between the nuclear and the cytoplasmic environments. The nuclear envelope is made up of two concentric lipid bilayers, the inner and the outer nuclear membranes. Separating both membranes is a 10-60 nm gap known as the perinuclear space (26). The outer membrane appears to be continuous with the endoplasmic reticulum (75). The inner nuclear membrane is devoid of ribsomes and is lined by the nuclear lamina, a specialized type of intermediate filament protein (82, 141). Both layers of the nuclear membrane are perforated by nuclear pore complexes (62). During the M phase of the cell cycle, it is believed that the nuclear membrane breaks down. This disassembling at the beginning of the mitotic stage of cell division and reassembling at the end of mitosis, represents only a minor portion of the cell's existence. Even though there are great variance in eukaryotic cells, from less than 7 hours to more than a year in adult mammals, the interphase represents the major period of cell life. During this interphase, there are distinct nuclear and cytoplasmic compartments. Assembly of the nuclear envelope involves the attachment of vesicles to the chromatin followed by the fusion of vesicles to produce the double lipid bilayer membrane system. There are a number of receptors such as steroid, thyroid, retinoic acid and vitamin D3 that belong to the superfamily of nuclear receptors. These nuclear receptors are closely associated with the nuclear envelope. The lipid composition of the nuclear membrane in rats can be altered by diet, genetics and by in vitro manipulation (49, 168, 202, 209).

b. Nuclear pore complex

One of the most obvious features of the nuclear envelope is the presence of nuclear pores. Hertwig was the first to propose the existence of nuclear pores, using a light microscope in 1876. It was only relatively recently (1949) that the ultrastructure of nuclear pores were observed by Callan with an electron microscope (30). Nuclear pores allow selected molecules to be actively transported between the nucleus and cytoplasm. In eukaryotic cells, pores have been shown to occur at a density of about 11 pores per square micrometer of membrane area (3-4000 pores/nucleus) (10). However, it is now believed that this density is dependent upon the metabolic activity of the cell (8). Nuclear pores are fixed in large disclike structures known as nuclear pore complexes. Pore complexes perforate the double membrane, bringing together the inner and outer margins of each pore. The mass of the nuclear pore complex has been determined to be approximately 125 x 10^6Da (170). The architecture of the pore complex has been revealed by image analysis, ultrastructural studies and averaging techniques.

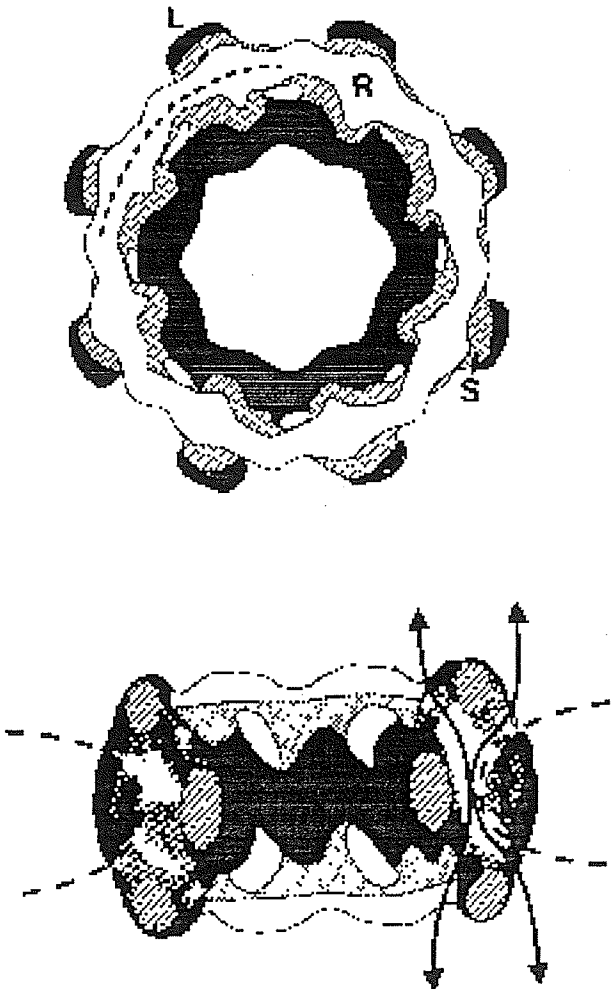


Figure 2: THE NUCLEAR PORE COMPLEX ARCHITECTURE

Surface views of 3D electron density map of NPC calculated from negative stained samples. (a) Top and (b) cut away side view of the NPC. The dotted line in (b) represents the position of the nuclear envelope, while the double-headed arrows show the possible alternate routes for passive diffusion through the peripheral channels. The

hatched areas indicate the cut ends of the rings and annular regions of the NPC. (Adapted from *Hinshaw J. E., 1994*)

A widely accepted model of the architecuture of the nuclear pore is that by Hinshaw et al. (Figure 2) (94). There is a central spoke-like assembly that is connected to two rings that are located on both the nucleoplasmic and cytoplasmic sides (95). It was hypothesized that the nuclear pore complex may function as a gene gating organelle that can interact with specific segments of the genome (23). Attached to the inside of the spokes, is a central channel complex that is responsible for mediated transport (170). This central channel complex, also known as the plug or transporter, is the site of transport of large molecules such as proteins, ribonucleic acids, and protein-nucleic acid complexes (79). These gated channels, the function of which are ATP and GTP dependent, can enlarge to a diameter of approximately 25 nm (79). Others have reported conflicting values for the diameter of nuclear complexes. The aperture of the pore complexes was reported, for example, to be 10 nm in the closed, and 40 nm during the open state in which the translocation of macromolecules occurs (9, 64). Nuclear pore complexes also contain aqueous channels (different size and function than the central channel complex) for the exchange of ions and small molecules between the nucleus and the cytoplasm. These channels are 10 nm in diameter and allow passive diffusion (79). It is believed that the nuclear pore complexes can also serve as rivets to hold together the inner and outer layers

of the nuclear membrane (62). The nuclear pore complex interrupts the nuclear lamina and may even be attached to it.

Few nuclear pore complex proteins have been identified. Certain proteins disappear after extraction of nuclear pore complexes with Trition X-100, which indicate that they may be integral nuclear membrane proteins. One component of the pore complex was characterized as being a glycoprotein (Gp210). This glycoprotein was shown to be located on the periphery of the cytoplasmic ring and is believed to be serve in the attachment of both nuclear membranes to the pore complex. The bulk of this molecule lies in the perinuclear space, with its C-terminal domain containing a membrane-spanning segment (82, 83). Binding of a monoclonal antibody to the lumenal domain of Gp210 was shown to result in inhibition of signal mediated nuclear import and passive diffusion across the NPC in vivo (88). Using monoclonal antibodies towards Triton-extracted hepatic nuclei from the rat, Davis and Blobel also identified a nuclear pore complex glycoprotein (p62) that reacts with the lectin wheat germ agglutinin (WGA). This protein was identified as belonging to a family of glycoproteins all of which contain terminal O-linked N-acetylglucosamine (GlcNAc) residues (54, 55). Glycoproteins with this type of GlcNAc-linkage are particularly enriched in cytosolic and nuclear envelope fractions. Many monoclonal antibodies have been described which recognize all members of the glycoprotein family of rat hepatic liver nuclear envelopes. Definite localization of these glycoproteins called nucleoporins was achieved by electron microscopic localization methods using either antibodies or ferritin-labelled WGA (155). Another rat nuclear pore

complex protein known as NUP153 or p180 has been recently cloned and sequenced (197). Pore complexes have been shown to remain associated with the lamina after extraction with salt and non ionic detergent (1).

c. Nuclear Proteins

There are many proteins that are present in the nucleus, these include histones, nuclear enzymes (DNA and RNA polymerases), RNA-processing proteins, and gene regulatory proteins. Most or all of these proteins are imported into the nucleus from the cytoplasm via a selective transport process. Although the structural proteins (histones, preribosomal particles and ribsomal proteins) are better understood, the gene regulatory proteins are considered more important. Control of gene expression may reside in specific elements that are either nonhistone proteins or phosphoproteins (29).

Continuing efforts to isolate and identify individual nuclear proteins have led to a huge amount of information. A particularly interesting protein, A24, named because of its migration on 2-D gel electrophoresis, appears to be a polypeptide bound to histone 2A. A24 is a conjugated form of a histone (2A) and ubiquitin. The ubiquitin can be cleaved off physiologically. It has been suggested that the non histone part of the protein may serve as a lever by which the 2A histone can be a site for the protein association with DNA (85). Recently, Gilchrist and Pierce have identified a calcium binding protein in the nuclear membrane of rat hepatatocytes (84). This acidic 93-kDa protein (p93) was shown to possess high capacity calcium binding sites. It was determined that p93 is a major calcium

binding protein located on the inner nuclear envelope membrane. It is possible that p93 functions in the regulation of calcium transients between the nucleoplasm and the perinuclear space (84). Additional calcium binding nuclear proteins that may be involved functionally, have been identified. Proteins of 110, 93 and 35 kDa were observed in the pig cardiac nuclear envelope (47, 84). A large number of nuclear events such as timing of events in the cell cycle, and gene expression, have been shown to be modulated via calcium binding proteins (184, 189).

d. Nuclear Scaffold

The nucleoplasmic surface of the inner membrane of the nuclear envelope is supported by a peripheral framework called the nuclear scaffold. The nuclear scaffold is composed of both structural and functional proteins. The major structural backdrop of the nuclear envelope, the lamina is believed to function in the organization of chromatin (81). Lamina in higher animals has three major protein components, lamins A, B, C. These laminins form a highly polymerized and insoluble fibrous matrix during interphase of the cell cycle (200). The insoluble network has been shown to remain after nonionic detergents and salt treatments (80). Clinically, antibodies to nuclear lamin proteins have been detected. Using immunofluorescence, western blotting and enzyme-linked immunosorbent assay techniques, Konstantinov's group found antilamin antibodies and antiphospholipid reactivity among patients with polyarthritis (105). It was suggested that these antilamin antibodies can serve as markers for a subgroup of polyarthritic patients (105).

Functionally, the nuclear scaffold includes a 46-kDa nucleoside triphosphatase which is thought to participate in nucleocytoplasmic transport of mRNA. (200). This 46-kDa component shares an amino-terminal sequence with lamins A and C, indicating that proteolytic remodeling of the nuclear scaffold may contribute to the generation of nucleoside triphosphatase activity (40).

Neutral protease activity intimately associated with the nuclear scaffold is also a functional constituent. This activity has considerable selectivity for lamins as shown by self-digestion of scaffold preparations, and it may participate in the remodeling of the nuclear scaffold after treatment with carcinogens (200). A Ca^{2+}-regulated serine protease, which was found only in the nuclear scaffold and appears to represent a unique multicatalytic protease complex was identified. Nuclear scaffold protease activity, which shows considerable selectivity for lamins A/C may be involved in derivation of the 46 kD protein, nucleoside triphosphatase. Using a protease inhibitor (succinyl-AAPF-chloromethylketone, AAPFcmk), cellular transformation and growth of C3H/10T1/2 cells were shown to be decreased by 34% and 56% at 25 microM and 50 microM AAPFcmk, respectively. Growth inhibition occurred without any major change in DNA content distribution, suggesting effects throughout the cell cycle (42) .

.2 *Nucleocytoplasmic Transport*

The nucleus and cytoplasm are both functionally and compositionally distinct throughout interphase of the cell cycle. Although cell cycle duration varies widely in eucaryotic cells, the interphase represents the major phase of existence of the cell. Therefore, the physical and functional separation at this time is particularly important for our understanding of nucleocytoplasmic transport. Nuclear transport is one of the most important membrane trafficking systems within the cell. This is a very complex system in which proteins, mRNAs, and assembled ribonucleoproteins are transported between the cytoplasm and the nucleus. Most proteins and nucleic acids cannot move across the nuclear pore complex by passive diffusion. Only low molecular mass solutes and macromolecules that are smaller than approximately 20-40 Kd can passively diffuse between the nucleus and cytoplasm (158). Instead, proteins and nucleic acids are transported through the nuclear pore complex by ATP and signal-dependent mechanisms that involves gated transport channels that can enlarge to a diameter of ≤ 40-60 nm (79). The precise mechanisms for regulation of nuclear transport are unclear at the present time, however, the following treatise will review our current knowledge.

i. Nuclear Receptors

Cells contains receptors which, when activated can result in changes in the physiological status of the cell. The nuclear envelope has been shown to contain binding sites for intracellular receptors such as thyroid, retinoic acid, vitamin D3 and steroid hormones. Binding of these compounds to the intracellular receptors are selective, and

occurs with high affinity. This interaction results in the conversion of an inactive receptor to one that can interact with the regulatory regions of target genes and modulate the rate of transcription of specific gene sets. The nuclear hormone receptor DNA-binding domain consists of two zinc finger-like modules whose amino acids are highly conserved among the members of the receptor superfamily. Oechsli et al (144), have previously demonstrated that luteal nuclei in cows and humans contain human chorionic gonadotropin/luteinizing hormone (hCG/LH) receptors and that these gonadotropins can directly stimulate nuclear membrane enzyme activity (nucleoside triphosphatase). In a separate study, hCG was shown to cause increased chromatin solubility in a concentration-dependent manner. Further, the addition of hCG antiserum; denatured hCG had no effect and cyclic adenosine 3',5'-monophosphate could not mimic the hCG response (144). The response was specific to the tissue and hormone.

Numerous evidence exists for the presence of receptors in or around the nuclear pore complex (77, 91, 123, 173, 195). For example using colloidal gold particles coated with nuclear proteins, it has been shown that under conditions of depleted ATP, nuclear proteins bind to the nuclear pore complex (195). These experiments suggest that the receptor is located on the nuclear pore complex or on the fibrils that are associated with the nuclear pore complex. Lee and Melese have also identified a 67 kDa potein that is tightly associated with either the nuclear pore complex or the nuclear lamina (115). Regardless of this evidence, it is still difficult to find a receptor that is exclusive to the nuclear pore complex. It was suggested that all pores appear to be competent to import a given protein (68)

There are also evidence that supports the non-nuclear receptors model. Histone H1 was shown to be retained in the cytoplasm, whereas non-nuclear proteins of similar size diffuse into the nucleus (28). The cytoplasmic retention of H1 is overcome by injection of excess H1 (28). This saturable cytoplasmic retention supports the notion that H1 is localized to the nucleus by a cytoplasmic receptor that prevents diffusion. A cytoplasmic factor, NIF-1 was also shown to stimulate nuclear protein import in vitro (138).

The multistep model for carrier-mediated nuclear protein import in which the receptor shuttles a specific protein to the nucleoplasm from the cytoplasm also has supportive evidence. For example, extracts of rat liver cell were shown to contain two proteins that bind to a nuclear localization signal containing protein that is present in the cytosol, nuclear envelope, and the nucleoplasm (3).

ii. Protein Import

Although some proteins are localized specifically to the interior of the nucleus, others remain excluded. Previously, it was thought that molecules gain access to the nuclear interior by freely diffusing in and out of the nuclear pores. Once inside of the nucleus, it was thought that these proteins bound to some "retention proteins" (61, 70). This model of protein import was accepted until recently. The strongest evidence in support of this diffusion model showed that some particles (non nuclear proteins and dextrans) entered the nucleus at a rate based only upon their size (26, 153). Particles that had a diameter greater than 9 nm could not diffuse into the nucleus (26). Despite such

findings, there were two major drawbacks to the diffusion model of protein import. Firstly, proteins of much greater diameter than 9 nm can be found inside the nucleus. Secondly, nuclear proteins such as nucleoplasmin can accumulate in the nucleus very quickly, much faster than dextrans of similar size (60, 61).

Currently, a model of selective transfer is gaining acceptance. Selective transport refers to an active and specific process rather than a passive movement and retention of proteins in the nucleus. Several lines of supportive evidence exist for this model. High energy phosphates such as adenosine triphosphate (ATP) were shown to be required for nuclear protein accumulation (139). Depletion of ATP inhibited nuclear protein stores both in vivo and in vitro (138, 139).

The precise mechanism by which substances are transported across the nuclear envelope is not fully understood. However, the selective transport process involves two distinct steps. The first step is the binding of a ligand to a receptor either on the nuclear envelope or in the cytoplasm. The second step is the translocation through the nuclear pore complex (9).

a. Step 1: Binding of a ligand to a receptor

The receptors for nuclear transport must exist in one of three places. Firstly, the receptor can be located at the nuclear pore complex where it binds and transport proteins into the nucleus. Secondly, the receptor can be located in the cytoplasm where it can bind a ligand and consequently transport it to the nuclear pore complex for import into the nucleus. Finally, the possibility exists for the receptors to be located in the cytoplasm, the

nuclear pore complex and the nucleoplasm. Transport will include the binding of a protein in the cytoplasm followed by the binding of the protein complex to the nuclear pore complex where it is transported into the nucleus. Upon completion, intra-nuclear receptors would eventually be recycled back to the cytoplasm (207).

b. Step 2: Translocation through the nuclear pore complex

Translocation through the nuclear pore complex is the second step in the nuclear protein import process. Factors of critical importance in this step include high energy phosphates and physiological temperature. In situations of no ATP and low temperature, import substrates were shown to bind to the outside of the nuclear pore complex but were not translocated into the nucleus (138, 173). The dependence of nuclear protein import on temperature has been demonstrated in both in vivo and in vitro conditions (78, 173). Movement of ligand from its initial binding site at the nuclear pore complex periphery to the central region of the pore complex, has been estimated to be a distance of about 50-100 nm (79). This movement is speculated to occur either by conformational changes of a component or by movement along the structural elements of the nuclear pore complex (129).

Nuclear protein translocation is a complex process that may require many additional steps to those mentioned above. For example, after the nuclear protein ligand accumulates in the area of the central channel of the nuclear pore complex, the following may occur: firstly, interaction with the central channel; secondly, channel gating; and finally, translocation into the nucleus. Additionally, at the completion of the

nucleocytoplasmic transport process, the ligand must be released from its receptors. Thus far, very little is known about these steps.

Since the mechanistic aspects of nuclear transport remain unknown, researchers are relying on the use of in vitro systems for the analysis of transport requirements (131). Signals (cystolic components) have been shown to be a requirement of the transport process (4). These soluble factors were shown to be necessary for nuclear protein import in digitonin permeabilized cells (4)

iii. Nuclear localization sequence (NLS)

Nuclear signal sequences have been shown to be necessary for nuclear import of some nuclear proteins (138, 173). Signals that specify the nuclear import of proteins are termed nuclear localization sequences. Nuclear localization sequences (NLS) are usually small stretches of amino acids highly enriched in basic residues (62). Proteins that contain greater than one nuclear signal sequence have been shown to move at a faster rate from the cytoplasm to the nucleus than proteins containing only one nuclear signal sequence (60). NLS-mediated nuclear import can be broken down into two phases. The first phase involves the ligand being associated with the nuclear pore complex at the cytoplasmic side of the nuclear envelope. This can occur at low temperature and in situations in which ATP is depleted. The second phase which occurs at physiological temperature and has an absolute requirement for high energy phosphates, involves the translocation of the ligand through gated channels (138, 173). Signal-mediated nuclear import has been shown to be dependent on ATP in in vivo and in vitro studies (28, 138). Nuclear import of specific

proteins such as kinases and various transcriptional factors has been shown to be regulated by masking/unmasking of nuclear localization signals or by anchoring of these proteins to cytoplasmic structures (79). Five cytosolic factors have been indentified as being needed for NLS-mediated import of peptides. These factors include the NLS receptor/importin (2), a protein involved in the binding reaction (p97) (5), the heat shock protein HSP70 (185), NTF2/B-2, a 14 kDa protein that interacts with the pore complex protein p62 (157), and the GTPase Ran/TC4 (130, 133). It is very likely that other factors besides the identified ones are necessary for nuclear import (86).

iv. GTPase Ran/TC4

Human TC4 cDNA was originally isolated based on its similarities to Ras. This protein was later purified and called Ran (Ras related nuclear protein) (21). Although similar to Ras, Ran is distinct enough to be categorized into a separate family of small GTP-binding proteins. One feature that distinguishes Ran from other members of the Ras superfamily is that it contains no signals for lipid modification. Ran also appears not to be associated with intracellular membranes (134). Further, Ran is also different from Ras in that it is more abundant (at least two orders of magnitude) (134). At any given time, approximately 80-90% of cellular Ran can be found in the nucleus, with the remaining 10-20% being in the cytoplasm (134). GTP is hydrolysed by Ran (130). Recently, the GTPase Ran/TC4 was identified as a necessary factor for efficient nuclear protein import in vitro (130, 133). Mutations in Ran or its nucleotide exchange factor have been shown to result in defects in nuclear import (178). Although, there is no need for the exogenous addition of Ran for accumulation of NLS ligand at the nuclear envelope (87, 133), recent evidence indicates that the GTPase Ran/TC4 is involved in a releatively early transport step (86, 134). One indication of how the GTPase cycle of Ran may play a role in nuclear import was demonstrated by Melchior & Gerace (129). They observed that Ran GTP, but not Ran GDP, specifically binds to the periphery of the nuclear pore complex on the cytoplasmic side. The rate of GTP hydrolysis by Ran is low (21). It is possible that it interacts with a GTPase-activating protein (GAP) in the transport pathway (129). A

potential GAP involved in nuclear protein import was identified as Rna1p by Bischoff et al (22). This protein was previously thought to be involved in RNA export and processing.

v. RNA Efflux

Relative to nuclear import, not much is known about nuclear export from the nucleus into the cytoplasm. One possible reason for the existence of such little information is that at the present time, no in vitro assay has been developed to measure RNA efflux. An in vivo method of examining RNA exit from the nucleus has been to microinject different labeled RNA substrates in the nuclei of Xenopus oocytes and quantify their transport after manual dissection (50). Using microinjected colloid gold coated with RNA, for example, it was shown that RNA is exported through the nuclear pores by a facilitated process (69). Results from these type of experiments have indicated that the export of messenger RNA, transfer RNA, 5 S ribosomal RNA and ribosomal subunits is similar to nuclear protein import. Regardless of the type of RNA used, it was shown that the process is energy dependent and can be inhibited at low temperatures. Additionally, the rate at which a specific substrate is exported from the nucleus was shown to decrease upon co-injection of an excess of unlabeled substrate of the same type, indicating that export is a saturable process and therefore likely to be carrier mediated.

Another method that has proved useful in nuclear export involves that of light microscopic *in situ* hybridization with a labeled probe. This technique is particularly useful for detecting defects in mRNA export. Studies using this methodology have shown

that mutations in mammalian RCA and its *S. cerevisiae* homolog PRP20/SRM1/MTR1, resulted in an accumulation of messenger RNA inside the nucleus, indicating defects in the export mechanisms (50, 52, 97). However, these mutations also affect mRNA processing. Therefore, it was suggested that these mutations may affect nuclear export in an indirect method leading to the production of transport-incompetent substrate (74, 179).

A protein that is often isolated in a complex with Ran is the regulator of chromosome condensation protein (RCC1) (52). RCC1 is a 45 kDa chromatin-binding protein that was first isolated in mutant hamster cells defective in cell-cycle progression. Cells possessing the mutant form of RCC1 can enter mitosis prematurely, before replication is complete. In addition, RCC1 has been implicated in maintenance of nuclear structure, DNA replication, and RNA transcription, processing, and export (52). RCC1 may also maintain Ran in the GTP-bound form inside the nucleus and a related protein B2 may serve the same purpose in the cytoplasm. It was also shown that specific mutant alleles of an RCC1 homolog can be suppressed by the Ran homolog (52). It was, therefore, suggested that the role of Ran in RNA export may be similar to its role in import (134).

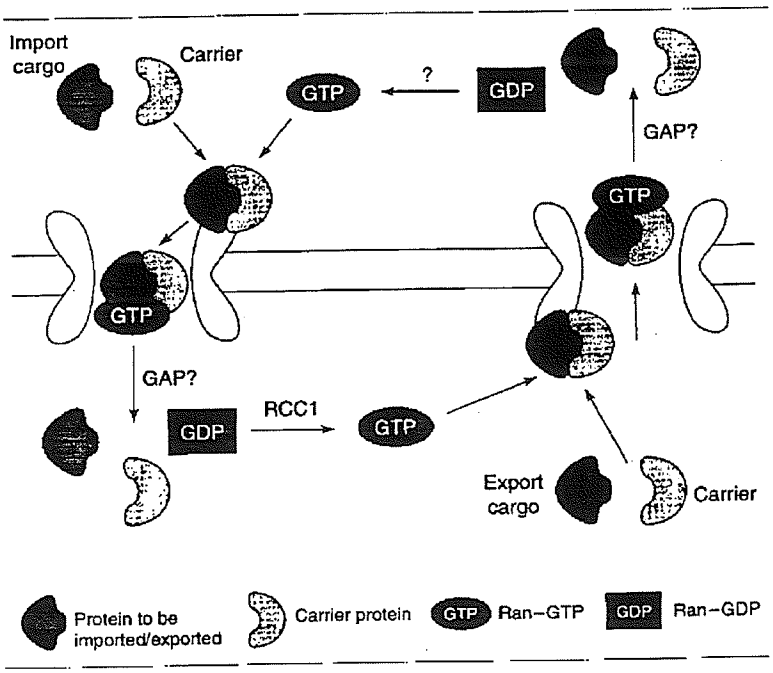


Figure 3: MODEL OF THE PATHWAY OF NUCLEAR IMPORT-EXPORT: THE FUNCTION OF RAN

A model for the possible function of Ran in import-export pathway. The binding of GTP-Ran to the cargo-carrier complex has one function - to release the complex from the docking site, while GTP hydrolysis has another - to dissociate the cargo carrier complex. (Adapted from *Moore,M.S. and Blobel,G., 1994*).

Several key observations regarding the role of RCC1 in transport have been made using a temperature-sensitive hamster cell line called tsBN2, which has a point mutation in RCC1. When these cells are shifted to the nonpermissive temperature, all of the pre-

existing RCC1 is rapidly degraded (52). Concomitant with the loss of RCC1, the cellular distribution of Ran shifts from primarily nuclear to over 50% cytoplasmic (171). At this point, an NLS-containing protein microinjected into the cytoplasm is still imported; however, mRNA export has been shown to be deceased (97). These results are consistent with the previous speculation that the B2 factor can regenerate active Ran in the cytoplasm. It has been suggested that in the absence of RCC1, the nuclear Ran might be primarily in the GDP-bound form and therefore unable to associate with export substrate. Ran might then be free to diffuse back into the cytoplasm where it can be regenerated (by B-2) to function in nuclear import (171).

Ran or RCC1 (or both) has been implicated in virtually every aspect of nuclear structure and function (52). One possible way of explaining these effects is to discard the notion that nuclear import and export are unrelated, and to view them instead as distinct stages of a continuous import-export pathway. Having a co-ordinated system for nucleocytoplasmic transport in which at least a subset of the transport factors are used in both directions seems much simpler and more efficient than using different mechanisms for each stage of transport. Each type of cargo (proteins, ribonucleoproteins, etc.) could have a different carrier, but these carriers might contain a common element that allows them to interact with Ran. The crucial role for Ran would be as a 'molecular switch' during transport.

Exactly how the GTPase cycle of Ran is related to transport is unknown. Currently, the accepted theory involves the likelihood that GTP-bound Ran is required for 'forward' movement of the cargo at any stage of transport. Figure 3 shows one model for

how the GTPase cycle of Ran might be linked to transport. In this model, the carrier recognizes an NLS-bearing substrate in the cytoplasm and docks at the cytoplasmic face of the NPC. GTP-Ran associates with this docked cargo-carrier complex, stimulating its release from the docking site for continued transport through the NPC. During this passage through the NPC, there are probably a number of biochemical events that must occur. For example, the initial docking of NLS-containing substrates along the NPC-associated fibers can occur some distance from the 'central transporter' region of the NPC (see Figure 2) (73, 173). Whether the NPC itself actively participates in the transport process by undergoing conformational changes that facilitate or are required for transport of the substrate, or whether it functions merely as a 'tunnel' through which substrates are transported, is unknown at present. Moore and Blobel (134) have postulated that GTP hydrolysis by Ran could stimulate a conformational change in the carrier, resulting in the release of the cargo. This conformational change might serve to prevent the carrier from rebinding NLS-containing proteins already inside the nucleus. GDP-Ran could then be regenerated to GTP-Ran by the action of RCC1, thereby starting a second phase of the import-export cycle (134).

vi. Nucleoside Triphosphatase (NTPase)

Several different nuclear enzymes possess NTPase activity. These include DNA-dependent RNA polymerases, helicases and topoisomerases (106). Thus, the identification of a specific enzyme as having NTPase activity does not determine function. The NTPase

activity of mammalian nuclear envelopes has broad substrate specificities towards nucleosides (191). Hydrolysis of high energy phosphates like ATP and GTP generates the energy necessary for nucleocytoplasmic transport (21, 50, 78, 138, 173). This hydrolysis is accomplished by a membrane bound nucleoside triphosphatase (7, 8, 42, 43, 90, 166, 175, 182, 200, 201). The NTPase is thought to exist as part of a laminar protein complex found on the inner face of the nuclear membrane (8, 42, 43, 103, 200, 201, 205). The NTPase, therefore, is a protein of critical importance in nucleocytoplasmic transport. Agutter et al, (6) showed that NTPase activity can be stimulated by RNA containing poly(A) or poly(G) sequences. This finding was confirmed by Bernd et al. (19). Inhibitors of NTPase activity also inhibited the efflux of prelabelled RNA from isolated nuclei (7, 38). Pea nuclei have an NTPase that is stimulated by phytochrome. This was shown in both isolated nuclei and intact pea plumules (36, 206). This stimulation was inhibited by chelators of calcium and calmodulin antagonists. Also, calcium-activated calmodulin stimulated NTPase activity three fold (35). Calcium and phytochrome are, therefore, considered important in pea nuclei NTPase activity. Mammalian nuclei also possess the NTPase. For example, the involvement of nuclear envelope nucleoside triphosphatase in nucleocytoplasmic translocation of ribonucleoprotein was shown in rat and pig liver nuclei (40, 132). Based on cytochemical studies of the relation of NTPase activity to ribonucleoproteins (RNP) in isolated rat liver nuclei, it was suggested that a connection exists between NTPase activity and the translocation of RNP particles through the nuclear envelope (205). Further, when RNP were applied to isolated rat liver nuclei and cytochemical tests carried out for NTPase, a positive reaction was detected. The

reaction was activated by Mg^{2+} and Ca^{2+} (205). Affinity labelling experiments localized an ATPase to the inside of rat liver nuclear envelopes. This was accomplished by the use of closed nuclear membrane vesicles and an ATP analogue that does not penetrate the nuclear membrane vesicles (103). Schroder et al purified and characterized the major nucleoside triphosphatase from rat liver nuclear envelopes and also showed that the hydrolysis of ATP and GTP by this enzyme parallelled energy dependent efflux of poly(A)-containing mRNA from nuclei in vitro (182). Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the NTPase from rats was shown to have an apparent molecular weight of 40,000 (182). The enzyme activity in the nuclear envelope was reported to be associated with the annular part of nuclear pore complexes (205). Also, the enzyme was reported by Clawson et al., to have a molecular weight to 46kD and is found on the nuclear scaffold. Studies by Tong et al., showed similar antigenic properties between the pea and rat liver nuclei (201). To definitively establish this identification, Clawson et al., undertook a series of photolabeling, proteolysis, and immunoprecipitation experiments. Mice were immunized with human lamin C expressed in bacteria, and monoclonal antibody-producing hybridomas were obtained. The purified monoclonal antibodies all recognized lamins A and C on immunoblots of the nuclear scaffold. In support of this proposed proteolytic cleavage site, specific assays with tyrosine-containing thiobenzyl ester substrate documented the presence of nuclear scaffold protease activity which cleaves at tyrosine residues. These findings indicate that the major Mr 46,000 photoaffinity-labeled protein in the nuclear scaffold represents the putative NTPase thought to participate in nucleocytoplasmic transport, and it is derived from lamin A or

lamin C by nuclear scaffold proteolytic activity which exposes a cryptic ATP-binding site near the highly conserved end of the coil (43).

Nuclear NTPase activity is responsive to various drug interventions. It has been shown that liver regeneration can by induced in rats by treating with carbon tetrachloride. The regenerative activity was substantial with a sharp mitotic response 2 days after treatment. There was a fourfold increase observed in nuclear nucleoside triphosphatase activity after drug treatment (41). In a study by Sidransky et. al., hepatic nuclear response to tryptophan was assessed on rats that were on a choline-deficient diet. Serum cholesterol was decreased in the choline deficient group. Hepatic nuclear nucleoside triphosphatase activity was increased in the choline supplemented plus tryptophan group (188). The administration of mitomycin C to rats resulted in stimulated ATPase activity in hepatic nuclei. Four days after injection of mitomycin C, hypotonic extracts of liver cell nuclei showed a 14 fold higher specific activity than that found in normal rat liver nuclei. (208). The properties of the ATPase were found to be similar to those of a nucleoside triphosphatase found in the nuclear matrix and envelope. This suggests that some energy-providing mechanisms involved in the repair processes of damaged DNA or cellular injury are induced by mitomycin C administration (208).

.3 *Cholesterol*

i. General Overview

Membranes constitute the structural organization of lipids in a bilayer in the cell. Functional proteins are either bound to the surface or inserted into the bilayer and interact within specific domains in the lipid environment. Variations in membrane cholesterol content affect the fluidity of the bilayer, thus altering its permeability and function. In biological membranes, under physiological conditions, a high cholesterol content rigidifies the bilayer decreasing its permeability. A lower cholesterol content induces the opposite effect. Both intrinsic and extrinsic factors form the basis for determination of membrane lipid composition and consequently membrane physicochemical properties. Further, many intrinsic metabolic controls, such as fatty acid desaturation and phospholipid biosynthesis, may be attenuated by a change in extrinsic influence (i.e. diet).

ii. Alterations of Cellular Cholesterol

It is estimated that greater than 90% of the cholesterol may be located in the plasma membrane of the cell. Smaller amounts of cholesterol are found in intracellular membranes. Within a specific membrane, cholesterol is not uniformly distributed between the inner and outer leaflets. Erythrocytes for example have been shown to contain greater

cholesterol content on the external surface relative to the cytoplasmic surface (113). Cholesterol is heterogeneously distributed in the the nuclear membrane. The outer leaflet of the nuclear membrane is more homogeneously distributed that the inner leaflet (12). Several factors contribute to the disproportional cholesterol content among the plasma and subcellular membranes. The composition of phospholipids and proteins contributes to this disproprotional cholesterol content in membranes. Model studies have suggested that cholesterol transfers between membranes (194). In the cell, intramembrane cholesterol transfer can occur via sterol carrier proteins. Cellular cholesterol can emanate from two sources: cellular biosynthesis and plasma lipoproteins such as low density lipoproteins. The cholesterol content in the cell is dynamic and both endogenous and exogenous hypercholesterolemic conditions can increase membrane cholesterol.

Endogenous: Endogenous methods of increased membrane cholesterol includes that of genetic models. The Yoshida Pittsburgh rat strain is an inbred strain of rats which has endogenous hyperlipidemia. These animals although without vascular atherosclerotic damage, have been shown to have elevated membrane cholesterol. (37). Watanabe hereditable hyperlipidemic (WHHL) rabbit is an animal model for human familial hypercholesterolemia. Marked accumulation of cholesterol in the aorta of WHHL rabbit can be seen as a function of time (151). As compared with that of normal rabbits, WHHL have decrease membrane fluidity (probably due to increase membrane cholesterol) and suffer from an increased incidence of athrosclerosis (151). Conversely, a decrease in

membrane cholesterol content has been observed in herediatary cardiomyopathy in hamsters (152).

Exogenous: Diet can alter membrane cholesterol. Rats were fed diets rich in lard, corn oil, or fish oil. Hepatocyte basolateral membrane cholesterol was found to be higher after ingestion of a fish oil diet compared with the corn oil and lard groups: Lipid phosphorus and the relative distribution of phospholipid classes were similar (143). When male Wistar rats were maintained on a 2% cholesterol diet for two months (58), liver mitochondrial cholesterol was enriched 70% over control. Erythrocyte membranes isolated from guinea pigs that were fed a diet supplemented with 1% cholesterol had a 2-fold increase in cholesterol relative to control (109).

In Vitro Methods for Altering Cholesterol Content: Acquisition or depletion, as well as variation of membrane cholesterol was shown to be highly selective (102). Liposomes and plasma lipoproteins are two in vitro methods of altering cholesterol content.

Liposomes are formed by sonicating phospholipids or phospholipids-sterol mixtures in an aqueous medium under suitable conditions (111, 168). Trapped cholesterol within liposomal vesicles can be transferred to membranes resulting in an increase in membrane cholesterol content (111, 168). Alternatively, by reversing the procedure, it is possible to measure the rate at which cholesterol diffuses from membranes by using cholesterol-free phosholipid liposomes.

Liposomes are often used effectively to increase or decrease the cholesterol content of various membranes. Using cholesterol enriched liposomes (cholesterol/phosphatidylcholine 2:1 mol/mol), Kutryk and Pierce (111) were able to increase cardiac sarcolemmal membrane cholesterol content by 96% over control values. The increase in membrane cholesterol was shown to be both time and concentration dependent (111). Using a different membrane (purified isolated nuclear membranes), Ramjiawan et. al., have also shown that it is possible to increase (three fold relative to control) the cholesterol content using cholesterol enriched liposomes (168). Alterations in membrane cholesterol using liposomes were used, for example, in a study by Ng et. al. to study the effect of cellular cholesterol depletion (with phosphatidylcholine liposomes) and enrichment (with cholesterol and phosphatidylcholine liposomes) on cellular pH regulation in SV-40 virus transformed human MRC-5 fibroblasts (140). After incubation of normal platelets with cholesterol rich liposomes, a higher membrane anisotropy and a higher cholesterol to phospholipid (C/P) molar ratio of the plasma membrane were found (147). Perfusion of arterial segments of the isolated carotid artery in vitro (4 h) with cholesterol-rich liposomes consisting of free cholesterol and phospholipid in a 2:1 molar ratio, resulted in a 60% increase in free cholesterol content without affecting phospholipid content (20). Aortic smooth muscle cells incubated with cholesterol-enriched multilamellar liposomes, resulted in a 6 fold increase of free cholesterol in the plasma membrane (34). Cultured human lymphoblasts incubated with lipososomes resulted in increase membrane cholesterol content (163). It is also possible to extract cholesterol from the plasma membrane (111). Plant phosphatidylcholine liposomes extracted 42% of red blood cell

membrane cholesterol, without causing haemolysis (100). This cholesterol extraction was responsible for the decrease of membrane microviscosity and for an increase in Na^+, K^+-ATPase activity (100).

Plasma lipoproteins can also be used to alter cholesterol content of various cells. Cholesterol-enrichment of cultured rabbit aortic smooth muscle cells was accomplished by incubating confluent smooth muscle cells with cationized low-density lipoprotein (204). Under in vitro conditions, the incubation of normal human erythrocytes in cholesterol-enriched plasma results in an increase in the cellular cholesterol content with no change in phospholipid composition (99). Incubation of cardiomyocytes with low-density lipoprotein induced a significant increase in cellular free cholesterol, cholesterol esters and total cholesterol within 30 minutes of treatment (121). Longer incubation times also resulted in a further, but modest increase in cholesterol.

Cholesterol Localization Within the Cell: In mammalian cells, unesterified cholesterol is confined largely to membranes due to its lipophilicity. Within a specific cell, membrane cholesterol content can differ greatly. It has been demonstrated that the density of filipin/cholesterol complexes located on the luminal plasma membrane of the columnar absorbing cells of the proximal colon was significantly higher (about twice) than in the distal colon (126). Subfractionation of mitochondria revealed that cholesterol was located primarily in outer membranes of both control and cholesterol-enriched preparations (58). Gap junction channels are embedded in relatively cholesterol-rich domains of cardiac

membranes. The fluidity of cholesterol-rich domains is of importance to gap junctional coupling.

iii. Functions of Cholesterol in Biological Membranes

Factors which alter the nuclear membrane lipid composition can consequently exert an effect on membrane proteins. Although the nuclear membrane is different from other subcellular membranes, it also exhibits similarities. Cholesterol is a key component in all biological membranes. Due to its amphiphilic properities, cholesterol is able to fit into a phospholipid bilayer with its polar hydroxyl group protruding into the polar surface region, and the hydrophobic steroid rings oriented parallel to, and buried in, the hydrocarbon chains of the phospholipids. Cholesterol creates a physical state which is intermediate between the liquid crystal and gel phases in the surrounding lipids of the membrane (32, 112, 169, 210). From its specific conformation in the membrane, cholesterol confers to the membrane a state of "intermediate fluidity", enabling motion of hydrocarbon chains in the gel phase while restricting motion in the liquid crystalline phase. Cell membranes usually work best when the lipid bilayer membrane is in the liquid crystalline state (210). To assess whether alterations in membrane fluidity of neonatal rat heart cells modulate gap junctional conductance, Bastiaanse et. al. (17), compared the effects of two substances that decrease membrane fluidity (heptanol and 2-(methoxy-ethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)-octanoate). Their results indicate that heptanol decreases gap junction by decreasing the fluidity of cholesterol-rich domains, rather than

by increasing the bulk membrane fluidity (17). Numerous evidence exists regarding the influence of membrane fluidity on membrane bound enzymes.

iv. Influence of Cholesterol on Enzymes

Observations on the plasma membrane as well as other subcellular membranes suggests that many proteins are free to move within the phospholipid bilayer, provided that the lipidds are in a suitably fluid state. Cholesterol being a critical component in the maintainane of membrane fluidity, can consequently affect membrane enzymes. Cholesterol may affect a particular membrane bound enzyme in one of three ways. Firstly, it can directly bind to the enzyme. Secondly, it can alter the enzyme activity through a special molecular ordering effect on the membrane. Finally, cholesterol can alter the activity of an enzyme via an indirect route. The effect of cholesterol on a specific enzyme depends on that particular enzyme. It is possible for an enzyme to be stimulated, inhibited or have no change in activity.

a. Enzyme Stimulation by Cholesterol

Although membrane cholesterol content in a given cell type is thought to be tightly regulated, some enzymes are stimulated as a result of altered cholesterol content. Numerous evidence exists for the stimulation of enzymes by increases in membrane cholesterol. Kutryk and Pierce have shown increases in cholesterol content of cardiac sarcolemmal membranes result in a stimulation of the Na^{+}-Ca^{2+} exchanger protein (111). Chronic administration of a high dose of acetylacetic acid (200 mg/kg body weight)

resulted in a significant increase in rat liver plasma membrane gamma-glutamyl transpeptidase activity. The changes in enzymatic activity were positively correlated to membrane cholesterol content (145). Hypercholesterolemia and elevated 15-lipoxygenase are associated. 15-Lipoxygenase is the principal mammalian enzyme that oxidizes polysaturated fatty acids in intact lipoproteins and in membrane phospholipids. Bailey et. al. reported activities of 15-lipoxygenase to be greater by 4 and 8 times in heart and lung respectively in cholesterol fed rabbits (15). Recent findings indicate that cholesterol-enrichment and plasma lipoproteins can enhance vascular contractile response. Cholesterol-enrichment of endothelial cells indirectly influenced the vascular response by disturbing the function of the Na^+/K^+ pump (114). Cellular cholesterol depletion (with phosphatidylcholine liposomes) led to an increased maximal velocity (Vmax) of the Na^+-H^+ antiporter, with no changes in the apparent dissociation constant (Kd) or Hill coefficient for intracellular H^+ (140). Cholesterol enrichment of carotid arterial cells increased endothelium-derived relaxing factor (EDRF) activity of the arterial endothelium and increased smooth muscle responses to the EDRF(s) (20).

Proteoglycan metabolism is altered in cholesterol-enriched cells. In a study to determine the effects of lipid accumulation on proteoglycan synthesis, cholesterol-enriched rabbit aortic smooth muscle cells in culture was used. Control and cholesterol-enriched cells were incubated with [35S]sulphate, [3H]glucosamine, or [3H]serine. Metabolically labelled proteoglycans in the cell layer and medium were quantified. During a 20 h incubation period, proteoglycan synthesis in cholesterol-enriched cells increased by 40-50% above that in control cells. The cholesterol-enriched cells also showed a 45-50%

increase over control rates in the intralysosomal accumulation of a large chondroitin sulphate proteoglycans (204).

b. Enzyme Inhibition by Cholesterol

Alterations of the cholesterol content can result in some enzymes being inhibited. The integrity of contractile cells is maintained by the regulation of Ca^{2+}. Ca^{2+}-ATPase is an enzyme that actively transports Ca^{2+} into the sarcosplasmic reticulum and thus lowers the cytoplasmic concentration of Ca^{2+} during muscle relaxation. Cholesterol has been shown to have an inhibitory effect on the Ca^{2+}-ATPase activity (14, 149, 150). Cholesterol enrichment inhibits Na^{+}/K^{+} pump and thus reduces cellular K^{+} content in endothelial cells. This reduction in cellular K^{+} may play a role in the endothelium-dependent vascular response induced by hypercholesterolemia (114). The sodium-hydrogen (Na^{+}-H^{+}) exchanger is a glycoprotein that is membrane bound. Inhibition of Na^{+}-H^{+} exchange activity was observed in cultured human lymphoblasts that had elevated membrane cholesterol content (163). A membrane-bound enzyme, 5'-nucleotidase, was inhibited after 24 to 48 hrs incubation with 25-hydroxy cholesterol. Depletion of membrane cholesterol content by the cholesterol oxide was suggested to be responsible for the membrane functional alterations (135).

c. Lack of Effect of Cholesterol on Enzymes

Some enzymes are unaffected by cholesterol. For example, increases in basolateral membrane cholesterol content did not affect Na^+-dependent taurocholate transport (143). Kutryk and Pierce have reported no change in the Na^+K^+-ATPase in cardiac sarcolemmal vesicles despite an increased membrane cholesterol content (111). Cholesterol enrichment of SV-40 virus transformed human MRC-5 fibroblasts had no effect on the activation of the Na^+-H^+ antiporter by intracellular acidosis (140). Using mitochondria isolated from normal rat liver and AS-30D hepatoma in addition to cholesterol-enriched mitochondria, Dietzen and Davis found that an increase in membrane cholesterol did not induce "truncation" of the citric acid cycle or any other mitochondrial abnormality in tumor cells (58).

d. Enzyme Stimulation and Inhibition by Cholesterol Within the Same Membrane System.

Stimulation and inhibition of particular enzymes can occur at the same time. Using cardiac sarcolemal vesicles that were enriched in cholesterol, Kutryk and Pierce have shown stimulation of Na^+-Ca^{2+} exchange, inhibition of Ca^{2+}pump activitiy and no change in activity of Na^+ K^+-ATPase (111).

v. *Influence of Cholesterol on Gene Expression*

Gene expression can be altered by changes in the cellular cholesterol content. The number of low density platelets was found to be increased in patients with hypercholesterolemia, as compared with the number in controls. The percentage increase of the low density platelet subpopulation was even more pronounced in patients with hypercholesterolemia when compared with that in patients suffering from myocardial infarction or angina (147). Alloxan-diabetic rabbits develop hypercholesterolemia and hypertriglyceridemia in response to cholesterol feeding. To determine whether alterations in apolipoprotein composition of plasma lipoproteins were due to changes in apolipoprotein gene expression, Lenich et. al. measured the steady state apoE mRNA levels in several tissues from both control and diabetic rabbits. It was found that apoE mRNA levels and tissue cholesterol content are altered in the diabetic cholesterol-fed rabbit.

In vitro studies with control platelets incubated with cholesterol rich liposomes also showed an increase in the subpopulation of low density platelets. After incubation of control platelets with cholesterol rich liposomes, a higher membrane anisotropy and a higher cholesterol to phospholipid (C/P) molar ratio of the plasma membrane were found. Furthermore, cholesterol-enriched platelets were more sensitive upon thrombin stimulation. The results suggest that a shift of platelet subpopulations to a higher number of low density platelets could be caused by either the level of plasma cholesterol or an in-vitro incubation with cholesterol rich liposomes (147). Investigation on the influence of cholesterol-enrichment of vascular smooth muscle cells on cytokine-induced nitric oxide

synthesis have revealed that cholesterol enrichment of arterial smooth muscle cells upregulates cytokine-induced nitric oxide synthesis (164). Dietary cholesterol and dietary saturated fatty acids were shown to affect the plasma concentrations of various HDL components and the hepatic and intestinal expression of the apolipoprotein (apo) A-I gene and the hepatic expression of the A-II gene differently in three inbred strains of female mice (193).

Following cholesterol enrichment of arterial smooth muscle cells, cyclooxygenase activity was found to be reduced approximately 8-fold in intact lipid-laden cells relative to untreated cells. These results have led to the conclusion that cholesterol enrichment alters posttranscriptional processing of cyclooxygenase (COX-1) expression, as well as altering cyclooxygenase-2 (inducible form of cyclooxygenase) gene expression (165). Cholesterol feeding to both normal and nephrotic rats has been shown to cause significant increases of glomerular TGF-beta 1 and fibronectin mRNA levels (59).

Tellurium is a compound that systemically blocks cholesterol biosynthesis at the squalene epoxidase step. Because of the high demand in developing peripheral nerves for newly synthesized cholesterol required for myelin assembly, this metabolic block can lead to demyelination of nerves. Using this model of demyelination, Morell et. al. studied cell specific responses during the processes of myelin breakdown. Schwann cells were were found to upregulate their cholesterol biosynthetic pathway, while liver (the main source of cholesterol for many tissues outside the nervous system) upregulated the synthesis of cholesterol to overcomes the metabolic block. The shortage of cholesterol in schwann

cells also resulted in an immediate secondary response of down-regulation of steady-state mRNA levels for specific myelin proteins.

The expression of genes coding for products involved in lipoprotein metabolism have a differential susceptibility to cholesterol. Animals maintained for 2 months on a high fat diet (40% w/w) containing 0.1% cholesterol demonstrated an increase in Apo A-IV mRNA by 66% to 127% in the cholesterol fed animals (151).

By studying the relationship between macrophage cholesterol content and apolipoprotein E (apoE) gene expression in cultured mouse peritoneal macrophages, Mazzone et. al. concluded that changes in macrophage apoE content and secretion after cholesterol enrichment or depletion can be attributed to changes in apoE mRNA levels and cholesterol-induced changes in apoE mRNA are associated with increased apoE gene transcription (128).

vi. Oxidized Cholesterol

Cholesterol can become oxidized by the enzyme cholesterol oxidase (3-beta-hydroxy-steroid oxidase). Cholesterol oxidase catalyzes the oxidation of cholesterol to 4-cholesten-3 one and other oxidized cholesterol derivatives such as 20 alpha-hydroxy cholesterol and 25 alpha-hydroxy cholesterol (110, 118, 122).

Cholesterol oxidase can be an effective tool in assessing the distribution of cholesterol within a particular membrane system. Treatment with cholesterol oxidases, for example, has revealed that cholesterol is heterogeneously distributed in brush border membranes isolated from the apical domain of the renal and intestinal epithelial cells (65).

The use of cholesterol oxidase have also lead to the finding that glutaraldehyde fixation, a commonly used process in the analysis of cholesterol distribution in cells, can mask the existence of cholesterol pools in membranes (66). Methods of cholesterol determination using cholesterol oxidase have been developed. For example, a simple method for the determination of cholesterol concentrations in bile and other bilirubin containing biological fluids was developed. This method utilizes cholesterol oxidase. Advantages of this method include the avoidance of bilirubin interferences, and high reproducibility (24, 45). Another developed method involves the enzymatic conversion of cholesterol to cholest-4-ene-3-one by cholesterol oxidase followed by the analysis of the sample by high performance liquid chromatography to detect this oxidized product (45). Cholesterol oxidized products has been measured by various techniques (45, 108, 110, 117, 118, 122).

The formation of cholesterol oxides have multiple biological effects. The oxidation of cholesterol in vivo may play an important role in the modification of LDL which could contribute to the generation of the lipid-laden foam cells and subsequently atherosclerosis. Oxidized cholesterol (4-cholesten-3-one and 20 alpha-OH cholesterol) has been identified in human subjects. Specifically, low density lipoprotein (LDL) and very low density lipoprotein from patients with proven coronary disease and in asymptomatic control subjects were examined. It was found that the content of 4-cholesten-3-one in LDL from patients was significantly increased in comparison to values from the control subjects (117). A high cholesterol diet can result in erythrocytes becoming sensitive to lysis by cholesterol oxidase, a protein not hemolytic to normal erythrocytes (116). Homogenized spleen, liver and kidney from the hyperlipidemic

animals were sensitive to in vitro cholesterol oxidation while tissues from non-lipemic animals were resistant to modification. Lysis was associated with conversion of membrane cholesterol to its oxidation product (4-cholesten-3-one) (116). The injection of 25-hydroxycholesterol (25-OH) or cholestane-3 beta, 5 alpha, 6 beta-triol (triol) intravenously, into rabbits, resulted in numerous balloon-like protrusions and crater-like defects indicative of endothelial damage to the aortic surfaces (136).

In vitro effects of cholesterol oxides are also multiple. Cholesterol oxidation of LDL have been shown to alter lipid deposition in vascular smooth muscle cells and also cell morphology (118). Oxysterols, especially 25-hydroxycholesterol, can reduce cellular cholesterol efflux in vitro. Therefore, oxysterols, either endogenous or derived from the diet, may influence cellular cholesterol efflux in vivo, the first step in reverse cholesterol transport (101). The effect of oxysterols may be sterol specific. Addition of 25-hydroxycholesterol to HepG2 cells resulted in an increase of the net accumulation of cholesteryl esters in cells and medium by 2-3-fold and decreased that of unesterified cholesterol by 50% in both compartments (51).

Cholesterol oxides have been shown to be cytotoxic. Alterations in membrane function caused by these cholesterol oxides could be the mechanism for their cytotoxic effects. Oxidation of cardiac sarcolemmal membrane cholesterol resulted in altered active and passive transsarcolemmal calcium movement (110). Membranes that are rich in sphingomyelin are more resistant to oxidation by cholesterol oxidase, primarly because sphingomyelin interacts tightly with cholesterol and this can prevent the accessibility of cholesterol for oxidation by cholesterol oxidase (89). Carrier-mediated hexose transport

by cultured rabbit aortic smooth muscle cells, measured using 2-deoxyglucose, was reversibly inhibited by an oxide of cholesterol within one hour (135).

4 Introduction

.1 *Cholesterol Stimulation*

i. Nuclear Membrane Cholesterol Can Modulate Nuclear Nucleoside Triphosphatase Activity

Although we are beginning to learn more about the factors which regulate the pore complex (8, 21, 79, 138, 155, 173), the relationship of the nuclear membrane itself to pore function is not understood as well. A biochemical approach to this problem is to examine the influence that the nuclear membrane has on nuclear NTPase activity. The NTPase has been proposed to regulate the opening of the nuclear pore complex (7, 8, 138, 166, 173, 182). If the NTPase protein is situated on the laminar proteins under the nuclear membrane and thereby physically removed from the nuclear membrane, one might expect that the lipid composition of the nuclear membrane would have no effect on NTPase activity (and pore function). This hypothesis is not consistent with previous work from our laboratory which has suggested that nuclear cholesterol content can increase in livers from JCR:LA-corpulent rats and this may induce an elevation in nuclear NTPase activity (49). Membrane integrity in these nuclei was also found to be abnormally susceptible to osmotic shock. Although the membrane cholesterol content was suggested as the

mechanism responsible for these changes, many other mechanistic interpretations are possible in an in vivo animal model of disease. It would be helpful to examine the direct interaction of cholesterol with nuclear function in an in vitro setting where the number of external variables is more controlled. Therefore, the purpose of the present study was to focus upon the effects on NTPase activity of in vitro enrichment of hepatic nuclei with cholesterol.

.2 *Cholesterol Oxidase Inhibition*

i. Oxidation of Nuclear Membrane Cholesterol Inhibits Nuclear Nucleoside Triphosphatase Activity

Oxygen derived free radicals have been implicated in the etiology of toxic liver injury, atherosclerosis, inflammatory disorders, tumor promotion and rheumatoid arthritis (92, 172). There are a variety of mechanisms by which oxygen derived free radicals mediate cell damage and death (148). For example, oxygen derived free radicals exert important effects on protein synthesis and gene expression (53). Free radicals induce these effects via strand breaking, cross linking and base pair modification in DNA (31, 63, 180)%. Ultimately, this will alter DNA synthesis, cell growth and division. However, another mechanism whereby free radicals may alter cell growth and viability is through oxidation of membrane lipids (13, 53, 122, 154). Lipid peroxidation is a typical product of free radical interactions with membranes (53, 154, 172). Membrane cholesterol is another target of oxygen derived free radicals (110, 183, 198). Oxidation of cell cholesterol can result in significant changes in cell growth (137).

Cell growth and gene expression is dependent upon the transport of mRNA out of the nucleus, and peptide and protein transport into the nucleus. This nucleocytoplasmic trafficking occurs in an energy-dependent manner through the nuclear pore complex (50, 78). The Ran TC4 GTPase protein has recently been implicated as a critical factor to provide energy for nucleocytoplasmic trafficking (130, 133). However, it is highly

unlikely that it is the sole factor responsible for the generation of energy (86). The nuclear nucleoside triphosphatase (NTPase) has been proposed to be involved in nucleocytoplasmic trafficking (7, 8, 138, 166, 173, 181). Energy liberated by the hydrolysis of nucleotides by the NTPase has also been shown to provide sufficient energy for nucleocytoplasmic transport (7, 8, 138, 166, 173, 182). Furthermore, modifications in NTPase activity (an increase or a decrease) result in corresponding changes in mRNA or peptide flux through the pore complex (7, 166). Recent work has demonstrated that cholesterol in the nuclear membrane exerts a significant modulatory effect on the nuclear nucleoside triphosphatase enzyme (49, 168). The purpose of the present study was to investigate the influence on NTPase activity of oxidative modification of nuclear membrane cholesterol. If oxidized cholesterol exerts an effect on nuclear NTPase activity, it raises the intriguing possibility that oxidation of nuclear membrane cholesterol may represent an alternative mechanism whereby free radicals can disrupt gene expression and cell viability.

5 Experimental Methods and Procedures

i. Membrane Isolation and Preparation

a. Nuclei Isolation

Isolated intact nuclei were obtained from the livers of male Sprague-Dawley rats weighing between 300 and 350 grams by a modification of the protocol described by czubryt et al (47). Typically, livers of 3-4 animals were excised and placed in an ice-cold solution of STM buffer (250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$) supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1μM leupeptin. Livers were scissors-minced and washed 3 times with STM buffer before homogenization in 5 volumes of STM (4 °C) in a Potter-Elvehjem tube using a loose-fitting teflon pestle. The homogenate was filtered through 6-ply gauze and then centrifuged at 3000 rpm for 10 minutes in a JA-20 rotor (Beckman Instruments). The pellets were gently rehomogenized by hand in 5 volumes of STM. Centrifugation was repeated as above, and pelleted material was resuspended in STM solution to a final volume of 12 ml. The crude nuclei suspension was then diluted with 24 ml of 2.3 STM (STM buffer with 2.3 M sucrose). The diluted suspension was layered onto 6 ml 2.3 STM cushions and centrifuged at 4 °C at a speed of 16,000 rpm for 45 minutes in a Beckman SW 28 rotor. The final pellet contained highly purified nuclei which were resuspended in

STM buffer to a final protein concentration of approximately 20 mg/ml. Nuclei were rapidly frozen in liquid nitrogen and stored at -85°C until required.

b. Endoplasmic Reticulum (ER) Isolation

For the isolation of endoplasmic reticulum (ER) membranes, one liver was homogenized in STM as described above. The homogenate was centrifuged at 3,000 rpm for 10 min in a Beckman JA-20 rotor. The pellet was discarded and the supernatant was filtered through 6-ply gauze and centrifuged at 8,500 rpm for 10 minutes in a Beckman JA-20 rotor. The supernatant from this step was then centrifuged in a Beckman 40.1 rotor at 40,000 rpm for 60 min. Pelleted material was resuspended in 8 ml of STM. Discontinuous sucrose gradients, containing 15 mM CsCl and 10 mM Hepes, pH 7.2, were constructed as described (187). Each tube contained 4 ml of 0.6 M sucrose, 6 ml of 0.75 M sucrose, 10 ml of 1.3 M sucrose. A 2-ml aliquot of crude ER was layered on top of each tube and this was centrifuged at 40,000 rpm for 2.5 hr. Rough ER membranes were obtained from the pelleted material and diluted using STM solution to 250 mM sucrose, pelleted in a 40.1 rotor as above, and resuspended in 1.5 ml of STM to a final protein concentration of 40 mg/ml. ER membranes were rapidly frozen in liquid nitrogen and stored at -85 °C until required.

c. Sarcolemmal Isolation

Animals were killed by decapitation and their hearts were immediately removed. Ventricular tissue was isolated from the atria, connective tissue, as well as major blood vessels, and then processed for the isolation of sarcolemmal membrane vesicles. Sarcolemmal membranes were isolated as described in detail (162), which is a modification of the technique of Frank and co-workers. This method has been extensively employed for the purification of cardiac sarcolemmal membrane vesicles (110, 111, 161, 162). Ventricular tissue from two to five hearts was minced and then homogenized twice in a Waring blender on high speed for 12 s in 20 ml of (in mM) 250 sucrose, 1 dithiothreitol, 100 KCl, 25 Na pyrophosphate, and 20 Trizma maleate (pH 7.6), 1.2 M KCl, and 25 mM Na pyrophosphate and then centrifuged at 48,400 g for 45 min. The resultant pellet was suspended in 15 ml of (in mM) 250 sucrose, 1 dithiothreitol, and 20 Trizma maleate (pH 7.6) and incubated with 30,000 U of DNase I (Cooper Biochemicals) for 45 min at 30 °C The suspension was homogenized three times at high speed with a Polytron for 7 s, diluted with 20 ml of (in mM) 250 sucrose, 1 dithiothreitol, and 20 Trizma maleate (pH 7.6), and then centrifuged at 17,400 g for 15 min. The supernatant was centrifuged at 150,000 g for 45 min, and the pellet was recovered and resuspended in 6 ml of a 40% sucrose solution. Three milliliters of this suspension was layered under a discontinuous gradient consisting of 2 ml 27% sucrose [containing 0.02 mM ethyleneglycol-bis (ß-animoethyl ether)- N,N,N^1,N^1 -tetraacefic acid (EGTA)], 1 ml 30% sucrose, 2 ml 34% sucrose, and 2 ml 37 sucrose. All sucrose solutions contained 2 mM

NaCl. Samples were centrifuged in a Beckman SW41 rotor at 154,000 g for 12-16 h. The sarcolemmal fraction was recovered as a diffuse band above the 30-34% interface.

d. Mitochondrial Isolation

Mitochondrial isolation was carried out as per technique previously described be Pierce et al. (159).

ii. Liposomal Vesicular Preparations

Phosphatidylcholine and phosphatidylcholine-cholesterol liposomes were prepared using a modified method of (111). Phosphatidylcholine and phosphatidylcholine with cholesterol (1:0.5, 1:1, 1:2) were dissolved in chloroform solvent. The mixture was then exposed to a constant stream of nitrogen gas in the dark to eliminate the solvent. Samples were lyophilized for 12 hrs to ensure no remaining residual chloroform. A solution containing 1.0 ml of 250 mM sucrose, 50 mM Tris-HCL (pH 7.4), 5 mM $MgCl_2$ and 0.2 ml/mol of phospholipid was added, and allow to swell for 10 min. Samples were then sonicated for 60 min. using a Bransonic 1200 sonicator. After sonication, the suspensions were centrifuged at 104,000 x *g* at 5°c for 1hr in a Beckman TL-100 tabletop ultracentrifuge to remove undispersed lipid and large vesicles. Small unilaminar vesicles were obtained from the supernatant and was used immediately.

iii. Membrane Cholesterol Modification

a. Cholesterol Incorporation

Phosphatidylcholine and phosphatidylcholine-cholesterol liposomes were prepared as described (111). Isolated purified nuclei were incubated for 16-18 hrs at 4°C in the presence of cholesterol/phosphatidylcholine (2:1 mol/mol) or phosphatidylcholine liposomes. The liposome/nuclei mixtures were then centrifuged at 104,000 x *g* for 25 min to separate the nuclei from the liposomal vesicles. The nuclei pellet was washed once and then resuspended in 250 mM sucrose, 50 mM Tris-HCL (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Phenylmethylsulfonyl fluoride, 1μM leupeptin to obtain a final protein concentration of 0.5-1.0 mg/ml.

b. Cholesterol Oxidation

Nuclear cholesterol oxidation was accomplished by incubating isolated nuclei with cholesterol oxidase *(Pseudomonas fluorescens)* in a solution of 250 mM sucrose, 50mM Tris-HCL (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Phenylmethylsulfonyl fluoride, 1μM leupeptin . Incubation times and cholesterol oxidase concentrations varied *see results*. Catalase was always included whenever cholesterol oxidase was used. There was excess amount of catalase to scavange H_2O_2 that was produced.

iv. Biochemical Assays

a. Nucleoside Triphosphatase Activity

Nuclear NTPase activity was assayed as follows. Nuclei (0.5 mg/ml) were pre-incubated for 10 minutes at 30 ^{0}C in 360 µl of buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and various concentrations of total $MgCl_2$ required for specified free Mg^{2+} concentrations in the presence of 5 mM GTP or ATP. Nucleoside dependence of NTPase activity was performed in the presence of 1 mM free Mg^{2+}. Calculations of bound and free ligands were performed using the computer based LIGAND program of Fabiato and Fabiato (67). Stability constants used for Mg.ATP and Mg.EDTA were 1.9×10^4 and 4.764×10^5 respectively. NTPase activity was initiated by the addition of the nucleoside triphosphate to the pre-incubation solution. NTPase activity was quenched by the addition of 10% SDS. Inorganic phosphate was measured according to the method of Raess and Vincenzi (167).

b. Mannose 6'-Phosphatase Activity

Nuclear membranes and ER (5 mg/ml) were assayed for mannose-6-phosphatase as described previously (84). For the determination of patent activity, 40-µl membranes were preincubated in 260 µl of 50 mM Mes (pH 6.5) containing 15 ml·ml^{-1} bovine serum albumin (fraction V) at 30 ^{0}C for 10 minutes. Mannose-6-phosphatase activity was initiated updon addition of 100 µl of mannose 6-phosphatase (from 100 mM stock in 50

mM Mes (pH 6.5). Mannose-6-phosphatase activity was linear up to 30 min and was terminated by the addition of 200 µl of 10% SDS to the reaction tube. Total (latent plus patent) activity was determined after prior incubation of membranes on ice for 30 minutes with 0.4% deoxycholate. Inorganic phosphate liberated was assayed by the method of Raess and Vincenzi (167). Absorbance was recorded at 660 nm.

c. K^+-pNPPase Activity

K^+-dependent *p*-nitrophenyl phosphatase activities were measured as described (84, 160). 50 mM $MgCl^2$, 1 mM EGTA, 5 mM *p*-nitrophenylphosphate, and 20 mM KCL, pH 7.8, at ^{0}C. The K^+-independent phosphatase activity, measured in the same reaction medium with KCl, was subtracted. The reaction volume was 1 ml and contained about 8 µg of sarcolemmal, nuclear and endoplasmic reticular protein. The reaction was quenched after 7 minutes with 2 ml of 1 N NaOH, and the absorbance at 410 nm was used to determine the amount of *p*-nitrophenol formed.

d. Na^+-K^+ ATPase Activity

Na^+-K^+ adenosine triphosphatase activities were measured as described (84, 160). Briefly, Na^+-K^+-ATPase activity was measured in a medium containing 50mM Tris, 120mM MgCl, 3.5mM $MgCl_2$, 1mM EGTA, 5mM NaN_3, 20 mM Kcl, 3mM ATP, pH 7.0, at 37 °C. The reaction volume was 1ml, and approximately 8-10 µg of protein was used.

The reaction time was 10 minutes. ATPase activity measured in KCl-free mixture was subtracted. Inorganic phosphate liberated was measured by the method of Fiske et. al (71).

e. Nuclear Membrane Integrity Assay

The integrity assay is typically carried out in 12 plastic eppendorf tubes, in a final volume of one milliliter each. First, aliquots of NaCl-STM are added to each tube such that a typical assay would have tubes containing 0, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300 or 1000 mM NaCl (final concentrations). If fewer than this number of tubes are to be used, it should be pointed out that the tube containing 1000mM NaCl is required, as this tube corresponds to maximum release of nuclear contents, and is necessary to determine percent release in all the other tubes. The tubes containing between 125 and 250 mM NaCl are critical as well because this is the usual range in which 50% release of nucleotides is obtained.

STM should be added next to make the volume up to 1 ml (after taking in to account the volume of nuclei to be added), followed by the nuclei. The final amount of nuclei added to each tube, as determined by protein assay (127), is approximately 0.5 to 1 mg. All tubes should have the same amount of nuclei for a given sample. It may be possible to use smaller amounts of nuclei, depending on the sensitivity of the spectrophotometer used. For practical reasons, the concentration of nuclei in the original stock should be at least 2-5 mg/ml as determined by protein assay. Once the nuclei have

been added to the tubes, they are capped and quickly inverted once or twice to mix the ingredients, then placed on ice for thirty minutes. Do not vortex the tubes, as this may cause physical disruption of the nuclei. It is necessary to create a set of blanks, containing only NaCl-STM, STM and protease inhibitors, with salt concentrations corresponding to those of the nuclei-containing tubes. The blanks are treated throughout the experiment exactly as the nuclei-containing samples are.

After incubation, the tubes are centrifuged in a microfuge at ~7500xg for ten minutes. The supernatants are collected and the absorbance measured at 260 nm in a spectrophotometer in non-UV absorbing quarts cuvettes, using the salt-only tubes as blanks. This wavelength was chosen because DNA released from the nuclei exhibits peak absorption at this wavelength (176). The absorbance values are normalized the maximum absorbance measured (this usually corresponds to the sample containing 1000 mM NaCl) and expressed as a percentage of maximum absorbance$_{260}$. Once this maximum has been determined, it is possible to interpolate the concentration of slat necessary to cause release of 50% of the nuclear contents (releasing concentration, or RC_{50}).

f. Succinic Dehydrogenase Activity

Succinic dehydrogenase activities were measured as described (57, 76).

g. Malondialdehyde Content

The oxidation of isolated hepatic nuclei was initiated through two different free radical generating systems. Various concentrations of AMVN, a lipophilic free radical

generator (56, 98), was incubated with hepatic nuclei at 37°C for 60 minutes. The nuclei were also oxidized by incubation with another free radical generating system, Fe^{3+} -ADP (1 mM $FeCl_3$ chelated by 0.5 mM ADP) and 0.3 mM DHF for 60 minutes at 37°C (107, 119, 120). Nuclear membrane lipid peroxidation was monitored by measuring the malondialdehyde (MDA) content of oxidized hepatic nuclei as assessed by the thiobarbituric acid-reactive substances method (72, 117). Freshly prepared malondialdehyde bis (dimethyl acetal 1,1,3,3-tetramethoxypropane) was utilized as a reference standard. Thiobarbituric acid reactive substances were expressed as MDA equivalents.

h. Protein Content

Protein was measured by the method of Lowry and colleagues (124).

v. Lipid Quantification

Cholesterol content of nuclear membranes was carried out after lipid extraction in chloroform:methanol (111) and determined by an enzymatic method (146). Phospholipid phosphorus measurements were carried out after lipid extraction as described (16).

vi. *High Performance Liquid Chromatography*

High performance liquid chromatography (HPLC) was used to identify the oxidated cholesterol content in isolated nuclei after treatment of nuclei with or without cholesterol oxidase. A modified technique as described by (110, 118, 122) was employed. Briefly, 200 µl of isolated nuclei were suspended in 1 ml of 2:1 chloroform:methanol, and was extracted at 4°C. Proteins were separated from chloroform:methanol solvent mixture and discarded. The samples were evaporated under a constant stream of nitrogen gas in the dark. The lipids were suspended in 100µl of methylene chloride and also evaporated using nitrogen gas in the dark. Samples were resuspended in 25µl of methylene chloride of which, 10µl was used for HPLC analysis. The HPLC system used was a Waters 484 tunable absorbance detector (Waters Chromatography Division, Millipore, Milford, Mass.), Waters 116 solvent delivery system, and Waters 501 pump. The mobile phase consisted of 97:3 hexane:isopropanol (vol:vol), and was held constant at 2.0 ml/min. The column used was a Waters Nova-Pak silica column (3.9 x 150 mm). The wavelength of absorbance was 208nm. The Waters Baseline 810 chromatography workstation was used in the controlling process.

vii. *Statistical Analysis*

Data were analyzed, where appropriate, by Student T´test and multiple analysis of variance tests for statistical significance. The significance level of $p<0.05$ was selected as

the minimum level for significance. Km and Vmax were determined using the Edie-Hofstee plot.

6 Results

i. Cholesterol Stimulation

Nuclear Membrane Cholesterol Can Modulate Nuclear Nucleoside Triphosphatase Activity

To insure purity and structural integrity, isolated nuclei were examined at the light microscopic level. The nuclei were morphologically intact and contained negligible amounts of cellular debris (Figure 4). Toluidine blue which reacts preferentially with DNA, also strongly stained the isolated nuclei. Biochemical examination of the nuclei was carried out to obtain a more sensitive analysis of nuclear purity. Table 1 shows specific biochemical markers indicative of cellular and subcellular membrane contamination. Minimal specific activities were observed for succinic dehydrogenase, K^+-pNPPase and Na^+-K^+ ATPase indicating negligible contamination from mitochondrial and plasma membranes. Mannose 6'-phosphatase activity was used as a marker for ER membranes (84). Mannose 6'-phosphatase activities were 17% of hepatic ER membrane activities. This relatively high activity is due to the apparent continuity of the outer nuclear membrane with the endoplasmic reticulum (75).

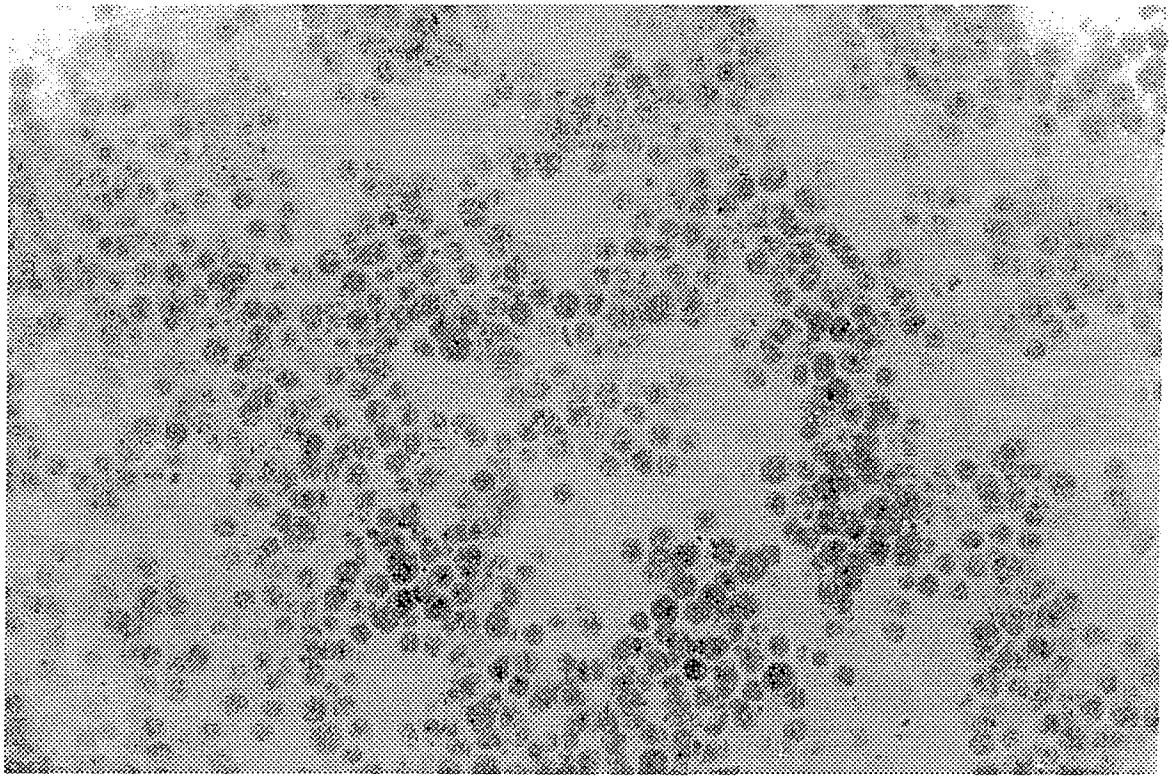


Figure 4: STRUCTURAL INTEGRITY OF THE HEPATIC NUCLEI

Isolated nuclei were stained with 0.05% toluidine blue for 10 mins and photographed using an orange filter under oil-immersion at 40x magnification. Isolated nuclei were free of cellular debris and stained strongly for toluidine blue.

Table 1: Comparison of marker enzymes for various membranes

Enzyme Activity	Nuclei	Sarcolemma	Endoplasmic Reticulum	Mitochondria
Succinic Dehydrogenase (nmol/mg/min.)	0.02 ± 0.01	n.a.	n.a.	0.53 ± 0.2
Mannose 6'-Phosphatase (nmol/mg/min.)	43.9 ± 2.75	n.a.	254 ± 14.6	n.a.
K^+-pNPPase (µmol phenol/mg/hr.)	0.16 ± 0.02	32.93 ± 2.7	n.a.	n.a.
Na^+-K^+ ATPase (µmol Pi/mg/hr.)	0.23 ± 0.69	27.10 ± 3.6	n.a.	n.a.

Values represent means ± S.E. (n = 4)

n.a. represents not assayed.

Purified isolated nuclei were incubated with phosphatidylcholine or cholesterol/phosphatidylcholine liposomes (cholesterol/phospholipid ratio initally of 2:1 mol/mol). The liposomal technique is a well established method to alter cholesterol content of a membrane (111, 156). At the end of the incubation period, the nuclei were separated from the liposomal vesicles by centrifugation. The treated nuclei pellet was then washed and spun down once more in order to be certain of a complete separation of nuclear from liposomal vesicles. The phospholipid content of the nuclei did not increase significantly after liposome treatment (Table 2). This would indicate that adherence of liposomes to the nuclei did not represent a problem. Furthermore, in separate experiments, [^{3}H] cholesterol-enriched liposomes transferred cholesterol to nuclei with a 2.5 fold greater efficiency than [^{14}C] cholesterol oleate-enriched liposomes. This preferential transfer of cholesterol versus cholesterol oleate from the liposomes to the nucleus would again indicate that adherence of the liposomes was not a major problem. In a series of experiments (data not shown) in which we incubated nuclei for various times, we found optimal incorporation of cholesterol occurred at 16-18 hrs of incubation with a 2:1 mol/mol liposomal ratio of cholesterol to phospholipid. Cholesterol content of treated nuclei increased three-fold over control values (Table 2). Incubation of phosphatidylcholine liposomes (cholesterol/phospholipid ratio of 0:1 mol/mol) with nuclei did not alter the cholesterol or phospholipid phosphate content of the nuclear membrane (Table 2).

Table 2: Modification of the cholesterol content of nuclear membranes

	Control	Phosphatidylcholine Treated	Cholesterol Treated
Cholesterol content (n mol/mg protein)	2.2 ± 0.3	2.3 ± 0.4	6.1 ± 1.8*
% of control		104 ± 7	275 ± 18*
Phospholipid content (n mol/mg protein)	21.8 ± 3.4	20.1 ± 2.8	20.9 ± 3.2
% of control		94 ± 5	96 ± 6
n	8	8	7

Nuclei were untreated (control), or incubated overnight with cholesterol free phospholipid liposomes (phosphatidylcholine treated) or with cholesterol / phospholipid liposomes (2:1 mol / mol). See "Experimental Procedures" for cholesterol and phospholipid quantification protocols. Values represent means ± S.E. * $p<0.05$ vs control.

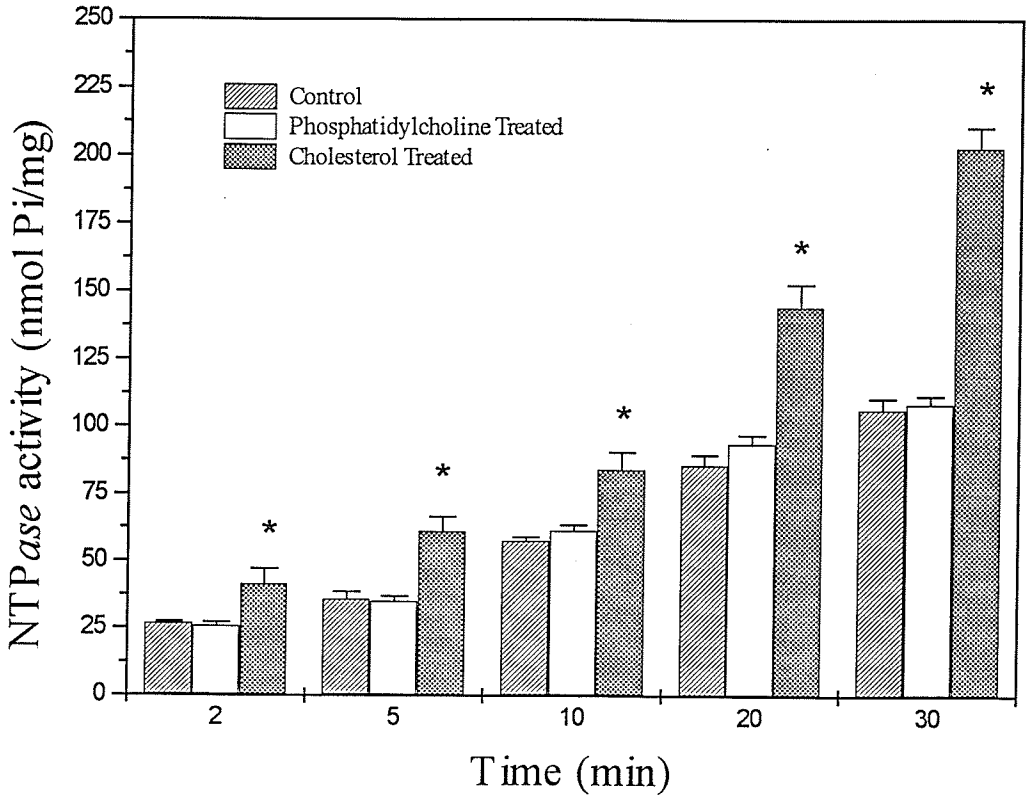


Figure 5: EFFECT OF CHOLESTEROL ON NTPase ACTIVITY OF NUCLEAR MEMBRANES AS A FUNCTION OF REACTION TIME

NTP*ase* activity was initiated upon addition of ATP to a final concentration of 5mM. Reactions were quenched at various times with 10% SDS. Significant differences are indicated (*$P<0.05$) between control and cholesterol enriched membrane preparations. Values represent means ± standard error measurements for 6 separate nuclear preparations.

NTPase activity was examined as a function of reaction time after the nuclei were enriched with cholesterol (Figure 5). ATP hydrolysis increased in all groups as the reaction time progressed. Significant differences were observed between control or phosphatidylcholine liposome treated groups and cholesterol enriched nuclei. This significance was apparent at all time points measured. There were no significant differences, however, between control and phosphatidylcholine-treated nuclei. The NTPase activity was not a result of cross contamination of the nuclei with another membrane system (Table 1). Furthermore, its kinetic biochemical characteristics are different than those exhibited by other nucleoside triphosphatases like actin (39). Others have also concluded that its presence in the nuclear fraction is not due to cross contamination (175). The NTPase has been purified (40, 96) from the nucleus and is now thought to be a part of the laminar protein situated on the inner face of the nuclei (42, 43, 103, 200, 201, 205).

ATP hydrolysis by control and cholesterol enriched nuclear membranes was examined as a function of varying [ATP] (Figure 6). There were significant differences between control and cholesterol enriched nuclei at each [ATP]. Cholesterol enrichment induced a significant change in the Vmax value for the enzyme without altering the Km for ATP (Table 3).

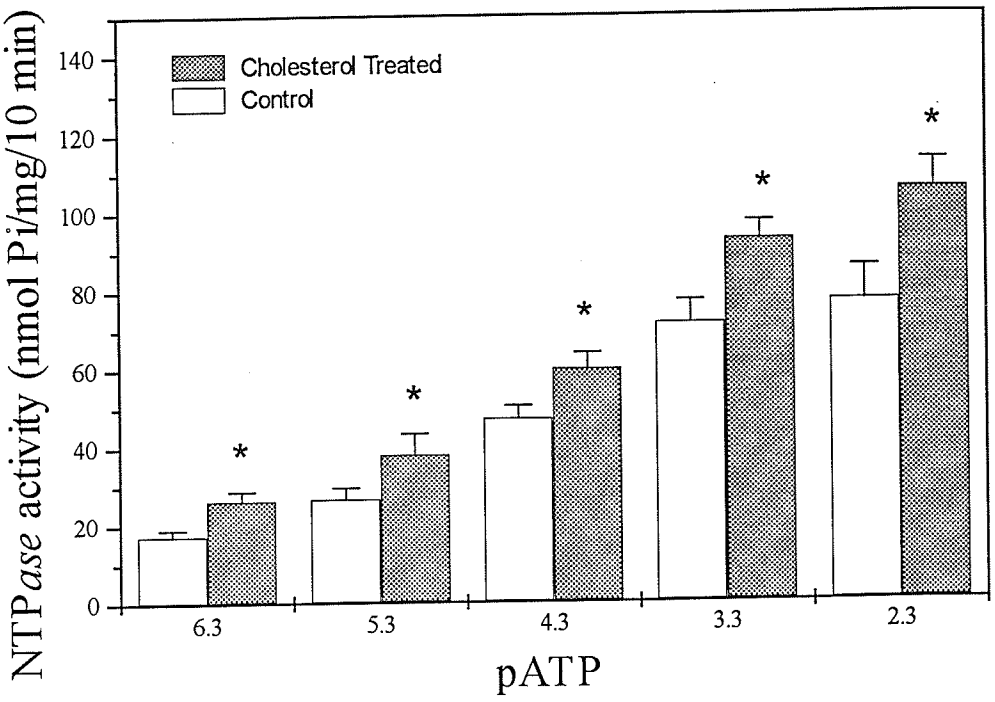


Figure 6: NTPase ACTIVITY OF CONTROL AND CHOLESTEROL ENRICHED NUCLEAR MEMBRANES AS A FUNCTION OF [ATP]

Reaction time was 10 min. Free Mg^{2+} was held constant at 1mM. Significant differences (*P<0.05) in NTP*ase* activity are indicated between control and cholesterol enriched preparations. Values represent means ± standard error measurements for 6 preparations.

Table 3: Effects of cholesterol enrichment on the kinetics of nuclear nucleoside triphosphatase activity.

	Control nuclei		Cholesterol enriched nuclei	
	Km (μM)	**Vmax** (nmol/mg/10 min)	**Km** (μM)	**Vmax** nmol/mg/10 min)
GTP	98.5 ± 5.3	107.6 ± 8.3	81.5 ± 7.4	141.7 ± 9.9*
ATP	91.3 ± 8.4	77.8 ± 5.4	83.6 ± 6.0	106.6 ± 7.2*

Cholesterol enrichment was carried out as described in Experimental Procedures. Data were calculated from Hanes plots. Values represent means ± S.E (n=5-6). *$p<0.05$ versus control values.

NTPase activity was also examined in the presence of GTP as a substrate instead of ATP. GTP*ase* activity of control and cholesterol enriched nuclear membranes increased with time (Figure 7). Significant differences were observed between control and cholesterol enriched membrane preparations (Figure 7). GTP*ase* activity of control and cholesterol enriched nuclear membranes was also studied as a function of [GTP] (Figure 8). Significant differences were observed between control and cholesterol enriched membrane preparations. Cholesterol enrichment of nuclei induced a significant change in the Vmax, but not the Km, of the enzyme in the presence of GTP (Table 3).

The Mg^{2+} dependence of the enzyme activity of control, phosphatidylcholine treated and cholesterol enriched nuclear membranes is shown in figure 9. Significant differences in NTP*ase* activities were observed between control or phosphatidylcholine treated nuclei and cholesterol enriched preparations. Statistical significance was observed at all [Mg^{2+}]. Maximum stimulation of activity was observed at approximately 5.0 mM free Mg^{2+}. There was an rapid increase in enzyme activity as free [Mg^{2+}] was increased from 1.0 mM to 5.0 mM for control, phosphatidylcholine and cholesterol treated nuclei. There were no significant differences between control and phosphatidylcholine treated nuclei.

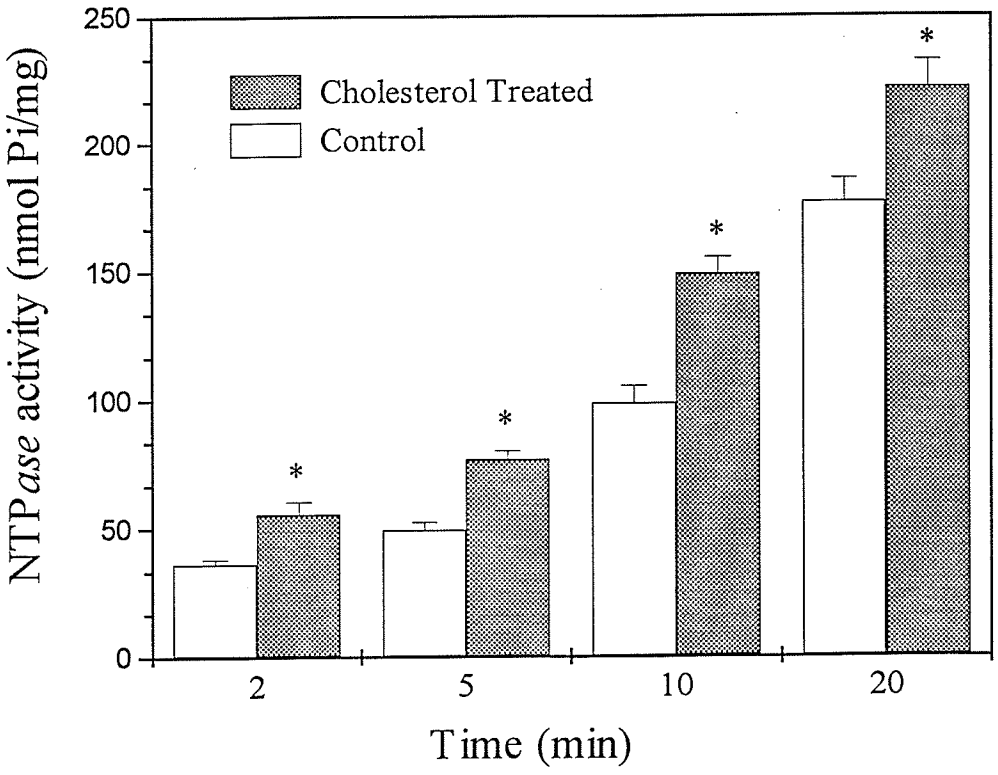


Figure 7: NTPase ACTIVITY AS A FUNCTION OF TIME IN CONTROL AND CHOLESTEROL ENRICHED NUCLEAR MEMBRANES IN THE PRESENCE OF GTP

NTP*ase* activity was assayed as in Figure 5 using GTP as the substrate. Free Mg^{2+} was held constant at 1mM. Significant differences in NTP*ase* activity are indicated (*$P<0.05$) between control and cholesterol enriched preparations. Values represent means ± standard error measurements for 5 preparations.

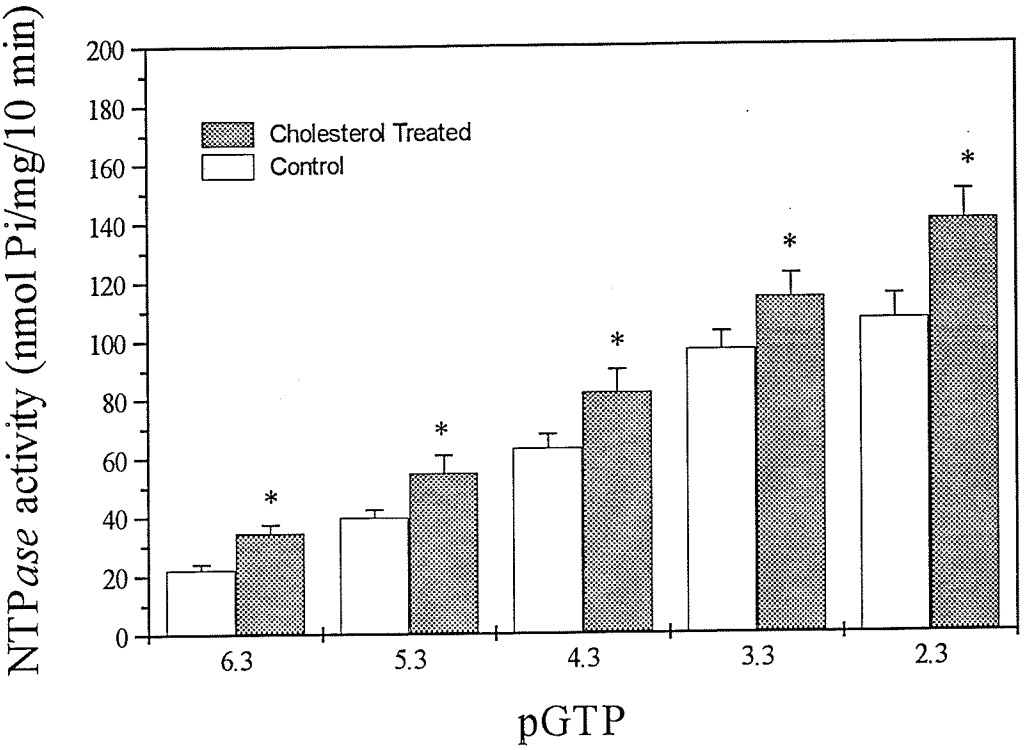


Figure 8: NTPase ACTIVITY OF CONTROL AND CHOLESTEROL ENRICHED NUCLEAR MEMBRANES AS A FUNCTION OF [GTP]

Reaction time was 10 min. Free Mg^{2+} was held constant at 1mM. Significant differences in NTP*ase* activity were indicated (*$P<0.05$) between control and cholesterol enriched preparations. Values represent means ± standard error measurements for 5 preparations.

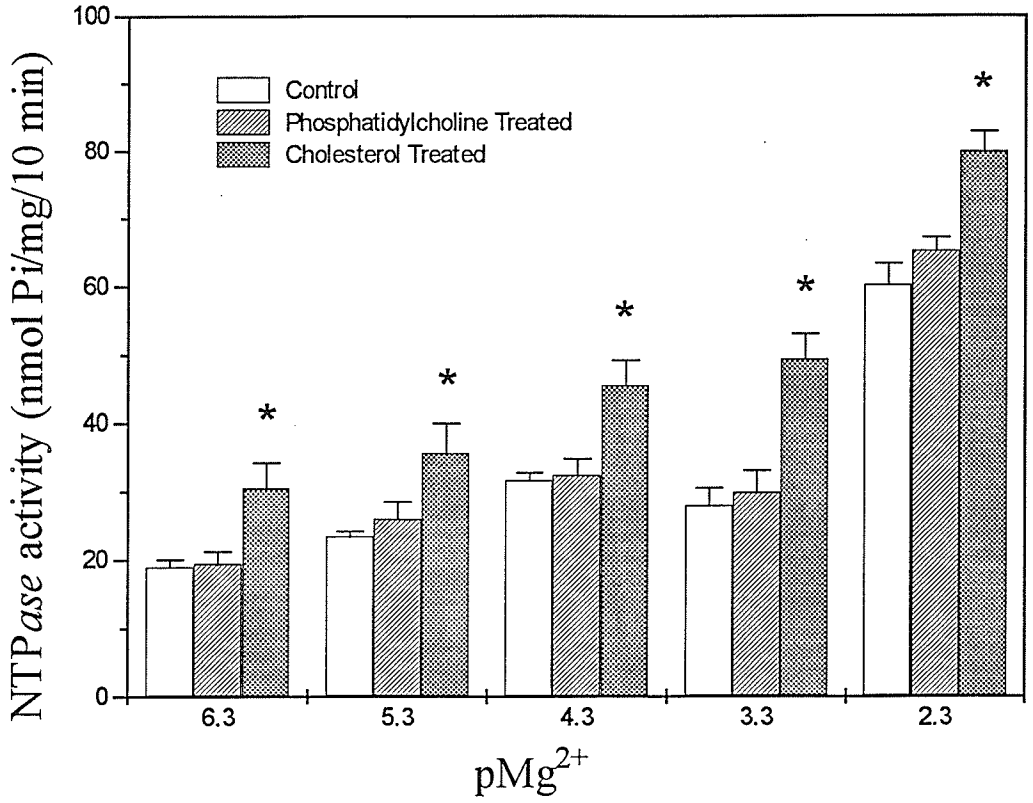


Figure 9: NTPase ACTIVITY OF CONTROL, PHOSPHATIDYLCHOLINE TREATED AND CHOLESTEROL ENRICHED NUCLEAR MEMBRANES AS A FUNCTION OF [Mg^{2+}]

NTP*ase* activity was assayed as in Figure 5. Reaction time was 10 min. ATP concentration was 5mM Significant differences in NTP*ase* activity were indicated (*$P<0.05$) between control and cholesterol enriched preparations. Values represent means ± standard error measurements for 6 preparations.

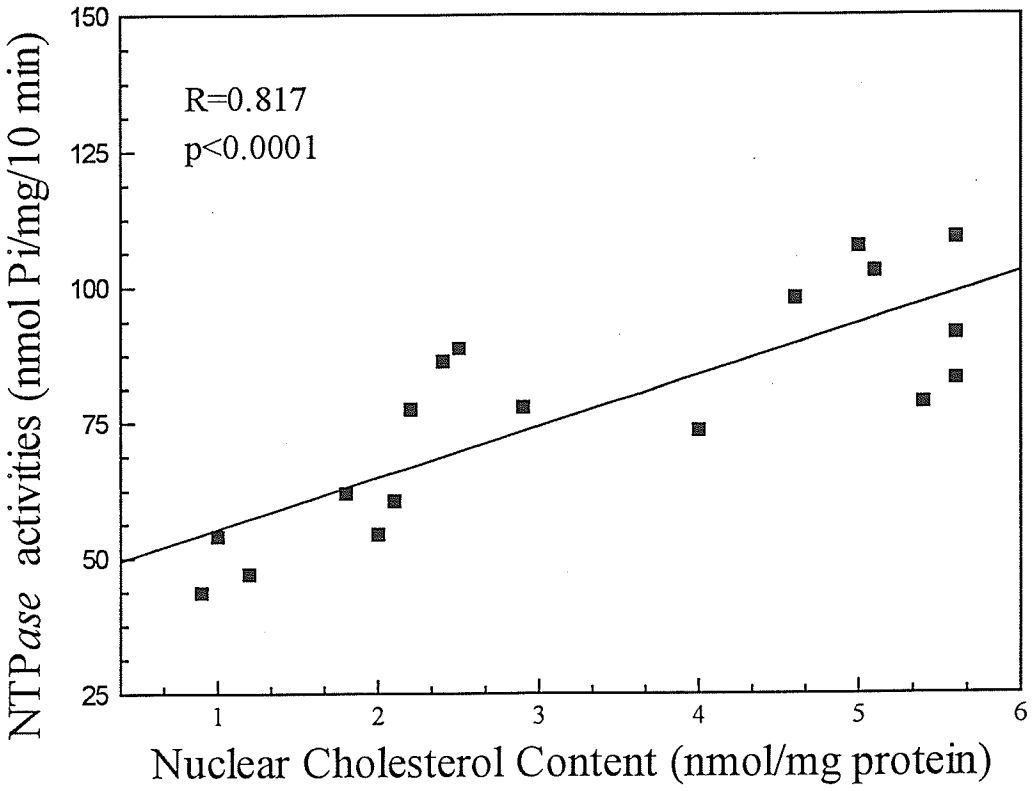


Figure 10: NTPase ACTIVITY AS A FUNCTION OF NUCLEAR MEMBRANE CHOLESTEROL CONTENT

The results from 18 experiments relating NTP*ase* activity to the nuclear membrane cholesterol content are depicted here. .NTP*ase* activity exhibited a significant correlation with cholesterol content (r = 0.803). NTPase activity was measured for 10 minutes in the presence of 5 mM ATP.

The relationship of enzyme activity to the cholesterol content of nuclei was plotted in Figure 10 from results obtained from a number of individual experiments. These data points include control and cholesterol enriched membranes. A significant relationship was demonstrated for nuclear cholesterol content and membrane NTPase activity ($r = 0.803$).

The effects of cholesterol incorporation on nuclear membrane integrity was also studied. Release of nucleotides from the nuclei in response to in vitro treatment with NaCl was used as an indicator of nuclear membrane integrity (48). Cholesterol-enriched nuclei released more nucleotides at a similar [NaCl] than untreated or phosphatidylcholine-treated nuclei (Figure 11). The concentration of NaCl at which 50% maximal nucleotide release was observed was also significantly lower in the cholesterol enriched nuclei in comparison to the two control groups (Table 4).

The relationship between nuclear membranes susceptability to disruption and that of membrane cholesterol content are depicted in Figure 12. Data from this and a previous study (49) involving in vivo elevation of cholesterol are plotted. The in vivo study involved using the JCR:LA-*cp* rat model. These animals are genetically hyperlipidemic and have increased nuclear membrane cholesterol content (49). The RC_{50} value in mM and cholesterol content in nmol/mg protein are plotted from both studies. An interdependent relationship exists ($r = -0.996$) between nuclear cholesterol content and the sensitivity of the nuclei to disruption ($p=0.004$).

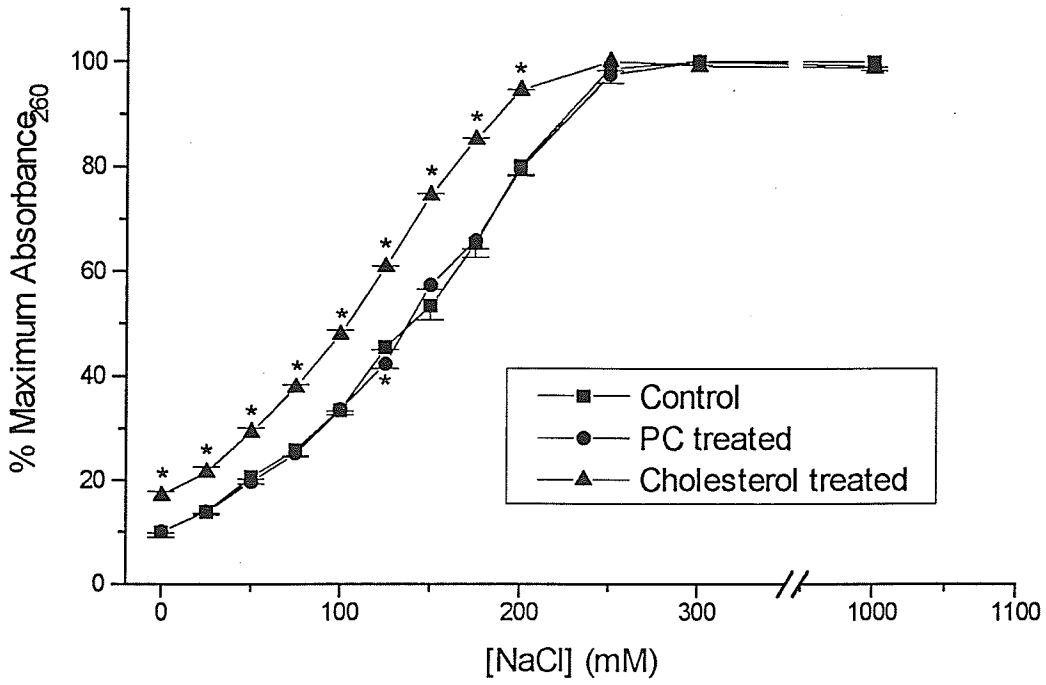


Figure 11: NORMALIZED A_{260} IN SUPERNATANTS FROM NUCLEI AFTER TREATMENT WITH INCREASING [NaCl]

Significant differences (*P<0.05) are indicated between control, cholesterol enriched and phosphatidylcholine treated nuclei. Values represent means ± standard error measurements for 3 preparations.

Table 4 : The effect of cholesterol enrichment on the integrity of isolated nuclei

	RC_{50} (mM)
Control	143 ± 6
Phosphatidylcholine	138 ± 1
Cholesterol	104 ± 1 *#

The NaCl concentration (mM) at which the intact nuclei population released 50% of their maximal nucleotide content due to osmotic shock was measured. Nuclei from control (untreated), cholesterol-free (phosphatidylcholine treated), or cholesterol-enriched (cholesterol treated) preparations were tested. Values represent means ± S.E. * $p<0.05$ vs control; # $p<0.05$ vs phosphatidylcholine treated.

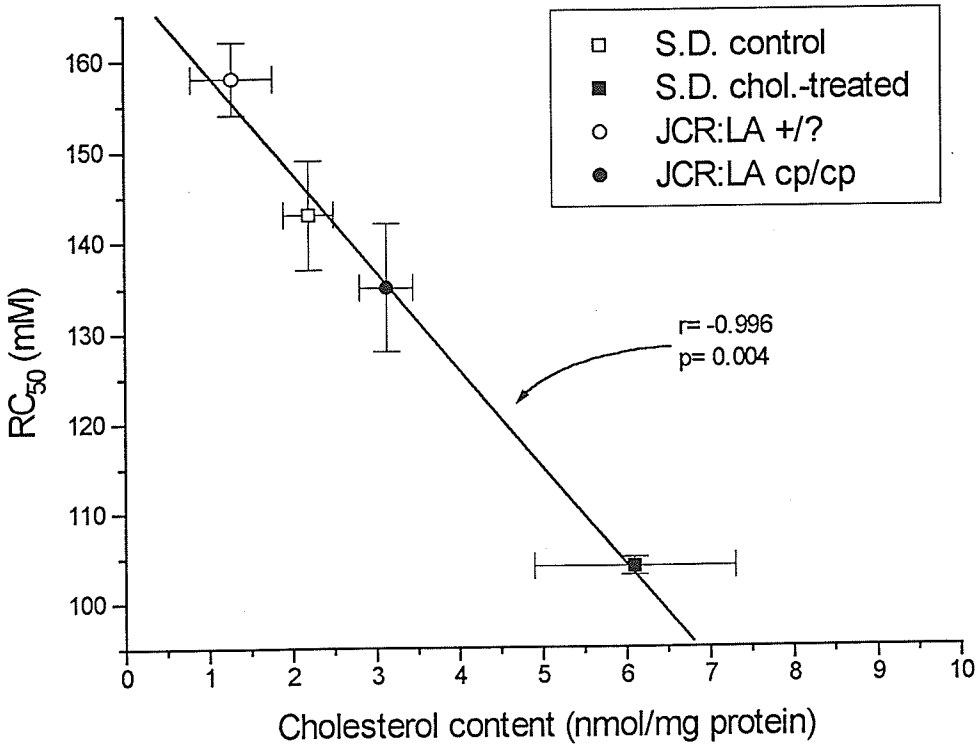


Figure 12: RELATION OF NUCLEAR MEMBRANE CHOLESTEROL CONTENT AND SUSCEPTABILITY TO RUPTURE DUE TO EXPOSURE TO INCREASING [NaCl]

RC_{50} (mM) and cholesterol content (nmol/mg protein) are plotted for control (open box) and cholesterol-enriched (shaded box) nuclei in the present study and JCR:LA-cp corpulent (open circle) and lean (shaded circle) nuclei from previous work (49). Values represent means ± standard error measurements for 7-8 preparations. A strong correlation ($r = -0.996$) exists between nuclear cholesterol content and the RC_{50} value ($p=0.004$).

ii. Cholesterol Oxidase Inhibition

Oxidation of Nuclear Membrane Cholesterol Inhibits Nuclear Nucleoside Triphosphatase Activity

Nuclear NTPase activity was examined as a function of reaction time in control and cholesterol oxidase treated nuclei. Control NTPase activity was similar to that reported previously (168). Cholesterol oxidation of the nuclear membrane resulted in a significant reduction of nuclear NTPase activity (Figure 13). The reduction of NTPase activity occured relatively rapidly. Significant differences were observed between control and cholesterol oxidase treated nuclei at time points of 2 minutes or greater.

Nuclear NTPase activity was also examined as a function of cholesterol oxidase on purified nuclei. Nuclei were treated with varying concentrations of cholesterol oxidase. There were significant decreases of NTPase activities in the cholesterol oxidase treated group relative to control (Figure 14). Lower concentrations of cholesterol oxidase ($\leq$ 6.5 units) reduced activity by 30-40%. Very high concentrations of cholesterol oxidase depressed the NTPase activities further but were not sufficient to inhibit the activity totally.

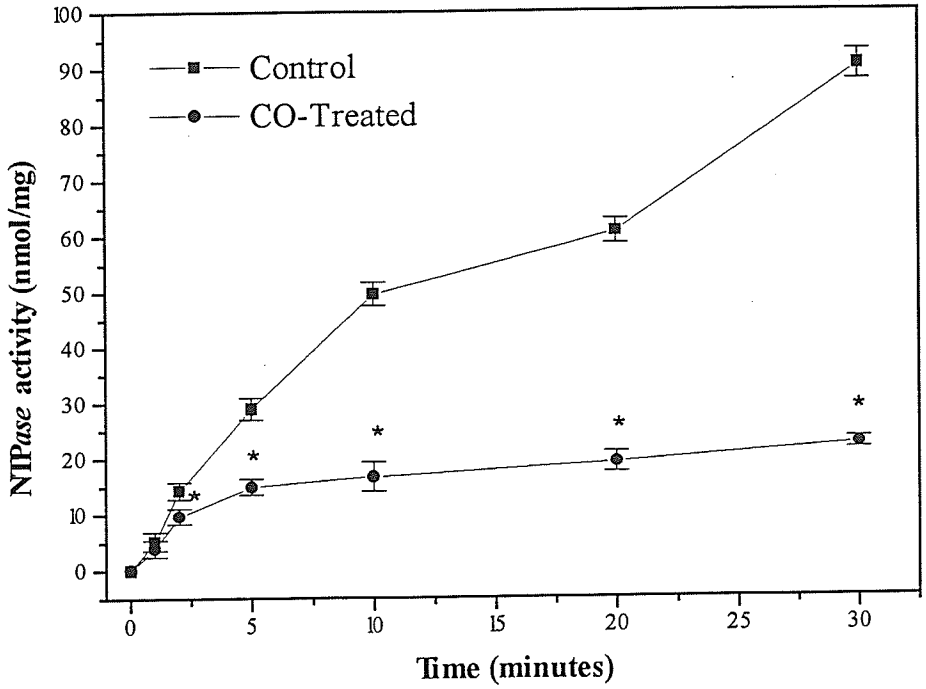


Figure 13: EFFECT OF CHOLESTEROL OXIDASE ON NTPase ACTIVITY OF NUCLEAR MEMBRANES AS A FUNCTION OF REACTION TIME

Nuclei 2mg/ml were treated with 6.5 units of cholesterol oxidase, then washed once to remove the enzyme before measuring NTPase activity. NTP*ase* activity was initiated upon addition of ATP to a final concentration of 5mM. Reactions were quenched at various times with 200 µl 10% SDS. Significant differences ($P<0.05$) were noted (*) between control and cholesterol oxidase treated preparations. Values represent means ± standard error measurements for 7 preparations.

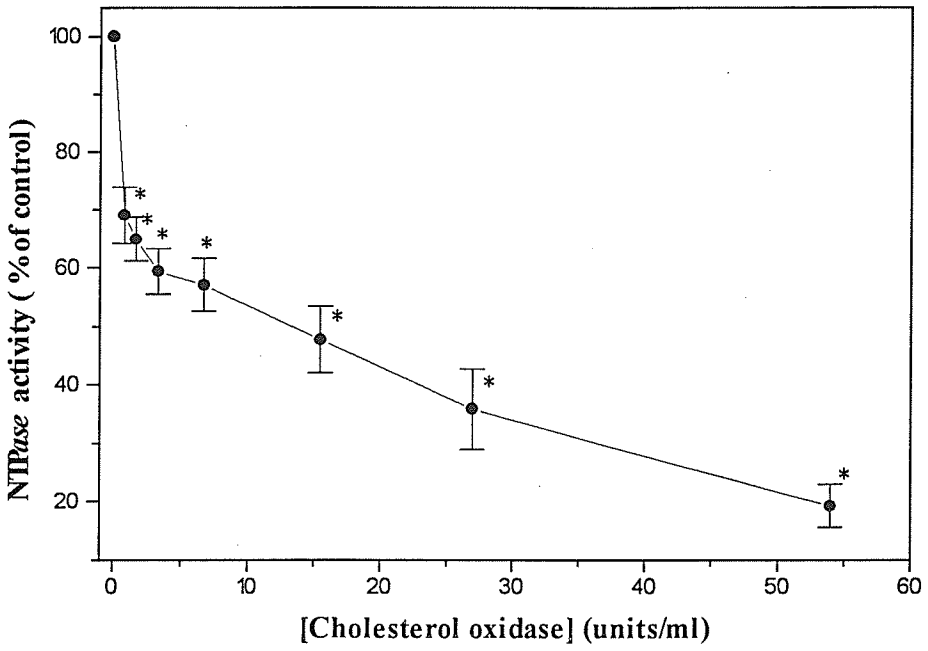
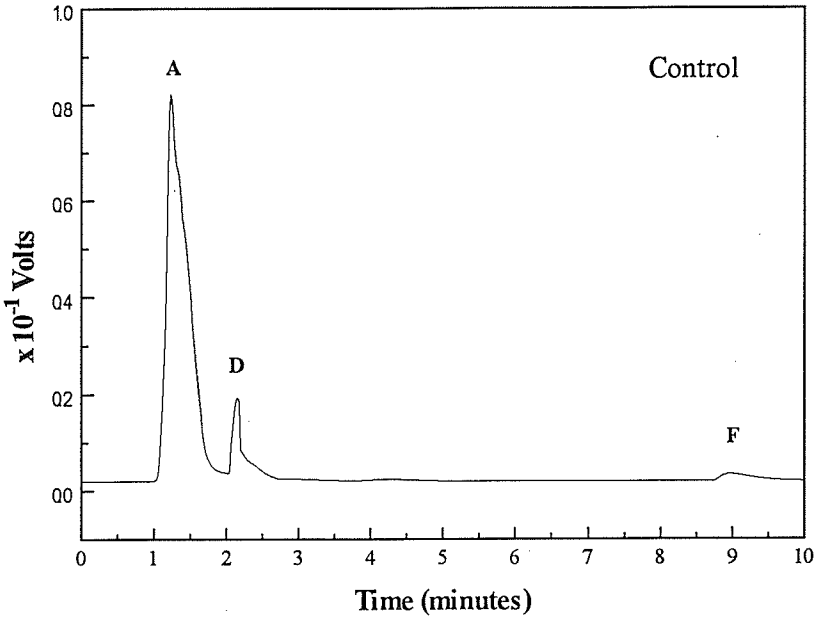


Figure 14: EFFECT OF VARIOUS CHOLESTEROL OXIDASE CONCENTRATIONS ON NTPase ACTIVITY OF NUCLEAR MEMBRANES

NTP*ase* activity was initiated upon addition of ATP to a final concentration of 5mM. Reaction time was 10 minutes. Significant differences ($P<0.05$) are indicated (*) between control and cholesterol oxidase treated preparations. Values represent means ± standard error measurements for 7 preparations.

To be certain that membrane cholesterol was being oxidized by cholesterol oxidation, HPLC analysis was carried out on control and cholesterol oxidase treated nuclei. Figure 15 shows representative chromatographs of both control and cholesterol oxidase treated nuclei. Both sets of samples were subjected to the same treatment and extraction procedures (see experimental procedures). Chromatographs from control and cholesterol oxidase treated samples show the typical solvent peak labelled as A (Figure 15). Peak D present in both sets was identified by standard samples as cholesterol (cholest-5-en-3β-ol). Peaks B,C and E present in the cholesterol oxidase treated group are cholesterol oxides. Specifically, peak B represents cholest-4-en-3-one, and peaks C and D refers to 20-α-hydroxy cholesterol and 25-hydroxycholesterol, respectively. The appearance of peak F, present in both control and cholesterol oxidase treated groups could not be identified.

ATP hydrolysis by control and cholesterol oxidase treated nuclei was examined as a function of varying [ATP] (Figure 16). There were significant differences between control and cholesterol oxidase treated nuclei at each [ATP]. Cholesterol oxidation induced a significant change in the Vmax value for the enzyme without altering the Km for ATP (Table 5).


Control
A
D
F
1.0
0.8
0.6
0.4
0.2
0.0
x 10⁻¹ Volts
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)

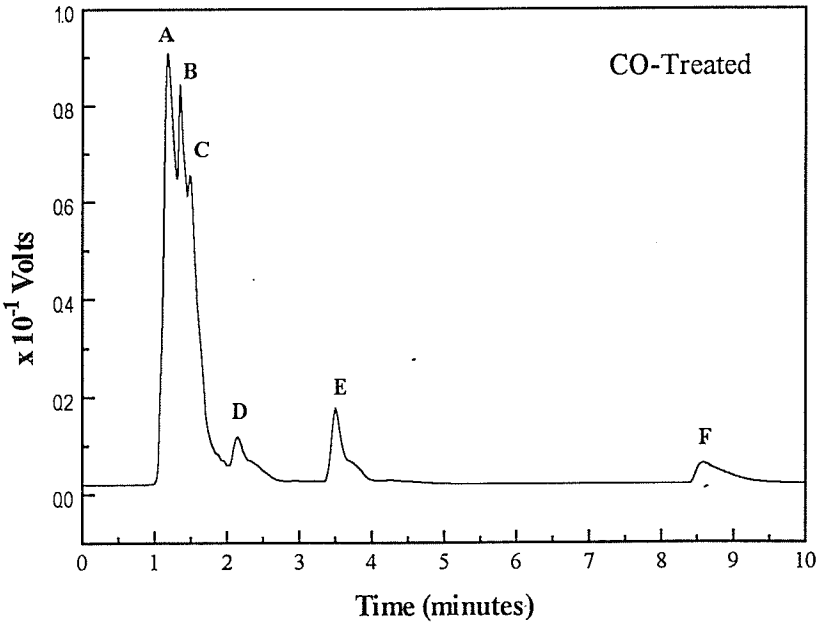

CO-Treated
A
B
C
D
E
F
1.0
0.8
0.6
0.4
0.2
0.0
x 10⁻¹ Volts
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)

Figure 15: REPRESENTATIVE HPLC CHROMATOGRAPHS OF CHOLESTEROL SPECIES IN CONTROL AND CHOLESTEROL OXIDASE TREATED NUCLEAR MEMBRANES

Upper chromatograph represent control, peak D is identified as cholesterol. Lower chromatograph represent cholesterol oxidase treated nuclear membranes, peaks B, C and E are cholesterol oxides.

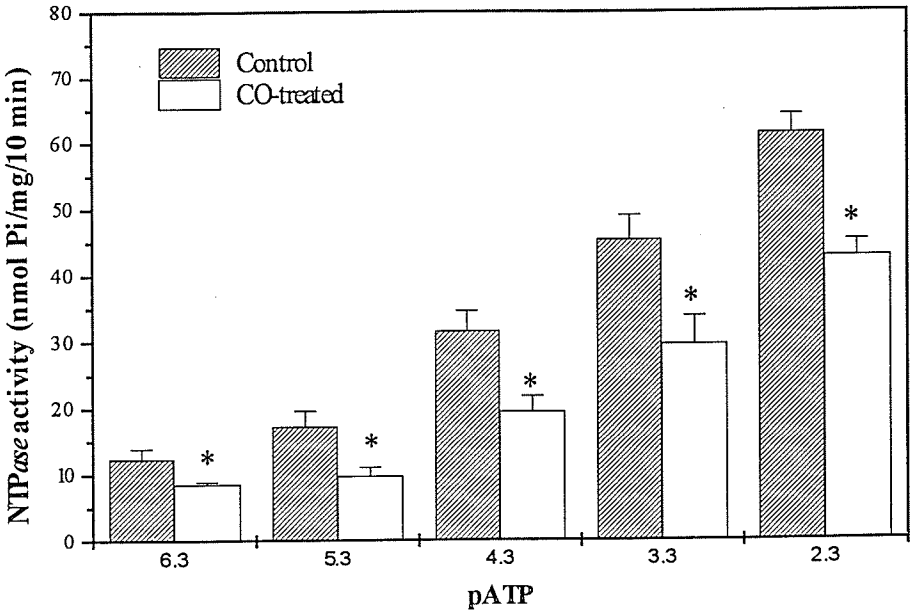


Figure 16: NTPase ACTIVITY OF CONTROL AND CHOLESTEROL OXIDASE TREATED NUCLEAR MEMBRANES AS A FUNCTION OF [ATP]

Reaction time was 10 min. Free Mg^{2+} was held constant at 1mM. Significant differences (*$P<0.05$) in NTP*ase* activity are indicated between control and cholesterol enriched preparations. Values represent means ± standard error measurements for 6 preparations.

Table 5: Effect of cholesterol oxidation on the kinetics of nuclear nucleoside triphosphatase activity

	Control nuclei		Oxidized nuclei	
	Km (μM)	**Vmax** (nmol/mg/10 min)	**Km** (μM)	**Vmax** (nmol/mg/10 min)
ATP	68.6 ± 5.4	62.3 ± 4.9	74.5 ± 6.5	43.3 ± 2.6*

Cholesterol oxidation was carried out as described in Experimental Procedures. Data were calculated from Eadie-Hofstee plots. Values represent means ± S.E (n=6). *$p<0.05$ versus control values.

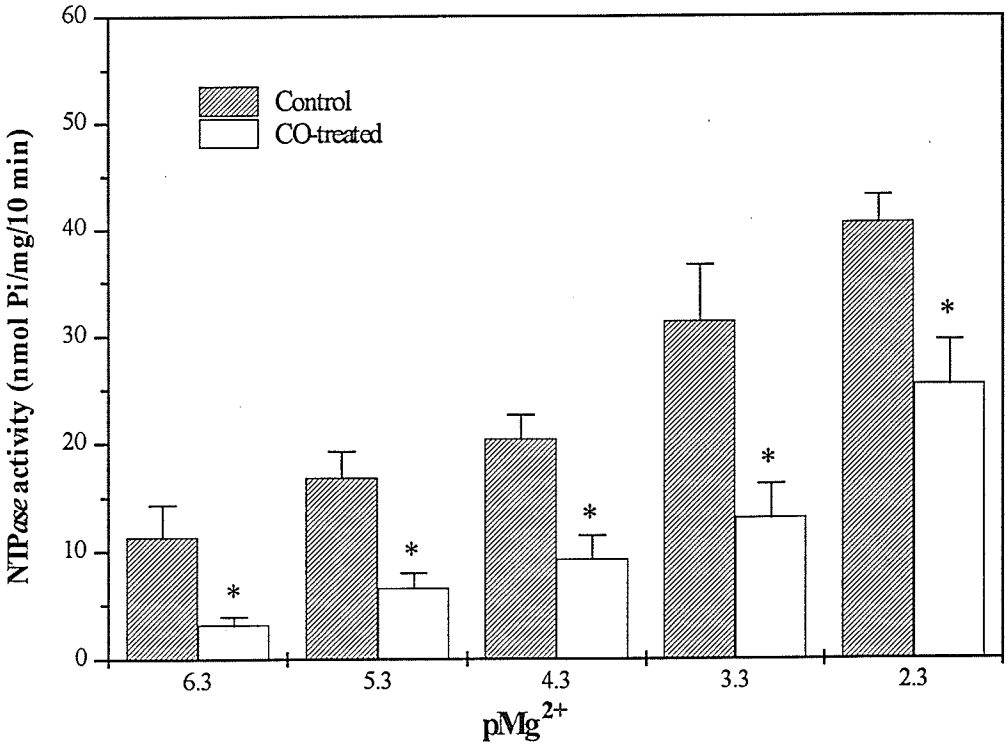


Figure 17: NTPase ACTIVITY OF CONTROL AND CHOLESTEROL OXIDASE TREATED NUCLEAR MEMBRANES AS A FUNCTION OF [Mg^{2+}]

NTP*ase* activity was assayed as in Figure 14. Reaction time was 10 min. ATP concentration was 5mM. Significant differences in NTP*ase* activity were indicated (*$P<0.05$) between control and cholesterol enriched preparations. Values represent means ± standard error measurements for 7 preparations.

The Mg^{2+} dependence of the NTPase activity of control and cholesterol oxidase treated nuclear membranes is shown in Figure 17. Significant differences in NTP*ase* activities were observed between control and cholesterol oxidized nuclei. Statistical significance was observed at various [Mg^{2+}]. Maximum stimulation of activity was observed at approximately 5.0 mM free $Mg^{2+}$for both groups.

7 Discussion

i. *Cholesterol Stimulation*

Nuclear Membrane Cholesterol Can Modulate Nuclear Nucleoside Triphosphatase Activity

Our results clearly demonstrate that the nuclear NTPase is sensitive to the cholesterol content of the nuclear membrane. The nuclear cholesterol content was 15-100 fold lower than values reported previously for plasma membranes, mitochondria and sarcoplasmic reticulum (76, 111). This low cholesterol content left the nuclei difficult to deplete of cholesterol any further. Long (24-48 hr) incubations with liposomes which did not contain cholesterol did not result in a significant reduction in nuclear cholesterol levels. The movement of cholesterol with the liposomal technique is via diffusion that is dependent upon concentration differences between the liposomes and the membrane (33). It is likely that the cholesterol concentration within the nuclei was insufficient to induce a significant movement of cholesterol out of the nuclear membrane. This in vitro finding would also suggest that if nuclear cholesterol is to be modified in vivo, it will be more likely to increase than to decrease. Work on nuclei obtained from hyperlipidemic rats supports this hypothesis (49). This study (49) showed that the nuclear membrane cholesterol increased in vivo and the NTPase activity increased with it. The present data provide significant direct evidence that nuclear membrane cholesterol can modulate

NTPase activity. It is also worth noting that the increase in nuclear cholesterol content previously observed in vivo (49) is very similar to the increase achieved in vitro in the present study (250% vs 275% of control, respectively).

Two possible mechanisms may be responsible for the effects of cholesterol on nuclear NTPase activity. Some enzymes are influenced by membrane cholesterol directly (111, 125). The cholesterol may be in close proximity to the enzyme imbedded in the membrane and this may have a direct effect on enzyme function. Alternatively, cholesterol is known to affect other enzymes in an indirect manner by altering the lipid fluidity in a general way thoughout the membrane (210). Increases in membrane cholesterol can increase membrane rigidity (or decrease fluidity) in a sphingomyelin-poor membrane like the nuclei or, conversely, depletion of the cholesterol content can increase the lipid fluidity of the membrane (46, 196). This change in the membrane's biophysical properties can dramatically affect enzyme function (27, 174, 210). The direction of this change (stimulation or inhibition of enzyme activity) is entirely dependent upon the enzyme examined. Some enzymes are stimulated by cholesterol enrichment in the same membrane that other enzymes are inhibited (111). The stimulation of nuclear NTPase activity by cholesterol in the present study probably represents an indirect effect of cholesterol on the enzyme. This conclusion is based upon the location of the NTPase within the nuclei. The NTPase catalytic site appears to reside as part of a laminar protein subcomponent (40, 201). The laminar proteins form a cytoskeletal mesh along the inner surface of the nuclear membrane (82, 104, 141). The NTPase activity is dependent upon a hydrophobic environment but does not rely upon the bulk phospholipid content of the membrane (191).

Without evidence, therefore, of the NTPase imbedding itself into the membrane bilayer in a conventional manner, it is difficult to imagine a cholesterol annulus forming around the enzyme resulting in a requirement of the enzyme for cholesterol. Alternatively, it is more likely that changes in membrane cholesterol alter membrane fluidity and consequently how proteins like the NTPase interact with the membrane. It is relevant to emphasize that others have shown that changes in the membrane fatty acid composition of the nuclei alters NTPase activity and mRNA transport (202, 203). This was suggested to occur through a modulation of the physical properties of the membrane. More directly, another study has shown that nuclear membrane lipid fluidity has important effects on nucleocytoplasmic RNA transport (93). It is not unreasonable to hypothesize, therefore, that the incorporation of cholesterol into the nuclear membrane in the present study alters NTPase activity via a change in membrane rigidity.

The functional and pathophysiological implications of the present study are two-fold. First, the increased nuclear cholesterol content may challenge membrane integrity. The response of cholesterol-enriched nuclei to increasing [NaCl] suggests that cholesterol incorporation has left the membrane integrity more susceptible to damage from stressful stimuli. This observation strengthens previous conclusions that cholesterol enrichment of the nuclei from JCR:LA-cp rats was responsible for the increased susceptibility to osmotic shock (49). Indeed, plotting the relationship between nuclear cholesterol content and the RC_{50} value for preparations from the present study and those observed in nuclei from JCR:LA-cp rats (49) demonstrates a strong association between the two parameters (Figure 12; r=-0.99). Secondly, and more importantly, is the relevance of the changes in

NTPase activity to nucleocytoplasmic transport. Translocation of mRNA from the nucleus into the cytoplasm occurs in an energy dependent manner through the nuclear pore complex (50, 78). The nuclear NTPase may be responsible for the gating of the nuclear pore complex (7, 8, 138, 166, 173, 182). Alterations in NTPase activity result in proportionate changes in mRNA flux through the pore complex (7, 38, 166). Based upon these previous observations, it is reasonable to propose, therefore, that the changes in NTPase activity induced by cholesterol in the present study will have significant effects on mRNA translocation and nuclear pore function in general.

ii. Cholesterol Oxidase Inhibition

Oxidation of Nuclear Membrane Cholesterol Inhibits Nuclear Nucleoside Triphosphatase Activity

Cholesterol oxidase treatment of purified nuclei resulted in a substantial decrease in nuclear NTPase activity. This decrease in NTPase activity was both time and concentration dependent. The cholesterol oxidation was identified not only via changes in the nuclear enzyme function but directly by HPLC. We have previously identified oxidation of nuclear lipids by free radicals using FT-IR spectroscopy (119).

The decrease in nuclear NTPase activity is due to oxidation of nuclear membrane cholesterol by cholesterol oxidase. Denatured cholesterol oxidase by itself had no effect on the nuclear NTPase (data not shown). H_2O_2 is produced as a side product during the oxidation of cholesterol by cholesterol oxidase (190). However, it is unlikely to be the cause of the change in enzymatic activity observed here for two reasons. First, even if all

of the nuclear cholesterol were oxidized it would not generate the millimolar H_2O_2 concentrations which were necessary to induce changes in other enzyme activities (110). Secondly, catalase was used at a concentration more than sufficient to ensure complete inactivation of the H_2O_2 generated (110).

The effects of oxidized cholesterol on NTPase in the present study were not a direct effect of oxidized cholesterol on the NTPase catalytic site. The NTPase enzyme appears to exist as part of a laminar protein subcomponent (40, 201). The laminar proteins form a cytoskeletal mesh along the inner surface of the nuclear membrane (82, 104, 141, 142). It is difficult, therefore, to conceive of a cholesterol annulus forming around the enzyme. Consistent with this hypothesis, previous work has suggested that native cholesterol interactions with the NTPase were not direct but via changes in membrane fluidity (168). Thus, a more suitable mechanistic hypothesis for the present study is that oxidation of membrane cholesterol exerted effects on the nuclear NTPase by causing an increase in fluidity and general disordering of the membrane. Previous studies have demonstrated that cholest-4-en-3-one (18) and 25-hydroxycholesterol (25, 199) can induce membrane permeability changes due to changes in membrane order or packing. Both of these oxidized cholesterol species were identified by HPLC in the present study. This change in the membrane's biophysical properties can dramatically affect enzyme function (27, 174, 210). The effects of oxidized cholesterol on other membrane-bound enzymes have also been reported (110).

The effects of cholesterol oxidase on nuclear NTPase activity provides information on the location of the cholesterol in the membrane which interacts with the NTPase. The

nuclear envelope is unusual because it is made up of not one but two separate, concentric lipid bilayers, the inner and the outer nuclear membranes. Separating both membranes is a 10-60 nm gap known as the perinuclear space (26). Nuclear NTPase activity was inhibited 30-40% by low concentrations of cholesterol oxidase (≤ 6.5 units). Thus, the majority of NTPase activity (60-70%) was resistant to the action of cholesterol oxidase. Even in the presence of extremely high concentrations of cholesterol oxidase approximately 20% of the NTPase activity remained. Cholesterol oxidase is not normally membrane-permeable (186) except after extensive incubation times and/or after the use of high enzyme concentrations. It is likely, therefore, that the inner nuclear membrane did not undergo significant oxidation of its cholesterol until relatively large cholesterol oxidase concentrations were employed. We conclude, therefore, that cholesterol located in the inner membrane is likely to be most important with respect to NTPase activity. This is also consistent with the location of the NTPase on the inner face of the inner nuclear membrane. This concept of the two membrane leaflets differing in their cholesterol composition would also support previous work which suggested that the double bilayer of the nucleus was comprised of two functionally different membrane systems (177).

The pathophysiological implications of the data are important. Oxygen derived free radicals are important mediators of cell damage and death. When free radicals interact with molecules such as lipids, DNA or proteins, their structure and function can be altered. Free radical interactions with DNA may be lethal to the cell or induce changes in cell growth (44). However, another mechanism by which free radicals may alter cell growth and viability is through oxidative modification of membrane lipids. Membrane

cholesterol is one target of oxygen-derived free radicals (110, 172, 192). Our data would suggest that cholesterol, when oxidized in the nuclear membrane, will have important effects on NTPase activity. In view of the suggested role for the NTPase in regulating nuclear pore function (7, 8, 138, 166, 173, 182), one would expect these changes to alter nucleocytoplasmic trafficking of substances in the cell. This would be expected to result in changes in gene expression and transcription. Consistent with this line of reasoning, oxidation of cell cholesterol has resulted in significant changes in cell growth (137). Considering the potential importance of oxygen derived free radicals in diseases involving altered cell growth (i.e. cancer, atherosclerosis), our data provide a novel alternative mechanism for these effects.

8 References

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