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THE UNIVERSITY OF ALBERTA

STUDIES ON THE ASSOCIATION OF PERTUSSIS TOXIN WITH THE SURFACES OF GOOSE ERYTHROCYTES AND CHINESE HAMSTER OVARY CELLS

AND

CHARACTERIZATION OF THE ADP-RIBOSYLTRANSFERASE ACTIVIT OF RADIOIODINATED PERTUSSIS TOXIN

by

GREGORY JOHN TYRRELL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA

FALL, 1988

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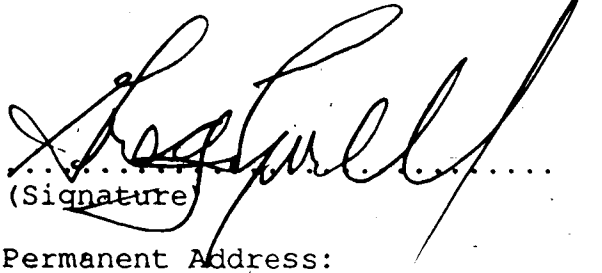

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(Supervisor)

Date: June 27, 1988

Dedication

This thesis is dedicated to my parents for all their help and guidance

and

to one person who has been with me throughout the course of my degree, whose presence has given me the motivation to continue on in future pursuits

Abstract

Although pertussis toxin (PT) catalyzed ADP-ribosylation has been characterized in a variety of systems, the receptor binding activity of this toxin is poorly understood. In this thesis I have identified PT receptor proteins in goose erythrocyte membranes and CHO cell membranes. This was done by passing solubilized membrane proteins through PT-Sepharose. The same receptor proteins were also found to bind to wheat germ agglutinin indicating the presence of GlcNAc or NeuNAc carbohydrate determinants. The PT receptor proteins from goose erythrocyte membranes were also competively eluted from the PT affinity column using fetuin, a glycoprotein which inhibits PT-mediated hemagglutination of goose erythrocytes. This is accomplished at a minimum inhibitory concentration of 0.3 ± 0.2 μM. In contrast, fetuin is only able to inhibit PT mediated CHO cell activity at much higher concentrations (50,000 x higher) indicating that the CHO cell receptor(s) for PT has a higher affinity for this toxin than does fetuin.

In the second part of this thesis I have characterized the ADP-ribosyltransferase activity of iodinated PT. This was done using two substrates, rat C6 glial cell membranes and transducin obtained from bovine retinas. It was demonstrated that, regardless of how PT

is radioiodinated, the ADP-riboslytransferase activity remains functional. This is in direct contrast to the observation that iodination destroys the binding activity of PT. Also, it was found that Zn^{++} ions are able to inhibit PT-mediated ADP-ribosylation in the μM range.

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Abbreviations

N-terminus	amino terminal
k_a	association rate constant
BSA	bovine serum albumin
C-terminus	carboxyl terminal
CHO	chinese hamster ovary
CT	cholera toxin
DPPC	dipalmitoylphosphatidyl choline
DT	diphtheria toxin
k_d	dissociation rate constant
DTT	dithiothreitol
EGTA	[Ethylenebis(oxyethylenenitrilo)] tetraacetic acid
EF-1	elongation factor 1
EF-2	elongation factor 2
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
LT	*E. coli* heat labile toxin
LT2	*E. coli* heat labile toxin 2
Gal	galactose
G_{M1}	ganglioside G_{M1}
Gi	guanyl inhibitory protein complex
G	guanyl nucleotide binding protein
Gs	guanyl stimulatory protein complex
GlcNAc	N-acetylglucosamine
NeuNAc	N-acetylneuraminic acid
NAD	nicotinamide adenine dinucleotide
PNA	peanut agglutinin
PT	pertussis toxin
PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate buffered saline (pH 7.2)
RME	receptor-mediated endocytosis
ROS	rod outer segments
SDS	sodium dodecyl sulfate
SASD	sulfosuccinimidyl 2-(p-azidosalicylamido) ethyl-1,3'-dithiopropionate
x g	times gravitational force
TPCK	L-1-Tosylamide-2-phenyl-ethlychloromethyl ketone
TCA	trichloroacetic acid
v/v	volume per volume
w/v	weight per volume
WGA	wheat germ agglutinin

1.0 INTRODUCTION

1.1 Definitions:

Many pathogenic bacteria cause disease by producing extracellular substances (exotoxins) which cause a variety of effects in host tissues. Eradication of the organisms responsible for exotoxin production via antibiotics, in some cases, does not alleviate the illness since the exotoxin may persist within the individual for periods extending from days to months. As a result, the patient who suffers from a bacterial infection potentiated by such long lasting exotoxin(s) may still feel the effects of the disease long after the bacteria have disappeared from the infected site (91,111,148,154).

The first event involved in intoxication of a host cell is binding of the exotoxin to specific or non-specific receptors on the eukaryotic cell membrane. The term receptors here is actually a misnomer in that the host cell does not "knowingly" produce a receptor for exotoxins to attach to. Rather, through evolutionary selection, the exotoxin itself has "learned" to usurp specific structures on the host cell surface as its attachment sites. These are structures made to serve a necessary function in the host cell and not intended for

exotoxin attachment. Therefore, these structures could be classed as binding targets in relation to exotoxin attachement (91,154).

Once the exotoxin has bound to the cell membrane it gains entry into the cell cytosol by a variety of mechanisms characteristic of the exotoxin involved. Almost all exotoxins possess some type of enzymatic activity which is directed at specific cytoplasmic cell targets. Of the numerous enzymatic reactions elaborated by exotoxins the most common is that of ADP-ribosylation or the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD) to acceptor groups on proteins. In 1978 only 6 prokaryotic ADP-ribosyltransferases had been identified and, of these, only diphtheria toxin and exoenzyme S from *Pseudomonas aeruginosa* were bacterial exotoxins. It was suspected that cholera toxin was an ADP-ribosyltransferase but until 1978, this activity had not been detected. Now there are 12 known bacterial exotoxins which catalyze the ADP-ribosylation reaction and more are being discovered annually (116,132).

All bacterial exotoxins that have ADP-ribosyltransferase activity can be classified as A-B exotoxins, A referring to the enzymatic component of the exotoxin and B, the binding component (45). A-B toxins can be defined as exotoxins whose individually

adminstered components are atoxic but whose collectively adminstered components are fully toxic. The ADP-ribosylation reaction is not restricted to bacterial exotoxins but rather it is a reaction that is found to occur in a variety of cell systems. Since its first description in 1966 the reaction has been found to take place in almost all forms of life and in almost all cellular organelles (23). Although, the ADP-ribosylation reaction is one of the most common methods by which organisms achieve post translational modification of their proteins, its role in regulation of cellular function has not been clearly established (144).

The remainder of my introduction will be divided into two parts. The first will consist of a brief review of the known ADP-ribosylating bacterial toxins; their mechanisms of binding to host cells and their enzyme activity. The second part will focus on pertussis toxin with an overview of the pathogensis of pertussis and an examination of the toxin itself.

1.2 Toxins with ADP-ribosyltransferase Activity

One of the first toxins to be discovered and probably the most extensively studied is diphtheria toxin. This exotoxin consists of two components, which together are secreted as a single inactive polypeptide chain of 58,000 daltons (25,101). The exotoxin is

activated by proteolytic cleavage and thiol agents which result in the two fragments becoming separated into an ADP-riboslytransferase polypeptide (fragment A) and a binding polypeptide (fragment B) (60,123). It can also be hydrolyzed by trypsin alone leaving the disulfide bond intact resulting in what is termed nicked toxin. Neither nicked toxin or unnicked toxin display ADP-ribosyltransferase activity (30). The intracellular target of diphtheria toxin is elongation factor-2 (EF-2), a polypeptidyl-tRNA translocase (50,60). In protein synthesis, EF-2 is required for the translocation step in which peptidyl-tRNA is moved from the acceptor site to the donor site on the ribosome. This translocation reaction is accompanied by the hydrolysis of GTP to GDP. In 1968, Honjo and coworkers provided evidence that diphtheria toxin was able to transfer ADP-ribose from NAD to EF-2 (60). The modified EF-2 does not hydrolyze GTP to GDP and a stable complex is formed with GTP and the ribosomes involved. Thus translocation of peptidyl tRNA from acceptor site to donor site is blocked (123). The precise location of ADP-ribosylation of EF-2 has been shown to be a modified histidine residue, 2-[3-carboxyamido-3-trimethylammonio) propyl] histidine more commonly called diphthamide because of its role as a substrate for diphtheria toxin (153).

The organism *Pseudomonas aeruginosa*, unlike *Corynebacterium diphtheria*, produces a number of toxins that are considered to be virulence factors, two of which are exotoxin A and exoenzyme S. Exotoxin A is a heat-labile toxin which is secreted into the growth medium as a 66,000 dalton single polypeptide chain and affects cells by inhibiting protein synthesis (63). Full activity of exotoxin A, like diphtheria toxin requires reduction of internal disulfide bonds. Exotoxin A's structure has been determined by X-ray crystallography. It is made up of three domains designated I, II and III. Domain I is further subdivided into Ia and Ib. Domain Ia is required for cell surface binding, domain II is involved in translocation of the exotoxin across the cell membrane and domain III together with Ib are required for the exotoxin's enzymatic activity (2). Exotoxin A acts on the same substrate as diphtheria toxin (62). The kinetics of the ADP-ribosylation reaction are also identical but when the two exotoxins are compared structurally there are considerable differences. The enzymatic domain of diphtheria toxin is contained in the amino terminal portion of the exotoxin whereas the exotoxin A active site is in the carboxy terminal region. Further, the amino acid sequences of exotoxin A and diphtheria toxin show little homology and

any immunological cross-reactivity can only be demonstrated with extremely sensitive methods of detection (51,125). Since the enzymatic activities of diphtheria toxin and exotoxin A have been described in detail one would expect that the binding properties of the two exotoxins would also be well established. Unfortunately, this is not the case.

Diphtheria toxin's B fragment consists of two parts; a C-terminal end with a mw of 17,000 which is responsible for cell surface recognition and a 23,000 mw N-terminal end which interacts with the membrane bilayer. Diphtheria toxin entry is believed to involve the process of receptor-mediated endocytosis (RME) (101). Although a glycoprotein of 153,000 mw has been identified as a receptor candidate in hamster thymocytes, the specific receptor for diphtheria toxin in host tissues still remains unknown (122). Recent evidence has shown that proteins with a molecular weight range of 10,000-20,000 are involved in the binding of diphtheria toxin to primate cell lines. Anion transport is inhibited upon binding of diphtheria toxin to its receptor in these cells and it is believed that the anion-antiporter protein in these cells is in non-covalent association with the diphtheria toxin receptor.

In addition, the structure(s) that bind diphtheria toxin is sensitive to trypsin and phospholipase C and D (126).

Vero cells are perhaps the most sensitive cell line to diphtheria toxin. Receptors in Vero cells and another primate cell line, BS-C-1 cells, have been identified using cross-linking experiments. These proteins have a mw. of 20,000 and the binding of ^{125}I-diphtheria toxin to these cells could be inhibited by the addition of 100 fold excess unlabelled diphtheria toxin providing evidence that the receptors are specific for this exotoxin (25).

Pseudomonas exotoxin A has also been shown to enter susceptible cells such as LM cells via receptor-mediated endocytosis. Electron microscopy studies using biotinyl-exotoxin A and colloidial gold particles show that after 15 minutes at 37°C exotoxin A can be found in the region of the Golgi apparatus and occasionally within the Golgi cisternae (38,102). It is thought that in order for toxicity to be expressed, the exotoxin must be processed through this cell organelle. It has been postulated that the active form of exotoxin A enters the cytosol where it expresses its toxicity during fusion of the Golgi-derived toxin laden vesicles with lysosmes after 30 minutes at 37°C. Diphtheria toxin has also been found to require trafficking through the Golgi apparatus to allow

efficient expression of toxicity in Vero cells (101,103).

Although diphtheria toxin and *Pseudomonas* exotoxin A are internalized via RME it is still uncertain how the exotoxins, once inside an endocytic vesicle, gain entry to the cell cytosol. Evidence to date indicates that in diphtheria toxin, fragment A inserts itself into the lipid bilayer and then somehow exits the bilayer on the cytoplasmic side. The insertion of fragment A into the lipid bilayer has been shown to be both pH and temperature dependent. Optimal insertion occurs at pH 3.6 in artifical membranes. It is possible that acidic pH within the endosome may induce a conformational change resulting in the exposure of hydrophobic domains on the exotoxin (61). A similar event may happen *in vivo*. Acidification of the prelysosomal vesicle takes place via the activity of an ATP dependent proton pump which transports H^+ ions from the cytoplasm of the cell to the vesicle. This could result in the insertion of fragment A into the vesicle membrane and translocation to the cytosol. In artifical lipid membranes containing diphtheria toxin, 1.8 nm pores were seen to form upon acidification. Fragment A could conceivably enter the cytoplasm through pores of this dimension (70).

A decrease in pH in endosomes has been found to lead to an increase in binding of exotoxin A to lipid membranes (similar to diphtheria toxin). At a pH of 4, pore formation was found to take place at a maximal rate, again similar to diphtheria toxin. Exoenzyme S, a heat-stable toxin also produced by *Pseudomonas*, has a molecular weight of 150,000. It does not ADP-ribosylate EF-2 like exotoxin A but it may ADP-ribosylate EF-1. It is partially inactivated under conditions which potentiate the enzymatic activity of exotoxin A (155). Characterization of its binding properties has yet to be accomplished.

Another bacterial exotoxin that catalyzes ADP-ribosylation is cholera toxin. Unlike diphtheria toxin or exotoxin A, its site of enzymatic activity is the adenylate cyclase system. To better understand how this occurs a brief description of G proteins and the adenylate cyclase system is given.

G proteins or guanyl nucleotide-binding proteins are involved in trans-membrane signalling to various target enzymes involved in regulation of cellular metabolism (145). G proteins are actually made up of three unique subunits designated α, β and γ. The trimer structure is contained within the membrane in contact with the cytoplasm. The α subunit binds GTP and

hydrolzyes it to GDP. This occurs when a hormone receptor which has been stimulated by the appropiate agonist is complexed with the α, β and γ subunits (23). It is these proteins, more specifically the α subunit, which are the target for the ADP-ribosyltransferase activity of cholera toxin (76).

The adenylate cyclase system regulates the intracellular concentration of cAMP. It is a bimodal system consisting of a stimulatory and an inhibitory arm (fig. 1) (31,104,115,144). In the case of the stimulatory arm, extracellular signals which interact with receptors on the extracytoplasmic face of the cell stimulate the β-γ subunit complex to interact with the Gs α subunit causing it to release its bound GDP which is in turn replaced with GTP. The binding of GTP promotes the release of the α subunit from the β-γ subunit complex and dissociation from the activated receptor complex. The β-γ subunits recycle to bind another α subunit containing GDP and the cycle is repeated. The α subunit plus GTP, once dissociated from the β-γ subunit complex, is now free to stimulate the cyclase catalytic component to effect the conversion of ATP to cAMP. The GTP bound to the α subunit is slowly hydrolyzed to

Figure 1: The Mammalian Adenylate Cyclase System

Rs - stimulatory receptor component

Ri - inhibitory receptor component

Gs α - alpha subunit of the stimulatory guanyl binding complex.

Gi α - alpha subunit of the inhibitory guanyl binding complex

G $\beta\gamma$ - beta and gamma subunits of the guanyl binding complex. These subunits can interchange with either the stimulatory arm or the inhibitory arm.

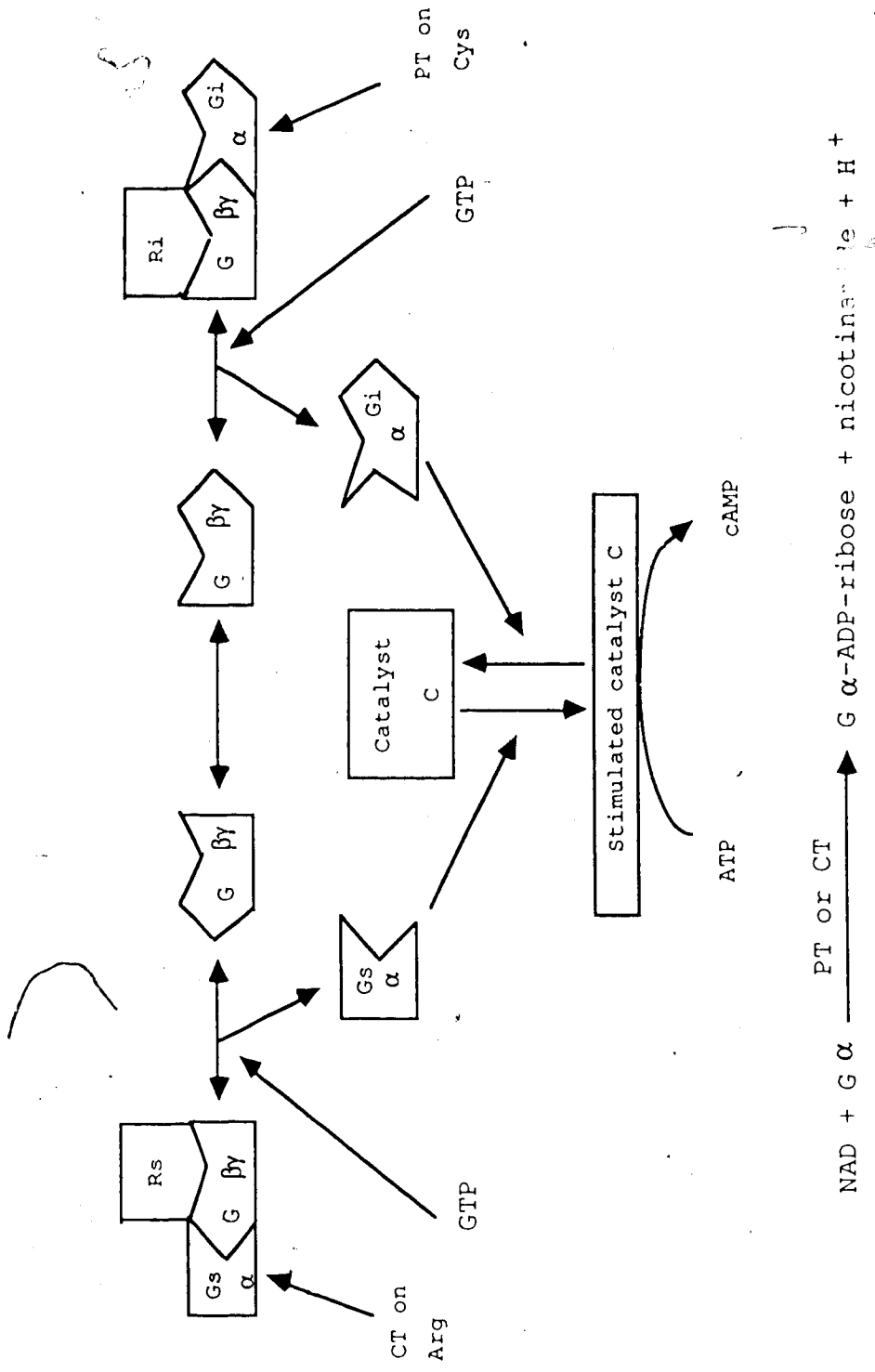
GTP - binds to the Gs α,β,γ or Gi α,β,γ complex causing the release of GDP. The Gs α or Gi α dissociate from the β,γ subunits allowing the G α subunits with bound GTP to stimulate catalyst

C - catalyst which upon stimulation hydrolyzes ATP to cAMP

CT - cholera toxin ADP-ribosylates an arginine residue in the Gs α subunit thereby preventing the hydrolysis of GTP to GDP.

PT - pertussis toxin ADP-ribosylates a cysteine residue in the Gi α subunit thereby preventing GDP from being released and exchanged for GTP.

NAD - nicotinamide adenine dinucleotide. PT or CT cleave ADP-ribose from NAD and attach it to a cysteine or arginine residue.


PT on Cys
GTP
Gi α
Ri
G βγ
Gi α
G βγ
G βγ
Catalyst C
Stimulated catalyst C
cAMP
ATP
Gs α
Rs
G βγ
Gs α
GTP
CT on Arg
NAD + G α
PT or CT
G α-ADP-ribose + nicotina
+ H+

GDP resulting in an altered α subunit which is available to be complexed by other β-γ subunits (107). The inhibitory arm works along the same principles as the stimulatory arm except that the Gi α subunit plus GTP inhibit the cyclase rather than stimulate it.

Cholera toxin ADP-ribosylates an arginine residue in the 43,000 molecular weight Gs α of the adenylate cyclase system (21,46). Cholera toxin is also able to ADP-ribosylate various proteins in addition to the α subunit involved in adenylate cyclase regulation. These include histone H1, a variety of cytoskelatal proteins, glycopeptide hormones and polyarginine (144).

The subunit structure of cholera toxin is different from that of diphtheria toxin or exotoxin A. It is actually composed of two functional subunits. The A subunit, with a mw of 28,000, can be dissociated into two sulfhydryl-linked polypeptide chains A1 and A2. A zinc-calcium metalloenzyme protease responsible for cleaving the nascent A subunit has been purified from *Vibrio cholera* (17). Only A1 is responsible for adenylate cyclase activation. The exotoxin becomes activated by treating it with thiol reagents to cleave the disulfide bond between A1 and A2. The second component, the B subunit mediates binding of the toxin to its host cell receptor. It is composed of five

identical polypeptide chains arranged in a pentameric structure.

Unlike diphtheria toxin or exotoxin A, the minimal receptor structure for cholera toxin has been determined to be ganglioside G_{M1}. It has now been demonstrated that this ganglioside is the structure that cholera toxin has the highest affinity for ($K_A \sim 1 \times 10^9$ mol^{-1}) (32). However, it is puzzling that although cholera toxin acts on intestinal cells, there is very little G_{M1} found in these cells. Galactoproteins of rat intestinal brush borders have also been found to bind cholera toxin at a much lower affinity than G_{M1}. It may be these glycoproteins that are binding cholera toxin in the intestine. The toxin has also been shown to bind rather weakly gastric mucins. Mucins may offer a possible explanation for the resistance of some animals to cholera toxin (101,137).

Once cholera toxin is bound to G_{M1} it must pass through the cell membrane to the cytoplasm. The most probable hypothesis about the mechanism of membrane pentration centers on the toroidal structure of the toxin's B subunit. It is a stable pentameric structure in the formation of a closed ring with a hole through the centre. X-ray crystallographic studies and electron microscopic work have shown that the A1 polypeptide

chain is located on the upper surface of the torus with polypeptide A2 extending some distance into the central hole. It is possible that, once the B subunit is bound, lipid-protein interactions result in unfolding of the B subunits which would then form a pore in the membrane through which the A subunit can pass (51). Another hypothesis is that one of the B components first binds to G_{M1} and anchors the exotoxin until the remaining B subunits are bound. This would result in a localized increase in ganglioside concentration at the membrane surface. It is thought that this would increase the chances of subsequent pentration of the lipid bilayer by fragment A. RME has not been ruled out as a mechanism for CT entry either. Upon binding, a lag period of 10 minutes occurs before toxic effects are seen and RME may account for this lag period. The exotoxin may then use the endosomal pH gradient discussed earlier to enter the cytoplasm (68).

An exotoxin from the bacterium *Escherichia coli* which has similar structure and enzymatic activity to cholera toxin is heat-labile (LT) enterotoxin. It possesses a 30,000 dalton enzymatic A subunit and a 11,000 dalton B subunit. The LT A subunit, unlike that of cholera toxin, can be isolated as a single polypeptide chain. However, the exotoxin must be cleaved

with trypsin and treated with a thiol reducing reagent to enhance its activity. This *in vivo* nicking process presumably occurs after the exotoxin has left the bacterial cell. The LT can be purified using a galactose-agarose affinity column. This suggests that galactose may be a receptor for LT (28).

It was determined in 1979 that LT had ADP-ribosyltransferase activity by demonstrating that a 43,000 dalton protein in pigeon erythrocytes was a substrate for the reaction (106). The erythrocyte protein has now been shown to be the stimulatory regulatory component of the avian adenylate cyclase system. It is uncertain as to what amino acid LT ADP-ribosylates. LT has other characteristics which are similar to cholera toxin's. The amino acid homology between cholera toxin and LT is 80%, both exotoxins cross react immunologically and in addition, their receptors may also be identical (17,34). Binding of LT to G_{M1} and LT inactivation by this ganglioside was first described in 1973 (58). However, it was found that the B subunit of cholera toxin was unable to inhibit the biological activity of LT in rabbit intestine. In contrast, binding of LT to G_{M1} deficient cells can be enhanced by the addition of G_{M1} to these cells in the same fashion as cholera toxin binding can be enhanced

(105,121). Also, in the dog, the B subunit of both exotoxins is able to inhibit the biological activity of LT. It would therefore seem that the B subunit of cholera toxin binds only to G_{M1} and therefore can block binding of whole cholera toxin to cell surface whereas LT may bind to G_{M1} and to other receptor sites not used by cholera (59). Also, LT may bind to glycoprotein receptors with a higher affinity than cholera toxin.

A new enterotoxin, termed heat-labile toxin 2 (LT2) is antigenically unrelated to either cholera toxin or LT but does possess trypsin-activated ADP-riboslytransferase activity. Unlike LT, LT2 production is not encoded by genes located on plasmids (52). Determination of the LT2 structure has only proceeded as far as demonstrating that it is composed of two subunits, an A subunit and a B subunit. LT2 has been shown to induce morphological changes in chinese hamster ovary (CHO) cells similar to that seen with cholera toxin and LT. It ADP-ribosylates the same proteins in human fibroblasts as cholera toxin, but the LT2 gene does not hy[illegible] with cholera toxin or LT genes (even at low str[illegible]evels) nor is its binding to susceptible [illegible]ted by G_{M1} (24).

The sp[illegible]cteria that produces perhaps the greatest ar[illegible]of ADP-ribosylating toxins is

Clostridium. All of the exotoxins produced by Clostridium can be classified as A-B toxins (112).

Clostridium botulinum produces seven antigenically distinct neurotoxins. These are designated A,B,C1,C2,D,E and F. All have been shown to possess a similar structure consisting of two polypeptide chains, a heavy and a light chain, with molecular weights of 100,000 and 50,000 respectively. The heavy chain, as in other bacterial exotoxins, is responsible for toxin binding whereas the light chain mediates the neurotoxic activity (131). The two chains are held together by noncovalent forces and at least one disulfide bond. The exotoxin is activated when the chains are proteolytically separated from one another but the light chain must still associate with the heavy chain in order to effect entry into the cell. Of the 7 known exotoxins of *C. botulinum*, only 3 have been found to possess ADP-ribosyltransferase activity; C1,C2 and D. C1 and D toxin have been shown to ADP-ribosylate proteins in membrane preparations of neural origin whereas C2 toxin ADP-ribosylates unpolymerized actin in intact cells and cell free preparations (93). In the case of C2 toxin the acceptor site for the ADP-ribose group is an arginine (ARG-177) (129). C1 and D toxins can be cross-neutralized with specific antisera and their production is governed by

lysogenic bacteriophages. The acceptor amino acids for the C1 and D toxin ADP-ribosyltransferase activites have yet to be determined. Both C1 and D toxins have been shown to ADP-ribosylate the same 21,000 dalton proteins in similar neural tissues. The ADP-ribosylation reaction of both exotoxins can be stimulated by the addition of GTP to the reaction. This suggests that the target protein may be a GTP-binding protein. It has also been demonstrated that toxin D is able to inhibit the release of catecholamine from cells. The possibility has also been considered that the 21,000 dalton protein may be involved in exocytosis and that ADP-ribosylation of this protein effectively stops catecholamine release by interfering with exocytosis (117). It has also been observed that guanidine hydrochloride, a compound used as a chemotheraputic agent in the treatment of clinical botulism, is an effective inhibitor of ADP-ribosylation catalyzed by exotoxins C1 or D (116).

Receptors for C1 and D toxin have not been clearly defined. Much work with respect to binding of *Clostridium* toxins has focused on tetanus toxin or botulinum toxin A and the results of these studies tend to be extrapolated to include all of the Clostridial toxins possessing a similar subunit structure. Electron microscopy studies have revealed that toxin A binds to

the presynaptic membranes of motor nerve endings in the cerebral and cerebellar cortices but not to postsynaptic membranes nor membranes of glial origin (57). Both toxins A and B bind to the ganglioside G_{T1b} but with different affinities. None of the other botulinum toxins were found to bind to this ganglioside. Toxins A and B, in addition to toxin C1, form channels in planar lipid membranes. Like diphtheria toxin, this process is pH dependent. The channels formed by botulinum toxins A and B conduct ions and their rate of formation is dependent on the square of exotoxin concentration (8). Receptor-mediated endocytosis has also been suggested as a possibile mechanism for entry by A and B toxins.

Lysosomotropic agents such as chloroquine have been found to protect the nerve from attack by toxin C (118). The binding of this exotoxin to the host cell has been shown to occur in a rapid and temperature dependent manner (132). Binding studies have found that C2 toxin's heavy chain binds to epithelial cells and brush border membranes in the mouse. The light chain of C2 will only bind to brush border membranes if the membranes are first treated with trypsinized heavy chain indicating that some form of proteolytic processing must occur before the exotoxin becomes active.

Another interesting ADP-ribosylating exotoxin from the clostridium species is iota toxin. This exotoxin was discovered over 4 decades ago but only now is its structure and enzymatic activity being determined. It is also composed of two separate polypeptide chains (154). The light chain is an ADP-ribosyltransferase that can ADP-ribosylate polyarginine and the heavy chain is thought to play a role in binding. The intracellular substrate for the exotoxin is unknown (133).

1.3 Pertussis Toxin

Pertussis toxin (PT), one of the virulence factors produced by the organism *Bordetella pertussis*, is the major immunogen in the killed whole cell vaccine and in the current acellular vaccine preparation (50). The history of whooping-cough immunization was a successful one until the last 10 years. Industrialized nations such as Canada employ a whole cell vaccine consisting of organisms which have been inactivated by heat and formalin or merthiolate. The whole cell vaccine in the past was combined with the diphtheria vaccine and first introduced into Canada in 1943 (149). Since this time there has been a steady decline in the incidence of whooping cough in this country. The vaccine is now adminstered with poliovirus and tetanus toxoids along with diphtheria toxoid adsorbed to an aluminum phosphate

adjuvant. Although this vaccine has been shown to be very effective in preventing whooping cough epidemics, it is not without its side effects. One child in approximatley every 100,000 to 150,000 are ultrasensitive to the vaccine and may develop acute encephalopathy with seizures and then coma. This may or may not be followed by permanent brain damage. In Canada, public compliance with the vaccine has not declined to the extent where the disease can manifest itself in a nationwide epidemic. However, this was not the case in Japan during the late seventies and early eighties.

Following introduction of pertussis vaccine in 1947 the incidence of whooping cough declined to almost negligible levels in Japan (148). In 1970 concerns over adverse reactions to the smallpox vaccine being adminstered at that time prompted the Japanese government to set up a system for reviewing claims associated with vaccine-related injuries. This subsequently drew increased attention to the adverse effects associated with the pertussis whole cell vaccine and in spite of its effectiveness, resulted in decreased compliance with the immunization program. Because of this, the incidence of whooping cough rose and with it the number of pertussis-associated deaths. As a result,

an acellular vaccine containing pertussis toxin and filamentous hemagglutinin (another virulence factor of *Bordetella pertussis*) was introduced (109). Clinical trials have demonstrated that the acellular product resulted in less local complications than the whole cell vaccine. This has restored public confidence and resulted in a decrease in the disease. Nonetheless, the procedure for inactivating PT in the acellular vaccine may partially destroy some epitopes which are necessary for protective immunity. There has now been an increase in activity aimed at better understanding the pathophysiological role of pertussis toxin in order to create an even safer vaccine containing PT which has been inactivated by site-specific mutagensis procedures.

PT is classified as an A-B exotoxin (140). It shares structural similarties with cholera toxin and *E. coli* LT. The exotoxin is a heterohexameric protein consisting of 5 subunits designated S_1 to S_5, with S_4 being repeated twice (87,140) (figure 2). The largest subunit, S_1 or the A protomer, has a molecular weight, based on the DNA sequence, of 26,024 and is the subunit responsible for the enzymatic activity of the exotoxin (71,72,74,80).

The enzymatic activity of the exotoxin has been examined in great detail. As discussed earlier, PT is

Figure 2: Schematic Representation of Pertussis Toxin (top view)

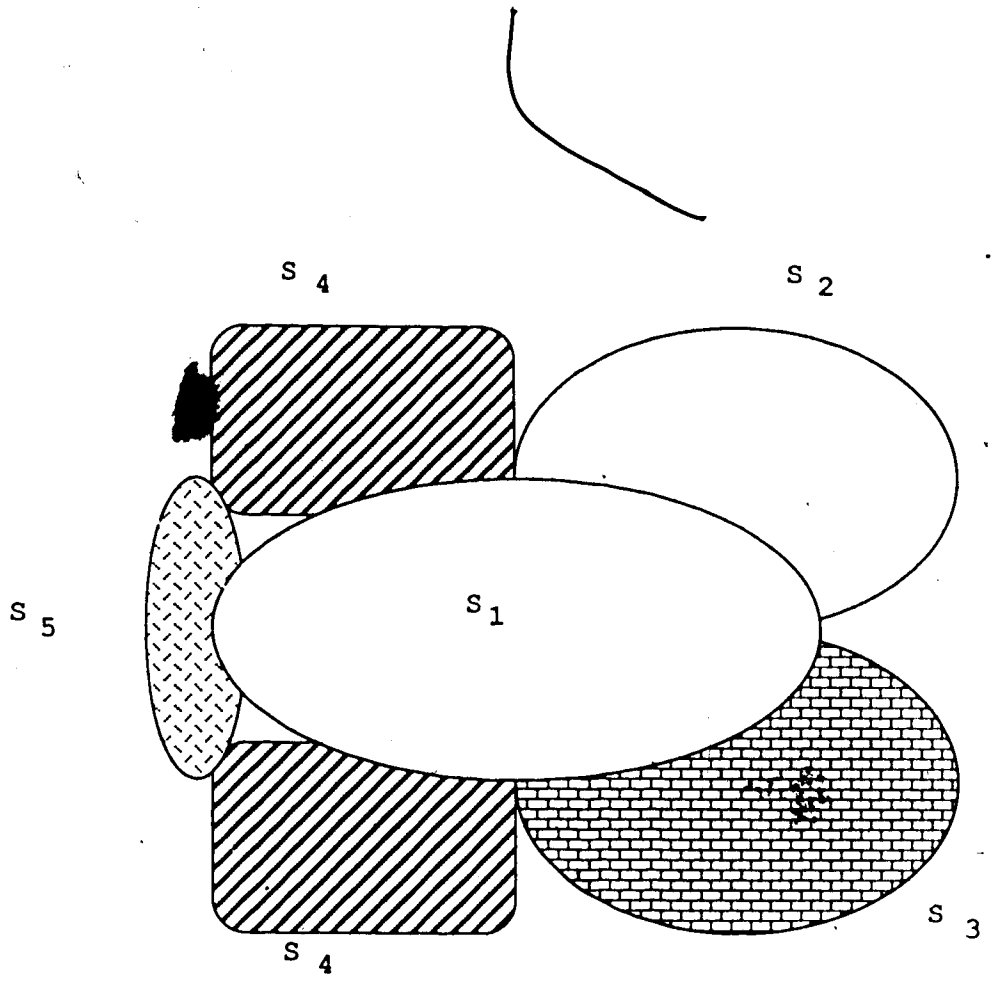
S_1 - The S_1 subunit of PT. Molecular weight 26,024. This is the subunit responsible for the toxin's enzymatic activity.

S_2 - The S_2 subunit of PT, Molecular weight 21,924. Forms a dimer with S_4. This subunit forms part of the base or binding component of the toxin.

S_3 - The S_3 subunit of PT. Molecular weight 21,873. Forms a dimer with S_4. This subunit also forms part of the base and mediates binding of the toxin.

S_4 - The S_4 subunit of PT. Molecular weight 12,058. This subunit is repeated twice. One subunit each forms dimers with S_2 and S_3. This subunit also plays a role in binding of the toxin.

S_5 - The S_5 subunit of PT. Molecular weight 11,013. Also referred to as the "C" subunit or connecting subunit. This subunit connects the two S_4 subunits that form the dimers, together.


S 4
S 2
S 5
S 1
S 3
S 4

able to ADP-ribosylate the Gi α subunit of the adenylate cyclase system. In addition, PT ADP-ribosylates transducin, a protein found in the visual excitation system. A brief examination of the visual excitation system (see fig.3) shows that it is similar to the mammalian adenyl cyclase complex. The exotoxin ADP-ribosylates a cysteine residue on the Gi α subunit modifying the protein to the extent that it is unable to release bound GDP resulting in loss of the inhibitory action.

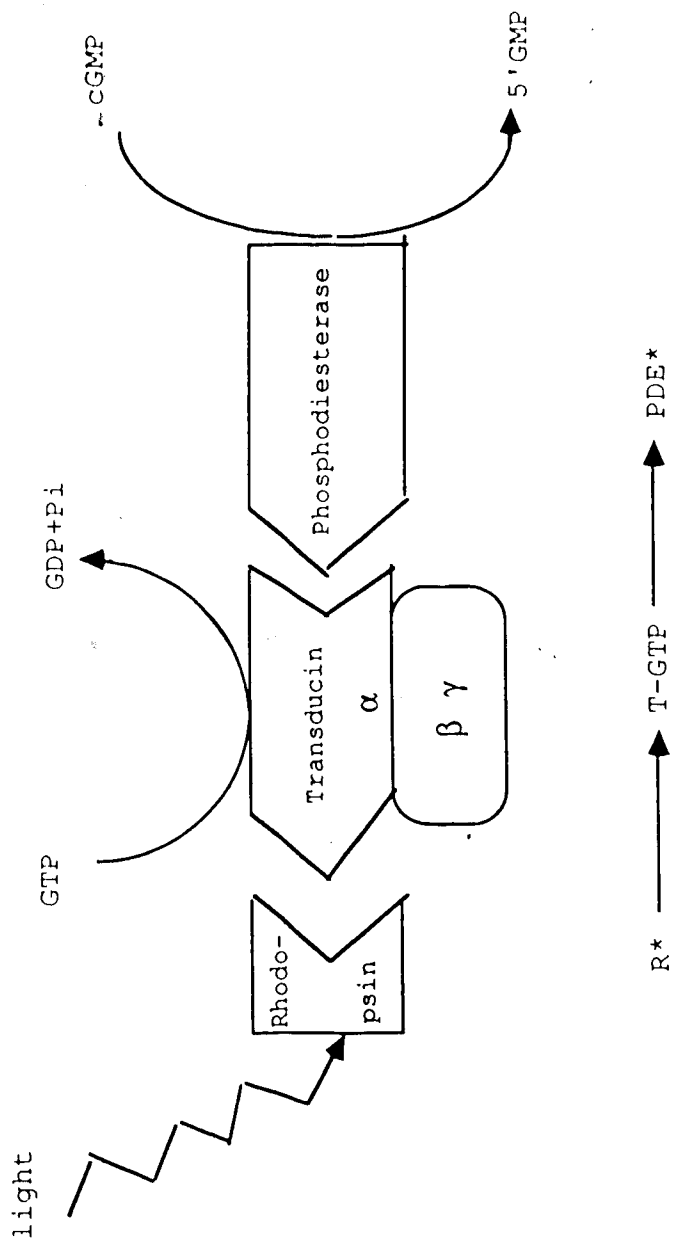
The transducin system is composed of three components; rhodopsin, transducin and phosphodiesterase (36,54,138). The receptor in this case is unphotolysed rhodopsin and the external stimulus is a photon of light. Once rhodopsin has been activated, it in turn activates transducin. Transducin, which is composed of three subunits; α, β and γ, is analogous to G proteins of the adenylate cyclase system. The β and γ subunits of transducin and the β and γ subunits of the adenylate cyclase can functionally substitute for each other. However, the Gs transducin, and Gi α subunits of the adenylate cyclase system are not interchangable. This indicates a high degree of functional homology between the β-γ subunits which is not evident in the α subunits.

Figure 3: The Visual Excitation Cycle

R* - Light stimulated rhodopsin. A single photon of light stimulates rhodopsin to interact with transducin.

T-GTP - Upon stimulation by rhodopsin, the transducin complex (α, β, γ subunits) releases GDP and binds GTP resulting in dissociation of the α subunit from the β, γ subunits.

PDE* - Stimulated phosphodiesterase. The α subunit of transducin with GTP bound in turn stimulates the third component in the cycle, phosphodiesterase. This causes the phosphodiesterase to hydrolyze cGMP to 5'GMP.


light
Rhodo-
psin
GTP
GDP+Pi
Transducin
α
β γ
Phosphodiesterase
cGMP
5'GMP
R*
T-GTP
PDE*

However, the G α subunits are similar in function in that they bind and hydrolyze GTP to GDP in all systems examined. They also are similar in size (41,000 to 37,000 molecular weight) and all interact with a catalyst contained within their respective systems. Inactive transducin consists of a complex of all three subunits with GDP bound to the α subunit. Upon stimulation by light-activated rhodopsin, GDP is exchanged for GTP and the β-γ subunits dissociate from the α subunit. The active α subunit with bound GTP now stimulates the phosphodiesterase which catalyzes the formation of 5'GMP from cGMP. The α subunit now slowly hydrolyzes GTP to GDP and reassociates with the β-γ subunit complex to repeat the cycle. The β-γ subunits are necessary for GTP-GDP exchange (105). PT is able to ADP-ribosylate an asparagine residue on the carboxy terminus of the α subunit of transducin (146). Another system in which PT has been found to act upon is the inositol phosphate system. This system also contains a GTP binding protein which is ADP-ribosylated by PT. The inositol phosphate pathway is similar to the adenylate cyclase system and the transducin system in that a receptor on the cell surface must first be stimulated by an extracellular messenger. This receptor is coupled to a G protein which functions to activate a

phosphodiesterase enzyme, phospholipase C. The phospholipase C then enzymatically acts on phosphatidylinositol-4,5-bisphosphate (PIP_2), one of the phospholipids that makes up the cell membrane. This is enzymatic activity results in the hydrolysis of PIP_2 to diacylglycerol and inositol triphosphate. Diacylglycerol stimulates C-kinase and inositol triphosphate indirectly mobilizing intracellular calcium which then stimulates a calcium-calmodulin-dependent protein kinase. ADP-ribosylation of the transducing G protein in this pathway by PT results in a loss of stimulation for these two kinases (12).

The S_1 subunit of PT also ADP-ribosylates a protein of molecular weight 41,000 daltons in both CHO cell and C6 glioma cell membrane preparations. ADP-ribosylation by PT is by no means restricted to these cell systems. It has also been found to enzymatically act on substrates in rat heart cells, human neutrophils and erythrocytes, 3T3 cells and spermatozoa (11,44,78,144).

The remaining subunits of PT form the B oligomer. The B oligomer, as in all A-B exotoxins, is responsible for binding the toxin to its receptor or receptors in eukaryotic systems and probably for entry of the exotoxin into the cell cytoplasm (6,141). However, unlike cholera toxin the B oligomer of PT can be divided

into two dimers (see figure 2). Dimer 1 consists of subunits S_2-S_4 with molecular weights of 21,924 and 12,058 respectively. Dimer 2 consists of subunits S_3-S_4 with a molecular weight of 21,873 for S_3 (87). Subunit S_5, sometimes called the connecting or "C" subunit, is also part of the base structure of the toxin and its molecular weight is 11,013 (87). In addition to binding to receptors on erythrocytes from various species, dimer 1 also interacts with the serum glycoprotein fetuin. It has recently been shown that acetamido-containing sugar groups on the non-reducing terminal position of fetuin's N-linked oligosaccharides are responsible for the toxin's binding (7). The B oligomer has been found to have properties other than its binding abilities. PT has been shown to have a number of immunopotentiating activities. It is a effective adjuvant for the production of antibodies. The active adjuvant in the pertussis vaccine is pertussis toxin. Pertussis toxin has also been found to be extremely active in enhancing the intensity and duration of the delayed type hypersensitivity response (DTH). This effect is antigen specific and is mediated by specific subsets of T cells, Thy+, L3T4+ and Ly- T-lymphocytes. Also an enhanced DTH response resulting from PT exposure has been found to correlate with an increase in the production of gamma

interferon. It has been suggested that the gamma interferon may contribute in vivo to prolonged DTH reactions induced by pertussis toxin. Pertussis toxin enhanced DTH also plays a role in the induction of experimental allergic encephalomyelitis (EAE) in mice and rats. Extracts from mice previously immunized with pertussis toxin has been shown to be effective in promoting EAE whereas those not previously immunized are not. Other properties of pertussis toxin include mitogenicity in T cells, stimulation of glucose oxidation in adipocytes and histamine-sensitizing activity (107).

The exotoxin also has a unique effect upon the morphology of CHO cells. Within 24 hours after the addition of exotoxin to these cells, they begin to present a clustered clumped morphology as opposed to their usual elongated dispersed appearance. This is believed to be caused by the A protomer's enzymatic activity upon the cell. Stimulation of insulin secretion and lipolysis, potentiation of adenylate c[illegible]ase, hypotensive activity and inhibition of epinephrine hyperglycemia are also attributable to activity mediated by the A protomer (43,113,114). It may be found in the future that all of these activites are a result of ADP-

ribosylation of particular signal transducing proteins contained within the cell systems examined.

1.4 Specific Aims of Research

The purpose of my studies was to better understand structure function relationships in PT to more clearly define the pathophysiological role of whooping cough. One aspect of my studies was to examine PT binding properties and identify receptors in goose erythrocytes and CHO cell membranes. Once purified, the receptors can be biochemically characterised in order to identify the molecular basis for the interaction with PT. This information may be able to aid in the development of receptor analogues that could conceivably be used as novel therapeutic reagents for hastening the recovery of patients in the paroxysmal phase of the disease. The second component of the work involved examining the ADP-ribosyltransferase activity of ^{125}I labelled PT. The ADP-ribosyltransferase activity of radiolabelled PT was characterized in order to determine whether or not fetuin-agarose protected PT still retained its enzymatic activity. It is important to determine what functions the radiolabelled exotoxin still retains if it is to be used for studies aimed at understanding the exotoxin's effect in a variety of systems. In addition, the studies will facilitate the identification of PT functional

domains which are targets for site specific mutagenesis. Biologically inactive mutant forms of PT will be useful for vaccines.

2.0 MATERIALS AND METHODS

2.1 Materials;

C6 rat glial tumour CCL 107 cells and Chinese Hamster Ovary-K1 CCL 61 cells were obtained from the American Type Culture Collection, Rockville, Maryland. All tissue culture medium and plastic ware were purchased from Gibco. Laboratories, Grand Island, N.Y., USA. and Flow Laboratories, McLean, Virgina, USA. Bovine eyes were purchased from a local slaughterhouse. Pertussis toxin was purchased from List Biological Laboratories Inc., Campbell, CA., USA or donated by Connaught Laboratories, Toronto, Ontario. Sodium chloride and dimethylsulfoxide were purchased from British Drug Houses Chemicals Canada Limited, Edmonton, Alberta. All other common reagents were purchased from Fisher Scientific Company, Edmonton, Alberta. Ethyleneglycol-bis-(B-amino-ethly ether) N,N'-tetra-acetic acid (EGTA), ethylenediaminetetracetic acid (EDTA), 4-(2-hydroxyethyl-1-piperazinee thanesulfonic acid (HEPES), L-1-Tosylamide-2-phenyl-ethlychloromethyl ketone (TPCK), phenylmethylsulfonyl fluoride (PMSF), L-cysteine (free base), ATP, GTP, thymidine, dithiothreitol, nicotinamide, bovine serum albumin, Trizma base, glycine, diphosphotidylcholine, $ZnCl_2$,

diethanolamine, α-methly-D-mannoside, imidazole, bovine pancreatic trypsin type III, neuraminidase (type X, 160 N-acetylneuramin-lactose units/mg protein) from Clostridium perfringens, fetuin type III, fetuin-agarose, α-1 acid glycoprotein, ceruloplasmin, polyvinylpyrrolidone and dipalmityl phosphatidyl-choline (DPPC) were purchased from the Sigma Chemical Company, St. Louis, MO., USA. α-2 macroglobulin and aprotinin were purchased from Boehringer Mannheim, Dorval, Quebec. Wheat-germ agglutinin and peanut agglutinin were purchased from E-Y Laboratories, San Mateo, CA., USA. Iodo-Gen and sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate (SASD) were obtained from the Pierce Chemical Co., Rockford, Il. ^{32}P-NAD was purchased from New England Nuclear, Boston, Mass., USA. ^{125}I was purchased from Edmonton Radiopharmaceuticals, Edmonton, Alberta. Aqueous counting scintillation fluid was purchased from Amersham, Arlington Heights, Illnois. Goose erythrocytes were purchased from Gibmar Laboratories, Ardrossan, Alberta. Monosialoganglioside G_{M1} was purchased from Supelco, Oakville, Ontario. Nitrocellulose sheets were obtained from Schleicher and Schunell, Keene, N.H., USA and from Bio-Rad, Richmond Calif., USA. Sodium dodecyl sulfate, TEMED, acrylamide, bis-acrylamide, SDS-PAGE

standards 14,500-94,000 daltons and Coomassie brilliant blue were purchased from Bio-Rad Laboratories. Amicon ultrafilters were purchased from Amicon Canada Ltd., 1226 White Oaks Boulevard, Oakville, Ontario. Sephadex G-25, agarose-wheat germ lectin, and activated CH-Sepharose 4B was purchased from Pharmacia, Dorval, Quebec. Spectrapore Dialysis Membrane (Mol. wt. cut off 6,000-8,000 and 14,000-16,000) was obtained from Spectrum Medical Industries, Inc., Los Angeles, CA. USA. Glutaraldehyde (70%) was purchased from Ladd Research Industries, Burlington, Vermont. Chloramine T, and Kodak X-omat AR X-ray film was obtained from Eastman Kodak Co., Rochester, N.Y. USA.

2.2 Preparation of Iodo-Gen Tubes;

Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) was dissolved at a concentration of 2 mg/mL in chloroform and 20 µL was added to the bottom of chromic acid-washed glass 12 x 75 mm culture tubes. The tubes were slowly agitated with a vortex mixer and the chloroform was evaporated using a gentle stream of nitrogen. This resulted in the even deposition of Iodo-Gen in concentric rings on the bottom inside surface of the tubes.

2.3 Preparation of Sephadex G-25 Gel Filtration Column;

Two grams of Sephadex G-25 was rehydrated in 10-20 mL of PBS for 30 minutes. A 10 mL disposable glass pipet with the constricted top removed was plugged at the bottom with a small amount of glass wool. The swollen Sephadex G-25 was then allowed to settle into the pipet. This resulted in a column size of 1.0 cm x 30.0 cm.

2.4 Iodination of Unprotected Pertussis Toxin, Cholera Toxin, Fetuin, Ceruloplasmin and Peanut Agglutinin;

Ten µg of protein was added to 100 µL of 0.33 M sodium phosphate buffer (pH 7.5) in an Iodo-Gen coated 12 x 75 mm glass culture tube. Twenty MBq of sodium 125-iodide (approximately 0.50 mCi) was then added to initate the iodination reaction. Iodo-Gen oxidizes the ^{125}I to a free radical form resulting in an iodide species which reacts with tyrosine residues in the toxin. The iodide atom becomes attached to the tyrosine phenolic ring in the ortho or para positions but very rarely both. After approximately 60 seconds, the solution was removed from the reaction tube and filtered through a glass wool-plugged pasteur pipet to remove Iodo-Gen which may have come off the glass. Two hundred µL of 1 mg/mL cysteine in PBS was passed through the

filter pipet to reduce the remaining oxidized ^{125}I and stop any further iodination. Two hundred µL of 0.1% BSA in PBS was then added to the mixture to stabilize the iodinated protein and prevent non-specific binding to surfaces during the remaining purification steps. Next, ten µL of this mixture was diluted into 2.5 mL of PBS in order to determine the total counts which were added to the reaction. One half mL of 1.0% BSA in PBS was then applied to a column of Sephadex G-25 (1 cm x 30 cm). The BSA solution was used to prevent non-specific binding of the radiolabelled protein to Sephadex G-25. The iodinated mixture was added to the column and allowed to drain into the gel bed. Following the addition of the iodinated protein solution, a further 0.5 mL of 1.0% BSA was added to the column. The column was then eluted with PBS and 16 half mL fractions were collected. Ten µL of each fraction was then counted in a LKB Rackgamma 1270 counter to locate the void volume fractions containing the iodinated protein. The Sepharose G-25 column was used to remove unreacted iodine from the reaction mixture. The percent TCA precipitable counts were determined for the reaction mixture and the pooled G-25 fractions containing iodinated protein as discussed below. The specific activity was calculated from TCA precipitable counts in the reaction mixture and the

total counts. Chloramine T radiolabelling was performed by a similar procedure. Briefly, 10 µg of protein was added to 100 µL of 0.33 M sodium phosphate buffer with 20 MBq of ^{125}I. This was incubated for 60 seconds in the presence of 20 µL of 1 mg/mL chloramine T in double distilled H_2O (ddH_2O). Twenty µL of a 2 mg/mL sodium metabisulfate in ddH_2O was added to stop the chloramine T reaction. After 30 seconds, 1.0% BSA was added to the mixture and the chloramine T iodinated samples were processed though a G-25 column as described earlier.

2.5 Iodination of Fetuin Protected Pertussis Toxin;

The procedure used was essentially the same as that described by Armstrong and Peppler (6). Briefly, before use fetuin-agarose was sedimented by centrifugation at 100 x g for 10 minutes and the resulting pellet was suspended in 10 volumes of 4.0 M $MgCl_2$ in PBS. The gel solution was then sedimented by centrifugation at 100 x g for 10 minutes. The $MgCl_2$ supernatant solution was discarded and the gel pellet was washed five times with PBS. Between washes, the gel was collected by centrifugation. Next, fifty µL of the washed gel pellet was mixed with fifty µL (5 µg) of PT in a 1.5 mL Eppendorf microfuge tube for thirty minutes on a table top rotator at room temperature. The gel slurry was

removed and placed into an iodo-gen coated tube (40 µg of iodo-gen/tube). Ten MBq (0.25 mCi) of sodium 125-iodine was added to the solution. This was incubated for 2 minutes at room temperature. Two hundred µL of cysteine (1 mg/mL) was added to the solution and after 60 seconds it was filtered through a glass-wool plugged pasteur pipet. The fetuin-agarose was washed in the pipet with 30-50 mL of PBS containing 10 mM potassium iodide. The ^{125}I-pertussis toxin was eluted from fetuin-agarose with 200 µL of 4.0 M $MgCl_2$ in PBS or 50 mM diethanolamine containing 0.15 M NaCl (pH 11.5). The diethanolamine was immediately neutralized with PBS (pH adjusted to 2.1 with 0.50 M HCl). One hundred µL of cysteine was then added and depending on the application, 300 µL of 0.1% BSA was also added. After the iodination procedure was completed, the percentage of counts incorporated into the toxin was determined by the TCA precipitation procedure described below.

2.6 Determination of Percent Incorporation of ^{125}I into Protein;

Five µL aliquots of the iodinated protein was mixed with 1 mL of 1% BSA in ddH_2O. This was done in duplicate tubes. One half mL of 10% trichloroacetic acid was added to one of the tubes and an equivalent amount of PBS was added to the other. The mixtures were then shaken

vigorously and placed in ice for at least 10 minutes. Next, the samples were centrifuged at 700 x g for 10 minutes to sediment the precipitated protein in the tube containing TCA. The amount of radioactivity remaining in the supernatant solutions was determined using a LKB Rackgamma model 1270 counter. The percentage of ^{125}I counts incorporated into protein was calculated from the difference in counts remaining in the TCA and control supernatant solutions.

2.7 Iodination of Wheat Germ Agglutinin;

The procedure used is similar to that of Bartles and Hubbard (10). Ten µL of wheat germ agglutinin (WGA) (1 mg/mL) was incubated for one half hour with 40 µL of 25 mM N-acetylglucosamine and 60 µL of 0.08 M sodium phosphate (pH 7.2) in a 12 x 75 glass culture tube pre-coated with iodo-gen. After the incubation time the WGA was iodinated by the conventional procedure discussed earlier. To determine whether binding activity of the lectin was retained, hemagglutination of chymotrypsin-treated goose erythrocytes was performed. Chymotrypsin-treated goose erythrocytes were prepared by first washing the cells in PBS by three cycles of centrifugation, 180 x g for 5 minutes. The cells were suspended at a concentration of 2 x 10^8 to 4 x 10^8 cells per mL. Chymotrypsin was added to a final concentration

of 1 mg/mL, and the cells were incubated at 37°C for 30 minutes. After treatment, 9 volumes of PBS containing PMSF (PMSF concentration was 100 times on a mole to mole basis that of chymotrypsin) were added and the cells centrifuged as described above. The treated cells were suspended in PBS and washed five times by centrifugation to remove residual enzyme activity and inhibitor. Chymotrypsin-treated goose erythrocytes were used because it was found that they hemagglutinate much more strongly than do untreated goose erythrocytes. The chymotryptic activity may expose cryptic sites for WGA to bind to as well as PT.

2.8 Procedure for Goose Hemagglutination Assay;

The hemagglutination assays were performed in plastic 96-well U shaped bottom microtitre plates obtained from Flow Laboratories Inc., McLean, Virgina, USA. Serial twofold dilutions of PT or WGA (starting concentration 1 µg/mL) were prepared in PBS. Prior to use, fetuin-agarose protected ^{125}I pertussis toxin or ^{125}I WGA were diluted ten fold in PBS and unprotected ^{125}I pertussis toxin was used without dilution. Fifty µL of the diluted samples was added to the wells. Fifty µL of untreated or chymotrypsin-treated goose erythrocytes ($1x10^7$-$4x10^7$ cells/mL) was added to all the wells and

the plates were incubated at room temperature for one hour.

2.9 Preparation of Goose Erythrocyte Membranes;

Twenty mL of packed goose erythrocytes was suspended in an equal volume of 0.9% sodium chloride. The cells were sedimented by centrifugation at 500 x g for five minutes. The supernatant solution was removed and discarded and the erythrocytes were washed twice with 0.9% sodium chloride. The cells were then suspended in eight volumes of SPIE buffer (5 mM sodium phosphate pH 7.6, 1 mM EDTA, 1 mM PMSF (phenylmethlysulfonyl fluoride)). The cell suspension was incubated in this buffer at 4°C for twenty minutes to lyse the erythrocytes. The erythrocyte membranes were washed with the SPIE buffer by three cycles of centrifugation (27,000 x g, 20 min/cycle). A 30 mL Wheaton glass tissue homogenizer was used to suspend the membrane pellets between each washing step. The erythrocyte membranes were then washed twice with PBS or until the resulting pellet was a light tan colour. This material was washed with 50 mM Tris-HCl pH 7.4. The pellet was then suspended in one volume of the 50 mM Tris buffer and a homogenous suspension was created using a Branson model 185 Sonifier Cell Disrupter at a setting of 2 for 30 seconds. The membranes were stored at -70°C until use.

2.10 Procedure for Determining Protein Concentrations;

A modification of the Lowry assay was used for membrane protein determinations using BSA as a standard for the procedure (88). The modification involved preparing all of the protein solutions in 1% SDS. Optical density measurements were performed at a wavelength of 600 nm with a Gilford model 250 Spectrophotometer.

2.11 Procedure for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE);

SDS-PAGE was carried out using the discontinous buffer system described by Laemmli (84). The 1.5 mm thick gels were prepared using a Bio Rad Protean II slab gel casting unit. The concentration of acrylamide: bisacrylamide stock solution was 30%: 0.8% (w/w). The final concentration of polyacrylamide in the stacking gels was 5% and in the separating gels 12.5% except where otherwise stated. Gradient separating gels were also employed using a concentration range of 7.5% to 15.0%. These were used to resolve proteins of higher molecular weight which could not be distinguished with the linear gel system. The electrophoresis buffer consisted of 120 mM Tris-HCl (pH 7.5), 960 mM glycine and 0.5% SDS. An equal volume of sample buffer (6.25 mM

Tris, pH 6.8, 2.5% SDS, 12.5% glycerol, 0.001% bromophenol blue, 6.5% β-mercaptoethanol) was added to each sample and the samples were heated in a boiling water bath for ten minutes prior to loading into the sample wells formed in the stacking gel. Five to 100 µg of protein (depending on the sample) was loaded per well. Gels were stained with 0.25% Coomassie Brillant Blue R-250 in 45% methanol, 9.0% acetic acid and destained in 10.0% methanol 10.0% acetic acid.

2.12 Autoradiography Procedure;

SDS-PAGE gels were stained with coomassie brillant blue and destained to visualize the proteins. The gels were then dried under a vacuum using a Bio Rad Dual Temperature Slab Gel Dryer Model 1125B. Next, the gels were placed in X-ray film cassettes containing Dupont Cronex Lightning Plus intensifying screens and Kodak X-Omat AR film. The films were exposed at -70°C and then developed to determine the location of radiolabelled proteins.

2.13 Western Blotting Procedure;

Proteins separated in polyacrylamide gels were electrophoretically transferred to nitrocellulose in 25 mM sodium phosphate buffer (pH 7.5) overnight at 27 volts at 4°C in a Bio-Rad Transblot Cell. To visualize proteins transferred to nitrocellulose, 0.1% amido black

in 40% methanol and 10% acetic acid was mixed with the nitrocellulose sheet for 1 minute. The sheets were washed with ddH_2O to destain areas which did not contain protein bands. Prior to incubaton with iodinated PT or plant lectins, unstained nitrocellulose sheets were placed in sealed plastic bags and incubated with a solution consisting of 3% BSA in 10 mM Tris-HCl (pH 7.5) (to block sites not containing protein) and 0.85% NaCl unless otherwise stated, overnight. The BSA-treated nitrocellulose sheets were then incubated with ^{125}I pertussis toxin, ^{125}I WGA, or ^{125}I PNA. After incubation with the radiolabelled probes, the nitrocellulose membranes were washed for 2 hours with 0.85% NaCl at room temperature with a minimium of 15 changes of the wash solution. The nitrocellulose sheets were then dried and exposed to Kodak X-ray film at -70°C as described earlier.

2.14 Periodate Treatment of BSA;

BSA solutions were treated with periodate to oxidize any oligosaccharide impurities which may have interfered with lectin binding experiments where BSA was used as a blocking agent. Five hundred mL of BSA solution consisting of 3% BSA in 0.1 M sodium acetate (pH 4.5) was incubated with 10 mM periodic acid for 6 hours at room temperature. Excess periodate was

inactivated by the addition of glycerol to a final concentration of 10 mM. The oxidized BSA solution was then dialyzed overnight against ddH_2O and stored with 0.01% sodium azide at 4°C.

2.15 Binding of ^{125}I Pertussis Toxin to Chinese Hamster Ovary Cells;

Chinese hamster ovary (CHO) cells were grown in Ham's F12 medium supplemented with 5% fetal calf serum in an atmosphere of 5% CO_2 at 37°C in 24 well tissue culture plates. When confluent monolayers were obtained, the cells were washed 3 times with unsupplemented Ham's F12 medium. The tissue culture plates were then placed onto a bed of ice and ^{125}I pertussis toxin, prepared in the presence of fetuin-agarose, was added in volumes specified in the results. After incubation, the unbound toxin was removed and the monolayers were washed with three 0.5 mL portions of Ham's F12 medium. After washing, the cells and bound toxin were solubilized in 1% SDS at 37°C, placed into 12 x 75 mm culture tubes and counted in the gamma counter.

2.16 Effect of Fetuin on Pertussis Toxin-Chinese Hamster Ovary Cell Activity;

Fetuin inhibition studies were performed to determine whether fetuin could compete for pertussis toxin binding to CHO cell receptors. CHO cells were

added to 96 well flat bottom tissue culture plates at a concentration of 5.0 x 10^4 cells/mL (5.0 x 10^3/well). After 18 to 20 hours the monolayers were approximately 50 to 60% confluent. The cells were washed three times with 100 µL of Ham's F12 medium. Native pertussis toxin was diluted in Ham's F12 to a concentration of 300 ng/mL. Eight serial two-fold dilutions of PT were prepared in Ham's F12. Ten µL of each PT dilution was then added sequentially to 100 µL of Ham's F12 medium in wells 1 to 12 of each row in the 96 well plates. Serial fold dilutions of fetuin were also prepared in Ham's and 10 µL of each dilution of fetuin was sequentially added to each column of wells in the 96 well plates. The CHO cells were then examined 24 hours later for the cytotonic effects of PT and the endpoint dilution for PT was determined for each concentration of fetuin.

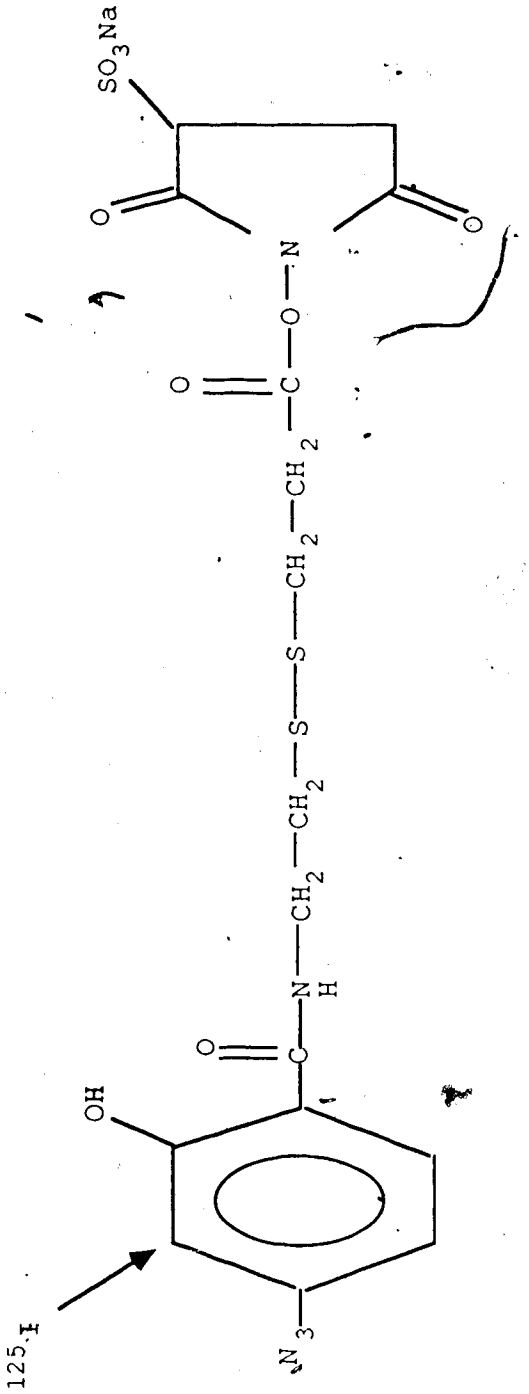
2.17 Procedure for Cross-linking Pertussis Toxin to Receptors in Chinese Hamster Ovary and VERO Cells;

The cross-link labelling procedure was used to identify receptors for pertussis toxin in Chinese Hamster Ovary cells. The bifunctional cross-linker choosen was SASD (sulfosuccinimidyl 2-(p-azidosalicylamido) ethyl-1,3'-dithiopropionate) obtained

from Pierce Chemical Co.. The utility of SASD is that it possess two dissimilar functional groups. The succinimidyl portion is specific for coupling to amino groups. The azido group covalently binds non-specifically into any carbon-carbon, carbon-hydrogen, or carbon-oxygen bond. We know that PT has abundant amino groups which are able to react with the succinimidyl group but the nature of the chemical groups on the receptor are unknown (see fig. 4). SASD was also selected because it can be iodinated in the position between the azide and hydroxyl moieties of the phenyl ring. The advantage of this is that the cross-linker can be iodinated rather than the toxin itself. It also has a disulfide bond between the two active groups. The disulfide bond is readily cleavable with β-mercaptoethanol in the Laemmli sample buffer. SASD was iodinated in an iodo-gen coated tube with 20 MBq of sodium 125-iodide for one minute in the dark. This was then incubated with pertussis toxin bound to fetuin-agarose. The fetuin-agarose was used to prevent the binding of the cross-linker to the amino groups of lysines in the binding site of the toxin. After the cross-linker was attached to PT, the fetuin-agarose gel slurry was placed into a foil-covered glass wool plugged pasteur pipet and washed to remove any unreacted ^{125}I or

Figure 4: Sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl 1,3'-dithiopropionate (SASD).

SASD is a photoreactive bifunctional cross-linker that can be iodinated between the azide and the hydroxyl moieties of the phenyl ring. The two functional groups are an azide group which will insert into proteins upon exposure to ultraviolet light (366 nm) and a succinimidyl group which reacts with primary amino groups.


SO3Na
O
O
O
N
O
O
C
CH2
CH2
S
S
CH2
CH2
CH2
N
H
O
C
OH
125I
N3

SASD. The complex is eluted off fetuin-agarose with 4.0 M $MgCl_2$ and the percent incorporation of ^{125}I into SASD coupled to pertussis toxin was determined by TCA precipitation as described earlier. Also, to determine whether the pertussis toxin was still capable of binding after the coupling reaction, the goose erythrocyte hemagglutination assay was performed. Ten µg of the ^{125}I-SASD-pertussis toxin suspension (as determined by goose hemagglutination assay) was incubated with a CHO cell monolayer for one half hour on ice and then exposed to a long wave ultraviolet lamp (366nm) for ten minutes. The CHO cell monolayer was washed three times with Ham's F12 to remove unbound ^{125}I-SASD plus pertussis toxin complexes and then solubilized in Laemmli sample buffer and subjected to SDS-PAGE. The gel was stained with coomassie blue stain, destained, dried, and subjected to autoradiography.

2.18 Preparation of C6 Rat Glial Cell Membranes;

C6 Rat glial cells were grown in Ham's F12 medium supplemented with 10% fetal calf serum in large roller bottles (1700 cm^2) which had been precoated with fetal calf serum. Approximately 1.5 x 10^8 cells could be obtained when the monolayers were confluent. The growth medium was then removed and the cells were lifted from the glass surface using 50 mL of a solution consisting

of 137 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1 mM EGTA and 5 mM Hepes (pH 7.4) (72). The cells were incubated with this solution for 10 minutes at 37°C, placed into a 15 mL Corning centrifuge tube and centrifuged in a table top centrifuge (IEC model Centra 4 centrifuge) at 200 x g for 10 minutes. The remaining steps were performed at 4°C. The cells were then transferred to a 10 mL Wheaton glass homogenizer with 2 mL of lysis buffer consisting of 1 mM $NaHCO_3$, 0.5 mM $CaCl_2 \cdot 2H_2O$ (pH 7.4) and a protease inhibitory solution consisting of 0.010 mM TPCK, 1 mg/mL α-2 macroglobulin, 1 mg/mL aprotinin and 1.0 mg/mL PMSF. The cells were slowly homogenized (50 strokes) and the homogenate was centrifuged in a 15 mL corning centrifuge tube for 10 minutes at 200 x g. The supernatant solution was discarded and the sedimented membranes were suspended in the lysis buffer. The membranes were then centrifuged in two, 25 mL Corex screw top tubes at 12,000 x g for 20 minutes. The resulting supernatant solutions were discarded and the sedimented membranes were saved. These were pooled and placed onto a 40% sucrose solution containing 40 mM imidazole and 4 mM EGTA (total volume 1.5 mL in a SW 40Ti centrifuge tube from Beckman). The remainder of the tube was filled with buffer consisting of 40 mM imidazole and 4 mM EGTA. This was then centrifuged for 90 minutes at 186,000 x g. The

opalescent membrane band at the sucrose buffer interface was then removed by careful suction and the pellet was discarded. Next, the membrane suspension was transferred to another SW 40Ti centrifuge tube and centrifuged at 186,000 x g for a minimium of 30 minutes. The pellet was suspended in imidazole buffer and protein concentration determined by the modified method of Lowry as described earlier (88).

2.19 Extraction of Lipids From Chinese Hamster Ovary Cells;

CHO cells were grown in large roller bottles and harvested as described earlier for the C6 glioma cells. Lipids were extracted by suspending the CHO cell pellet in 20 volumes of chloroform-methanol (2:1,v/v). The cell suspension was then centrifuged at 500 x g for 10 minutes and the supernatant solution removed and retained. The cell pellet was suspended in 20 volumes of chloroform-methanol (1:2, v/v) containing 5% water. This solution was centrifuged at 500 x g for 10 minutes and the supernatant solution was pooled with the first supernatant solution. The cell pellet was again suspended with twenty volumes of chloroform-methanol (1:2, v/v). The suspension was centrifuged at 500 x g for 10 minutes and the supernatant solution was pooled with the others. The chloroform-methanol was then

removed from the pooled supernatant solutions using a rotary evaporator. The dried lipids and glycolipids were dissolved in chloroform : methanol (2:1) and stored at -20°C until use.

2.20 Binding of Pertussis Toxin and Cholera Toxin to Chinese Hamster Ovary Lipids on Cellulose Coated Sheets;

A cellulose-coated plastic sheet was cut into 4 strips 2 cm wide and 10 cm long. Ten µg of ganglioside G_{M1} in chloroform : methanol (2:1) was applied to the strips such that the resulting spot was no larger than 2 mm in diameter. A second application of G_{M1} was made to the strips 3 cm away from the first spot. An aliquot of lipid extracted from the CHO cells (approx. 10 µg) was applied directly on top of this second G_{M1} spot. A third application consisting of CHO cell glycolipids was also made to the strips. The strips were then incubated with 3% BSA in a large petri-dish in the presence or absence of 10,000 fold molar excess of fetuin. The strips were then incubated with ^{125}I-PT or ^{125}I-CT overnight at 4°C and then washed with 0.85% NaCl solution 4 times to remove any unbound labelled toxin. The strips were dried and subjected to autoradiography as described earlier.

2.21 ^{125}I Surface Labelling of Goose Erythrocytes and Chinese Hamster Ovary Cells:

Two hundred µL of fresh packed goose erythrocytes were suspended in 100 µL of 0.33 M sodium phosphate (pH 7.2). This was incubated with 20 MBq of sodium ^{125}I in an iodo-gen coated tube for five minutes. Two hundred µL of 1 mg/mL cysteine in PBS was then added and the iodinated cells were washed seven times with PBS by centrifugation at 200 x g for 5 minutes at room temperature to remove unreacted ^{125}I. CHO cells were grown in Ham's F12 medium supplemented with 5% fetal calf serum in an atmosphere of 5% CO_2 at 37°C in two 150 cm^2 tissue culture flasks until confluent. The growth medium was removed and the cells were lifted from the plastic surface using 10 mL of a solution consisting of 137.0 mM NaCl, 5.0 mM KCl, 5.60 mM glucose, 1.0 mM EGTA and 5.0 mM Hepes (pH 7.4). The cells were incubated in this solution at 37°C until lifted. They were then placed into a 15.0 mL Corning centrifuge tube and centrifuged in a table top centrifuge (IEC model centra 4 centrifuge) at 200 x g for 10 minutes. The cells were then transferred to an iodogen tube and 20.0 MBq of sodium ^{125}I was added. This was incubated for 5 minutes. Two hundred µL of 1 mg/mL cysteine in PBS was then added and the surface iodinated

CHO cells were washed 7 times with PBS by centrifugation at 200 x g for 5 minutes to remove unreacted ^{125}I.

2.22 Use of Pertussis Toxin Antibody to Isolate Receptors from Goose Erythrocyte Membranes;

Antibody specific to pertussis toxin was prepared by immunizing rabbits with an emulsion of 100 µg of pertussis toxin in 1.0 mL incomplete Freud's adjuvant, distributed equally into each hind footpad. Twenty-one days latter, the rabbits recieved a booster dose of 10 µg of pertussis toxin in 100 µL adjuvant, distrubted equally into each popliteal lymph node. Rabbits were bleed eleven days later and the sera was tested for precipitation with pertussis antibody in gel diffusion tests. Sera giving good reactions in the precipitation test was pooled and absorbed with packed organisms from the avirulent strain of B. pertussis, Sakairi, and the IgG fraction isolated by a protein A-Sepharose column (119). The anti-pertussis toxin IgG was shown to be free of anti-FHA.

Two hundred µL of freshly surface iodinated goose erythrocytes were dissolved in buffer consisting of 50.0 mM Tris-HCl pH 8.1, 110.0 mM sodium chloride, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.10% Triton X-100, 0.010 M TPCK, 1.0 mg/mL α-2 macroglobulin, 1.0 mg/mL aprotinin and 1.0 mg/mL PMSF. This mixture was incubated for 30 minutes at

37°C. The solubilized membranes were then centrifuged at 100,000 x g for one hour. If the resulting supernatant solution did not contain at least 2/3 of the total ^{125}I counts, the pelleted membrane material was solubilized in buffer containing 0.2% Triton X-100 and the solubilization procedure was repeated. Next, the solubilized membrane proteins were incubated in a 1.5 mL Eppendorf tube with 50 µg of pertussis toxin for six to eight hours at 4°C. Seventy µg of pertussis toxin antibody was then added and left to bind to the pertussis toxin overnight at 4°C. Two hundred µL of Protein A-Sepharose (from Pharmacia) was then added and this was incubated for two hours at room temperature on an end over end table top rotator. The gel slurry was transferred to a glass wool plugged pasteur pipet and washed with 10 volumes of Tris-HCl pH 7.4. The antibody-antigen complexes were eluted from the Protein A with 0.1 M acetic acid and then neutralized with sodium hydroxide. The protein A bound counts were analysed by SDS-PAGE and subjected to autoradiography as described earlier.

2.23 Preparation of Pertussis Toxin Affinity Columns;

Pertussis toxin was covalently attached to activated CH-Sepharose 4B in a ratio of 100 µg of toxin

to 0.033 g of activated CH-Sepharose 4B according to the manufacturer's (Pharmacia) instructions. This gave a final gel volume of 100 µL. Briefly, the activated CH-Sepharose was swollen in ice cold 1 mM HCl for 15 minutes and then washed with 200 mL of 1 mM HCl on a sintered glass funnel. The pertussis toxin was dissolved in 0.1 M sodium bicarbonate (pH 8.0) (coupling buffer) or dialyzed into this buffer overnight. The pertussis toxin suspension was mixed with the activated CH Sepharose 4B overnight at 4°C on a table top rotator. After the coupling reaction, the suspension was placed into a glass wool plugged pasteur pipet and unbound toxin was removed with the bicarbonate coupling buffer. To be certain that the pertussis toxin bound to the Sepharose, a goose erythrocyte hemagglutination assay was done using the solution washed from the Sepharose column. No hemaaglutination by pertussis toxin was dectable in this solution indicating that 100% of the toxin was coupled to the Sepharose. The remaining active ester groups were blocked b treatment with 1 M ethanolamine (pH 9) for 1 hour. The columns were then washed alternately with 20 volumes of 0.1 M acetate buffer (pH 4) and 0.1 M Tris-HCl buffer (pH 8) containing 0.5 M NaCl five times. The columns were stored with 0.1% azide at 4°C until use. Experimental

control columns consisting of ethanolamine-inactivated CH Sepharose 4B were also prepared.

2.24 Use of Pertussis Toxin and WGA Affinity Columns to Isolate Receptors in Goose Erythrocytes and Chinese Hamster Ovary Cells;

The affinity columns were washed with PBS and pre-blocked with 0.1% BSA for one half hour to prevent non-specific interactions of puntative receptors with the Sepharose. Next, the 0.1% BSA was allowed to drain out and 200 µL of the solubilized ^{125}I surface labelled goose erythrocytes or ^{125}I surface labelled CHO cells or ^{125}I fetuin or ^{125}I ceruloplasmin was added to the columns. The labelled proteins were incubated with the gel for one half hour. After binding, the columns were washed with 3 to 4 mL of 50 mM Tris-HCl (pH 7.4) until background counts were consistent. Bound material was eluted with 0.1% Triton X-100 containing 100 µg of fetuin or 100 µg of α-1 acid glycoprotein in ddH_2O, or 200 µL of 50 mM diethanolamine in 0.15 M NaCl (pH 11.5). The ^{125}I fetuin and the ^{125}I ceruloplasmin fractions were counted in a gamma counter. The diethanolamine and NaCl in the goose erythrocyte and CHO cell fractions was dialyzed away overnight and replaced with 0.1% SDS. This was necessary to prevent the salt from becoming concentrated in the next step and interfering with the

SDS-PAGE analysis. The fractions are then concentrated to dryness in a Savant model SVC-100H Speed Vac Concentrator and dissolved in 50 µL of Laemmli sample buffer. They were then analysed by SDS-PAGE. The gel was dried and autoradiography was perform as described earlier.

2.25 Comparison of Pertussis Toxin Subunits S_2 and S_3 Amino Acid Sequences to the Amino Acid Sequence of WGA;

The analysis of sequence homologies between PTS_2, PTS_3 and WGA was done on a IBM model XT PC using the MicroGenie computer software.

2.26 Preparation of Transducin for Pertussis Toxin Mediated ADP-Ribosyltransferase Activity Measurements;

Transducin was purified according to the method of Kuhn (83). Freshly dissected bovine retinas were vigoursly agitated in a buffer solution consisting of 70 mM potassium phosphate (pH 7.5), 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 65 mM NaCl (buffer 1) containing 45% sucrose. The retinas were centrifuged for 10 minutes at 2000 x g. The supernatant solution was removed and the pelleted material was suspended in the same buffer using a 30 mL Wheaton glass tissue homogenzier and centrifuged for an additional 10 minutes at 2000 x g. The supernatant

solution was removed and pooled with the first. This solution was then diluted 3 times with buffer 1 and centrifuged at 13,000 x g for 40 minutes. The pellet was suspended in buffer 1 containing 24% sucrose. Next, the suspension was layered onto sucrose step gradients consisting of 11 mL of buffer 1 containing 34% sucrose, 9 mL of buffer 1 plus 30% sucrose and 9 mL of buffer 1 plus 26% sucrose and centrifuged at 140,000 x g for 30 minutes. The orange material at the 26%/30% sucrose interface was harvested and diluted 2.5 X with buffer 1. This was centrifuged at 27,000 x g for 20 minutes. The sedimented rod outer segments (ROS) were suspended using a 30 mL Wheaton glass tissue homogenizer in buffer 1 containing 12% sucrose and then diluted 10 X with 5.0 mM Tris-HCl (pH 7.5), 0.5 mM $MgCl_2$ and 1.0 mM DTT (buffer 2). This mixture was centrifuged at 27,000 x g for 50 minutes. The supernatant solution was discarded and the ROS pellet was washed once more. Again the supernatant solutions were discarded and the pellet was saved. Next, the pellet was suspended in buffer 2 containing 10 mM GTP and then centrifuged at 27,000 x g for 50 minutes. This step was repeated twice and the supernatant solutions containing transducin were pooled and concentrated to 10 mL using an Amicon Model 52

concentrator fitted with a PM30 (30,000 molecular weight cut off) filtration membrane.

2.27 ADP-Ribosylation of C6 Rat Glial Membranes;

The ADP-ribosylation procedure was similar to that of Katada *et al.* (78). The reaction was perfomed using 10 µL of 100 mM thymidine, 10 µL of 10 mM ATP, 10 µL of 1 M nicotinamide to inhibit endogenous ADP-ribosyltransferases and 5 µL of 100 mM DTT. ^{32}P-NAD was used at a concentration of 30 nM [α-^{32}P] NAD (0.185 MBq/assay) and the membrane concentration was 100 µg/assay. The entire reaction volume was brought up to 100 µL using 10 mM Tris buffer (pH. 7.4). The reaction was allowed to proceed at 37°C for one hour in a 1.5 mL Eppendorf tube and then terminated using 100 µL of 10% trichloroacetic acid. The precipitated protein was sedimented in an Eppendorf model 5413 centrifuge at 8,800 x g for 2 minutes. The supernatant solution was removed and the pellet was suspended in Laemmli sample buffer. The sample was neutralized with 0.1 N NaOH. The mixtures were heated for 10 minutes in a boiling water bath and analysed by SDS-PAGE. The gel was stained with Coomassie blue, destained, dried and subjected to autoradiography as described earlier.

2.28 ADP-Ribosylation of Transducin;

The ADP-ribosylation reactions were performed according to the method of Moss *et al.* (105) with a few modifications. Briefly, the reactions were carried out for 1 hour at 30°C in a total volume of 100 µL. The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 5 mM thymidine, 5 mM DTT, 10 mM GTP, 10 mM ATP, 0.5 mM DPPC, 200 µM transducin, 30 nM [α-^{32}P] NAD (0.185 MBq/assay). Pertussis toxin was preactivated with 100 mM DTT for 10 minutes prior to the assay. The reaction was stopped after one hour by the addition of 500 µL of 10% TCA. The solution was then centrifuged as described above and the precipitated protein was solubilized in 10% SDS or Laemmli sample buffer. Five mL of scintillation counting solution was added to the samples containing the 10% SDS. These were counted in a Beckman model LS 6800 liquid scintillation counter. The samples dissolved in Laemmli sample buffer were analysed by SDS-PAGE. After electrophoresis, the gels were stained with Coomassie blue, destained, dried and subjected to autoradiography as discussed earlier.

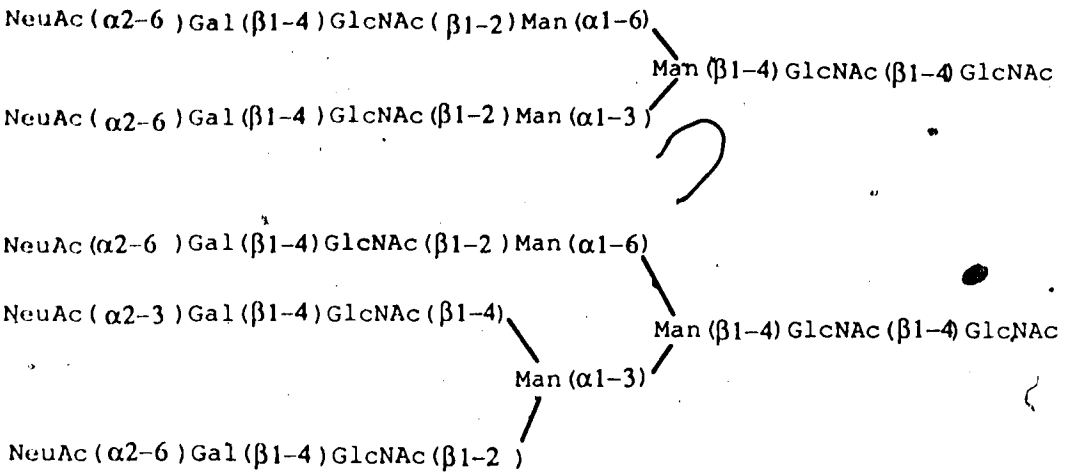
3.0 RESULTS

3.1 Investigation of PT Receptors in Goose Erythrocytes and Chinese Hamster Ovary Cells:

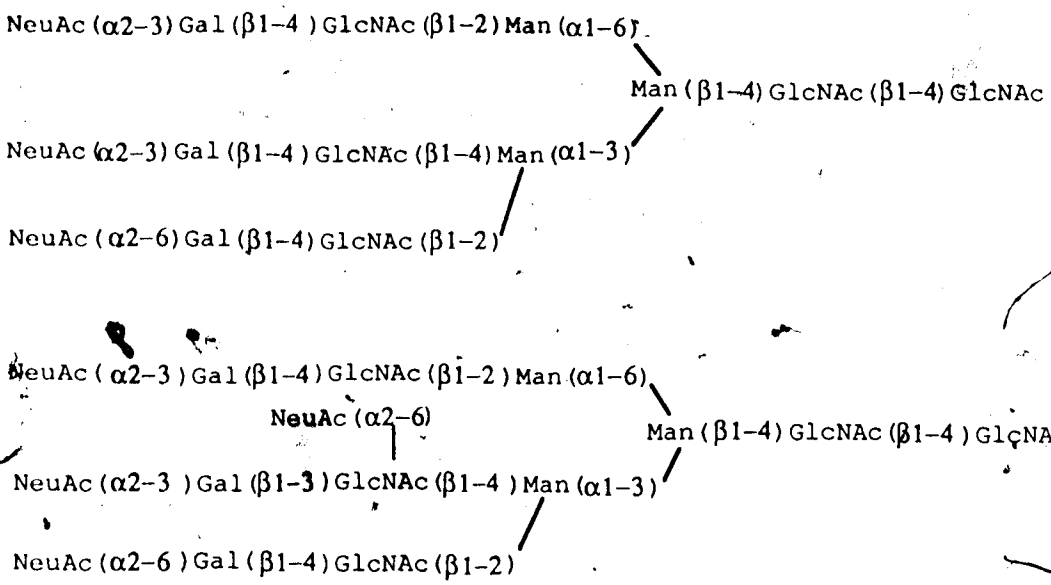
Western blots of goose erythrocyte membranes were incubated with ^{125}I labelled PT to identify receptors for pertussis toxin. When the membranes were incubated with fetuin-protected ^{125}I PT, two sets of protein bands were observed (fig. 6). One set of bands had molecular weights ranging from 36,000 to 31,000. A second set of proteins displayed molecular weights ranging from 18,000 to 14,400. Another group of proteins in the 93,000-62,000 molecular weight range were only observed when freshly prepared goose erythrocyte membranes were used in the experiment. These protein bands were not observed when the goose erythrocyte membranes were stored at minus 70°C for a period longer than one week. It has also been established that PT binds to the oligosaccharide domains of glycoproteins such as fetuin. As expected, ^{125}I-labelled PT bound to fetuin on western blots (fig. 6). Previously it was reported that PT bound to GlcNAc groups in fetuin's asn-linked oligosaccharide (see fig. 5) (133). More recently, it was shown that terminal sialic acid groups in fetuin's oligosaccharide units were also important for PT binding activity

Figure 5. Diagram illustrating the asparagine-linked carbohydrate structures of ceruloplasmin, fetuin and α-1 acid glycoprotein. (37,134,139).

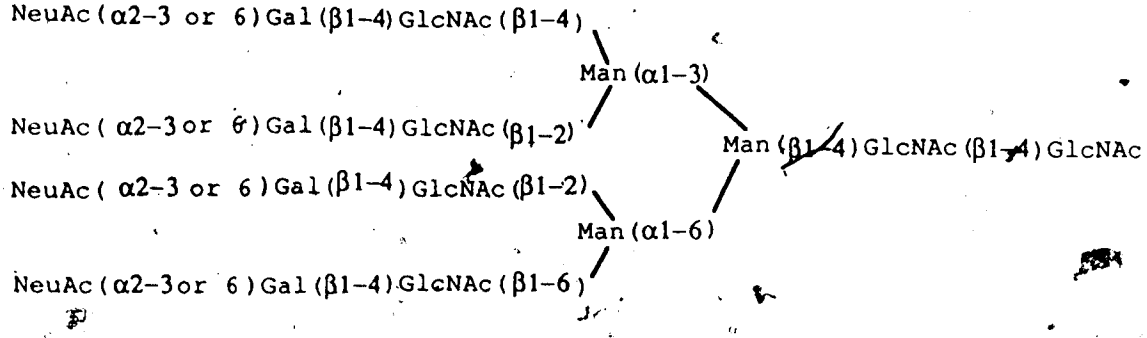
CERULOPLASMIN



FETUIN



α-1 ACID GLYCOPROTEIN



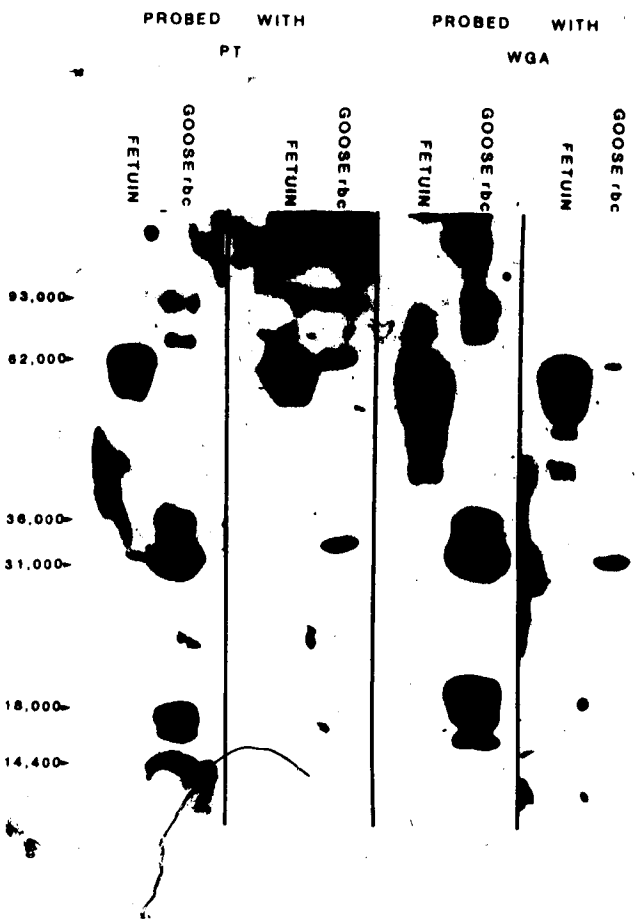


Figure 6. A composite photograph of western blots probed with ^{125}I PT and ^{125}I WGA. Each blot contains 100 µg of goose erythrocyte membranes and 5 µg of fetuin. The blots are in duplicate to illustrate the inconsistencies in banding patterns which were observed when different membrane preparations and labelled probes were used. Also, the blots illustrate the similarities seen when one blot is probed with ^{125}I PT and another with ^{125}I WGA. The molecular weights of the proteins that bind ^{125}I PT and ^{125}I WGA are indicated at the left side of the figure and are for the first panel only.

(7,18). These observations suggested that PT's lectin-like properties were similar to those of WGA. The suggestion was supported by the observation that ^{125}I-labelled WGA and ^{125}I-PT bound to the same goose erythrocyte proteins (fig. 6).

I thought different lectins would be useful for further establishing the specificity of PT and WGA for the goose erythrocyte receptors. PNA was choosen because of its specificity for β-D-Gal(1-3)β-D-GalNAc present in O-linked oligosaccharides. ^{125}I-PNA also bound to similar bands as PT and WGA (fig. 7). In competition binding studies, galactose was unable to inhibit the binding of ^{125}I-PNA plant lectin to the lower molecular weight goose erythrocyte proteins (fig. 7). High concentrations of galactose are known to block the interaction of PNA with its receptors. Fetuin and α-1 acid glycoprotein were also subjected to SDS-PAGE and blotted onto nitrocellulose. The α-1 acid glycoprotein, like fetuin, contains sialic acid residues and I was interested in determining whether ^{125}I pertussis toxin would bind to this protein with the same intensity as to fetuin or any of the goose erythrocyte proteins. It was determined that the minimum inhibitory concentration of fetuin in the PT-mediated goose hemagglutination assay was 0.3 ± 0.2 μM (n=11). However, the minimum inhibitory

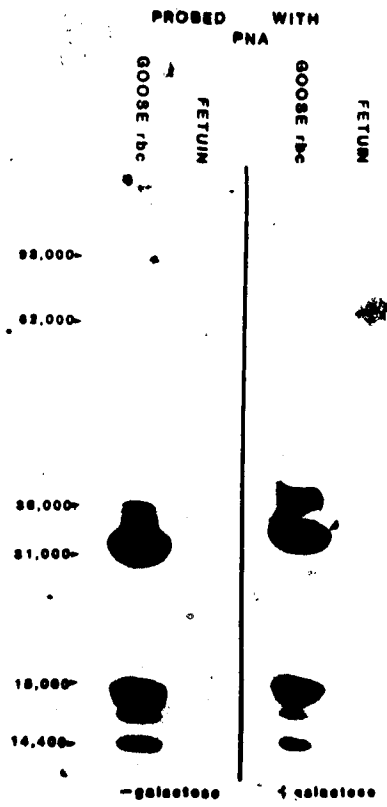


Figure 7. Western blot of 100 µg of goose erythrocyte membranes and 5 µg of fetuin. ^{125}I PNA is used as the radiolabelled probe. The ^{125}I PNA binds to similar protein bands in goose erythrocyte membranes as does PT and WGA but it does not bind to fetuin. The blots were incubated with ^{125}I-PNA probe in the presence and absence of 10,000 x molar excess galactose. The molecular weights of the visible proteins are also given at the left of the figure.

concentration of α-1 acid glycoprotein was 13.5 ± 0.5 μM (n=4). When the western blots were incubated with ^{125}I-PT, the resulting autoradiograms demonstrated that the α-1 acid glycoprotein bound ^{125}I pertussis toxin with the same intensity if not higher than that of fetuin (see fig 8.). Therefore, the results of my investigations suggested that the western blotting procedure was unable to distinguish between low and high affinity PT receptor species. This difficulty was compounded by different amounts of each erythrocyte receptor species applied to the gels. Low affinity binding to the lower molecular weight erythrocyte receptors may have been exaggerated because of the large amount of protein in these bands (fig. 9). Binding to the high molecular weight receptors, although less prominent, may represent higher affinity interactions with species representing a minor fraction of goose erythrocyte protein. In coomassie blue stained gels, the lower molecular weight species clearly represented the bulk of the protein material in goose erythrocyte membranes. In contrast, the higher molecular weight receptors were less intensly stained with coomassie blue. Alternately, binding to the lower molecular weight species in western blots may have been the result of ionic or hydrophobic protein-protein interactions which

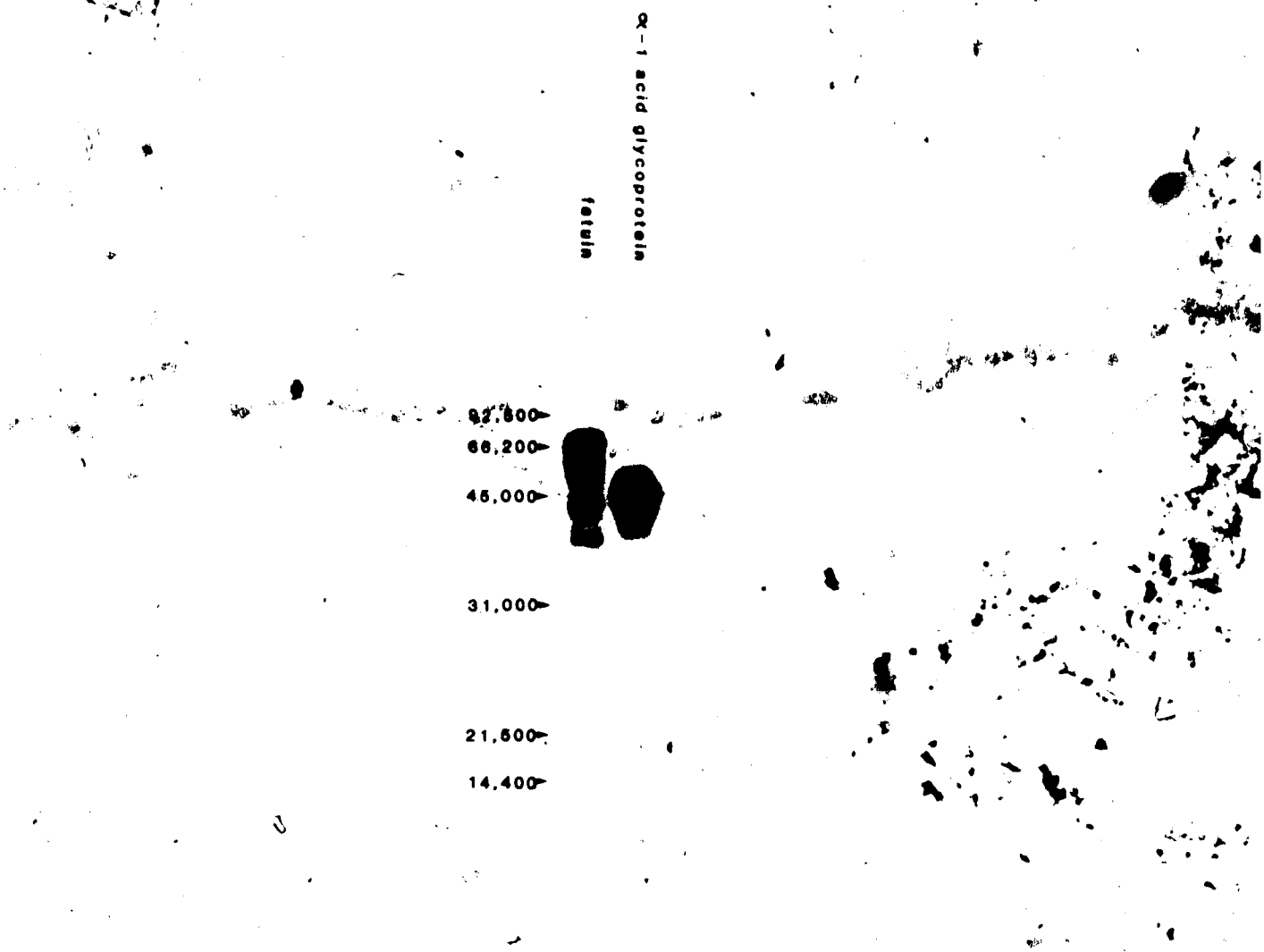


Figure 8. Western blot of fetuin and α-1 acid glycoprotein. Five µg of fetuin and 5 µg of α-1 acid glycoprotein were transferred onto nitrocellulose and probed with fetuin agarose protected ^{125}I PT. The probe was sonicated in a water bath sonicator for 20 seconds and filtered through a 0.22 µm filter to break up PT aggregates before being added to the blot.

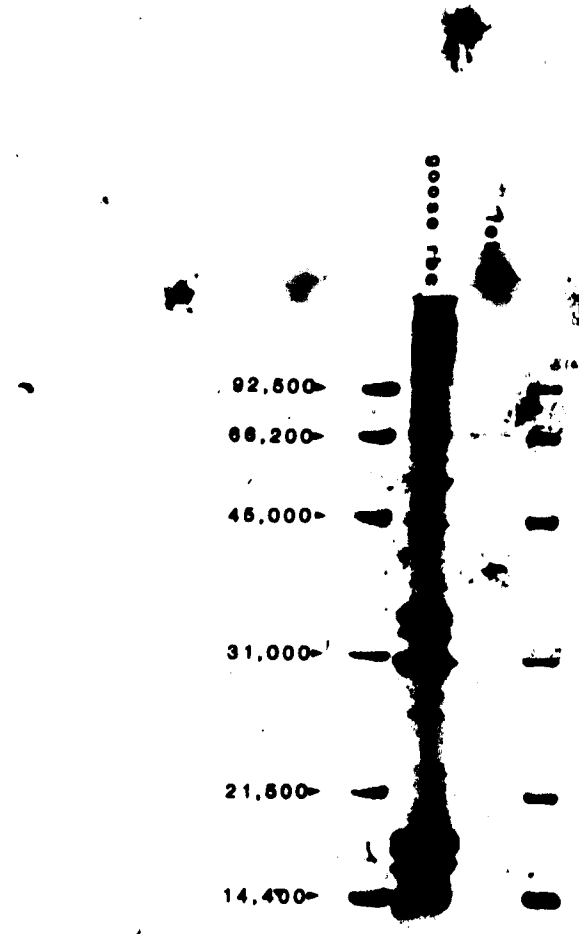


Figure 9. Coomassie blue stained SDS-PAGE of molecular weight standards, 100 µg of goose erythrocyte membranes and 5 µg of fetuin. The molecular weight standards are on both sides of the figure.

were unrelated to the receptor binding domains of the lectin probes. The inability to compete for PNA binding to low molecular weight receptors with galactose indicated there may be some merit to this alternate conclusion.

Another problem encountered with the western blotting procedure was the inability to obtain autoradiograms with low amounts of background binding to regions not containing SDS-PAGE separated proteins. To remedy this problem different blotting membranes were used and different blocking agents were also tried. These included polyvinylpyrrolidone in concentrations of 1% and 3% and the ionic detergent nonident P-40 at a concentration of 0.5%. Even periodate destruction of potentially contaminating glycoproteins in the BSA was performed. None of these modifications were able to reduce the background to any significant extent. A procedure which resolved the problem consisted of first sonicating the ^{125}I pertussis toxin for 30 seconds in a water bath sonicator and then filtering the toxin through a 0.22 µm filter. This may have served to break up the aggregates of pertussis toxin before it was added to the blot (fig. 8).

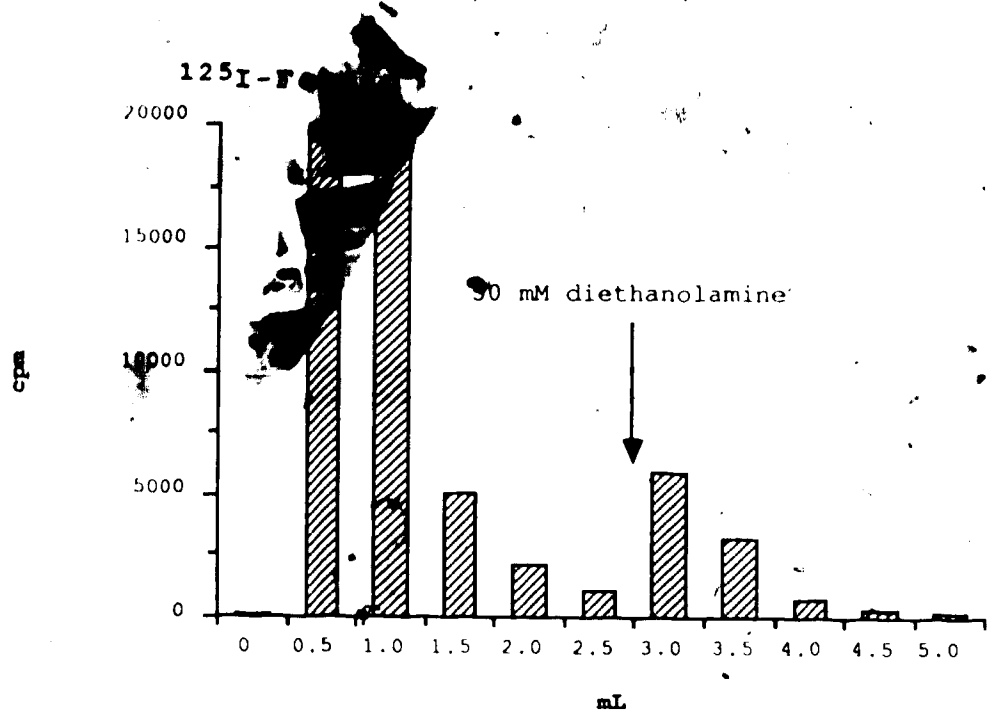
3.1.1 Pertussis Toxin-Antibody Immuno-Affinity Chromatography:

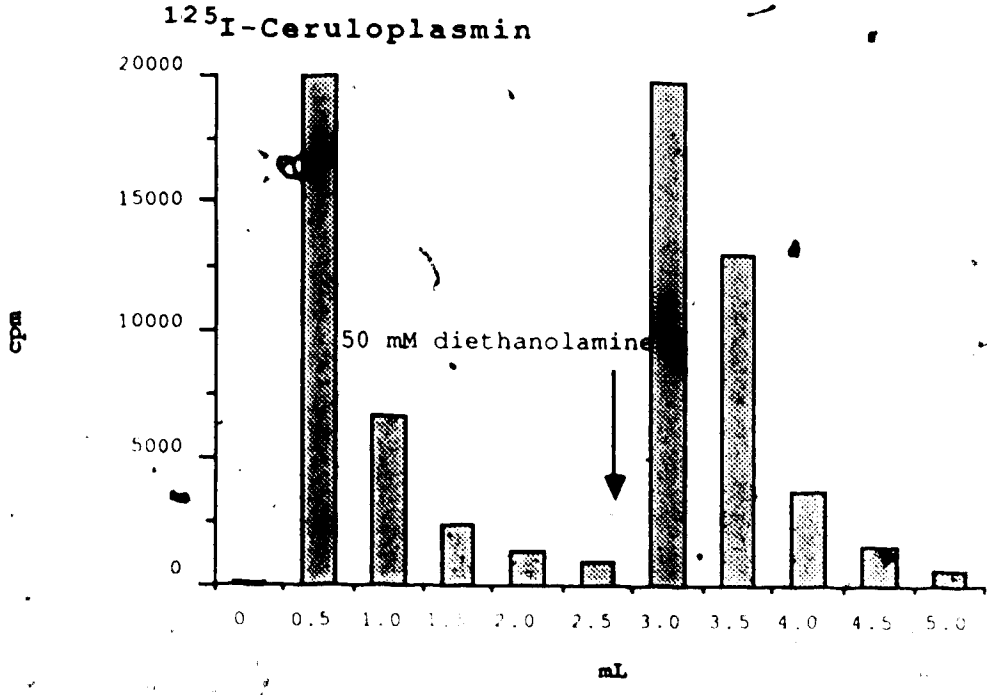
Because of the ambigous results obtained with the western blotting procedure it was decided to try and identify receptors using an affinity chromatography approach. Immunoaffinity chromatography was chosen as an initial approach to the problem because the procedure required only small amounts of purified PT. ^{125}I surface labelled goose erythrocytes solubilized in Triton X-100 were incubated with pertussis toxin. After incubation, pertussis toxin specific antibody was added to the [redacted]on to form antibody-antigen-receptor complexes. Complexes were then collected on protein A-Sepharose and analysed by SDS-PAGE. However, it was not possible to identify PT receptors which bound to protein A-Sepharose only in the presence of PT-specific antibody. To overcome this problem, it was felt that a direct approach was required. This involved the attachment of PT directly to activated CH-Sepharose 4B.

3.1.2 Binding of ^{125}I Fetuin and ^{125}I Ceruloplasmin to PT Affinity Columns:

^{125}I labelled fetuin and ^{125}I labelled ceruloplasmin were applied to pertussis toxin affinity columns to examine the functional integrity of receptor binding domains of Sepharose-bound PT (fig. 10). Ceruloplasmin,

Figure 10. Binding of ^{125}I-fetuin and ^{125}I-ceruloplasmin to PT-Sepharose columns. PT-Sepharose was prepared as described in Materials and Methods. Radiolabelled fetuin and radiolabelled ceruloplasmin were applied to two separate PT-Sepharose columns (50,000 cpm ea.) and allowed to bind for 30 min.. The unbound material was eluted with, 50 mM diethanolamine. The fractions were collected and counted in a gamma counter.


125I-F
20000
15000
10000
5000
0
cpm
50 mM diethanolamine
0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
mL


125I-Ceruloplasmin
20000
15000
10000
5000
0
cpm
50 mM diethanolamine
0 0.5 1.0 2.0 2.5 3.0 3.5 4.0 4.5 5.0
mL

like fetuin, is a serum glycoprotein containing terminal sialic acid residues. It has been previously demonstrated that ceruloplasmin is able to inhibit PT mediated goose erythrocyte agglutination at a lower concentration than fetuin (7). Therefore, I was interested in determining which glycoprotein bound the best to the PT-Sepharose. When ^{125}I-fetuin was applied to the PT affinity columns, 14.20% of the counts applied to the columns were eluted with diethanolamine. The observation that so few ^{125}I-fetuin counts were bound to the PT affinity columns was not unexpected since a similar result was obtained when ^{125}I-fetuin was passed through WGA-agarose. Fetuin oligosaccharide units are not homogenous and consequently not all fetuin molecules may be capable of binding to PT. When ^{125}I ceruloplasmin was applied to the PT-Sepharose columns, almost 50.0% of the counts were eluted with 50.0 mM diethanolamine. This indicated that more of the ceruloplasmin is able to bind to the PT affinity columns. The possibility that PT has a higher affinity for ceruloplasmin than for fetuin in the PT-Sepharose column was not unrealistic because the minimum inhibitory concentratin of ceruloplasmin was lower than fetuin in the goose hemagglutination assay (7).

3.1.3 Affinity Chromatography Using Pertussis Toxin Affinity Columns:

^{125}I surface labelled goose erythrocyte membranes were solubilized in Triton X-100 as described in Materials and Methods. The insoluble material was sedimented by centrifugation and the remaining soluble material was applied to a PT-Sepharose column, a WGA-Sepharose column and a blank Sepharose column. (fig. 11). All three columns were eluted with 50 mM diethanolamine (pH 11.5). The only protein band that binds to all three column types (nonspecific) has a molecular weight of 10,000. The proteins that bind to the PT-Sepharose are almost identical to those that bind to the WGA-Sepharose. A protein with a higher molecular weight than 92,500 binds with to both the PT and WGA-Sepharose columns. The molecular weight of this protein can not be determined with accuracy on a linear gel because it is higher than the largest molecular weight standard. Five other proteins also bind to both PT-Sepharose and WGA-Sepharose. These have molecular weights of 81,000, 55,000, 49,000, 27,000 and a protein band of 19,000 daltons which is of weak intensity. One band is specific for the WGA-Sepharose. It has a molecular weight of 66,200 and is as intense as the 81,000 protein band. The large bands seen at the bottom

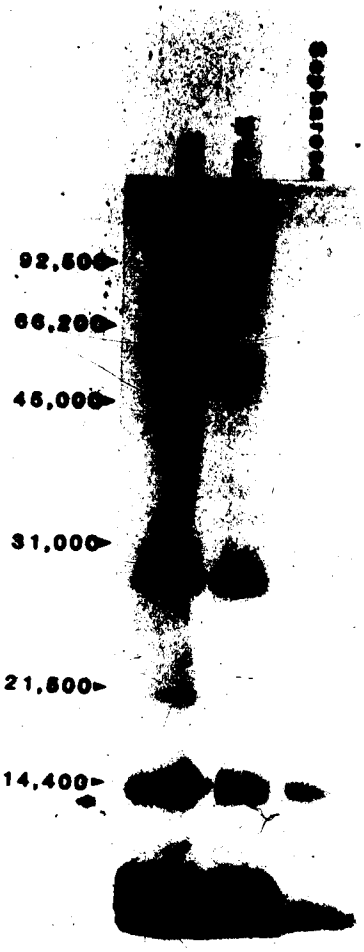


Figure 11. Comparison of ^{125}I surface labelled and solubilized goose erythrocytes eluted from a PT affinity column, a WGA affinity column and a blank Sepharose column. One hundred µg of PT was attached to CH-Sepharose 4B as was 100 µg of WGA. Equal numbers of cpms were applied to all three columns and the bound material was eluted with 50 mM diethanolamine pH 11.5. The eluted fractions were dialyzed and dried. Laemmli sample buffer was added and the fractions subjected to SDS-PAGE. Autoradiography was then performed.

of the SDS-PAGE have progressed beyond the dye front indicating that they are proteins of a very low molecular weight or some other ^{125}I labelled cellular constituent that is not protein in nature or free ^{125}I. Because goose hemagglutination assays had previously demonstrated that fetuin is able to inhibit PT-mediated hemagglutination and α-1 acid glycoprotein is not, I decided to determine whether either of these glycoproteins could elute any of the ^{125}I-surface labelled and solubilized goose erythrocyte proteins. For comparison, one PT-Sepharose column was also eluted with 50 mM diethanolamine (pH 11.5) (fig. 12). The 50 mM diethanolamine essentially eluted off the same protein bands as seen in figure 11 however, differences were detected when 100 μg of fetuin or 100 μg of α-1 acid glycoprotein were used as eluents. The fetuin elutes all the same proteins as does the diethanolamine with the exception of one protein with a molecular weight of approximately 92,500. The α-1 acid glycoprotein elutes off the same proteins as the fetuin and diethanolamine with the exception of the 92,500 dalton protein and a 49,000 dalton protein which is eluted with either fetuin or diethanolamine. The intensity of the protein bands also varies depending on which eluent is used. The proteins eluted from the column with the diethanolamine

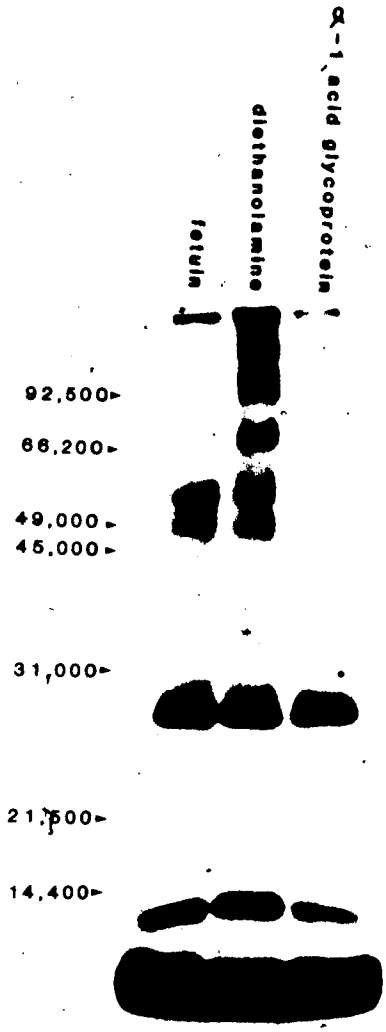


Figure 12. Elution of PT affinity columns with fetuin, diethanolamine and α-1 acid glycoprotein. ^{125}I surface labelled goose erythrocytes were solubilized with Triton X-100 and then passed through columns containing 100 µg of PT attached to CH-Sepharose 4B. After washing away unbound material, the affinity columns were eluted with either 100 µg of fetuin, 100 µg α-1 acid glycoprotein or 50 mM diethanolamine. The eluted fractions were dialyzed, dried, subjected to SDS-PAGE and autoradiography.

display more intensity on the autoradiogram than the proteins eluted with fetuin. The proteins eluted with α-1 acid glycoprotein have the weakest intensity of the three. This is not unexpected knowing that fetuin competes more efficiently for PT binding sites in the goose hemagglutination assay than does α-1 acid glycoprotein. The 49,000 dalton protein selectively eluted off the PT-Sepharose column with fetuin is the best candidate for being the PT-receptor on goose erythrocytes of all of the other proteins seen on the autoradiogram.

3.2 BINDING ACTIVITY OF PERTUSSIS TOXIN TO CHINESE HAMSTER OVARY CELLS

3.2.1 Binding of ^{125}I Pertussis Toxin to Chinese Hamster Ovary Cells:

I also wished to characterize PT receptors in the CHO cell system in which a physiological response to the toxin could be measured. Upon addition of native pertussis toxin CHO cells undergo a unique morphological change (55).

The binding of pertussis toxin to CHO cells can be demonstrated through the use of ^{125}I-PT prepared in the presence of fetuin-agarose (6). To determine the rate of

association of the toxin to CHO cell monolayers, a time course binding assay was performed (fig. 13) using the highest concentration of PT which could be obtained from the iodination procedure. This was done to determine the initial rate of association at this concentration. The data displayed in figure 14 demonstrated that the association rate was linear for approximately 5 minutes after which time the rate declined until the binding reaction reached equilibrium. When the initial association rates of ^{125}I-PT were plotted against concentration a straight line was obtained whose slope represented a K_a of $5.6 \pm 2.3 \times 10^{-3}$ min^{-1} (fig 14). The linear relationship between the concentration of PT and initial association rate indicated that PT binding to CHO cells is a second order reaction.

3.2.2 Inhibition of Pertussis Toxin Chinese Hamster Ovary Cell Activity by Fetuin:

An experiment was performed to determine if fetuin could compete with CHO cell receptors for pertussis toxin. Only at extremely high concentrations of fetuin was any inhibition of CHO cell activity seen. The lowest concentration at which fetuin was able to inhibit pertussis toxin activity in CHO cells was 50,000 x greater than that of pertussis toxin. In comparison, fetuin is able to completely inhibit goose erythrocyte

Figure 13. Binding time course of fetuin-agarose protected ^{125}I-PT to CHO cell monolayers. Binding experiments were performed at 4°C on confluent CHO cell monolayers. Approx. 20,000 cpm of ^{125}I-PT was added to the wells at the beginning of the experiment. After the times indicated, unbound ^{125}I-PT was removed and the monolayers were solubilized in 10% SDS to determine the amounts of bound ^{125}I-PT. Binding at each time point was performed in triplicate and the error bars represent the standard deviation of the mean.

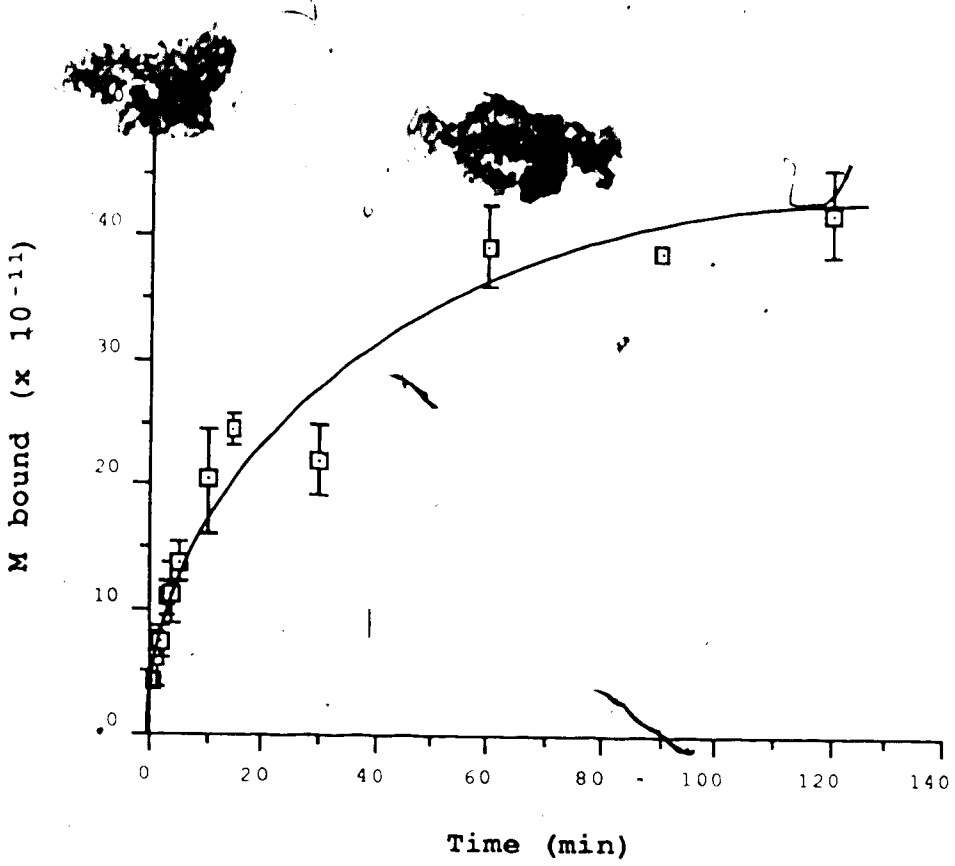
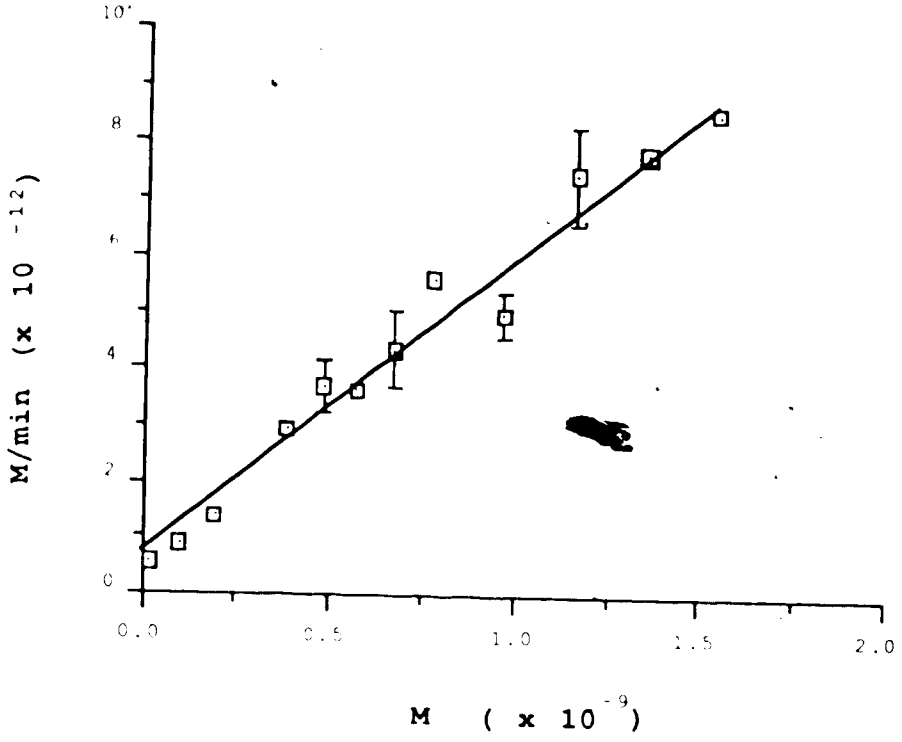

M bound (x 10⁻¹¹)
40
30
20
10
0
0
20
40
60
80
100
120
140
Time (min)

Figure 14. CHO cell association rate. The association rate was determined at 4°C by allowing increasing amounts of fetuin-agarose protected ^{125}I-PT to bind to CHO cells for 5 minutes. Unbound ^{125}I-PT was removed and the monolayers were solubilized in 10% SDS to determine the amounts of bound PT. Binding at each concentration of PT was performed in triplicate and the error bars represent the standard deviation of the mean.


M/min (x 10 -12)
10
8
6
4
2
0
0.0
0.5
1.0
1.5
2.0
M (x 10 -9)

agglutination at a concentration 30 fold in excess of PT (7). This indicated CHO cells contain receptors which have a higher affinity for PT than fetuin or goose erythrocytes.

3.2.3 Cross-linking of PT to Chinese Hamster Ovary Receptors:

A cross-linking procedure was employed to identify PT receptors in CHO cells. The cross-linker, SASD, was choosen because it has a cleavable disulfide bond between the two functional groups (fig. 4). It can also be iodinated between the azido and hydroxyl moieties of the phenyl ring thus providing an indirect method of iodinating the receptor proteins into which the azido group can insert. Vero cells do not display morphological change in the presence of PT. To determine if the insensitivity was at the level of receptors, PT complexed with SASD was applied to Vero cells which acted as a negative control.

After iodination and reacting SASD with PT, TCA precipitation indicated that 55% of the ^{125}I-SASD had inserted into protein. A hemagglutination assay using chymotrypsin treated goose erythrocytes was done to be certain that the insertion of the succinimidyl group on the cross-linker into the toxin had not destroyed the toxin's ability to bind. The toxin titred out to an

equivalent amount of native toxin indicating that at least the goose erythrocyte receptor binding site had not been compromised by the insertion of the cross-linker. However, the results of the cross-linking experiment (fig. 15) showed that introduction of the ^{125}I-labelled azido group into CHO cell proteins did not occur. The only bands visible on the autoradiogram were those of the five subunits belonging to pertussis toxin.

3.2.4 PT Binding to Chinese Hamster Ovary Cell Glycolipids:

A dot blot assay was used to determine whether glycolipids could function as receptors for pertussis toxin in CHO cells. Lipids were purified from CHO cells using a chloroform-methanol extraction procedure (see Materials and Methods) and applied to cellulose coated plastic sheets (fig. 16). This was done in quadruplicate. Fetuin-agarose ^{125}I-labelled PT was used to probe two blots and ^{125}I cholera toxin was used to probe the other two. Ten thousand fold molar excess fetuin was added to one blot for each of the toxins to determine whether it would be able to inhibit lipid-toxin interactions. The autoradiograms of the dot blots showed that the cholera toxin binds well to the samples containing G_{M1} and to the purified CHO cell lipids but pertussis toxin failed to bind at all to any of the lipids. The cholera toxin

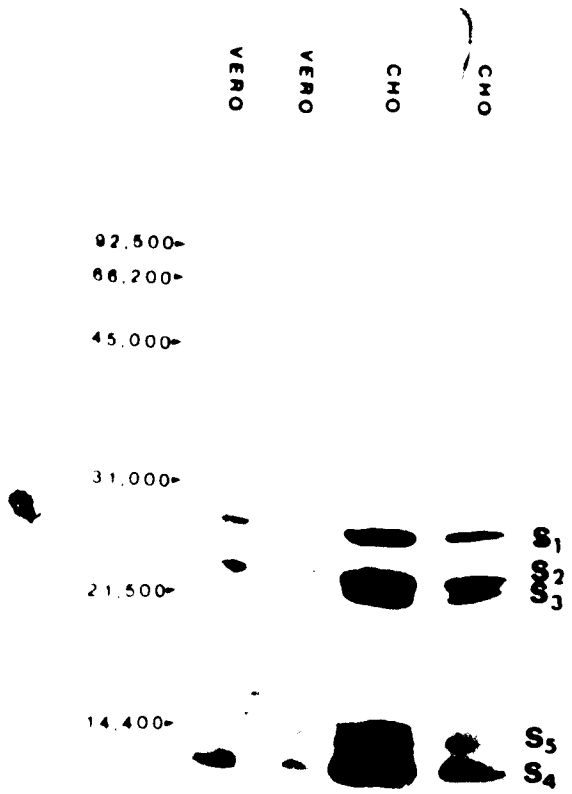


Figure 15. Cross linking PT to receptors in CHO and Vero cells. ^{125}I radiolabelled cross linker SASD was reacted with PT and the mixture applied to monolayers of CHO and vero cells at 4°C in the dark in duplicate. The cells were then washed before exposure to remove unbound PT and then exposed to ultraviolet light (366 nm) for 10 minutes. The monolayers were solubilized in Laemmli sample buffer and subjected to SDS-PAGE. The gel was then dried and subjected to autoradiography.

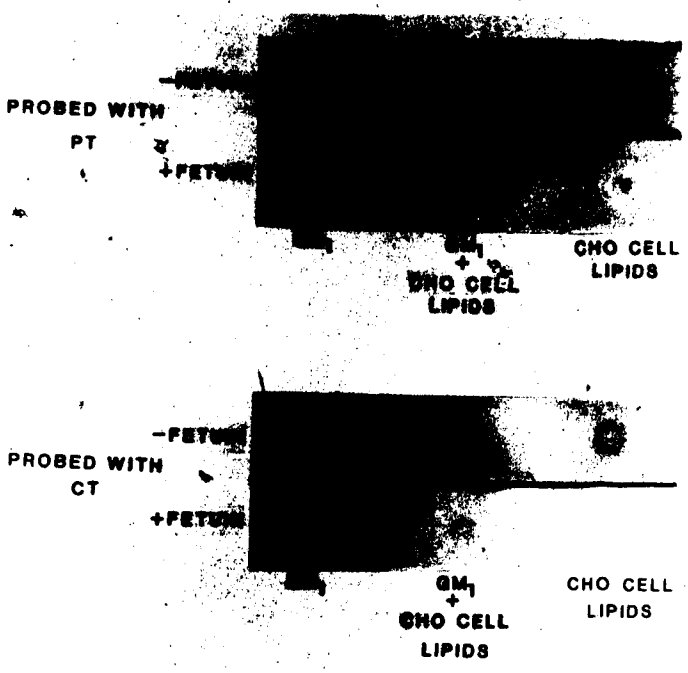


Figure 16. Comparison of the abilities of ^{125}I PT and ^{125}I CT to bind to ganglioside G_{M1} and CHO cell lipids. CHO cell lipids were purified as described in Materials and Methods. CHO cell lipids and ganglioside G_{M1} were spotted onto cellulose-coated plastic sheets in the positions indicated in the figure. The cellulose sheets were then incubated in a 3% BSA solution plus or minus fetuin. ^{125}I-PT or ^{125}I-CT was then added to the cellulose sheets while incubating in the BSA. The concentration of fetuin used was 10,000 fold molar excess in relation to the amount of ^{125}I-toxin used. The toxins were incubated with the cellulose sheets overnight and then washed to remove unbound toxin. The sheets were dried and autoradiography was performed for 48 hours.

binding to G_{M1} is also only slighty inhibited in the presence of fetuin. This result was in agreement with the known binding affinities of CT for fetuin and G_{M1} (33). This experiment strongly indicates that pertussis toxin does not use glycolipids as receptors in the CHO cell system.

3.2.5 Pertussis Toxin Affinity Chromatography:

^{125}I surface labelled CHO cells which had been solubilzed in 0.1% Triton X-100 was applied to three columns consisting of 1) 100 µg of pertussis toxin covalently attached to activated CH Sepharose 4B, 2) 100 µg of wheat germ agglutinin covalently attached to agarose beads and 3) a column consisting of activated CH Sepharose 4B treated with 0.1 M ethanolamine for 1 hour to destroy the activated ester groups. The wheat germ agglutinin was used to determine whether this lectin had a similar affinity for the same proteins in CHO cells as pertussis toxin. The bound material was eluted off using 50 mM diethanolamine pH 11.5 with 0.1% Triton X-100. The resulting autoradiogram (fig. 17) shows that there are a number of proteins that bind to all three column types, pertussis toxin, WGA, and Sepharose. What is significant though, is that there are predominatly 2 PT-binding species. The lower molecular weight protein is approximately 66,200 daltons. The molecular weight of

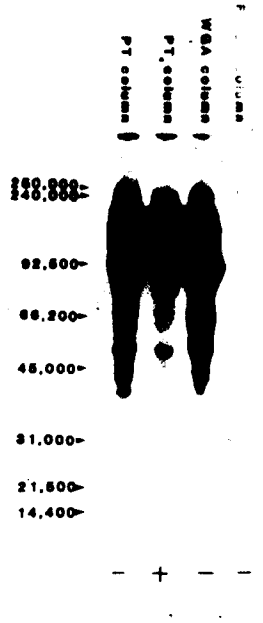


Figure 17. Comparison of ^{125}I surface labelled and solubilized CHO cells, eluted from two PT affinity columns, a WGA affinity column and a fetuin-agarose column. One hundred µg of PT was attached to activated Sepharose 4B. One hundred µg of WGA and 100 µg of fetuin was attached to agarose. An equal amount of radioactivity was applied to all four columns. Binding to PT took place in the presence of 10 mg/mL fetuin in the column marked (+). Binding to the remaining columns took place in the absence of fetuin (-). The bound material was eluted with 50 mM diethanolamine, pH 11.5. The eluted fractions were dialyzed and dried. Laemmli sample buffer was added and the fractions were subjected to SDS-PAGE using a gradient gel system of 7.5% to 15%. The gel was dried and autoradiography was then performed. The molecular weights at the left of the figure, indicate positions of standard proteins. The 250,000 and the 240,000 dalton protein standards are the α and β spectrin bands from human erythrocytes (86).

the larger protein can not be determined accurately because it is higher on the gel than the highest molecular weight standard.

3.3 ADP-RIBOSYLATION STUDIES

3.3.1 Chinese Hamster Ovary Cell Assay for PT Activity:

When PT is radiolabelled in the presence of fetuin, it's binding activity is maintained in the CHO cell system. In addition, fetuin-protected PT caused the same biological effects in CHO cells as untreated toxin. These data demonstrated that the fetuin-agarose iodination procedures had not altered the tyrosine residues critical for binding to goose erythrocytes or CHO cell receptors, but gave no indication of the integrity of the toxin's ADP-ribosyltransferase activity. Toxin that was labelled via the iodogen or chloramine T procedures with binding activity compromised (unprotected) did not induce any visible morphological change upon the CHO cells. This left me with two possible explanations for ^{125}I-PT activity in CHO cells. Either (1) PT activity is determined directly by the binding of pertussis toxin to the CHO cell receptors or (2) the A-protomer is responsible for CHO

cell activity as suggested by Burns *et al.* (20) but still requires active B oligomer for binding to CHO cells and allowing the A protomer to enter the cytoplasm. These two possibilites were further explored by examining the ability of native and iodinated pertussis toxin to ADP-ribosylate GTP binding proteins.

3.3.2 PT Mediated ADP-Ribosylation of Rat C6 Glial Cells:

C6 rat glial cells were choosen as a substrate system for the PT-mediated ADP-ribosyltransferase assay because Katada *et al.*(79) demonstrated that pertussis toxin is able to ADP-ribosylate a 41,000 dalton protein in C6 rat glial cell membranes. Crude membrane preparations were prepared according to Materials and Methods. These were then ADP-ribosylated with increasing concentrations of pertussis toxin to determine the sensitivity of the assay. The lowest concentration of PT tested was 2.5 µg/mL (fig. 18). The sensitivity is beyond this as determined by the intensity of the bands on the autoradiogram. These concentrations of PT were not sensitive enough because the maximum concentration of ^{125}I-PT obtained from the iodination procedure was 1.5 µg/mL. To increase the sensitivity, I purified the C6 glioma membranes by centrifugation onto a sucrose cushion. This enabled me to detect less than 0.1 µg/mL

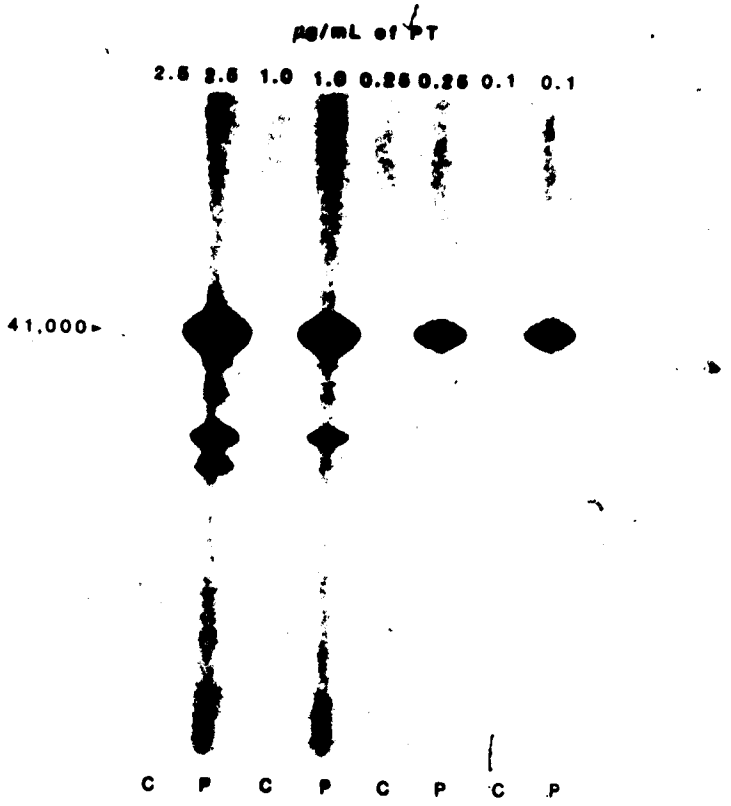


Figure 18. Comparison of PT-mediated ADP-ribosylation of crude and sucrose cushion purified C6 glioma cell membranes. Crude (C) and sucrose purified (P) cell membranes were used as substrates for the reaction described in the Materials and Methods. The reaction time was one hour and the concentration of membrane protein used was 1 mg/mL. After the reaction, the samples were subjected to SDS-PAGE and autoradiography was performed for a period of 24 hours. The substrate ADP-ribosylated had a mw of 41,000. The concentrations of PT used are indicated at the top of each lane.

of PT (fig. 18). Nonetheless, even with increased sensitivity, I was still unable to demonstrate ADP-ribosylation activity in ^{125}I-PT. Subsequently, the problem was traced to the presence of Mg^{++} ions in ^{125}I-PT (fig. 19). Four molar Mg^{++} was used to elute ^{125}I-PT from the fetuin-agarose. The final concentration of Mg in the ADP-ribosylation assay mixture containing ^{125}I-PT was 30 mM. Examination of figure 19 reveals the inhibitory effect 30 mM Mg^{++} had on the ADP-ribosyltransferase activity of native PT. Dialysis was used to reduce the concentration of Mg^{++} in ^{125}I-PT preparation. However, the dialysis procedure resulted in the loss of PT activity. This may have been due to ^{125}I-PT aggregation or binding to the dialysis tubing. EDTA was also used to try and chelate the Mg^{++} in the ^{125}I-PT preparation. Unfortunatlely, this was also unsuccessful. It was therefore clear that an alternate way would have to be found for eluting ^{125}I-PT from fetuin-agarose. A more sensitive assay would allow me to dilute the Mg^{++} to levels which would not interfere with ADP-ribosylation. ADP-ribosylation of transducin was found to be much more sensitive than the C6 glioma cell membranes. In the transducin assay, the minimum concentration of PT tested was 10 ng/mL (fig. 20). The sensitivity of this assay clearly extends beyond this

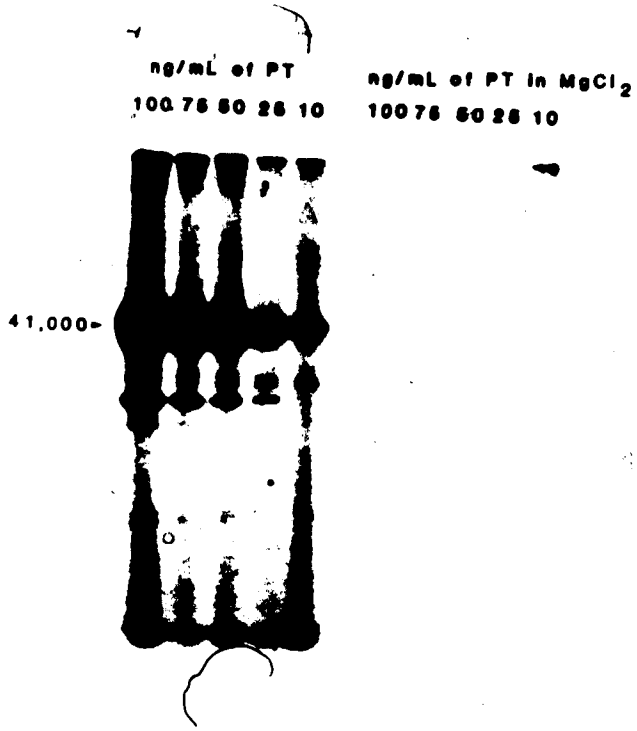


Figure 19. Effect of Mg on the PT-mediated ADP-ribosylation of sucrose cushion purified C6 glioma membranes. Reaction mixtures with and without $MgCl_2$ were compared to determine the extent of inhibition caused by Mg. The concentration of $MgCl_2$ used in the assay was 30 mM. The reaction was carried out as described in Materials and Methods. The samples were subjected to SDS-PAGE and autoradiography was performed for a period of 48 hours. The concentrations of PT used are as indicated.

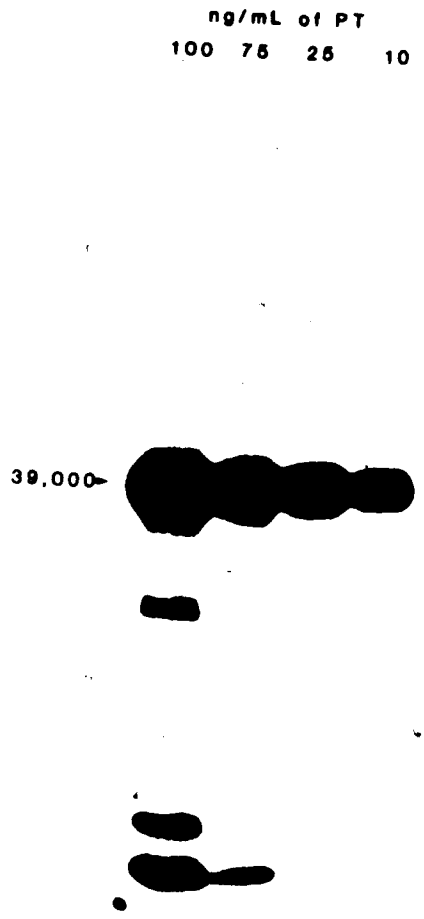


Figure 20. ADP-ribosylation of transducin. The α subunit of transducin was ADP-ribosylated by decreasing concentrations of PT. The reaction was carried out as described in Materials and Methods. The samples were subjected to SDS-PAGE and autoradiography was performed. The α subunit of transducin (39,000 daltons) which is ADP-ribosylated by PT is indicated by the arrow.

value. Since greater quantities of transducin than C6 membranes could be prepared, the remaining ADP-ribosylation studies were performed with transducin as the substrate.

3.3.3 Determination of Optimal Eluent For Removing ^{125}I-PT From Fetuin-Agarose:

Since Mg inhibited PT mediated ADP-ribosylation of the 41,000 dalton protein in C6 glioma cells, an alternate procedure for eluting ^{125}I-PT from fetuin-agarose was needed. Of all reagents examined, diethanolamine (pH 11.5) appeared to be the best (fig. 21). However, it was imperative to neutralize the pH of the solutions immediately after elution from fetuin-agarose to avoid inactivating the PT. NaSCn and $ZnCl_2$ were found to completely inhibit the reaction under the conditions tested and the other cations were intermediate between Zn^{++} and Mg^{++}. Further experiments were performed to investigate the inhibitory effect of Zn. $ZnCl_2$ was found to inhibit the reaction between concentrations of 10 µM and 1 mM (fig. 22).

3.3.4 Determination of the Number of Moles of ^{32}P NAD Incorporated per Mole of Transducin via PT Mediated ADP-Ribosylation of Transducin:

To be certain that the transducin assays accurately reflected the concentration of active PT, the time

Figure 21. Determination of the effect of various solutions on the ability of untreated PT to ADP-ribosylate transducin. The solution that had the least inhibitory effect (diethanolamine) was then used as the eluent for removing ^{125}I-PT from fetuin-agarose. The concentration of native PT was the same in each experiment. The concentration of each substance was determined by the dilution required to deliver the required amount of PT to the reactions. After one hour at 30°C, the amount of ^{32}P NAD incorporated into transducin was determined and expressed as mole/min/µg of PT (10^{-12}). Each chemical was tested in duplicate. The error bars represent the standard deviation of the mean.

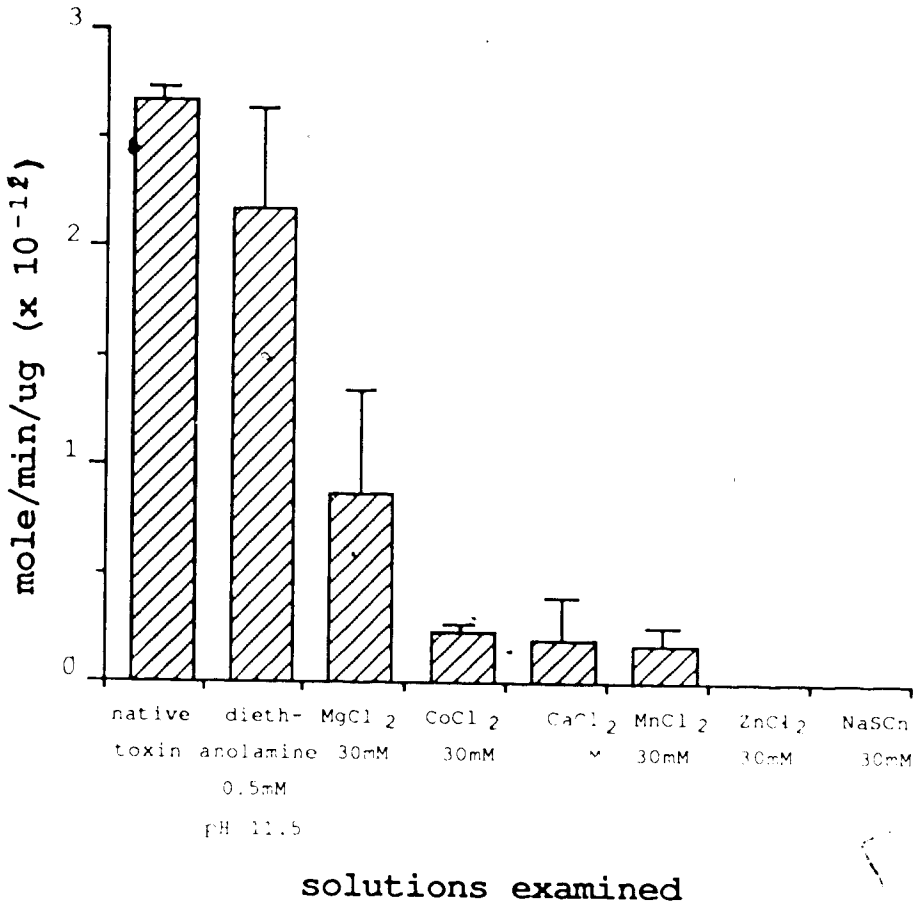
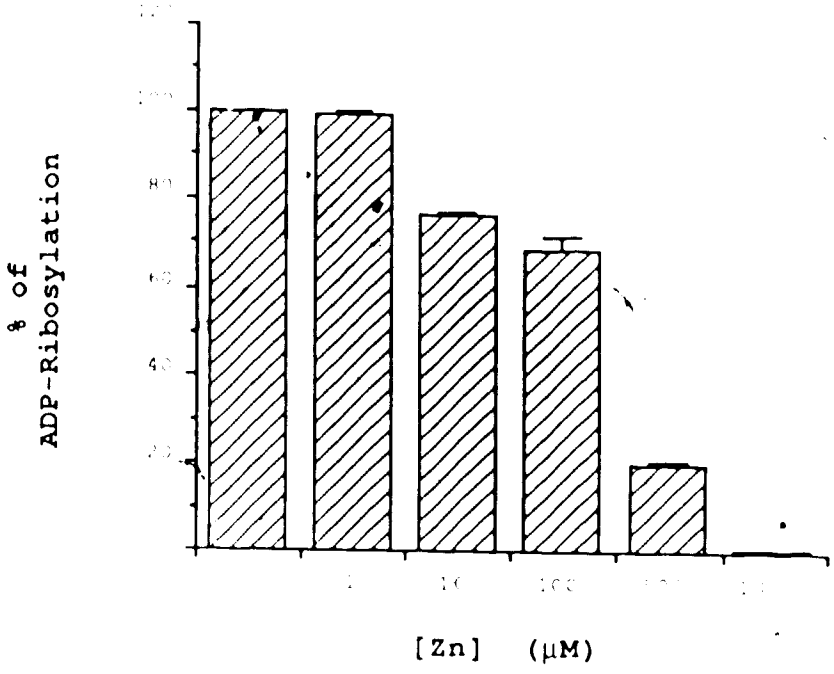

3
2
1
0
mole/min/ug (x 10-12)
native toxin
dieth- anolamine 0.5mM pH 11.5
MgCl 2 30mM
CoCl 2 30mM
CaCl 2
MnCl 2 30mM
ZnCl 2 30mM
NaSCn 30mM
solutions examined

Figure 22. Effect of increasing concentrations of $ZnCl_2$ on the ability of 100 ng/mL PT to ADP ribosylate 100 µg/mL transducin. Increasing amounts of $ZnCl_2$ were added (in duplicate) to the ADP-ribosylation assay mixture prior to initiating the reaction. The reaction was then allowed to occur at 30°C for 1 hour after which the proteins were precipitated with TCA, washed and then solubilized in 10% SDS. The counts were determined and expressed as a percentage of ADP-ribosylation by native toxin. The error bars represent the standard deviation of the mean.


% of
ADP-Ribosylation
[Zn] (μM)

course of the reaction was investigated at a maximum PT concentration of 100 ng/mL. Under the reaction conditions chosen, the reaction failed to reach equilibrium after 120 minutes (Fig. 23). After one hour 10.79×10^{-5} moles of ^{32}P ADP-ribose was incorporated per mole of transducin. This is far below the 1:1 molar ratio expected if all available ADP-ribose acceptor sites had been utilized (transducin molecule only has one ADP-ribosylation site for pertussis toxin) (36,146). Therefore, with a reaction time of 1 hour at 100 ng/mL of PT the reaction rate was not limited by the number of available ADP-ribose acceptor groups. Any changes in activity observed in the ^{125}I-PT would therefore accurately reflect the ADP-ribosyltransferase activity of PT present in those preparations.

3.3.5 Effect of Iodogen and Chloramine T Radiolabelling on Enzymatic Activity of The A-protomer of Pertussis Toxin;

As can be seen by the results shown in figure 24, neither iodogen, a mild iodinating reagent, nor chloramine T, a harsher iodinating reagent, had any significant effect on ADP-ribosyltransferase activity. Further, ADP-ribosyltransferase activity appeared to be retained even when binding activity was destroyed by conventional iodination procedures as demonstrated by

Figure 23. A. Effect of concentration of PT on the rate of ADP-ribosylation of the α subunit of transducin. An ADP-ribosylation assay using transducin was carried out in triplicate as described in the Materials and Methods. The concentration of PT used was 10, 100, 1000 ng/mL. The reaction time was one hour. The reaction was halted with 10% [illegible] and the moles of NAD incorporated per 100 µg of transducin was determined. B. Calculation of the number of moles of ^{32}P NAD incorporated per mole of transducin as determined by the ADP-ribosylation of tranducin by PT. An ADP-ribosylatin reaction was carried out as described in A. The concentration of PT used in the assay was 100 ng/mL. The error bars for both figures represent the standard deviation of the mean.

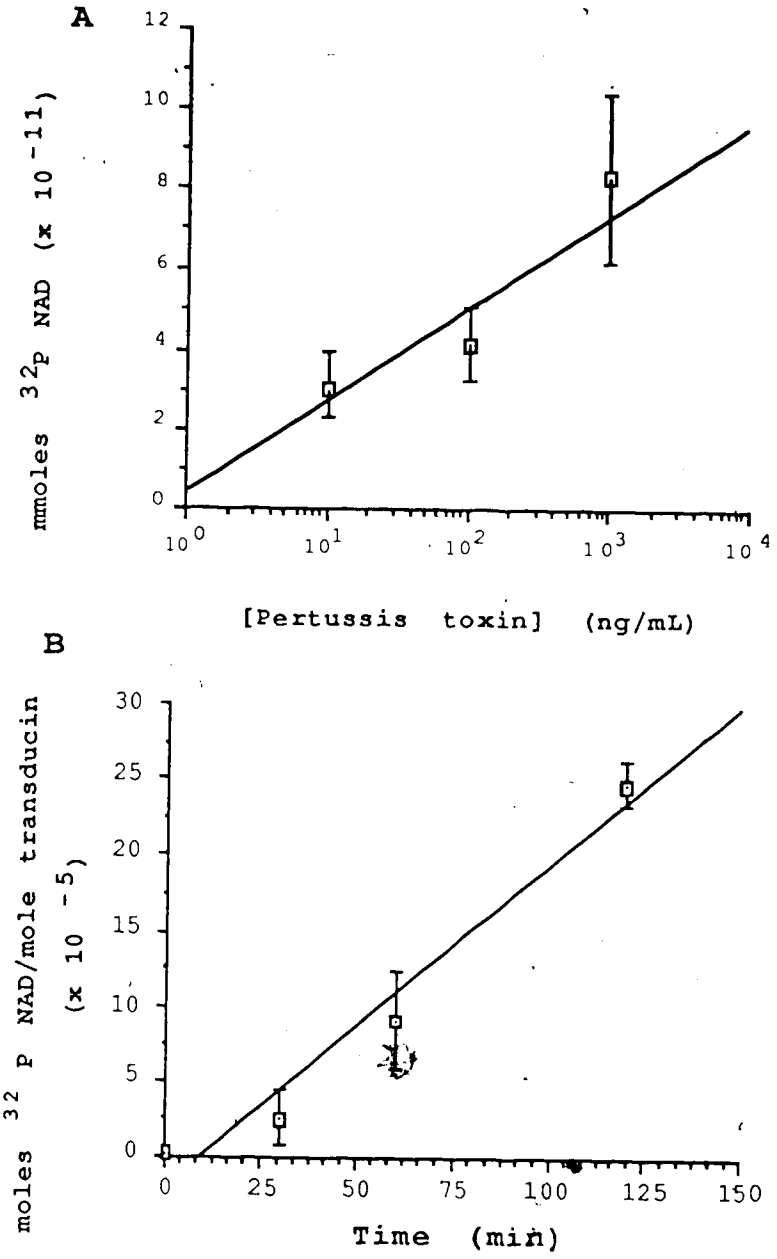
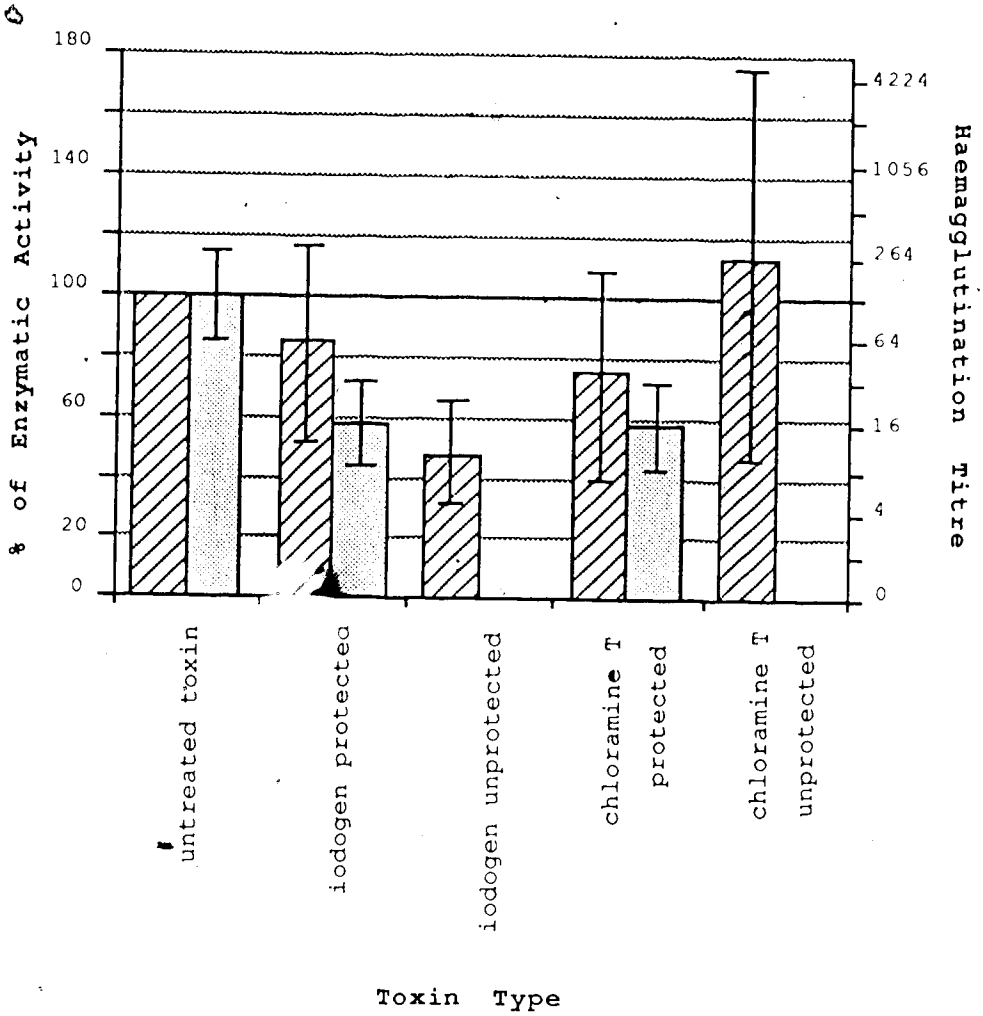

A
12
10
8
6
4
2
0
mmoles ^{32}P NAD ($\times 10^{-11}$)
10^0
10^1
10^2
10^3
10^4
[Pertussis toxin] (ng/mL)
B
30
25
20
15
10
5
0
moles ^{32}P NAD/mole transducin ($\times 10^{-5}$)
0
25
50
75
100
125
150
Time (min)

Figure 24. Effect of different PT iodinating techniques on the ability of PT to ADP-ribosylate transducin and agglutinate goose erythrocytes. Four different methods were used to iodinate PT (iodinate of protected PT by iodogen or chloramine T and iodination of unprotected PT by iodogen or chloramine T). The concentration of I-PT was determined by the heamagglutination titre of goose erythrocytes (stippled columns). For unprotected ^{125}I-PT in which no heamagglutination was detectable, the specific activity was used to determine a toxin concentration. The ^{125}I-PT was then used at a uniform concentration, in an ADP-ribosyaltion assay of transducin as described in the Materials and Methods. The reactions were repeated 5 times and the counts converted to percent enzymatic activity (cross hatched columns). The error bars represent the standard deviation of the mean.


% of Enzymatic Activity
180
140
100
60
20
0
4224
1056
264
64
16
4
0
Haemagglutination Titre
untreated toxin
iodogen protected
iodogen unprotected
chloramine T protected
chloramine T unprotected
Toxin Type

the loss of hemagglutinating activity. This indicated that, unlike the B oligomer which possess tyrosine residues in critical areas of functional domains required for binding, the tyrosine residues in the A protomer are probably not in the critical functional regions required for ADP-ribosyltransferase activity.

DISCUSSION

4.1 PT-Receptor Binding Studies;

In this thesis I have used Western blotting and a PT affinity procedure to investigated receptors for PT in goose erythrocytes and chinese hamster ovary cells. PT receptors in other cell systems have not been identified although a number of serum glycoproteins have been shown to have an affinity for PT. These include fetuin, ceruloplasmin, α-1 acid glycoprotein and haptoglobulin (6,7,41,64,143). Although the study of receptors for PT is incomplete, a more advanced understanding of the structural components responsible for binding of PT has been achieved.

In an elegant series of experiments, Nogimori *et al.* (114) demonstrated that PT may contain at least two binding sites located in the B subunit dimers. This was accomplished through acetamidation and reductive methylation. Reductive methylation of dimer 1 had no effect on PT mediated goose hemagglutination or mitogenesis in the mouse lymphocyte system. In contrast, reductive methylation of dimer 2 destroyed mitogenic activity, although the goose hemagglutination activity remained unaltered. More evidence supporting the role of dimer 1 in binding to goose erythrocytes was provided by

a monoclonal antibody obtained against an epitope on subunit S_2 of dimer 1. This monoclonal antibody effectivly inhibited PT mediated hemgglutination of goose erythrocytes (3).

Dimer 1 was also found to play a more important role than dimer 2 in the transmembrane insertion of the A protomer. This was directly demonstrated by the binding of PT to adipocytes and subsequent glycerol release due to ADP-ribosylation of Gi of the adipocyte adenylate cyclase system (114). Both dimers bind to adipocytes, however dimer 1 was more potent than dimer 2 in suppressing glycerol release mediated by whole PT. Therefore, it was concluded that monovalent binding of PT to adipocytes via dimer 1 was sufficient for the transmembrane insertion of the A protomer.

The studies reported in this thesis have advanced our understanding of receptors for PT. Goose erythrocyte and CHO cell receptors identified in the PT affinity chromatography experiments were identical to the glycoproteins to which PT bound. This was not surprising because it had previously been demonstrated that PT, like WGA, binds proteins containing GlcNAc or NeuNAc determinants. Although the results were inconclusive, PT and WGA also bound to similar proteins in the western blot experiments. PT may be binding to

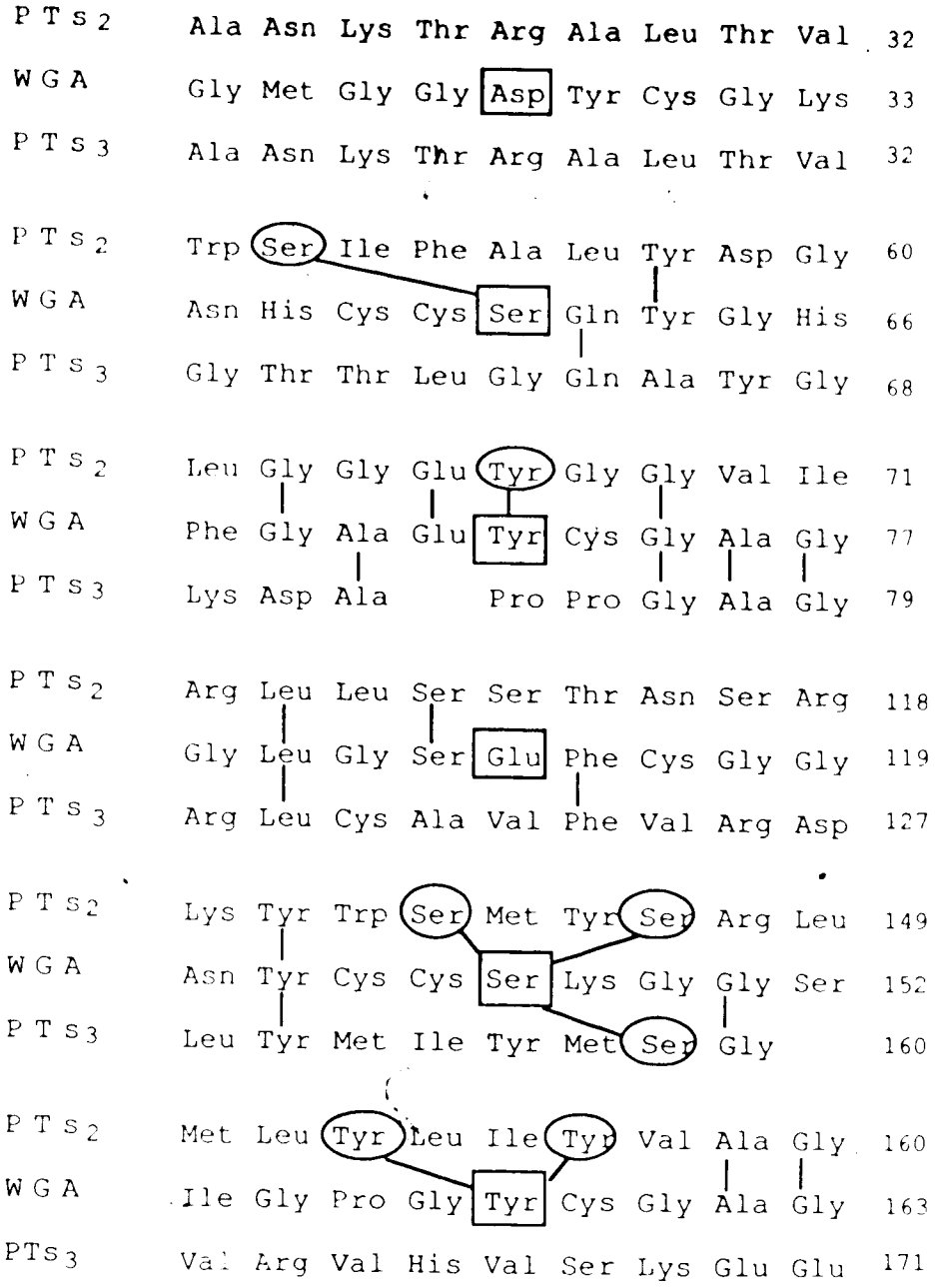
the goose erythrocyte glycoproteins in the affinity chromatography and western blot experiments via dimer 1 since this is the component of PT which is responsible for hemagglutination (114). In addition, the same surface labelled components of CHO cells bound to PT and WGA affinity columns. Since it has been suggested that PT is binding to the glycoproteins in goose erythrocytes via dimer 1 and that WGA binds to the same glycoproteins in both systems, it is possible that PT is binding to CHO cells via dimer 1 interaction with glycoprotein receptors. The CHO cell system may be analogous to the adipocyte system in which monovalent dimer 1 binding is sufficient for transmembrane insertion of the A protomer (114).

Because WGA and PT bind to the same glycoproteins in CHO cells, it can be concluded that these proteins contain GlcNAc or NeuNAc moieties. This is further supported by previous experiments in which nitrocellulose filters containing CHO cell lysates were treated with sialidase and then incubated with pertussis toxin followed by ^{125}I-labelled anti-pertussis toxin. Removal of sialic acid resulted a loss of binding activity of the receptor glycoproteins in CHO cell lysates (18). However, GlcNAc or NeuNAc carbohydrate determinants cannot be the only factor determining the

binding of PT to these cells otherwise fetuin would have been able to compete for PT in the CHO cell-fetuin inhibition assay. There must be other factors besides the oligosaccharide structure which is involved in determining the relative affinities of these receptors for PT. The secondary structure of the glycosylated protein, the number of branches in the oligosaccharide structures, or the number of glycosylation sites on the glycoprotein itself may all play an important role in PT binding (7).

Because of the similarity in the binding specificity between PT dimer 1 and WGA, I thought it would be interesting to examine the known amino acid sequences of the binding subunits of PT and the amino acid sequence of WGA (fig. 25). When the sequences of S_2 and S_3 were aligned with WGA, very little homology was found. However, if only the amino acids responsible for WGA binding activity were considered, subunit S_2 has a higher degree of similarity than S_3 to WGA. In subunit S_2 of dimer 1, 4 of the six amino acids are closely matched with the binding amino acids in WGA. In contrast, subunit S_3 of dimer 2 has only one amino acid closely matching one of the binding amino acids of WGA. This is surprising since subunits S_2 and S_3 are 70-80% homologous. The matching amino acids in S_2 may be

Figure 25. Amino acid sequence segments of PT-S_2, PT-S_3 and WGA. The boxed in amino acids in the WGA sequences represent the amino acids responsible for WGA binding (153). Five residues flanking either side of these residues are also shown. The circled amino acids represent the same residues from PT subunits S_2 and S_3 that were close to those involved in WGA binding when the total sequences of all 3 proteins were aligned for maximum homology.


PTs2 Ala Asn Lys Thr Arg Ala Leu Thr Val 32
WGA Gly Met Gly Gly Asp Tyr Cys Gly Lys 33
PTs3 Ala Asn Lys Thr Arg Ala Leu Thr Val 32
PTs2 Trp Ser Ile Phe Ala Leu Tyr Asp Gly 60
WGA Asn His Cys Cys Ser Gln Tyr Gly His 66
PTs3 Gly Thr Thr Leu Gly Gln Ala Tyr Gly 68
PTs2 Leu Gly Gly Glu Tyr Gly Gly Val Ile 71
WGA Phe Gly Ala Glu Tyr Cys Gly Ala Gly 77
PTs3 Lys Asp Ala Pro Pro Gly Ala Gly 79
PTs2 Arg Leu Leu Ser Ser Thr Asn Ser Arg 118
WGA Gly Leu Gly Ser Glu Phe Cys Gly Gly 119
PTs3 Arg Leu Cys Ala Val Phe Val Arg Asp 127
PTs2 Lys Tyr Trp Ser Met Tyr Ser Arg Leu 149
WGA Asn Tyr Cys Cys Ser Lys Gly Gly Ser 152
PTs3 Leu Tyr Met Ile Tyr Met Ser Gly 160
PTs2 Met Leu Tyr Leu Ile Tyr Val Ala Gly 160
WGA Ile Gly Pro Gly Tyr Cys Gly Ala Gly 163
PTs3 Val Arg Val His Val Ser Lys Glu Glu 171

important for the lectin-like binding properties of dimer 1. It should also be noted that two of the amino acids responsible for binding of WGA are tyrosine residues, which are closely matched to two tyrosines in the S_2 subunit. These may be the tyrosines that, if not protected during radioiodination, become radiolabelled compromising the binding of WGA and PT. It is important to identify amino acids in pertussis toxin that may be responsible for binding of the toxin since it is these amino acids that could be used as targets for site-specific mutagensis studies.

The second class of PT binding is multivalent. Unlike monovalent binding, PT multivalent binding is thought to involve both dimers. As discussed early, only methylation of dimer 2 destroyed the toxin's ability to stimulate mitogenesis whereas methylation of dimer 1 did not (114). This must not be taken to mean that only dimer 2 mediates mitogenesis. Neither dimer 1 or dimer 2 by themselves are able to stimulate mitogenesis (114). This indicates that multivalent binding of PT to receptors on the lymphocyte is necessary for mitogenesis. Multivalent binding may occur if both dimer 1 and dimer 2 bind to two different types of lymphocyte receptors or it may be the result of aggregation of PT resulting in two or more copies of dimer 2 being present

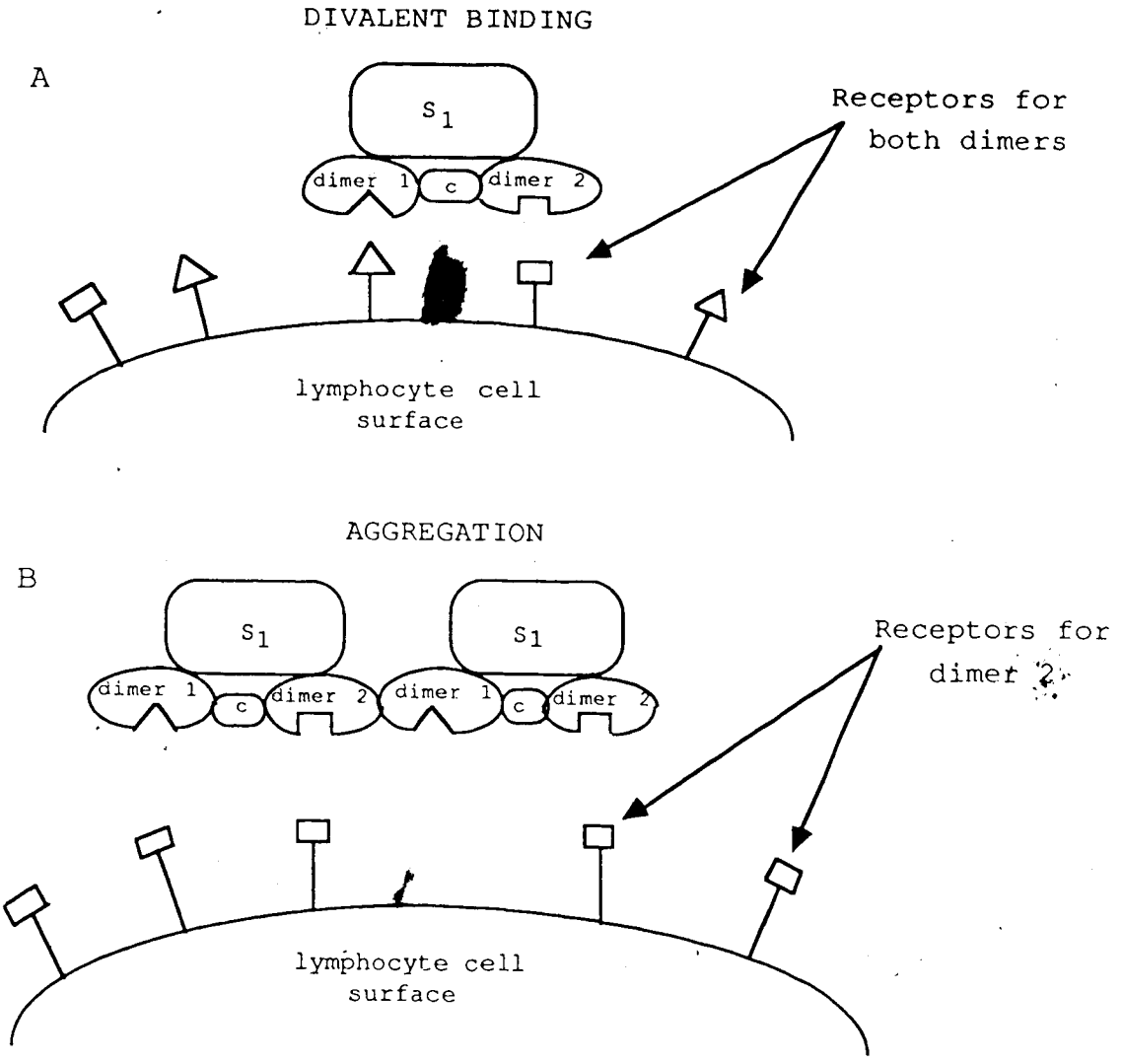
to cross-link transmembrane lymphocyte glycoproteins. (fig. 26) (114,141).

The involvement of dimer 1 in mitogenesis is suggested by a further comparison between WGA and PT. Both WGA and PT have the ability to stimulate mitogenesis in T cells. However, the exact mechanism by which WGA stimulates mitogenesis of T cells is not completely understood and whether or not it can even be classified as a classical mitogen is a matter of controversy (13,49,143).

Future experiments may involve the differential elution of ^{125}I surface-labelled detergent-solubilized CHO cell membranes from the PT Sepharose columns. By eluting the column with fetuin and then with 50 mM diethanolamine, receptors with a higher affinity than fetuin can be identified. Also, the increased sensitivity of chymotrypsin-treated goose erythrocytes to PT-mediated agglutination should be examined at the receptor level using PT-Sepharose affinity columns. It would be interesting to see if ^{125}I surface labelled, detergent solubilized-chymotrypsin treated goose erythrocytes gave the same PT receptor banding pattern as the untreated goose erythrocytes.

Future research may also involve the peptide sequences in subunit S_2 which are involved in binding

Figure 26. Diagrams illustrating 2 possible methods by which PT may bind to lymphocytes resulting in cross-linking of glycoproteins and stimulation of mitogenesis. In A. lymphocytes may contain receptors for both dimer 1 and dimer 2 as illustrated, or it may contain receptors for both dimers. In A., mitogenesis results from the cross-linking of receptors for dimer 1 and dimer 2. In B., only dimer 2 receptors need to be crosslinked to stimulate mitogenesis. Since each PT molecule only contains one dimer 2, crosslinking results from aggregation.


DIVALENT BINDING
A
S_1
dimer 1
c
dimer 2
Receptors for both dimers
lymphocyte cell surface
AGGREGATION
B
S_1
S_1
dimer 1
c
dimer 2
dimer 1
c
dimer 2
Receptors for dimer 2
lymphocyte cell surface

activity. The sequences related to WGA are candidates for this. These could be used in binding competition assays or used to obtain antibodies to be utilized in binding competition experiments. Still further work may involve site-directed mutagensis experiments in which the amino acids identified in figure 25 are substituted for different amino acids and then examined to see if the toxin is still capable of binding to receptors.

4.2. ADP-ribosylation Studies;

As discussed in the introduction, PT has the ability to enzymatically catalyze ADP-ribosylation of the inhibitory α subunit of the adenylate cyclase system and the α subunit of transducin. It is believed that the morphological changes induced by PT on CHO cells is a result of PT mediated ADP-ribosylation of a 41,000 dalton protein believed to be the inhibitory α subunit of adenylate cyclase (20). PT radiolabelled with ^{125}I without protection of the binding sites, cannot induce a morphological change in CHO cells. However, if the binding sites are protected by fetuin-agarose before radiolabelling, the toxin is able to bind to CHO cells and induce the morphological change (6). Although this demonstrated the lability of the binding activity of the B oligomer the effect of iodination on the enzymatic activity of the A protomer was unknown. As previously

discussed, both cholera toxin and pertussis toxin ADP-ribosylate a regulatory component of the adenylate cyclase system. Cholera toxin ADP-ribosylates Gs and pertussis toxin ADP-ribosylates Gi. Even though these two toxins enzymatically alter different proteins in this system, the net effect is the same, an increase in cAMP levels in the cell. However, when either of these two toxins are incubated with CHO cells, two distinct morphological changes are seen. CT causes the CHO cell to become elongated and spindle shaped whereas PT causes a clumping morphology to predominate. If the net result of incubation of CT or PT with CHO cells is an increase in intracellular cAMP due to toxin mediated ADP-ribosylation, then why should two different morphological differences be seen? One possible answer is that the B oligomer may also play a role in altering CHO cell morphology by these two toxins. The B oligomers show a difference in binding, CT binds to ganglioside G_{M1} whereas PT binds to glycoproteins. This difference in binding may result in the altered CHO cell morophology. Other evidence which suggests that enzymatic activity is responsible for altered CHO cell morphology is provided by Burns *et al.* (20) in which it was shown that alteration of CHO cell morphology paralleled ADP-ribosylation of a 41,000 dalton protein.

I sought to provide more direct evidence by using the fetuin-agarose protected radioiodinated PT. It had been previously demonstrated that this toxin still retained the ability to alter CHO cell morphology (6) but it was uncertain if the ^{125}I-PT still possessed an unaltered A protomer. If it did not, then CHO cell activity could be attributed to the B oligomer only. If the A protomer was unaffected by the radioiodination then it would not be possible to assign CHO cell activity only to the B oligomer. Also, if enzymatic activity was still functional, was it because of the protection afforded the toxin by the fetuin-agarose or was it because the A protomer was simply unaffected by ^{125}I? Direct evidence for this was only obtained by using an assay system in which the enzymatic activity of the A protomer could be measured regardless of the condition of the B oligomer. Two systems were explored as possible candidates which fit this criteria, the adenylate cyclase system of rat C6 glioma cells and the G-protein transducin obtained from the bovine visual excitation system.

C6 Rat glial tumour cell membranes (C6 membranes) were the first source of G protein used. C6 glioma membranes possess a 41,000 dalton membrane G protein which is ADP-ribosylated by PT (76,78,79). Moreover, the C6 membrane protein has the same molecular weight as a

protein recently found in CHO cells which can also be ADP-ribosylated by pertussis toxin. Conceivably, these two proteins are the α subunit of the inhibitory arm of the adenylate cyclase system in these two cell lines. First attempts at using C6 glioma membranes as a source of G protein for ADP-ribosylation by radiolabelled PT were unsuccessful. The major problem was the inhibitory effect caused by the presence of $MgCl_2$ in the radiolabelled toxin preparation.

Because of this problem, it was decided to try and increase the sensitivity of the assay by further purifying the membranes on a sucrose cushion. By adding this extra purification step, I was able to increase the sensitivity greater than 250 fold (fig. 18). However, even with this increased sensitvity the $MgCl_2$ still inhibited the reaction at the concentration in which the ^{125}I PT would be suspended in.

As mentioned earlier, transducin is a GTP binding protein of the retina which can be used as a substrate for PT mediated ADP-ribosyltransferase (36,138,146). The A protomer of the toxin catalyzes the ADP-ribosylation of the α subunit of transducin in a direct stoichiometric reaction. Although the transducin assay was able to detect as little as 10 ng/mL of PT, the reaction was severly restricted by the presence of Mg^{++}

used to elute ^{125}I-PT from fetuin-agarose. A series of other substances were examined to determine an alternate elution buffer. Diethanolamine was found to affect the reaction the least provided neutralization of pH took place immediatly whereas $ZnCl_2$ and NaSCn completely inhibited the reaction under the conditions tested. The $ZnCl_2$ proved interesting in that it had not been previously known that Zn^{++} was an effective inhibitor of PT mediated ADP-ribosyltransferase activity. Further studies showed that it was able to inhibit in the μM range.

Using diethanolamine instead of $MgCl_2$ in the elution buffer, I was able to demonstrate the ability of ^{125}I PT, labelled on fetuin-agarose and via the iodogen procedure, to ADP-ribosylate the α subunit of transducin. PT radiolabelled without fetuin-agarose protection was also enzymatically active in this assay. It would therefore seem that, unlike the binding sites in the B oligomer, the A protomer is unaffected by radiolabelling. Since I wished to generate PT which contained full binding activity but no ADP-ribosyltransferase activity, I used chloramine T to determine whether a harsher iodinating reagent would destroy the ADP-ribosyltransferase activity of the A protomer. The chloramine iodination reaction however had

little effect on the A protomer's enzymatic activity. The same results were obtained whether toxin was protected with fetuin-agarose or not. It would seem then that the crucial biologically active sites of PT which are sensitive to radiolabelling are located in the B oligomer's receptor binding sites and that the A protomer is resistant to the iodination reaction.

Future experiments may involve site-directed mutagensis on the S_1 subunit as was proposed for the subunits of the B oligomer. Amino acids in the S_1 subunit that can be considered as prime targets are those that exhibit homology to the cholera toxin A subunit and the *E.coli* LT A subunit (87). Because of the homology between these three ADP-ribosyltransferase toxins, it is suspected that these are the amino acids which may be functionally responsible for enzymatic activity. Recent experiments using site-directed mutagensis have already shown that the substitution of amino acid arginine-9 in the S_1 subunit of pertussis toxin results in loss of NAD glycohydrolase activity and ADP-ribosyltransferase activity. Also, monoclonal antibodies directed against this region still bind indicating that a vaccine containing a genetically altered inactive S_1 subunit would be suitable in providing protection against the disease (87). The

inhibitory activity of zinc could also be examined in greater detail. It would be interesting to determine if cholera toxin and LT mediated ADP-ribosylation can also be inhibited by Zn^{++}.

5.0 CONCLUSIONS

In the first and second part of this thesis, I have identified receptor species for PT in both goose erythrocytes and CHO cells. These receptors are protein in nature containing GlcNAc or NeuNAc determinants which are involved in PT binding. These receptors may also be binding to the same dimer, dimer 1. Also, identification of a potentially important sequence homology in PT S_2 subunit with WGA was found. In the third part of the thesis I have determined optimal conditions for ^{125}I PT mediated ADP-ribosylation at low toxin concentrations. Also, Zinc at μM concentrations has been found to have an inhibitory effect upon PT mediated ADP-ribosylation. The ADP-ribosylation studies have demonstrated that, regardless if PT is protected or unprotected by fetuin during the radiolabelling procedure the S_1 subunit is still able to ADP-ribosylate transducin.

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