1995

Some aspects of glutathione and L-arginine/nitric oxide metabolism in the maintenance of platelet function.

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SOME ASPECTS OF GLUTATHIONE AND L-ARGININE/NITRIC OXIDE METABOLISM IN THE MAINTENANCE OF PLATELET FUNCTION

by

Daniel Joseph Sexton

A Dissertation
Submitted to the Faculty of Graduate Studies and Research through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada

1994
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ABSTRACT

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by

Daniel Joseph Sexton

Evidence is presented here for the regulation of platelet activation by intra-platelet levels of glutathione (GSH) and L-arginine. Our data is consistent with the intra-platelet GSH ([GSH]_{ip}) modulation through the regulation of the thromboxane A_2 (TxA_2) biosynthesis. The reduction of [GSH]_{ip} by 100 μM 1-chloro-2,4-dinitrobenzene (CDNB) enhanced thrombin-induced platelet aggregation and ADP-induced platelet aggregation was inhibited by the elevation of [GSH]_{ip} through a facilitative GSH-specific transport system. Platelet facilitative GSH uptake was subsequently characterized as being Na\(^+\)-independent, concentration dependent (K_M and V_{max} for GSH uptake in platelet plasma membrane vesicles (PPMV) is 18.2 ± 3.6 μM and 178 ± 27 pmol/min/mg protein, respectively), inhibited by GSH analogs, enhanced by KCl-induced membrane depolarization and sensitive to the intraplatelet thiol redox status since the K_M and V_{max} for GSH uptake in intact platelets changed from 137 μM and 42.2 pmol/min/10^9 platelets, respectively, to 31.7 μM and 31.3 pmol/min/10^9 platelets, respectively, on reducing intra-platelet GSH with 100 μM CDNB. Glutathione reductase (GR) was found to be inhibited by physiological levels of GSH with species-dependent differences. With respect to varying GSSG, GSH inhibited GR from human platelets in an apparent uncompetitive manner (K_i = 6.6 mM), while bovine intestinal mucosa and yeast GRs displayed apparent mixed
hyperbolic inhibition ($K_i = 2.9$ and $2.4 \text{ mM}$, respectively), and the *E. coli* enzyme exhibited an apparent competitive inhibition ($K_i = 12.1 \text{ mM}$). In the course of this study it was observed that 1-(4-chlorophenyl)-4,4-dimethyl-5-diethylamino-1-penten-3-one hydrobromide (CDDP) is an alpha class selective glutathione S-transferase (GST) substrate and that certain analogs of CDDP are GST inhibitors with $K_i$s ranging from 7.5 to 53.4 $\mu$M. Exogenous L-arginine inhibited ADP-induced platelet aggregation which suggests that platelet-derived NO regulates platelet activation. Platelet L-arginine uptake was via the cationic amino acid transporter, system $\text{y}^+$, which appears to be regulated by NO. S-nitrosoglutathione (GSNO) was found to be photolyzed by visible light. The release of NO by GSNO photolysis resulted in an enhanced NO-dependent cytotoxicity towards HL-60 cells. GSNO, or related compounds, may therefore find use as photochemotherapeutic agents.
Dedicated to my beloved wife
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LIST OF ABBREVIATIONS

ADP: adenosine diphosphate;

BIM: bovine intestinal mucosa;

CDDP: 1-p-Chlorophenyl-4,4-dimethyl-5-diethylamino-1-penten-3-one hydrobromide;

CDNB: 1-chloro-2,4-dinitrobenzene;

DNP-SG: S-dinitrophenyl glutathione;

DTT: dithiothreitol;

G-site: glutathione S-transferase glutathione binding site;

γ-GT: γ-glutamyltranspeptidase;

[GSH]₀, intra-platelet GSH concentration;

GP: glycoprotein;

GPX: glutathione peroxidase;

GR: glutathione reductase;

GSH: reduced glutathione;

GSNO: S-nitrosoglutathione;

GSSG: oxidized glutathione;

GST: glutathione S-transferase;

H-site: glutathione S-transferase electrophile binding site;

HEPES: N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid);

12-HETE, 12-hydroxy-eicosatetraenoic acid;

12-HPETE, 12-hydroperoxy-eicosatetraenoic acid;
MOAT: multispecific organic anion transporter;
iNOS: inducible form of nitric oxide synthase;
NMDA: N-methyl-D-aspartate;
NOS: nitric oxide synthase;
PBS: phosphate buffered saline;
PGG2: prostaglandin G2;
PGH2: prostaglandin H2;
PMSF, phenylmethanesulfonyl fluoride,
PPMV: platelet plasma membrane vesicles;
SDS: sodium dodecyl sulfate;
TxA2: thromboxane A2;
vWF: von Willebrand factor
Chapter 1

INTRODUCTION

1.1 Overview

Platelets are fragments of megakaryocytes that circulate in the blood at high concentrations. In response to exposure of the subendothelial layer from a ruptured blood vessel, platelets become activated. When activated, platelets undergo a shape change from disc-like to spiny spheres followed by adhesion, aggregation and secretion of platelet agonists and clotting effectors. A platelet plug formed at the injured site prevents bleeding (reviewed in Siess, 1989). Pathological platelet conditions such as hypoaggregation and hyperaggregation do exist (Bick, 1992). Hyperaggregation, for example, is believed to be associated with diabetes mellitus and is linked to an increased risk of thrombosis (Winocour, 1992). Platelet hyperaggregation may be the result of either an increased sensitivity to agonist(s) or a dysfunctional regulatory mechanism(s). To discover the defective mechanisms present in platelet pathological syndromes a thorough understanding of the events in the normal platelet governing both the induction and inhibition of platelet activation is essential. The generalities and intricacies of most of the various signal transduction pathways leading to platelet activation have previously been the focus of much attention and consequently the induction of platelet activation is a fairly well understood process. The regulatory mechanisms responsible for inhibiting or in some cases reversing platelet activation are not as well characterized.

The intra-platelet glutathione level ([GSH]_i) has been implicated as being a regulator of platelet aggregation (Bosia et al., 1985; Hofman et al., 1980; Matsuda et al.,
The influence of $[\text{GSH}]_p$ homeostasis on platelet activation has to date only been determined using GSH depletion experiments. GSH depleting agents are thiol reagents and as such are expected to have intracellular targets other than GSH. The effects of some of these reagents may, therefore, not be attributed solely to the depletion of GSH. The role of GSH in platelets may be probed through the use of specific GSH-depleting reagents or via the elevation of $[\text{GSH}]_p$. We utilized both types of experiments in an attempt to settle the controversial issue of whether or not GSH regulates platelet function. Our efforts to elevate intra-platelet GSH led to the novel finding that platelets contain a transport system for intact GSH which was subsequently characterized.

There is irrefutable evidence that nitric oxide (NO) of exogenous origin is a potent inhibitor of platelet aggregation. It was recently demonstrated in our laboratory that platelets contain an isoform of nitric oxide synthase (NOS) and are consequently capable of synthesizing NO. The effect of endogenous platelet-derived NO on platelet function is not as well known but it very likely participates in the regulation of aggregation. The events responsible for the regulation of NO production are currently the subject of much interest. The regulation of platelet NO production has not yet been examined. It is known, however, that the intra-platelet L-arginine concentration can be rate-limiting towards the production of NO in platelets. We decided to characterize L-arginine transport across the platelet plasma membrane with the intention of determining whether or not L-arginine transport is regulated in some manner by NO production.
1.2 Platelet Structure, Composition, and Function

1.2.1 Platelet Structure and Composition

Platelets are small anucleated, discoidal blood cells (Siess, 1989). They are fragments of megakaryocytes. Their size ranges from 0.5 - 1 μm in thickness and from 1.5 to 2.5 μm in length. The normal concentration of platelets in the human circulatory system ranges from 150 to 450 x 10^9/mL with each platelet surviving approximately 10 days. Recently, evidence was presented for the existence of two classes of platelets in humans and in rats on the basis of the presence or absence of a certain acid phosphatase (Behnke and Forer, 1993).

Platelets are composed of a microtubular cytoskeleton, a large number of secretory storage granules, a small number of mitochondria and an internal membrane network called the dense tubular system that is derived from smooth endoplasmic reticulum. The dense tubular system serves as the intracellular calcium storage site in platelets. The different types of platelet granules are α-granules, lysosomal granules, dense granules, and glycogen granules.

The following lists of granule contents were compiled by Packham (1994). Components of the α-granules include fibrinogen, von Willebrand factor, fibronectin, platelet factor 4, β-thromboglobulin, thrombospondin, albumin, factor V, platelet-derived growth factor, transforming growth factor-β, high molecular weight kininogen, plasminogen, plasminogen activator inhibitor-1, α2-antiplasmin, α1-antitrypsin, histidine-rich glycoprotein, α2-macroglobulin, and C1 inhibitor. The lysosomal granules contain N-acetylglucosaminidase, β-glucuronidase, β-galactosidase, aryl sulfatase, heparitinase,
elastase, collagenase, and cathepsin. In the dense granules ADP, ATP, serotonin, and Ca\(^{2+}\) have been identified. The dense granule of some species also contains Mg\(^{2+}\) and histamine.

The surface of the platelet is very porous with many relatively large invaginations of the plasma membrane towards the platelet interior which are known as the open cannicular system. The open cannicular system is believed to be composed of multiple channels for the uptake of calcium and the secretion of granular contents. The platelet surface membrane consists of an amorphous outer coat that is rich in carbohydrate called the glycocalix. Underneath the glycocalix is a bilaminar membrane composed mainly of proteins and lipids.

1.2.2 Platelet Function

Hemostasis is the physiological process to arrest bleeding. Hemostasis may be divided into four stages (Ranney and Rapoport, 1990):

1) transitory vasoconstriction;
2) platelet aggregation;
3) coagulation;
4) fibrinolysis.

Of primary interest to this study is platelet aggregation and consequently the other stages will not be described. When a blood vessel is ruptured, platelets become activated in response to the exposure of subendothelial collagen. Platelet adhesion to subendothelial collagen initiates platelet activation. Through a series of events platelet activation
culminates in the formation of a platelet plug that prevents the loss of blood. The following is a list of sequential overlapping events involved in platelet activation:

1) platelet shape change;
2) liberation of inositol 1,4,5-triphosphate and diacylglycerol;
3) exposure of platelet membrane procoagulant phospholipids;
4) initiation of primary reversible platelet aggregation (cohesion);
5) release reaction: liberation and oxidation of arachidonic acid;
6) release reaction: secretion of granular contents;
7) secondary irreversible platelet aggregation;
8) actomyosin-mediated centipetal consolidation of platelet aggregate.

Besides preventing the loss of blood due to vessel rupture, platelets are also important in closing the gaps that continually form between adjacent endothelial cells of capillaries. These gaps form as a result of minimal degrees of stress upon the capillaries which can occur, for example, in those capillaries in the lower legs of an individual upon standing. Platelets rush in between the endothelial cells to fill the gaps. When the stress is no longer present, the platelets are released as single cells back into circulation. The process is by necessity reversible and consequently the platelets are not fully activated.

Platelet responses to agonists can be either reversible or irreversible depending on the dose and the potency of a particular agonist. For hemostasis irreversible platelet aggregation is required. It appears that only if an agonist is capable of inducing granule secretion and arachidonic acid liberation will irreversible aggregation occur. Although platelet stimuli are quite diverse there are a finite number of activation mechanisms
present. There is much information on the molecular mechanisms of platelet activation but
many questions remain unsolved (reviewed by Siess, 1989). For the purpose of this study
a brief description of general platelet activation events is presented. The platelet
activation mechanisms that will be described are shown in Fig. 1. The effects of
glutathione (GSH) on platelet activation that are shown in Fig. 1 will be described in
Sections 1.3.8.

1.2.2.1. Platelet Adhesion

To prevent the loss of blood, platelet adhesion to collagen must occur within 50
ms (Born and Richardson, 1980). Due to the high shear stress in flowing blood vessels
mere adhesion to collagen is not sufficient for the accumulation of platelets at the site of
injury. In vivo platelet adhesion to collagen requires the presence of the protein von
Willebrand factor (vWF). Pathophysiological conditions associated with the absence of a
functional vWF or vWF receptor exhibit a prolonged bleeding time (Ruggeri and
Zimmerman, 1985). The vWF is a negatively charged protein comprised of a 200 kDa
subunit assembled into multimers of dimers or tetramers to yield a combined molecular
weight that ranges from 800 to 20 000 kDa. In addition to the normal plasma levels of
vWF, the protein is secreted from the platelet α-granules. The protein aids in the adhesion
process by binding both to collagen and to two receptors on the platelet surface
membrane, GPIb and GPIIb/IIIa. The presence of the GPIIb/IIIa receptor on the platelet
surface is induced by many stimuli but not collagen. Fibrinogen also binds to the
GPIIb/IIIa receptor. It is thought that GPIb is the physiologically important vWF receptor
Fig. 1. Some important platelet activation mechanisms. The scheme is a depiction of some of the general events governing the onset and regulation of platelet activation. The sites where GSH interacts with certain platelet activation mechanisms are indicated. Abbreviations not defined in the text are as follows: RI, prostacyclin or PGD2 antagonist receptor; RS, any of several agonist receptors; AC, adenylate cyclase; 5,7-LOX, 5-lipoxygenase; LA4, leukotriene A4; LC4, leukotriene C4; PI, phosphatidylinositol; PIP2, inositol bisphosphate; IP3, inositol trisphosphate; PLC, phospholipase C; DAG, diacylglycerol; PL, phospholipid; COX, cyclooxygenase; POX, peroxidase activity of cyclooxygenase enzyme; 12-LOX, 12-lipoxygenase; HHT, 12-hydroxyheptadecatrienoate; MDA, malondialdehyde.
since the high plasma levels of fibrinogen are expected to enable fibrinogen to compete successfully over vWF for binding (Pietu et al., 1984). GPIb is constantly present on the platelet surface but only binds vWF when complexed to collagen. Presumably, vWF complexation to collagen induces a conformation change in vWF that promotes GPIb receptor binding (Bolhuis et al., 1981).

1.2.2.2. Platelet Shape Change

Platelet adhesion is followed by platelet shape change, an early event in platelet activation (Siess, 1989). Platelets change in shape from flattened discs to spheres with multiple projecting pseudopods (spiney spheres). The process is initiated by elevated intracellular calcium levels from intracellular stores (dense tubular system). Extracellular calcium is not a requirement of shape change since this process has been observed in the presence of high concentrations of the chelators EDTA or EGTA. All platelet stimuli except epinephrine and phorbol esters, which do not increase cytosolic calcium, induce full platelet shape change. Both of these agonists induce some pseudopod formation without spheration which suggests that the two types of shape change (spheration and pseudopod formation) are separate events and occur through separate mechanisms (Patscheke, 1980). Spheration has been found to precede pseudopod formation (Hantgan, 1984).

During shape change a Ca\(^{2+}\)-dependent polymerization of monomeric G actin (42 kDa) to form parallel double helical microfilaments, called F actin, occurs. A Ca\(^{2+}\)-dependent contraction of the platelet membrane occurs during spheration and involves an increased association of myosin with the F actin filaments to produce actomyosin
(Hartwig, 1992). The resulting actomyosin complex activates myosin ATPase which generates the contractive force required for shape change from discoidal to spherical. Myosin is a large protein composed of one pair of heavy chains (200 kDa) and two pairs of light chains (20 and 15 kDa). Platelet stimulation with several agonists is associated with the phosphorylation of the 20 kDa myosin light chain as catalyzed by Ca\textsuperscript{2+}/calmodulin-dependent myosin light chain kinase (Siess, 1989). Myosin light chain phosphorylation leads to an increase in actin-activated ATPase activity, the association of myosin with the cytoskeleton, and the assembly of myosin into filaments.

The formation of pseudopods is partially mediated by bundles of F actin that cause the membrane projections (pseudopods) from the activated platelet. Two platelet proteins responsible for the stabilization of actin filaments are the actin-binding protein, which cross-links the filaments, and α-actinin which may serve to anchor the filaments to the inner membrane surface.

1.2.2.3. Liberation of Inositol 1,4,5-Triphosphate and Diacylglycerol

Another early event in the platelet activation sequence is the phospholipid liberation of inositol 1,4,5-triphosphate and diacylglycerol. When platelets are stimulated, the agonist:receptor complex activates phospholipase C through a G-protein system. Phospholipase C cleaves the phospholipid phosphatidyl inositol 4,5-bisphosphate to produce inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate mobilizes calcium from the dense tubular system while diacylglycerol activates protein kinase C which promotes aggregation possibly by the phosphorylation of a 47 kDa protein
(Bachelot et al., 1992; Nishizuki, 1984). The function of this 47 kDa protein is not known but it is consistently phosphorylated on platelet activation.

1.2.2.4. Exposure of Platelet Membrane Procoagulant Phospholipids

During shape change a transbilayer movement of membrane phospholipids occurs. This phospholipid flip brings procoagulant phospholipids to the extracellular surface of the platelets where they activate two steps of the intrinsic coagulation pathway that facilitates the generation of thrombin on the platelet surface (Mann et al., 1992; Ehrman et al., 1978). Phosphatidylyserine is the most potent procoagulant phospholipid (Schroth and Zwaal, 1991). The main function of the platelet membrane in coagulation is to provide a catalytic surface for the assembly of different coagulation factors.

1.2.2.5. Platelet Cohesion

The activated, spiny spherical platelets are said to be “sticky” due to their increased ability to encounter each other more closely and to the appearance of many different receptors on the extracellular surface of the platelet membrane. Crucial to the aggregation or cohesion process is the assembly of the fibrinogen receptor, the GPIIb/IIIa complex, at the platelet surface. The GPIIb/IIIa complex is absent in the unstimulated platelet. The assembly of the GPIIb/IIIa complex in the platelet membrane is prevented by the elevation of intracellular cAMP which inhibits aggregation (Peerschke, 1985). The GPIIb/IIIa receptor is an integral membrane protein complex that accounts for ~ 18 % of the total platelet membrane proteins (Newman et al., 1982).
Fibrinogen is a hexameric glycoprotein (340 kDa) consisting of three pairs of nonidentical chains (Aα, Bβ, and γ) covalently linked by a cluster of disulfides (Doolittle, 1984). Fibrinogen can be considered the glue between aggregated platelets since different sites on the same fibrinogen molecule can interact with the GPIIb/IIIa receptor of one platelet and the GPIIb/IIIa receptor of another platelet. In total fibrinogen contains three GPIIb/IIIa receptor interaction sites (Doolittle, 1984). The presence of one or both of the divalent cations Mg$^{2+}$ or Ca$^{2+}$ is required for fibrinogen receptor binding. Prolonged incubation of platelets with EGTA or EDTA prevents aggregation by promoting a dissociation of the GPIIb/IIIa receptor into monomers (Pidard et al., 1986). Fibrinogen receptor exposure and shape change are distinct events since epinephrine, a stimulus that does not evoke shape change, induces fibrinogen receptor exposure (Shattil et al., 1986). The GPIIb/IIIa receptor also binds thrombospondin, fibronectin, and vWF which are released from the α-granules. Thrombospondin also binds to fibrinogen.

1.2.2.6. Release Reaction: Liberation and Oxidation of Arachidonic Acid

The liberation of arachidonic acid and its subsequent metabolism occurs together with granule secretion to form a process known as the release reaction. The induction of the release reaction determines whether or not a stimuli is sufficient to evoke secondary irreversible aggregation. The activation of phospholipase A$_2$ by a G-protein system results in the phospholipid liberation of arachidonic acid. Platelet phospholipid-derived arachidonic acid is then converted to either prostaglandin G$_2$ (PGG$_2$) by the enzyme cyclooxygenase or through the actions of 12-lipoxygenase to 2-hydroxy-eicosatetraenoic
acid (12-HPETE). The reactive metabolite 12-HPETE is stabilized by reduction to 12-hydroperoxy-eicosatetraenoic acid (12-HETE). The role of 12-HPETE and 12-HETE in platelet function is uncertain since they have been shown to be either inhibitors (Aharony et al., 1982) or stimulators of platelet activation (Hemler and Lands, 1980).

In addition to cyclooxygenase activity, the cyclooxygenase enzyme also contains peroxidase activity which converts PGG$_2$ to PGH$_2$. Cyclooxygenase can be irreversibly inhibited by acetylsalicylic acid (aspirin). PGH$_2$ is subsequently transformed to several metabolites including PGD$_2$, PGG$_2$, PGF$_{2\alpha}$, and thromboxane A$_2$ (TxA$_2$). TxA$_2$ is a very potent platelet agonist that contributes greatly to the potentiation of an in vivo stimuli, such as subendothelial collagen exposure, by the activation of platelets not in direct contact with the stimuli. Receptors for TxA$_2$ are present in the platelet membrane and their activation by complexation mobilizes calcium from intracellular stores. TxA$_2$ is short-lived as it is rapidly degraded nonenzymatically to TxB$_2$.

PGE$_2$ and PGF$_{2\alpha}$ are weak platelet agonists while PGD$_2$ is an inhibitor of platelet activation. The physiological significance of PGD$_2$ inhibition of platelet activation is questionable since only trace amounts are produced. The binding of PGD$_2$ to its platelet membrane receptor results in the activation of adenylate cyclase by a G-protein system. The resulting increase in cytosolic cAMP activates the calcium pump which restores calcium levels to that of the basal state.
1.2.2.7. Release Reaction: Secretion of Granular Contents

The secretion of the lysosomal and α-granular contents is a later event in platelet activation that occurs only with relatively strong agonists. Lysosomal granule contents, in particular, are only secreted in response to very potent agonists such as high concentrations of thrombin. Secretion can occur by two mechanisms. One mechanism is initiated early in the platelet activation process and involves a centralization of the granules during platelet spheration (Carrol et al., 1982). If the agonist is sufficiently strong a membrane fusion event occurs between the granule membrane and the membrane of the open canalicular system with platelet extrusion of the granular contents. Alternatively, granules may move to the platelet periphery where they may fuse with the plasma membrane (Polasek, 1987). Some of the granular substances contribute to platelet activation while others affect the vessel wall tone (serotonin, TxA2, or ATP) or stimulate smooth muscle cell proliferation (platelet-derived growth factor).

1.2.2.8. Centripetal Consolidation of Platelet Aggregate

The final event of platelet activation involves the consolidation of the mass of platelet aggregates. This consolidation involves an orientated centripetal contraction of actomyosin. The contraction stabilizes the platelet aggregate to result in the formation of a platelet hemostatic plug.
1.2.2.9. Measurement of Platelet Aggregation

Platelet aggregation is most often studied using a turbidometric approach that is based on the observed increase in light transmission through a stirred platelet suspension on aggregating (Zucker, 1989). The turbidometric method is very simple and useful in many applications. Accurate measurements of the rate of platelet aggregation or adhesion, however, cannot be obtained turbidometrically since it cannot detect the initial formation of aggregates. Within 10 seconds 50% of single platelets may be aggregated with little or no indication of aggregation by the turbidometric method. To measure early events in platelet aggregation a stopped-flow resistive particle counting method has been developed that measures the loss of single platelets from an aggregated suspension (Gear, 1994).

1.2.2.10. Physiological Platelet Agonists

In vivo the normal initial stimulus is, as mentioned, subendothelial collagen. There are several other platelet agonists that are produced by the platelet and potentiate the overall platelet response to the initial stimulus. This positive feed-back response is largely the consequence of platelet arachidonic acid oxidation and granule secretion and contributes significantly to the growth of the platelet aggregate. There are several endogenous platelet agonists as shown by the following list.

Some examples of platelet agonists other than collagen are thrombin, ADP, TxA₂, platelet-activating factor (1-O-alkyl-2-acetyl-glycerophosphocholine), serotonin, epinephrine, and vasopressin (Packham, 1994). On its own serotonin is a very weak
agonist but it contributes synergetically with other agonists. Viruses, bacteria, tumor cells, and immune complexes have also been shown to be stimuli of platelet activation (Packham, 1994). Most of the aforementioned agonists mediate their effects through specific receptors and GTP-binding proteins (G-proteins). The role of G-proteins in platelet activation has been well documented (reviewed in Manning and Brass, 1991). In the following paragraphs of this section general aspects of the mechanisms of some important platelet agonists will be described.

Collagen induced platelet aggregation proceeds through an initial lag period in which arachidonic acid metabolites are synthesized and released to promote aggregation. Apparently collagen on its own is not able to induce aggregation but is dependent on the release reaction. Inhibitors of cyclooxygenase strongly inhibit collagen-induced platelet aggregation (Kinlough-Rathbone, 1977). Collagen is still an important physiological platelet agonist but generally not in its monomeric form. Monomeric collagen or tropocollagen (~300 kDa) is a triple stranded coil composed of one α and two β chains, the latter being intramolecularly crosslinked. There are several types of collagen and the three that support platelet adhesion (types I, III, and IV) are found in the subendothelium of the vasculature. Human platelets are activated only by polymerized microfibrillar human collagen or high concentrations of the α1(I)-chain of denatured chick skin collagen. Platelet adhesion to collagen is dependent on Mg²⁺ while plasma concentrations of Ca²⁺ are inhibitory (Santoro, 1986). Several collagen receptors have been proposed but the currently accepted collagen receptor is GPlα (Santoro, 1986).
Thrombin is the most potent of the known platelet agonists and its receptor has been identified and cloned (Vu et al., 1991). As an agonist thrombin is unique in that it has hormone and enzyme activities. In fulfilling its hormone role thrombin binds to the receptor GPIb which is an integral membrane protein that spans the membrane (Phillips and Agin, 1977). GPIb is composed of the following two polypeptide chains, GPIbα (143 kDa) and GPIbβ (22 kDa). The activated thrombin-receptor complex induces full aggregation.

As an enzyme thrombin is a serine protease involved in the formation of fibrin clots from fibrinogen that surround and support platelet aggregates. GPV (89 kDa) is a thrombin substrate on the surface of the platelet membrane that is cleaved to release the hydrolytic fragment GPVf1 (69.5 kDa) (McGowan et al., 1983). Thrombin hydrolysis of GPV correlates with platelet aggregation through an unknown mechanism.

Arachidonic acid and the arachidonic acid metabolites TxA₂, PGG₂ and PGH₂ are potent but labile agonists. Platelet activation with arachidonic acid is dependent on cyclooxygenase activity, while TxA₂ is the most potent arachidonic acid metabolite. The effects of the above agonists appear to be mediated by one receptor designated as the TxA₂/PGH₂ receptor. Since these compounds are short-lived under physiological conditions, stable analogs are often utilized to investigate their effects on platelet function (Siess, 1989).

ADP is a platelet agonist that exhibits two distinct effects. In one instance ADP induces shape change, the exposure of fibrinogen receptors and aggregation. A second effect of ADP is the inhibition of adenylate cyclase. As mentioned elevated intracellular
cAMP, the product of adenylate cyclase, inhibits aggregation. It has been proposed that these two effects may be mediated by two distinct receptors or by different sites on the same receptor. In any event controversy exists over the identity of the ADP receptor as either a 43 kDa protein (Christalli and Mills, 1993) or a 100 kDa protein that has been termed aggregin (Colman, 1990).

ADP-induced aggregation in the presence of physiological levels of calcium is only comprised of the primary reversible stage of aggregation regardless of the concentration of ADP. At low calcium concentrations (~ 50 μM) ADP can induce full aggregation. The mechanism responsible for this dependence on extracellular calcium is unknown but it serves to demonstrate an example of a difference in the activation pathways among different agonists (Packham, 1994).

1.2.2.11. Physiological Regulation of Platelet Activation

The strict regulation of platelet activation is necessary to prevent thrombus formation. The uncontrolled hyperaggregation of platelets is the most common proximate cause of myocardial infarctions and cerebral infarction. The prevention of irreversible platelet aggregation is required when platelets fill in gaps between endothelial cells as described in Section 1.2.2. Platelet antagonists, substances that inhibit activation, have been identified and some aspects of their mechanisms are known (Siess, 1989). It is not known, however, how these antagonists or others may induce reversible platelet aggregation.
Endothelial cells release two substances known to inhibit platelet aggregation, prostacyclin (PGI₂) and nitric oxide (NO). PGI₂ activates adenylate cyclase and NO activates guanylate cyclase resulting in the intracellular elevation of the secondary messengers cAMP and cGMP, respectively. The inhibitory mechanism of NO on platelet function will be described in Section 1.4.2. PGI₂ inactivates platelets through interaction with a receptor (PGI₂/PGD₂ receptor). PGD₂ is produced by platelet arachidonic acid metabolism as described above and inhibits aggregation in the same manner as PGI₂. PGE₁ is another platelet inhibitory arachidonic acid metabolite produced by the platelet that mediates its actions through the PGI₂/PGD₂ receptor.

Elevated intracellular levels of cAMP and cGMP result in the activation of cAMP and cGMP-dependent protein kinases. The resulting protein phosphorylation distribution mediates platelet relaxation. The elevation of cAMP by PGI₂, PGD₂, PGE₁ results in the phosphorylation of proteins with the following molecular weights 22, 24, 50, and >400 kDa (reviewed in Siess, 1989). The 22 kDa protein is a membrane bound protein that may be involved in the uptake of calcium by the dense tubules (Fox et al., 1979). The 24 kDa protein has been identified as GPIIbβ, which is a component of the thrombin receptor (Fox et al., 1987). The 50 kDa protein is also a major cytosolic protein phosphorylated in intact platelets upon exposure of the NO-generating reagent sodium nitroprusside which suggests that its phosphorylation is important in the inhibition of platelet function (Takai et al., 1982).
1.3 Glutathione Metabolic Functions

Glutathione (L-γ-glutamyl-L-cysteinylglycine) (GSH) is present at high intracellular concentrations (1-10 mM) in most cells including platelets (Till et al., 1988). The high intracellular concentration of GSH gives it the distinction of being the most abundant intracellular nonprotein thiol. GSH has been utilized by cells to perform many different functions. Some of the more well characterized functions of GSH include the protection against oxidative damage, the detoxication of foreign compounds, the formation of peptidoleukotrienes, involvement in protein disulfide formation and RNA reduction to DNA, and the maintenance of an intracellular reducing environment for redox sensitive enzymes and proteins to remain active (reviewed in Meister, 1992; Deneke and Fanburg, 1989). In addition to the reduction of protein thiols, GSH can also participate in the maintenance of various compounds such as ascorbic acid and α-tocopherol in their reduced forms. Some of these functions will be described in the following sections on GSH.

Acquired intracellular GSH deficiency has been associated with some diseases such as AIDS, lung and respiratory disorders, liver cirrhosis, malnutrition (reviewed in White et al., 1994) and diabetes mellitus (Winocour, 1992). Hereditary GSH deficiencies are uncommon and are associated with a deficient GSH metabolic enzyme. In the following sections on the GSH metabolic enzymes references will be made, if available, to cases of hereditary enzyme deficiency.
1.3.1 Glutathione Biosynthesis

GSH is synthesized intracellularly from its constituent amino acids by most cells. The first step in the synthesis involves the formation of a peptide bond between the γ-carboxyl group of glutamate and a cysteine amine and is catalyzed by the enzyme γ-glutamylcysteine synthetase (Fig. 2). The resulting γ-glutamyl linkage to cysteine is very resistant to hydrolysis by normal peptidase activity. The enzyme γ-glutamylcysteine synthetase requires 1 mole of ATP per mole of product. Another mole of ATP is consumed by the peptide addition of glycine in the formation of γ-glutamylcysteinylglycine (GSH) as catalyzed by the enzyme glutathione synthetase. The control point in the biosynthesis is believed to be mediated primarily through the regulation of γ-glutamylcysteine synthetase. γ-Glutamylcysteine synthetase has been shown to be feedback inhibited by GSH (Seelig et al., 1984).

1.3.1.1 γ-Glutamylcysteine Synthetase

γ-Glutamylcysteine synthetase (γ-GCS) from certain sources has been previously characterized to some degree. The 3-dimensional structure of γ-GCS, however, has not been determined. γ-GCS catalyzes the following reaction.

\[ \text{Glu} + \text{Cys} + \text{ATP} \xrightarrow{\gamma}\text{-GluCys} + \text{ADP} + \text{P}_i \]

The enzyme displays strict specificity towards glutamate but will accept some amino acids other than cysteine with a reduced activity particularly when Mn\(^{2+}\) is substituted for Mg\(^{2+}\) (Seelig and Meister, 1985). The reaction is thought to occur via an enzyme bound γ-glutamyl phosphate intermediate (Seelig and Meister, 1985).
Fig. 2. Glutathione metabolism. This figure depicts the events leading to GSH biosynthesis and some functional consequences of GSH. The events shown are described in the text. The different transport systems involved in GSH metabolism are shown using filled ovals. The filled ovals that contain a question mark (?) represent transport systems that have not been characterized. Abbreviations not defined in the text are as follows: AA, any of a number of amino acids or small peptides; RX, an electrophile; GSR, glutathione electrophilic conjugate; g-glutamyl cycle, γ-glutamyl cycle; g-GT, γ-glutamyl transpeptidase; g-Glu-AA, an amino acid with a γ-glutamyl peptide linkage; DPT, dipeptidase; CysR, cysteine electrophilic conjugate, CT, cyclotransferase; 5-OP, 5-oxoprolinase; γ-GCS, γ-glutamylcysteine synthase; GSH S, glutathione synthase; HMS, hexose monophosphate shunt.
Rat kidney γ-GCS has been shown to be composed of a heavy subunit (~ 73 kDa) covalently linked through a disulfide bond to a light subunit (~ 27.7 kDa) (Huang et al., 1993a; Huang et al., 1993b; Yan and Meister, 1990). The heavy rat kidney subunit is believed to contain most, if not all, of the structural requirements for enzyme activity and for feedback inhibition by GSH (Huang et al., 1993a). GSH inhibits γ-GCS by a reduction of the enzyme and by competing with glutamate for the glutamyl binding site on the enzyme (Huang et al., 1993b). The function of the light subunit is uncertain but it has been suggested that it may exert a stabilizing effect on the enzyme and it has been shown that the light subunit reduces the degree of inhibition by GSH (Huang et al., 1993b). The light subunit regulates the activity of γ-GCS by promoting a higher affinity for glutamate than for GSH binding at the glutamyl binding site on the enzyme. Rat liver γ-GCS was similar to the rat kidney enzyme in that it had a light and heavy subunit but differed in molecular structure and in the degree of inhibition by GSH (Chang and Chang, 1994). γ-GCS from human astrocytoma cells was also found to be very similar in subunit structure and arrangement to the rat kidney enzyme (Sriram and Ali-Osman, 1993). The E. coli enzyme is composed a single polypeptide chain that exhibits sequence homology to the heavy subunit of rat kidney γ-GCS (Huang et al., 1993b).

Though uncommon, cases of hereditary γ-GCS deficiency have been reported (Konrad et al., 1972; Beutler et al., 1990). Symptoms associated with this enzymatic deficiency include hemolytic anemia as a consequence of irregularly shaped red blood cells and spinocerebellar degeneration.
1.3.1.2 Glutathione Synthetase (EC 6.3.2.3)

Various aspects of the enzyme, glutathione synthetase (GSHase), have been studied in detail. GSHase catalyzes the following reaction.

\[
\gamma\text{-GluCys} + \text{Gly} + \text{ATP} \rightarrow \gamma\text{-GluCysGly} + \text{ADP} + \text{P}_\text{i}
\]

The enzyme from rat kidney is strict in its recognition of glycine but not towards the \(\gamma\)-GluCys moiety in that it will accept amino acids sterically similar to cysteine (alanine and serine) (Meister, 1985). The reaction mechanism proceeds by an ordered mechanism through the formation of a \(\gamma\)-GluCys phosphate intermediate and there is a requirement for K\(^+\) ions (Meister, 1985).

The quaternary structure of GSHase is not conserved among the enzymes from different sources. Rat kidney GSHase is a homodimer of 59 kDa subunits (Meister, 1985). In *E. coli* the enzyme is a tetramer of identical subunits with a molecular weight of 35.5 kDa (Guishima *et al.*, 1983). GSHase from yeast is also a tetramer but is comprised of two 33 kDa subunits (A) and two 26 kDa subunits (B) in a A\(_2\)B\(_2\) arrangement (Nakagawa *et al.*, 1993). The large subunit of the enzyme from *Xenopus laevis* has been found to be homologous to that of the yeast (Habenicht *et al.*, 1993).

The 3-dimensional structure of the enzyme from *E. coli* has been determined by X-ray crystallography to a resolution of 2.0 Å (Yamaguchi *et al.*, 1993). The structural topology of the ATP binding site of *E. coli* GSHase was found not to resemble that of a Rossman-fold or a P-loop motif. The ATP binding site was found to be located in the cleft formed between two \(\beta\)-sheets from different domains. There is also a loop in the
enzyme from Ile-226 to Arg-241 that is essential for activity as it apparently protects the acyl phosphate intermediate from spontaneous hydrolysis (Tanaka et al., 1993).

Cases of a GSHase deficiency have also been reported (Meister and Anderson, 1983; Weller et al., 1974; Mohler et al., 1970). Hemolytic anemia has also been associated with these patients as has 5-oxoprolinuria which leads to a severe metabolic acidosis (Meister and Anderson, 1983). Various neurological defects have also been found in these patients.

1.3.2 Protection Against Oxidative Damage

An important function of GSH is its ability to reduce reactive oxygen metabolites such as hydroperoxides. The transformation of hydroperoxides and other reactive oxygen species to even more reactive species such as OH·, ferryl complexes, RO·, and RO2·, occurs in vivo as catalyzed by physiological levels of metal ions (Benasson et al., 1993). Biological thiols such as cysteine, cysteamine and GSH can quench radicals produced from ionizing radiation including radicals derived from the sugar moiety of DNA (Tamba and Quintiliani, 1984). In this manner biological thiols offer radioprotection from such cytotoxic events as radical induced DNA strand breaks. Apparently, the thyl radicals produced combine in a diffusion controlled process and are of lessened but not entirely diminished cytotoxicity (Schoneich et al., 1989).

Hydroperoxides are continually produced by many basal cellular processes. For example several enzymes such as glycolate oxidase, D-amino acid oxidase, urate oxidase and superoxide dismutase produce H2O2 directly. An increased production of
hydroperoxides and other reactive oxygen species often coincides with a cellular activation event such as during phagocytosis where they are mediators of inflammation (Weiss, 1986). The cellular accumulation of reactive oxygen species and radicals is cytotoxic largely due to membrane phospholipid oxidation which destroys the integrity of the plasma membrane. It is essential for viable cells to possess a system for limiting the concentration of these reactive species. The reduction of many hydroperoxides is catalyzed by the enzyme glutathione peroxidase.

1.3.2.1 Glutathione Peroxidase (EC 1.11.1.9)

Glutathione peroxidase (GPX) is a well characterized enzyme and the best characterized selenoprotein. GPX utilizes the reducing potential of GSH to reduce hydrogen peroxide (H$_2$O$_2$) as well as many different organic hydroperoxides (ROOH) regardless of whether or not they are of exogenous or endogenous origin. GPX displays very poor activity towards endoperoxides (ROOR). In contrast to the broad specificity towards hydroperoxides, GPX is not active in the presence of biological thiols other than GSH. As will be described in Section 1.3.4.1, some isozymes of glutathione S-transferases display selenium-independent glutathione peroxidase activity but only towards organic hydroperoxides. The GSH-mediated reduction of hydrogen peroxide can be used to monitor selenium-dependent GPX activity. The general reaction catalyzed by GPX is shown below where ROOH can also be H$_2$O$_2$ (Tappel, 1978).

$$2\text{GSH} + \text{ROOH} \longrightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O}$$
Reports of the molecular weight of the homotetrameric GPX molecule range from 76 kDa in rat liver to 110 000 in rat erythrocytes (Styblo, 1992) which corresponds to an approximate subunit size of between 19 and 27 kDa, respectively. GPX is primarily a soluble cytosolic enzyme but it has been identified in the mitochondrial matrix and a distinct isoform has been identified in rat plasma that apparently is comprised of several disulfide linked tetramers in a 700 kDa complex (Styblo, 1992). GPX from all sources contains a catalytically important selenocysteine at the active site of each subunit. Selenocysteine is the selenium analog of cysteine in which a selenium atom has replaced the cysteine sulfur.

The 3-dimensional structure of the enzyme from bovine erythrocytes (84 kDa) has been determined by X-ray crystallography to a resolution of 2 Å (Epp et al., 1983). The amino acid sequence of bovine erythrocyte GPX was determined after the 3-dimensional structure was solved by the same laboratory (Gunzler et al., 1984). In bovine erythrocytic GPX the active site was located in a flat depression on the molecular surface. The selenocysteine was located near the N-terminus within a βαβ substructure in which the selenocysteine was positioned between the first β-sheet and the α-helix.

The catalytic mechanism of GPX has been shown to be approximated by a “ping pong” kinetic scheme. The mechanism as suggested by Epp and coworkers (1983) can be summarized by the following scheme.
In the absence of GSH, GPX exists in a sluggish oxidized form (E-SeOOH) that is not shown in the scheme and is likely not present in vivo in the presence of millimolar GSH concentrations. The selenolate form of the enzyme (E-Se-) is capable of reducing many hydroperoxides to alcohols with the formation of the selenenic acid form of the enzyme (E-Se-OH). Two reducing equivalents are required to reduce the enzyme through a selenosulfide (E-Se-SG) back to its active selenolate form (Epp et al., 1983).

In the 3-dimensional GPX structure the amido groups Gin-70 and Trp-148 are located a distance of 3.3 and 3.4 Å from the selenocysteine-35 at the active site (Epp et al., 1983). It has been found using a compound that mimics GPX activity that these proximate nitrogens stabilize the otherwise elusive selenenic acid intermediate (Iwaoka and Tomoda, 1994).
The GSSG produced by the turn-over of GPX is recycled back to GSH through the actions of glutathione reductase as shown in Fig. 2. Glutathione reductase will be described in the following section (Section 1.3.3.1.)

1.3.3. Maintenance of Intracellular Reducing Environment

As a reducing agent, GSH serves as a source of electrons for several enzymes and proteins. GSH participates in a pathway for the reduction of ribonucleotides to the DNA precursors, deoxyribonucleotides, by supplying reducing electrons to the protein glutaredoxin which transfers them to ribonucleotide reductase (Eklund et al., 1984). GSH also serves an enzyme cofactor role for some enzymes involved in catalyzing some biological cis-trans isomerase reactions. One such cis-trans isomerization is the conversion of the aromatic amino acid metabolite maleylacetoacetate to fumarylacetoacetate via the enzyme maleylacetone cis-trans isomerase (Seltzer and Lin, 1978).

GSH reduces protein disulfides through a transhydrogenation reaction that may be catalyzed by a thiol transferase. Alterations in the protein thiol to disulfide status were shown over 40 years ago to affect the activity of some enzymes (Guzman Baron, 1951). The following transhydrogenation reaction depicts the GSH-mediated reduction of a protein thiol where the protein disulfide may be intermolecular, if R is some other molecule, or intramolecular, if R represents another cysteine in the same protein.

$$2\text{GSH} + \text{Protein-S-S-R} \longrightarrow \text{Protein-SH} + \text{RSH} + \text{GSSG}$$
Alternatively, GSSG, or any disulfide, can react with protein thiols through a transhydrogenation to form a mixed disulfide as shown in the following reaction.

\[
\text{GSSG} + \text{Protein-SH} \rightarrow \text{Protein-S-SG} + \text{GSH}
\]

Which of the above transhydrogenation reactions occurs predominantly \textit{in vivo} depends on the relative intracellular proportion of GSH to GSSG. The \textit{in vivo} activity of the enzyme glutathione reductase determines the intracellular GSH:GSSG ratio.

1.3.3.1. Glutathione Reductase (E.C. 1.6.4.2)

GR is a well-characterized flavoprotein that utilizes the reduction potential of NADPH to catalyze the reduction of GSSG. Briefly, two electrons are transformed from NADPH to FAD. FADH$_2$ reduces an active site disulfide to generate the reduced enzyme (EH$_2$) which is capable of reducing GSSG to 2 molecules of GSH. The enzyme remains tightly bound to FAD throughout its catalytic cycle which requires the binding of NADPH and release of NADP$^+$.

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

The equilibrium constant for the GR-catalyzed reaction at pH 7.0 and 25°C is (Chance \textit{et al.}, 1979)

\[
K = \frac{[\text{GSH}]^2[\text{NADP}^+]}{[\text{GSSG}][\text{NADPH}]} = 200 M
\]

Based upon a likely intracellular GSH concentration of 5 mM and a likely NADPH/NADP$^+$ ratio of 100:1 the equilibrium GSSG concentration can be calculated as approximately 1 nM and the GSH:GSSG ratio as $5 \times 10^6$. The calculated equilibrium GSH:GSSG ratio is far from the experimentally determined values which range from 20 to
1000 (Akerboom et al., 1982). One reason for the discrepancy between experimental and calculated GSH:GSSG ratios may be due to the high $K_M$ of GR towards GSSG (typically 30 to 100 $\mu$M). GR will not reduce GSSG when GSSG is present at concentrations much below its $K_M$ which permits the accumulation of GSSG to greater, than otherwise expected, intracellular levels.

GR has been isolated and characterized from several sources but most studies have been carried out using the enzyme from yeast and human erythrocytes and more recently from *E. coli* since this isoform has been cloned (Greer and Perham, 1986). In general, GR from different sources are very similar in structure and catalytic properties. GR is a homodimer of approximately 50 kDa subunits. The amino acid sequence of GR from several sources, including human erythrocyte and *E. coli* is known (Krauth-Siegel et al., 1982; Greer and Perham, 1986). The 3-dimensional structure of GR from human erythrocytes and *E. coli* has been determined by X-ray crystallography where the lowest resolution obtained was 1.54 Å and 3.0 Å, respectively (Karplus and Schultz, 1987; Ermler and Schulz, 1991).

As a result of the studies on GR, mentioned above, a model for the structure and catalytic cycle has been determined largely based on the human erythrocyte enzyme. Each GR subunit contains a FAD binding domain, a NADPH binding domain, a central domain and a subunit interface domain. The chain folds of the FAD and NADPH nucleotide binding domains are similar to those found in other proteins (Schirmer et al., 1989). GR is very selective towards GSSG reduction but some other disulfides such as disulfides of GSH analogs, the mixed disulfide between GSH and coenzyme A, 5-5'-dithio-bis-(2-
nitrobenzoate) (DTNB), and D,L-lipoate among others (Schirmer et al., 1989) have been shown to be poor substrates. The enzyme displays a strict specificity towards NADPH over NADH. Interestingly, Scrutton and coworkers (1992) have, through protein engineering techniques, transformed the substrate specificity of *E. coli* GR from NADPH to NADH by a seven amino acid mutation.

There are two active sites per molecule of GR and they are located at opposite ends of the subunit interface domains of the two subunits. In the human erythrocyte enzyme there is a flexible N-terminal extension of 18 amino acids that contains a Cys-2 thiol which may be important in enzyme regulation or in anchoring the enzyme to other intracellular structures (Schirmer et al., 1989). This N-terminal extension is absent in the *E. coli* enzyme. Another difference between the human erythrocyte and *E. coli* enzyme is the presence of an intersubunit disulfide bridge between Cys-90-Cys'-90 in human erythrocyte GR that is absent in *E. coli* GR. This disulfide occurs at a flexible region of the interface and may therefore stabilize the enzyme and possibly be involved in a regulatory mechanism (Schirmer et al., 1989).

Kinetic studies have shown that the GR-catalyzed reaction proceeds via a ping pong mechanism at low GSSG concentrations or an ordered sequential mechanism at high GSSG concentrations (Mannervik, 1973). At either concentration of GSSG the first substrate to encounter the enzyme is NADPH. After reduction of the flavin by NADPH the positive charge of Lys-66 promotes NADP⁺ dissociation (Karplus and Schultz, 1987; Pai and Schultz, 1983).
Figure 3 is a schematic representation of the events that occur during GR catalysis. Upon binding NADPH there is a large movement in Tyr-197 and two electrons are transferred from NADPH to the flavin moiety of FAD. The nicotinamide ring of NADPH lies flat on the flavin ring system while the two adenosine moieties of NADPH and FAD are far apart with an N-1 to N-1 distance of 2.9 nm. The reduced FADH₂ is surrounded by several basic residues and ion pairs which act to stabilize the transitory negative charge on the ring. For example Arg-291, the ion pair Lys-66:Glu-201, and Asp-331 together with a trapped water molecule are all in the vicinity of the flavin. In addition, the positive dipole of the α-helix from residues 338-354 further stabilize the flavin intermediate.

The reduced flavin then performs a two electron reduction of a catalytically important disulfide at the active site (Cys-58 and Cys-63 in the human erythrocyte enzyme) to result in the formation of the reduced enzyme. The pKa of the Cys-58 thiol is lowered by hydrogen bonding to a protonated His-467' which is associated with Glu-472'. Cys-58 is inside the GSSG binding site. Upon binding, GSSG interacts with Cys-58 through disulfide exchange to liberate one molecule of GS which abstracts a proton from the protonated His-467'. The Cys-58-S-SG mixed disulfide intermediate is then subjected to a second disulfide exchange reaction with Cys-63 that results in the production of a second molecule of GSH and the oxidized form of the enzyme.

The requirement of NADPH in the recycling of GSSG to GSH implicates the hexose monophosphate shunt, the major source of NADPH production, in intra-cellular GSH homeostasis (Hofman et al., 1980). Glucose-6-phosphate dehydrogenase oxidizes glucose-6-phosphate to 6-phosphoglucono-δ-lactone and concomittantly reduces NADP⁺
Fig. 3. Structure of human erythrocyte glutathione reductase. Panel A: The diagram demonstrates the domain organization of GR and the subunit interface. The approximate positions of the ligand binding sites for FAD, NADPH, and GSSG are indicated. The Cys-90-Cys-90' inter-subunit disulfide is also shown at the center of the structure. The active site can be seen at either end of the subunit interface. Panel B: This diagram is a sketch of the GR active site. The details of GR catalysis are described in the text. Both panels were reproduced with permission (Shirmer et al., 1989).
to NADPH + H⁺. Another NADPH can be generated following the enzyme-mediated hydrolysis of 6-phosphogluconolactone to 6-phosphogluconate which is subsequently oxidized and decarboxylated by phosphogluconate dehydrogenase to form ribulose-5-phosphate along with NADPH + H⁺. The three enzymes GR, glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase occur in human erythrocytes in a ratio of 1:1:10 and tend to form molecular associations in vitro (Rosemeyer et al., 1978). There is no direct evidence, however, for the in vivo presence of such a dodecameric multi-enzyme complex.

There are more than 300 variants of glucose-6-phosphate dehydrogenase deficiencies which in general have been accompanied by decreased erythrocyte GSH and varying degrees of hemolytic anemia (Beutler, 1989). There are also cases of individuals exhibiting a GR deficiency. In these cases patients were symptomized by severe hemolytic anemia following ingestion of fava beans (Loos et al., 1976), a shortening of the respiratory burst in activated leukocytes (Schirmer et al., 1989), cataract formation in the eye lens (Loos et al., 1976), and deafness at an early age (Schirmer et al., 1989).

1.3.3.2 Disulfide Exchange as a Potential Signal Transduction Mechanism

It has been suggested by Ziegler (1985) and others (Gilbert, 1990) that GSSG, or other small molecular weight disulfides, may be a novel type of secondary messenger. This suggestion is based on the findings that many enzymes catalyzing opposing reactions in a metabolic pathway are activated or inactivated in a reciprocal fashion through disulfide exchange with thiols and disulfides. For example, various aspects of carbohydrate
metabolism have been implicated as being regulated by the intracellular thiol and disulfide concentrations (reviewed in Ziegler, 1985). The switching point between glycolysis and gluconeogenesis is determined by the relative activities of phosphofructokinase which promotes glycolysis and fructose 1,6-biphosphatase which promotes gluconeogenesis. It has been found from *in vitro* studies that phosphofructokinase is inhibited by small molecular weight disulfides (Froede *et al.*, 1968; Gilbert, 1984) while fructose 1,6-biphosphatase is activated by small molecular weight disulfides (Pontremoli and Horecker, 1970). Evidence, however, for the direct demonstration of intracellular GSSG elevation in response to a hormone:receptor complex is lacking possibly due to the difficulties associated with accurate measurements of intracellular GSSG and protein-glutathione mixed disulfides.

Since GSH is the most abundant intracellular nonprotein thiol, it is likely that GSSG is the most abundant small molecular weight disulfide. The intracellular ratio of GSH to GSSG is therefore expected to be very important in the prevention or induction of GS-S-protein mixed disulfide formation. A portion of GSH is constantly being oxidized to GSSG as it has been shown that hydroperoxide is generated extensively by all cells (Chance *et al.*, 1979). The glutathione peroxidase-mediated reduction of peroxides is the major source of GSSG. In addition, there are certain cellular events that subject the cell to varying degrees of oxidative stress which is accompanied by an increase in the intracellular level of GSSG. For example, as already mentioned in Section 1.3.2, there is an oxidative burst during phagocytosis in leukocytes that results in the activation of collagenase by thiol oxidation (Weiss, 1986). Of particular interest to the present study is the observation
that during platelet activation some hydroperoxide metabolites of arachidonic acid are generated that are reduced by GPX and lead to an increase in intra-platelet GSSG levels (Burch and Burch, 1990). In most cases the intracellular rise in GSSG is transient due to reduction to GSH by GR. It is conceivable, however, that the elevated GSSG level may be sustained long enough to temporarily affect the activity of some redox sensitive proteins or enzymes.

One way that the GSH:GSSG ratio may be lowered to a level where the regulation of protein activity by GSH mixed-disulfide formation is possible is through the physiological inhibition of GR. If this inhibition could be induced in response to a hormone:receptor complex it may be a means for implementing GSSG secondary messenger activity. The physiological regulation of GR is considered in the following section.

1.3.3.3. Regulation of Glutathione Reductase Activity

Despite the wealth of structural and mechanistic information available for GR, little is known about the physiological regulation of its activity. There are many known inhibitors of GR but few are naturally present inside cells (Schirmer et al., 1989). The cytotoxic and pharmacological effects of compounds such as 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), nitrofurantoin, nitrofurimox, p-hydroxymercurybenzoate, and 1-chloro-2,4-dinitrobenzene (CDNB) may be at least partially attributable to their potent inhibition of GR (reviewed in Schirmer et al., 1989). Several thiol alkylating reagents also inhibit GR including iodoacetamide but not iodoacetate (Schirmer et al., 1989).
The heavy metal ions Zn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, and Hg$^{2+}$, have also been shown to inhibit GR to varying extents (Cartana et al., 1989; Serafini et al., 1989; Schirmer et al., 1989). Some of these metal ions (Zn$^{2+}$, Cu$^{2+}$ and Ni$^{2+}$) are naturally occurring biological trace elements. With respect to a varied GSSG concentration the reported $K_i$ for Zn$^{2+}$ ranges from 0.8 to 6.5 μM (Schirmer et al., 1989), for Cu$^{2+}$, ranges from 5 to 10 μM (Schirmer et al., 1989), and for Ni$^{2+}$ is approximately 260 μM (Cartana et al., 1989). It is conceivable, therefore, that an intracellular elevation in the concentration of those metal ions with the lowest $K_i$ (Zn$^{2+}$ or Cu$^{2+}$) may represent one physiological mechanism of elevating GSSG through the inhibition of GR. This hypothesis is completely unsubstantiated and is highly dependent upon the existence of hormone:receptor activated mechanisms for the regulation of intracellular metal ion concentration which has not been demonstrated.

There has been some previous consideration of substrate and product GR in vivo regulation. NADP$^+$ has been shown to be a competitive inhibitor with respect to NADPH and a noncompetitive inhibitor with respect to GSSG (Worthington and Rosemeyer, 1976; Carlberg and Mannervik, 1975). The calculated $K_i$ for NADP$^+$ with respect to NADPH was 70 μM for the human erythrocyte enzyme. The physiological significance of NADP$^+$ product inhibition is questionable since the normal intracellular [NADP$^+$] is much less than the $K_i$ (Schirmer et al., 1989).

Preincubation of GR with NADPH or NADH inactivated the enzyme in a time dependent manner (Pinto et al., 1984; Pinto et al., 1985). The inactivation was prevented in the presence of GSSG or GSH. The suggested mechanism for the NADPH-mediated
inactivation has been the formation of an erroneous intramolecular disulfide with one of the active site cysteines of the reduced enzyme and another thiol in the enzyme, possible Cys-2 (Pinto et al., 1985). The physiological significance of this inactivation is also questionable, since it is only observed in the absence of GSSG and GSH. GSH and GSSG, as previously mentioned, are present at high levels in most cells.

There have been several reports hinting at the in vitro inhibition of the enzyme by GSH. No details of the inhibition were provided in these early reports (Mannervik, 1969; Worthington and Rosemeyer, 1976). The first such detailed report (Chung et al., 1991) on the inhibition of glutathione reductase by GSH appeared in the literature while our manuscript was in preparation. In the previous study GSH was shown to be a non-linear, non-competitive inhibitor of rat liver GR. We expanded upon their findings by examining the effect of physiological levels of GSH on GR from human platelets, bovine intestinal mucosa, yeast, and E. coli and found that the inhibition of GR by GSH appears to be a widespread phenomenon that should be considered in any assessment of GSH metabolism or GSH:GSSG homeostasis (Sexton and Mutus, 1992). The extent and the type of inhibition, however, appears to be dependent on the enzyme source. The species-dependent difference in inhibition patterns for GR by GSH is, to the best of our knowledge, the first example of an observed functional difference in the generally highly conserved GR enzyme. Physiologically, the consideration of this inhibition has important consequences because the enzyme can be regulated by normal levels of GSH. Presumably, the enzyme will be inhibited by GSH under normal cellular conditions and will therefore be
less able to convert GSSG to GSH providing a concentration of GSSG to be maintained at higher than otherwise expected concentrations.

1.3.4 Glutathione Conjugation to Electrophiles

Another fate of intra-cellular GSH is conjugation to an electrophile. Nucleophilic attack of the GSH thiolate anion upon various electrophiles can lead to a stable conjugate. This electrophilic conjugation may occur non-enzymatically or through the enzymatic assistance of one or more of the glutathione S-transferases. The electrophile may be of endogenous origin such as the arachidonic acid metabolite leukotriene A₄ which upon conjugation to GSH forms the bioactive peptidoleukotriene C₄ (Mannervik and Danielson, 1988). Interestingly, all of the leukotrienes are, in fact, GSH conjugates or conjugates of GSH fragments. Alternatively, the electrophile may be a foreign compound that upon conjugation to GSH is detoxicated to some degree. There are examples, however, where the GSH conjugation to xenobiotic results in a more toxic conjugate. One example where GSH conjugation activates the mutagenic potential of a compound is the GST catalyzed GSH conjugation to ethylene dibromide where the resulting 1-bromo-2-S-glutathione product reacts with DNA to produce a S-[2-(N⁷-guanyl)ethyl]-glutathione (Cmarik et al., 1990).

1.3.4.1 Glutathione S-Transferases (EC 2.5.1.18): Structure and Function

Glutathione S-transferases (GSTs) are cytosolic or microsomal enzymes that catalyze either the conjugation of various electrophiles with glutathione (GSH) or the
selenium-independent reduction of organic hydroperoxides by GSH. That few endogenous GST substrates have been discovered suggests that either more physiological substrates remain to be discovered or the primary function of GST is in detoxication, or both. The endogenous GST substrates that have been discovered include the highly toxic lipid peroxidation products 4-hydroxyalk-2-enals (Danielson et al., 1987), leukotriene A₄ (Soderstrom et al., 1985), and prostaglandin D₂ (Ujihara et al., 1988) among others (Pickett and Lu, 1989). GST also displays ligand activity towards the binding of a variety of hydrophobic compounds such as heme, bilirubin, polycyclic aromatic hydrocarbons and dexamethasone. These activities and several other important aspects of GST have been very well reviewed (Awasthi et al., 1994; Dirr et al., 1994; Wilce and Parker, 1994; Rushmore and Pickett, 1993; Tsuchida and Sato, 1992, Schecter et al., 1992; Armstrong, 1991; Pickett and Lu, 1989; Mannervik and Danielson, 1988). Some examples of GST catalyzed reactions are shown below.

1) Nucleophilic substitution where X may may represent a halogen, CN or other good leaving group:

$$\text{GSH} + \text{RX} \rightarrow \text{GSR} + \text{HX}$$

2) Organic hydroperoxide reduction:

$$2\text{GSH} + \text{ROOH} \rightarrow [\text{GSOH}] + \text{ROH}$$

$$\text{GSH} + [\text{GSOH}] \rightarrow \text{GSSG} + \text{H}_2\text{O}$$

3) Nucleophilic attack on strained oxirane rings:

$$\text{GSH} + \overset{\text{O}}{\text{GS}} \rightarrow \overset{\text{OH}}{\text{GS}}$$
4) Michael addition to $\alpha,\beta$-unsaturated compounds:

\[
\text{GSH} + \begin{array}{c}
\text{\text{O}}
\end{array} \rightarrow \begin{array}{c}
\text{\text{G}}
\end{array}
\]

5) Nucleophilic attack on electrophilic nitrogen (nitrenium ion):

\[
\text{GSH} + R_2N^+ \rightarrow R_2NSG + H^+
\]

6) Reaction with a nitroso compound:

\[
\text{GSH} + RNO \rightarrow RNSG \rightarrow RNSG
\]

7) GSH-dependent isomerization of $\Delta^5$-androst-3,17-dione to $\Delta^4$-androst-3,17-dione:

The cytosolic GST enzymes are the products of 4 distinct gene families and are classified according to their isoelectric points and substrate affinities as either pi (acidic), mu (near neutral), alpha (basic) (Mannervik and Danielson, 1988) or theta (Meyer et al., 1991). Class theta is the most recently discovered class of GSTs. There is also a membrane associated form of the enzyme that has been identified in microsomes and is the product of a fifth gene family (Lundqvist et al., 1992). Each enzyme class can be further differentiated into several isozymes. The cytosolic GST classes alpha, mu and pi can be
separated on the basis of the differences in their net charges by ion-exchange chromatography. The isozymes within each class can be further resolved by chromatofocusing. It would appear that new GST isozymes are continually being discovered. The tissue distribution of many GST isozymes is highly specific. The expression of GST isozymes is selectively induced following exposure to certain compounds, including carcinogens (Tsuchida and Sato, 1992; Pickett and Lu, 1989). GSTs appear to be ubiquitous and are abundant especially in liver where they comprise 5% of the total cytosolic protein (Wilce and Parker, 1994).

GSTs are homodimers or heterodimers of approximately 25 kDa subunits. GSTs are composed of subunits from the same class. Dimerization of GST subunits from different classes are not known to occur in nature. Each subunit contains a GSH binding site or G-site and an electrophilic substrate binding site, the H-site. The G-site is very specific towards GSH while the H-site exhibits a broad specificity towards a number of structurally unrelated electrophiles. In fact, the structural determinants for H-site substrate recognition have not been determined. However, photoaffinity labelling (Hoesh and Boyer, 1989) and site-directed mutagenesis (Zhang and Armstrong, 1990) indicate that residues near the amino and carboxyl ends are involved. There is a very high sequence identity among subunits of the same class (60-80%) while inter-subunit identities are considerably less (25-35%) (Ji et al., 1992). The subunit folding topology, however, is similar among all known GST subunits for which the 3-dimensional structure is known.
The 3-dimensional structures of a porcine pi class GST complexed to glutathione sulfate (Reinemer et al., 1991), a human placenta pi class GST complexed with S-hexylglutathione (Reinemer et al., 1992), and the rat mu class (3-3 isozyme) (Ji et al., 1992) have been determined by X-ray crystallography to high resolution (2.3, 2.8, and 2.2 Å, respectively). Each structure revealed that the GST subunit was folded into two domains of different structures. In the rat mu class isozyme the first domain was comprised of residues 1-82 and contains four β-strands and three α-helices arranged in a βαβαβα motif. The second domain spans from residue 90-217 and contains five α-helices. The first domain contains the G-site while the second domain harbors the H-site.

The search for catalytically important residues has only recently met with success. Various amino acids have been implicated as being important in catalysis by kinetic studies and chemical modifications that were later ruled out by site-directed mutagenesis experiments (reviewed in Rushmore and Pickett, 1993). In each of the crystal structures the hydroxyl group of a tyrosine (Tyr-6 in the rat mu class enzyme) was within hydrogen bonding distance to the thiolate anion of bound GSH. Hydrogen bonding to the tyrosine hydroxyl stabilizes the thiolate anion which lowers the GSH thiol pKa from about 8.6 in aqueous solution to between 6 and 7 when bound to GST (Graminski et al., 1989) and enhances the electrophilic conjugation rate. The Y6F mutant of the same isozyme was found to exhibit less than 10% of the wild type activity (Liu et al., 1992).

Several mechanisms have been proposed to explain the catalytic mechanism of GST including ping-pong, random sequential, and ordered sequential (reviewed in Pickett and Lu, 1989). The determination of the exact kinetic mechanism is complicated by
deviations from Michaelis-Menten steady state kinetic properties. These deviations may be characterized by a nonhyperbolic substrate saturation curve or a nonlinear reaction rate and may be the result of time dependent conformational changes in the enzyme or the presence of structurally related isozymes in the purified enzyme preparation. Recent studies have led to the widely accepted view that the GST catalysis proceeds via either a random or ordered sequential mechanism (Pickett and Lu, 1989). Since the intracellular GSH concentration is about three orders of magnitude higher than the dissociation constant between GSH and the enzyme, GST would be expected to bind GSH first in vivo. It has been proposed that the initial binding of GSH results in a conformational change that promotes the binding of an electrophile (Mannervik and Danielson, 1988).

Human placenta pi class GST has been shown to contain a regulatory thiol. CDNB irreversibly inhibited the enzyme due to covalent modification at Cys-47 (Caccuri et al., 1992). The same isozyme was also shown to be inhibited by GSSG, cystine, and cystamine in a manner that indicated the formation of a GST mixed disulfide (Nishihara et al., 1991). Catalytic amounts of a thiol transferase and GSH reversed the inhibition induced by the small molecular weight disulfides (Terada et al., 1993). In contrast to the above results, rat liver microsomal GST has been shown to be activated by sulfhydryl alkylating reagents (Morgenstern et al., 1979). GSTs therefore appear to be another example of a redox sensitive enzyme.
1.3.4.2. Substrate-Dependent Glutathione S-Transferases Class Differentiation

There is currently much interest in the assessment of GST isozyme tissue distribution (Castro et al., 1990; Tu et al., 1983). The patterns of GST isozyme distribution may lead to the discovery of new endogenous substrates and may reveal novel approaches for chemotherapeutic treatment (as will be described in Section 1.3.4.2). The identification of tissue GST isozyme typically involves a combination of immunological procedures using antibodies raised against a particular GST class together with kinetic analysis using different electrophilic substrates.

Although the different classes of GST have overlapping substrate affinities, certain substrates are used to help differentiate the enzyme classes (reviewed in Mannervik and Danielson, 1988). The most commonly employed GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB), displays high activity levels towards most, but not all GST isozymes. Normally, initial tissue GST activity assessments are performed using CDNB but no information is obtained as to the identity of the isozyme present and the possibility exists that an isozyme not active towards CDNB will be overlooked. Consequently, activity measurements using several substrates that display class selectivity are normally performed in the classification of tissue GST.

Of the known GST substrates a few have been characterized as being class selective. Alpha class GST is primarily responsible for the selenium-independent glutathione peroxidase activity towards organic hydroperoxides. The common alpha class selective GST substrate is cumene hydroperoxide. Mu class GST displays the highest activity towards the GSH conjugation to epoxides such as trans-stilbene oxide. The
presence of pi class GST can be assessed on the basis of high specific activity towards the GSH conjugation to ethacrynic acid which contains a GSH reactive α,β-unsaturated carbonyl group. The presence of an α,β-unsaturated carbonyl group in a compound, however, is not sufficient to impart pi class selectivity. The discovery of new substrates selective to a particular enzyme class is useful to the more accurate determination of GST enzyme classification.

1-p-Chlorophenyl-4,4-dimethyl-5-diethylamino-1-penten-3-one hydrobromide (CDDP) and a related compound (NC 1109) are Mannich bases of α,β-unsaturated ketones that were originally developed as candidate anticancer ageats (Dimmock et al., 1989).

\[
\begin{align*}
\text{Cl} & \quad \text{N(C}_2\text{H}_5)_2\cdot\text{HBr} \\
\end{align*}
\]

1-(4-Chlorophenyl)-4,4-dimethyl-5-diethylamino-1-penten-3-one hydrobromide (CDDP)

\[
\begin{align*}
\text{Cl} & \quad \text{N} \quad \cdot\text{HCl} \\
\end{align*}
\]

1-(4-Chlorophenyl)-4,4-dimethyl-5-(1-piperidyl)-1-penten-3-one hydrochloride

(NC 1109)

The reaction of CDDP with some biologically important thiols has been previously examined and has shown CDDP to be a unique thiol reagent in that it is capable of
reacting differentially with protein and non-protein thiols (Mutus et al., 1989). On observing the effects of CDDP and similar compounds on the enzymes of GSH metabolism, it was found that the conjugation of CDDP to GSH is catalyzed by GST from horse liver but not human platelet GST. Horse liver contains many GST classes while human platelets have been shown to only express pi class GST. Subsequently, the GST-catalyzed GSH conjugation to CDDP was further examined and found to be a class-selective GST substrate that possesses high activity towards the alpha class of the enzyme and low activities towards the pi and mu isoforms (Sexton et al., 1993).

1.3.4.3. Inhibition of Glutathione S-Transferases: Potential Chemotherapeutic Applications

Quests for GST inhibitors have often been initiated with the application of combination chemotherapy in mind. GSTs are not only responsible for the detoxication of potential carcinogens but also, unfortunately, for the diminished efficacy of some chemotherapeutic agents. For example, the conjugation of the anti-cancer drug BCNU to GSH has been shown to be catalyzed by a GST isozyme and the resulting conjugate is a less effective cytotoxic agent (Smith et al., 1989). Other chemotherapeutic agents conjugated to GSH have been reviewed by Tsuchida and Sato (1989). Increased GST isozyme expression following the exposure of some cells to certain carcinogens and anticancer drugs has been shown. This increased GST level has been associated with increased cellular resistance to those compounds and has been implicated in the
development of the multidrug resistant (mdr) phenotype. The role of GST in mdr, however, is controversial (Tsuchida and Sato, 1989).

If it were possible to selectively inhibit only the GST isozyme present in the targeted cancerous tissue, not only would the potency of a drug like BCNU be enhanced but also the tissue selectivity of the drug would be accentuated. The GST isozymes present in the surrounding tissues would, in the best case scenario, not be inhibited and consequently the drug would be less cytotoxic to them and possibly exhibit fewer side effects. Isozyme specific GST inhibitors may find use in combination chemotherapy since GST isozyme tissue distribution is somewhat specific (Tsuchida and Sato, 1989). The development of GST isozyme specific inhibitors has been reviewed (van Bladeren and van Ommen, 1991).

We initiated preliminary studies on the inhibition of horse liver GSTs by a group of Mannich bases of an α,β-unsaturated ketones. These compounds are from a series of 3,5-bis (benzylidene)-4-piperidones that were originally developed as candidate anti-cancer agents (Dimmock et al., 1990).

3,5-bis (benzylidene)-4-piperidone hydrochloride (compound 1)
1-methyl-3,5-bis (benzylidene)-4-piperidone hydrochloride (compound 2)

1,1-dimethyl-3,5-bis (benzylidene)-4-piperidonium bromide (compound 3)

They were found to display between 100 and 9700 times the activity of BCNU towards P388 leukemia cells (Dimmock et al., 1989). If these compounds were to prove useful as isozyme specific GST inhibitors they may find use in combination chemotherapy. As combination chemotherapeutic agents they may exhibit two desirable properties, namely tumor cell directed cytotoxicity and GST inhibition.

1.3.4.4. The Fate of Glutathione Conjugates

Conjugates between GSH and certain electrophiles have been shown to transported out of the cell via a specific transport system. In human erythrocytes this transport is mediated by an ATP dependent process (Bartosz et al., 1993; Akerboom et al., 1992). Once outside the cell it is believed that the conjugate encounters the outer
membrane bound enzyme γ-glutamyl transpeptidase which hydrolyzes the γ-glutamyl moiety of the GSH conjugate and in the process transfers this group to an acceptor amino acid. The resulting cysteinylglycine conjugate is further degraded by an outer membrane bound dipeptidase to yield free glycine and a cysteine conjugate. This conjugate is generally believed to be transported back inside the cell where the cysteine conjugate is N-acetylated by the enzyme acetylase. The resulting mercapturic acid is excreted in the urine.

1.3.5. Glutathione Membrane Transport

There are many different transport systems present in GSH metabolism (Fig. 2). Not all, however, have been characterized. In general, the amino acid transport systems have been well characterized while there is very little available information on the membrane transport of peptides. GSH is the best characterized example of peptide membrane transport. The following is a list of GSH-linked transport systems:

1) γ-glutamyl amino acid transport;
2) glutamate/cystine transport;
3) cysteine transport;
4) glycine transport;
5) GSH conjugate/GSSG transport;
6) cysteine conjugate transport;
7) intact GSH transport.
1.3.5.1. γ-Glutamyl Amino Acid Transport

The transport of γ-glutamyl amino acids is the basis of the γ-glutamyl cycle. This cycle (Fig. 2) may be a means of cellular amino acid uptake. Evidence has been obtained to demonstrate that the γ-glutamyl cycle does, in fact, participate in the cellular uptake of some amino acids (Smith et al., 1991). Central to this transport mechanism is the activity of the enzyme γ-glutamyl transpeptidase (γ-GT) which is described in Section 1.3.5.2. γ-GT produces γ-glutamyl amino acids which are simply amino acids with a γ-glutamyl linkage. These γ-glutamyl amino acids are transported into cells by a specific carrier-mediated process which depends on the recognition of the γ-glutamyl moiety (Meister and Anderson, 1983). To date, the γ-glutamyl amino acid transport system has not been characterized.

1.3.5.2. γ-Glutamyl Transpeptidase (EC 2.3.2.2)

γ-Glutamyl transpeptidase (γ-GT) is an extracellular membrane bound enzyme that hydrolyzes the γ-glutamyl linkage of GSH or any γ-glutamyl peptide and transfers this linkage to another acceptor amino acid or small molecular weight peptide even GSH itself. When the acceptor is water hydrolysis of the γ-glutamyl linkage occurs.

\[
\text{GSH} + \text{amino acid} \rightleftharpoons \text{γ-glu-amino acid} + \text{CysGly}
\]

\[
\text{GSH} + \text{GSH} \rightleftharpoons \text{γ-glu-GSH} + \text{CysGly}
\]

\[
\text{GSH} + \text{H}_2\text{O} \rightarrow \text{glutamate} + \text{CysGly}
\]

γ-GT appears to be a widespread enzyme but the kidney exhibits the highest activity. The enzyme from rat kidney is the best studied. In general, high γ-GT is seen in
cells that exhibit intense secretory or absorptive functions (Meister et al., 1976). In addition to the \( \gamma \)-glutamyl cycle, the enzyme plays a key role in conversion of GSH S-conjugates to mercapturic acids and in the formation of leukotrienes (Hammarstrom, 1985). The enzyme is also a useful marker of pre-neoplastic changes of cell differentiation and cell aging (Meister and Anderson, 1983).

\( \gamma \)-GT is an integral membrane heterodimeric glycoprotein. The heavy subunit (51 kDa) contains a hydrophobic domain that spans the membrane and serves to anchor the holoenzyme in the membrane. The lighter subunit (22 kDa) is the catalytic subunit. The enzyme is synthesized as a single polypeptide chain (\( \sim 78 \) kDa) that is post-translationally proteolyzed (Nash and Tate, 1984). \( \gamma \)-GT can be solubilized using either Triton X-100 or papain. Papain cleaves a 52 amino acid hydrophobic domain from the heavy subunit. The catalytic properties of the enzyme solubilized with either papain and Triton X-100 are identical (Tate and Meister, 1985). The purified rat kidney enzyme exhibits considerably heterogeneity on SDS PAGE as a result of different degrees of sialylation (Tate and Meister, 1985).

The \( \gamma \)-glutamyl binding site on the enzyme tolerates many different L or D-\( \gamma \)-glutamyl compounds. The most common \( \gamma \)-glutamyl donor substrate for the \textit{in vitro} enzymatic assay is L-\( \gamma \)-glutamyl-p-nitroanilide which exhibits approximately twice the transpeptidase rate of GSH. The enzyme acceptor binding site also exhibits broad specificity but does not accept D-amino acids, L-proline or \( \alpha \)-substituted amino acids. Low transpeptidase activity is observed with branched chain amino acids. The most active amino acid acceptors of the \( \gamma \)-glutamyl moiety include cystine and glutamine while the
most common dipeptide acceptors are methionylglycine, glutaminylglycine, alanylglucose, cystinylbisglycine, serylglucose, and glyclglycine (Meister et al., 1981). GSH and GSSG can also be acceptor substrates.

The catalytic mechanism of γ-GT is approximated by a ping-pong kinetic scheme. A γ-glutamyl donor compound is the first substrate to bind resulting in the formation of a γ-glutamyl-enzyme intermediate and the release of the first product, CysGly. When the acceptor substrate encounters the γ-glutamyl-enzyme intermediate the γ-glutamyl moiety is transferred to the acceptor substrate (Allison, 1985).

Specific inhibitors of γ-GT include serine-borate and acivicin. Serine-borate reversibly inhibits the enzyme by a forming serine-borate complex that approximates the enzyme transition state (Tate and Meister, 1978). Acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid or AT-125, irreversibly inhibits the enzyme most likely by a covalent modification of an amino acid residue at the γ-glutamyl binding site (Reed et al., 1980).

1.3.5.3. Transport of Glutathione Component Amino Acids

Glutamate, cysteine and glycine are all GSH fragments and their transport systems have been well characterized (reviewed in Christensen, 1984). Amino acid transport systems, in general, have been studied extensively using mainly kinetic membrane transport methods. It has been possible to resolve several amino acid transport systems that display different, though often overlapping, substrate specificities. The uptake of amino acids by these systems may or may not be dependent on the cotransport of sodium.
Glutamate is taken up either by system $X^{-}_{AG}$ or by system $X^{-}_{c}$. System $X^{-}_{AG}$ is sodium dependent and specific for the anionic acids glutamate and aspartate while system $X^{-}_{c}$ is sodium independent and is specific for glutamate and cystine but not aspartate. Once influxed, cystine is reduced to cysteine and partially targeted towards the resynthesis of GSH. The transport of cysteine occurs either via system ASC which is sodium dependent and specific for neutral amino acids with small side chains such as cysteine, serine, and glutamine or via system A which is also sodium dependent and is specific for neutral amino acids with unbranched side chains such as cysteine, serine, glutamine, and glycine. Glycine is also transported by system Gly which is sodium dependent and specific for sarcosine.

1.3.5.4. Glutathione Conjugate, Glutathione Disulfide, and Cysteine Conjugate Transport

In recent years the GSH conjugate transport system has been the subject of much interest. Most of the studies on the transport of GSH conjugates have been performed using hepatocytes or erythrocytes as well as with the membrane vesicles of these cells using the model GSH conjugate S-dinitrophenyl glutathione (DNP-SG). In liver it has been shown that two GSH conjugate transport systems exist, one for the secretion across the canalicular membrane into the bile and another for the secretion across the basolateral membrane into the sinusoidal fluid.

Transport of GSH conjugates across basolateral membranes is better characterized where it has been shown to occur via a multispecific organic anion transporter (MOAT).
This transporter is ATP-dependent and distinct from other basolateral membrane ATP-dependent transporters such as P-glycoprotein and the taurocholate (Vore, 1993). In addition to GSH conjugates MOAT catalyzes the cellular efflux of several non-bile acid organic anions such as glucuronide conjugates, leukotriene C4, and GSSG. The canalicular membrane MOAT system also appears responsible for the uptake of certain organic ions and deposition in the bile (Hinchman et al., 1993). The rat liver MOAT protein has been purified and found to be similar to that observed in human erythrocytes on the basis of immunological analysis and molecular weight (37-38 kDa) (Zimmniak et al., 1992; Sharma et al., 1990). The human erythrocyte transport of DNP-SG has been shown to occur via a low (4 μM) and a high (1.6 mM) \( K_M \) transport system (Akerboom et al., 1992). The low \( K_M \) DNP-SG transporter appears to correspond to the liver MOAT system while the high \( K_M \) DNP-SG transporter is active mainly towards the efflux of monovalent organic anions (Bartosz et al., 1993).

Transport of DNP-SG across rat liver basolateral membranes is not ATP-dependent and appears to driven by the membrane potential (Kobayashi et al., 1990). This transport system, however, has not been studied to the same extent as that of canalicular membranes. The substrate specificity between the two transport systems differ. For example basolateral DNP-SG is not inhibited by GSSG (Kobayashi et al., 1990). However, the transport of GSSG across the basolateral membrane, has been observed by a transport system distinct from MOAT (Masuda et al., 1993).

The efflux of GSSG has been observed under conditions of oxidative stress in several cells such as eye lens (Srivastava and Beutler, 1968), erythrocytes (Srivastava and
Beutler, 1969), perfused liver (Jaeschke, 1990), isolated hepatocytes (Oude Elferink, 1990), and perfused heart (Ishikawa et al., 1986). GSSG efflux is considered to be a reliable index of oxidative stress. Liver GSSG efflux occurs across the canalicular membrane via MOAT and across the basolateral membrane through a transport system that has been shown to be driven by the potassium chemical gradient (high [K⁺] inside, low [K⁺] outside) (Masuda et al., 1993).

A cysteine conjugate is the result of a γ-GT-mediated hydrolysis of a GSH conjugate followed by exposure to a cysteinylglycine dipeptidase. The cysteine conjugate is believed to be transported into the cell through a carrier mediated process, which to date, has not been characterized.

1.3.5.5. Intact Glutathione Transport

Some cells have been shown to be capable of intact GSH facilitative transport. Inter-organ GSH transport has been hypothesized to be a mechanism for the generalized protection against oxidative stress (Hagen et al., 1987). GSH is effluxed primarily from the liver into the plasma which makes it available for the uptake by other tissues. The properties of carrier-mediated GSH uptake, however, are not conserved among different cells. The uptake of intact GSH may be a more economic method for the elevation of intracellular GSH than the γ-glutamyl cycle since it does not involve many enzymes and does not rely on the direct consumption of ATP. Indirectly, intact GSH uptake may be ATP dependent as the transport across some membranes is associated with sodium
cotransport. The electrochemical sodium gradient is maintained by an ATPase, the Na⁺/K⁺ pump.

Hepatocytes are the primary cells responsible for maintaining plasma GSH levels and consequently GSH transport in these cells favors efflux (Garcia-Ruiz et al., 1992). GSH transport in kidney cells is primarily in the form of uptake (Hagen et al., 1988). GSH uptake has also been observed with alveolar cells (Brown et al., 1992) and intestinal cells (Vincenzini et al., 1992; Hagen et al., 1991) where it is believed that these cells require exogenous GSH to aid in their defense against exposure to environmental toxins. Several foods such as cauliflower and broccoli are rich in GSH and their increased consumption has been associated with increased plasma GSH levels (Hagen et al., 1990a; Hagen et al., 1990b).

As mentioned, the liver is the main organ responsible for making GSH available to other tissues. Due to the importance of the liver in inter-organ GSH transport, the systems responsible for GSH transport in the liver have been the focus of some attention. In the liver three GSH transport systems have been identified: one system associated with the canalicular membrane, another with the basolateral membrane, and a third system in the inner mitochondrial membrane which influxes cytosolic GSH into this organelle (Fernandez-Checa et al., 1991; Martensson et al., 1990). The kinetics of liver mitochondrial GSH uptake is biphasic being comprised of a high affinity transport system ($K_M = \sim 60 \mu M$) and a low affinity transport system ($K_M = \sim 5.4 \text{ mM}$) (Martensson et al., 1990). The sodium dependence of GSH uptake by liver mitochondria was not determined.
Since liver GSH transport across the plasma membranes is primarily in the form of efflux a dependence on sodium cotransport is not possible.

The canicular membrane system releases GSH into the plasma while the basolateral membrane system deposits GSH into the bile. The properties of canicular and basolateral GSH transport systems are: low affinity, saturable efflux, competitive inhibition by various compounds, trans-stimulation by GSH and GSH conjugates, and dependence on membrane potential (Fernandez-Checa et al., 1990; Fernandez-Checa et al., 1988; Aw et al., 1986). Depolarization of the membrane potential inhibits efflux while hyperpolarization stimulates efflux. This dependence of liver GSH efflux on membrane potential has been disputed (Wright, et al., 1988).

GSH canicular transport is distinct from the aforementioned canicular membrane anion transporter MOAT (Fernandez-Checa et al., 1992). Transport at both liver poles (canicular and basolateral) differ in their affinity for GSH (basolateral $K_M = 7$ mM and canicular $K_M = 16$ mM) (Fernandez-Checa et al., 1992; Fernandez-Checa et al., 1989) and in their inhibition by BSP-SG. BSG-SG inhibits basolateral but not canicular GSH transport (Fernandez-Checa et al., 1992; Fernandez-Checa et al., 1989). The basolateral transport system has been shown to be bidirectional but under the low physiological concentrations of GSH present extracellularly only GSH efflux is expected to occur (Garcia-Ruiz et al., 1992). GSH efflux from intact hepatocytes was found to be influenced by the intracellular redox status (Lu et al., 1993). In that study increased intracellular cystine inhibited GSH efflux while influxed DTT stimulated GSH efflux suggesting that the liver GSH efflux transport system may be another example of a redox

...
sensitive protein (Lu et al., 1993). Recently, the rat liver canicular GSH transporter cDNA has been cloned and found to encode for a 835 amino acid protein (95, 785 Da) (Yi et al., 1994). Northern blot analysis revealed the presence of the rat liver canicular GSH transporter mRNA in kidney, intestine, lung and brain (Yi et al., 1994).

Liver GSH efflux has been shown to be under hormonal control but the mechanism(s) responsible is controversial. A cAMP-dependent system has been implicated since cAMP-dependent factors such as chlorella toxin, dibutyl cAMP, forskolin, and glucagon stimulated sinusoidal GSH release from cultured hepatocytes by hyperpolarization of the plasma membrane potential while the protein kinase C-dependent vasopressor hormones phenylephrine and vasopression had no effect (Lu et al., 1990). Contradictory results have been obtained from studies that utilized perfused rat livers where the vasopressor hormones were found to stimulate GSH efflux by a mechanism that involves the increased permeability of tight junctions (Raiford et al., 1991). Additional studies have implicated a protein kinase C-dependent mechanism since the activation of protein kinase C using phorbol esters stimulated hepatocyte GSH efflux while staurosporine, an inhibitor of protein kinase C prevented the phorbol ester-induced stimulation of GSH efflux (Sato et al., 1992a). Further support for the protein kinase C mechanism of hormone action on enhanced GSH efflux from liver has been put forth by Sies and Graf (1985) and Sato et al. (1992b).

The kidney is important in the recycling of inter-organ GSH. Renal extraction of GSH occurs by a glomerular filtration and a nonfiltering peritubular mechanism (Anderson et al., 1980). The latter of the two GSH extraction mechanisms is predominant and
involves GSH uptake. In the kidney two distinct intact GSH transport systems are present. As in the liver the basolateral GSH transport system is not identical to the brush-border system. In the kidney the basolateral membrane is in contact with plasma while the brush-border membrane faces the tubular lumen. The basolateral GSH transport system has been shown to be an electrogenic low affinity system \((K_m = 3 \text{ mM})\) that takes up GSH in a sodium dependent manner (Lash and Jones, 1984). The low affinity for GSH uptake by the kidney basolateral transporter suggests that it does not operate under normal plasma GSH concentrations (2-10 \(\mu\text{M}\): Flagg et al., 1993; Svardal et al., 1990; Lash and Jones, 1985). It has been shown with \(\gamma\)-GT-inactivated animals that elevated plasma GSH concentrations is not associated with increased renal GSH (Inoue et al., 1986). The abundance of \(\gamma\)-GT and dipeptidase in the kidney provides further evidence for the contention that GSH uptake across the basolateral membrane is primarily mediated by the \(\gamma\)-glutamyl cycle (Inoue et al., 1986). GSH uptake across the kidney brush-border membrane is independent of sodium, electrogenic, and of a higher affinity than the basolateral system \((K_m = 0.21 \text{ mM})\) (Inoue and Morino, 1984).

In order to make use of the low concentrations of GSH in the plasma other organs must possess high affinity GSH uptake systems. Several cells have been shown to be protected from the consequences of oxidative stress by the administration of near plasma GSH concentrations. For example rabbit type II alveolar cells have been shown to be protected from paraquat-induced cytotoxicity by 20 \(\mu\text{M}\) GSH (Brown et al., 1992; Hagen et al., 1986). Furthermore, the protective effect of GSH was abolished in the presence of the GSH transport inhibitor \(\gamma\)-glutamylglutamate (Brown et al., 1992; Hagen et al., 1986).
Upon further examination it was found that these cells do in fact express an intact GSH uptake system (Hagen et al., 1986). In these cells GSH uptake is sodium dependent (Hagen et al., 1986).

As already mentioned, increased dietary GSH was found to be accompanied by increased plasma GSH concentrations (Hagen et al., 1990a; Hagen et al., 1990b). The utilization of dietary GSH is attributed to the uptake of GSH by the gastrointestinal epithelium (Martensson et al., 1990b). The intestinal epithelium forms the first line of defense against the ingestion of toxic compounds. As with the aveolar cells, intestinal cells were protected from oxidant injury induced by t-butylhydroperoxide or menadione in a manner indicative of intact GSH uptake (Lash et al., 1986). Intestinal GSH uptake has been characterized using rats and rabbits and found to be comprised of two different high affinity GSH transport system at opposite cellular poles (reviewed in Vincenzini et al., 1992).

The GSH transport system in the intestinal basolateral membrane which in vivo takes up GSH from plasma is not the same as the intestinal brush-border membrane GSH transport system which takes up GSH from the lumen. Across the basolateral membrane GSH uptake was sodium dependent and inhibited by γ-glutamyl compounds, probenecid and ophthalamic acid (Lash et al., 1986). In contrast GSH uptake in the brush border membrane was sodium independent and not inhibited by γ-glutamyl compounds, probenecid or ophthalamic acid (Vincenzini et al., 1992). On the basis of these characteristics it has been suggested that the intestinal basolateral membrane GSH transport system resembles that system in the basolateral membranes of kidney and liver.
while the intestinal brush-border membrane GSH transporter is more characteristic of the kidney brush-border and of the liver canalicular system (Vincenzini et al., 1992). The dependence of the intestinal GSH transport system from either membrane on membrane potential has been implicated but not been clearly established (Vincenzini et al., 1992). The $K_M$ of GSH uptake by both membranes is much lower than that of other cells previously examined at approximately 20 $\mu$M (Vincenzini et al., 1992). As with liver GSH transport, hormones effected GSH transport in the perfused rat intestine (Hagen et al., 1991). In the intestine, however, GSH influx was stimulated as opposed to efflux by liver, as mentioned above. Intestinal GSH uptake was stimulated by the $\alpha$-adrenergic hormone phenylephrine but not by the $\beta$-adrenergic agonist isoproterenol (Hagen et al., 1991).

Platelets circulate in plasma which bathes them in liver-effluxed GSH. The average plasma GSH concentration ranges from 2-10 $\mu$M (Flagg et al., 1993; Svardal et al., 1990; Lash and Jones, 1985). It may be advantageous for platelets to possess a high affinity GSH transport system capable of influxing the typically low concentrations of GSH available in plasma in the maintenance of normal function and in the regulation of platelet activation. The uptake of GSH by platelets from the plasma may be important in maintaining an intraplatelet GSH concentration capable of preventing platelet hyperaggregation by mechanisms that will be described in Section 1.3.8.

Some characteristics of GSH transport systems in different cells are compared in Table 1.
Table 1
A Summary of Some Characteristics of GSH Transport Systems

<table>
<thead>
<tr>
<th>Source</th>
<th>Na(^+)-Dependence</th>
<th>Membrane Potential Dependence</th>
<th>(K_M)</th>
<th>trans-Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver canalicular</td>
<td>na</td>
<td>yes</td>
<td>16 mM (efflux)</td>
<td>nd</td>
</tr>
<tr>
<td>liver basolateral</td>
<td>na</td>
<td>yes</td>
<td>7 mM (efflux)</td>
<td>yes</td>
</tr>
<tr>
<td>liver mitochondria</td>
<td>nd</td>
<td>nd</td>
<td>60 (\mu)M, 5.4 mM (influx)</td>
<td>nd</td>
</tr>
<tr>
<td>kidney brush-border</td>
<td>no</td>
<td>yes</td>
<td>0.21 mM</td>
<td>nd</td>
</tr>
<tr>
<td>kidney basolateral</td>
<td>yes</td>
<td>yes</td>
<td>3 mM</td>
<td>nd</td>
</tr>
<tr>
<td>alveolar type II cells</td>
<td>yes</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>intestine brush-border</td>
<td>no</td>
<td>nd</td>
<td>17 (\mu)M</td>
<td>nd</td>
</tr>
<tr>
<td>intestine basolateral</td>
<td>yes</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

The abbreviations used in above table are na: not applicable and nd: not determined. References for the above results are provided in the text.
1.3.6. Role of Glutathione in Platelet Function

Intra-platelet glutathione ([GSH]_p) is involved in the regulation of platelet function largely due to its participation in arachidonic acid metabolism (described in Section 1.2.2.6) (Bosia et al., 1985; Hofman et al., 1980; Matsuda et al., 1979). The thiol reagent-mediated reduction of [GSH]_p has previously been shown to influence platelet aggregation (reviewed in Bosia et al., 1989). The following list summarizes the GSH interaction sites in arachidonic acid metabolism that may affect platelet function:

1) indirect inhibition of cyclooxygenase and 12-lipoxygenase;

2) potential isomerization PGH_2 to PGD_2;

3) conversion of 12-HPETE to 12-HETE;

4) leukotriene A_4 conversion to leukotriene C_4

As shown in Fig. 1 cyclooxygenase and 12-lipoxygenase, two important enzymes in arachidonic metabolism, are both activated by trace amounts of hydroperoxide (Lands, 1984; Hwang, 1982). Indirectly, GSH regulates both of these enzymes by reducing hydroperoxide in concert with glutathione peroxidase (GPX). Cyclooxygenase in mouse macrophages has been recently shown to be activated by nitric oxide (NO) (Salvemini et al., 1993), an event which may occur within platelets especially since platelets are capable of L-arginine-dependent NO synthesis (Muruganandam and Mutus, 1994; Radomski et al., 1987). GSH can further regulate cyclooxygenase by limiting the intra-cellular concentration of free NO by conjugation to form S-nitroso glutathione (GSNO) (Stamler et al., 1992). A decrease in [GSH]_p is therefore expected to enhance the production of
the cyclooxygenase and 12-lipoxygenase products, PGG₂ and 12-HPETE, respectively, while an increase should result in the opposite occurrence.

PGG₂ is subsequently transformed to several metabolites including the weak platelet antagonist PGD₂ and the most potent platelet agonist of the release reaction, TxA₂. Pathways that affect the production of TxA₂ will surely result in an altered degree of platelet activation. The isomerization PGH₂ to PGD₂ has been shown to be catalyzed by serum albumin (Watanabe et al., 1980; Christ-Hazelhof et al., 1976), a 26-34 kDa GSH-independent enzyme (Christ-Hazelhof and Nugteren, 1979), a 80-85 kDa GSH-dependent enzyme and by GST (Christ-Hazelhof et al., 1976). The enzyme catalyzing the formation of PGD₂ in the platelet has not been characterized so it is not known if it is dependent on GSH.

The reactive 12-lipoxygenase metabolite, 12-HPETE, can be stabilized by reduction to 12-HETE by platelet selenium-dependent GPX (Bryant et al., 1982) although the physiological involvement of GPX has been refuted (Hill et al., 1989). Indirect evidence for the involvement GPX in 12-HPETE reduction was obtained from the finding that platelet activation is associated with a transient increase in GSSG that was inhibited by aspirin (Burch and Burch, 1990). The function of 12-HPETE and 12-HETE, as mentioned in Section 1.2.2.6, is uncertain.

Arachidonic acid can also be converted to leukotriene A₄ by 5-lipoxygenase, an enzyme present in neutrophils but not platelets. Neutrophil-derived leukotriene A₄, however, can be converted to leukotriene C₄ by an enzyme in platelets that catalyzes the
conjugation of leukotriene A4 to GSH but is not GST (Soderstrom et al., 1992). The role of leukotriene C4 in platelet function is also unknown.

The involvement of GSH in arachidonic acid metabolism signifies a definitive, though indirect, role for GSH in the regulation of platelet activation. Since the most influential GSH-sensitive event in arachidonic acid metabolism is the production TxA2 a reduction of [GSH]ip is expected to enhance platelet aggregation. Previous studies have shown that when [GSH]ip is reduced aggregation is stimulated (Bosia et al., 1989). Reagents that reduce [GSH]ip by oxidization to GSSG, such as diamide, t-butyl hydroperoxide and cumene hydroperoxide, also induce the reversal of platelet aggregation (Bosia et al., 1989). The finding that diabetic platelets are deficient in [GSH]ip (Muruganandam et al., 1992) and exhibit hyperaggregation (Thomas et al., 1986b) is suggestive of a regulatory role for GSH in platelet activation. In activated diabetic platelets there is an increase production of thromboxane B2, a stable metabolite of TxA2 (Thomas et al., 1986b).

A more substantial decrease in [GSH]ip has been shown to inhibit platelet aggregation (Bosia et al., 1989). This suggests that in addition to its role in arachidonic acid metabolism, GSH is likely required for the maintenance of certain redox sensitive protein(s) that are crucial to some aspect(s) of the activation process. The identification of redox sensitive proteins that are involved in the platelet activation process may be initiated by a determination of which, if any, agonist pathways are the most sensitive to [GSH]ip depletion. As alluded to earlier (Section 1.3.3.2), redox regulation of protein activity is a potential signal transduction mechanism. The existence of such a pathway in
platelets has not been demonstrated. The elevation of GSSG during platelet activation (Burch and Burch, 1990), however, suggests the possibility that platelet function may be modulated by a redox regulatory mechanism.

Attempts to delineate the role of $[\text{GSH}]_i$ in platelet function, with the aid of GSH-depleting agents have been complicated by the fact that the agents employed are not specific towards GSH conjugation. The thiol oxidizing reagent, diamide, for example has been shown to inhibit platelet aggregation partially by crosslinking cytoskeletal proteins (Caruso et al., 1984). 1-Chloro-2,4-dinitrobenzene (CDNB) is a thiol alkylating reagent that is commonly used to deplete intracellular GSH yet it is an inhibitor of glutathione reductase (GR) and GPX (Hill et al., 1989; Bilzer et al., 1984). Thiol reagents that exhibit more GSH specificity are obviously required.

The influence of GSH on platelet activation has to date only been determined using GSH depletion experiments using thiol reagents. The elevation of $[\text{GSH}]_i$ may be beneficial to demonstrate the role of $[\text{GSH}]_i$ on platelet function without the side effects of thiol reagents.

1.4 L-Arginine/Nitric Oxide Metabolism

The finding that the relatively unstable and potentially toxic, diatomic free radical nitric oxide (NO) is a physiological regulator of cell function amazed the world of biological science. In the early 1980s a substance, termed endothelium-derived relaxing factor (EDRF) was reported to be released from vascular endothelium that induced vascular relaxation (Ignarro et al., 1981; Furchgott and Zawadzki, 1980). The unknown
substance had a half-life of only a few seconds and was inhibited by hemoglobin. In the later part of the 1980s the discovery was made that endothelial cells synthesize NO as part of a signal transduction pathway and that the EDRF was NO (Furlong et al., 1987). Since the discovery of NO an enormous interest in the determination of its physiological roles has developed. NO is a novel type of inter-cellular signaling agent in that it does not mediate its effects through receptor complexes since it can diffuse through membranes. The effects of NO are regulated solely by its production. The effects of NO are believed to be localized since the high reactivity and short half-life in a physiological milieu limit its diffusion distance. The many functions of NO have been extensively reviewed (Butler and Williams, 1993; Galla, 1993; Madison, 1993; Lancaster, 1992). Some of the better characterized functions of NO are listed below:

1. vasodilator;
2. platelet aggregation inhibitor;
3. mediator of actions of the excitatory neurotransmitter glutamate;
4. mediator of tumor cell-directed macrophage cytotoxicity.

1.4.1. Some Biologically Important Aspects of the Chemistry of Nitric Oxide

Before examining some of the biological roles of NO in greater detail, some biochemically pertinent aspects of the chemistry of NO will be considered. NO is a colorless gas at room temperature that displays a similar aqueous solubility to O₂ and CO. The solubility of NO at 25°C and 1 atmosphere pressure is 1.8 mM. NO contains an
unpaired electron in its 2p-π antibonding orbital and as such is paramagnetic. The resonance forms of NO are as shown below.

\[ \text{N}^\cdot\text{O}^- \leftrightarrow \text{N}=\text{O} \leftrightarrow \text{N}=\text{O}^- \leftrightarrow \text{N}^\cdot=\text{O} \]

In addition to NO other redox forms, such as NO\(^+\) and NO\(^-\), have been implicated to possess biological activity. NO\(^+\) has a higher bond order (~3) than NO (~2.5) which has a higher bond order than NO\(^-\) (~2). Stamler \textit{et al.} (1992) have implicated these forms in some of the important biological reactions of NO. Except where noted, NO represents the radical NO. One of the most important biological reactions is the aqueous reaction between NO radical and O\(_2\) to produce NO\(_2^\cdot\):

\[ 4\text{NO} + \text{O}_2 + \text{H}_2\text{O} \rightarrow 4\text{NO}_2^\cdot + \text{H}^+ \]

The rate law for the above reaction is second order in [NO] \((4k_{\text{eq}}[\text{NO}]^2[\text{O}_2])\) and \(4k_{\text{eq}} \approx 8 \times 10^6 \text{ M}^{-2} \cdot \text{s}^{-1}\) (Ford \textit{et al.}, 1993). Since the above reaction is second order with respect to NO, the half-life of NO in oxygenated aqueous solutions is largely dependent on the concentration of NO. Based solely on the reaction of NO with oxygen, the low concentrations of NO that are generated by many biological processes may be expected to last for as long as 100 - 500 seconds (Ford \textit{et al.}, 1993). Possibly because NO has other \textit{in vivo} fates than merely the reaction with oxygen, the observed half-life of NO in a biological system is generally only a few seconds (Stamler \textit{et al.}, 1992). NO also rapidly reacts with superoxide anion to produce peroxynitrate (OONO\(^-\)) (Gryglewski \textit{et al.}, 1986). NO binds strongly to Fe(II)-hemoglobin and unlike CO or O\(_2\) binds to Fe(III)-porphyrins. The Fe(III)NO-heme complex undergoes a charge transfer to form Fe(II)NO\(^+\) which is subject to attack by ambient nucleophiles leading to a liberation of NO.
(Stamler et al., 1992). NO also reacts with iron-sulfur centers in proteins, thiols, amines and possibly aromatic amino acids.

The reaction of NO with thiols to produce relatively stable S-nitrosothiolates is of interest to us owing to our ongoing investigations on GSH metabolism. The formation of S-nitrosothiols involves the transfer of nitrosonium ion to the thiolate ion.

\[ \text{RS}^- + \text{NO}^+ \longrightarrow \text{RSNO} \]

S-Nitrosothiolates, such as S-nitrosocysteine and S-nitrosoglutathione (GSNO), are easily prepared (Hart, 1985) and presumably exist in vivo as they were identified along with S-nitrosoproteins in mammalian plasma (Keaney et al., 1993). GSH is especially likely to form a S-nitrosothiol in vivo since GSNO is more stable than S-nitrosocysteine (Hart, 1985) and since GSH exists both in plasma and inside all cells where it is the most abundant nonprotein thiol (Meister and Anderson, 1983). S-Nitrosothiolates, GSNO in particular, exhibit many NO-dependent cellular activities (Clancey et al., 1992; Radomski et al., 1992; Stamler et al., 1992; Ignarro et al., 1981). Due to their increased stability over NO, S-nitrosothiolates have been postulated to function as NO reservoirs that may prolong the cellular response to NO (Stamler et al., 1992). Conversely they may function as scavengers of NO that limit the cellular NO response.

1.4.2. Some Examples of the Physiological Roles of Nitric Oxide

Nitric oxide is an important physiological vasodilator. As will be described in more detail in Section 1.4.3.1, NO is produced in an L-arginine-dependent manner by the enzyme nitric oxide synthase (NOS). One form of NOS in endothelial cells is activated by
calcium/calmodulin. Agents such as bradykinin elevate endothelial Ca\textsuperscript{2+} levels and activate NOS. In many cells the NO target is the heme iron of guanylate cyclase. Upon binding NO, guanylate cyclase becomes activated and the subsequent intracellular rise in intracellular cGMP induces specific protein phosphorylation through the activation of cGMP-dependent protein kinases. The result of cGMP-dependent protein phosphorylation is smooth muscle relaxation and in endothelial cells vasodilation (reviewed in Schmidt et al., 1993).

The inhibition of platelet aggregation by NO in situ, has been reported with either endothelium-derived NO (Busse et al., 1987, Furlong et al., 1987; Radomski et al., 1987; Azuma et al., 1986) or platelet-derivered NO (Malinski et al., 1993; Pronai et al., 1991; Radomski et al., 1990). Authentic NO and NO-releasing agents also inhibit platelet aggregation through a cGMP-dependent mechanism. In vivo the inhibition of platelet aggregation may result from endothelium-derived NO or platelet-derived NO. Which source of NO, if either, is predominant in the physiological inhibition of platelet aggregation has not been determined. Endothelium-derived NO must diffuse out of the endothelial cell, through the plasma before entering the platelet. Plasma and endothelial cells contain many possible NO targets such as protein sulphhydryls, amines and heme groups that would compete with the platelet for available endothelium-derived NO. Therefore, NO production within the platelet may be advantageous in inducing an immediate and localized response to NO. The importance of platelet-derived NO in the regulation of platelet activation has been refuted (Mollace et al., 1991). Convincing evidence, however, has implicated an important role for platelet-derived NO in the
regulation of platelet activation. The inhibition of collagen-induced platelet aggregation by L-arginine added to the platelet suspension (Radomski et al., 1990) as well as the electrochemical detection (Malinski et al., 1993) and the electron paramagnetic resonance detection (Pronai et al., 1991) of NO released from activated platelets strongly suggests that the platelet L-arginine/NO pathway is involved in the regulation of platelet activation.

NO is also involved in neurotransmission through a cGMP-dependent process. During neurotransmission the presynaptic neuron secretes the excitatory neurotransmitter, glutamate. Glutamate then binds to a N-methyl-D-aspartate (NMDA) receptor on the postsynaptic neuron and the activated receptor complex causes a channel in the membrane to influx extracellular calcium. The elevated intracellular calcium activates the neuronal Ca\textsuperscript{2+}/calmodulin dependent NOS. The NO that is produced can activate the guanylate cyclase in the neuron in which it is produced or in another neuron to propagate neurotransmission or in the presynaptic neuron. The activation of guanylate cyclase in the presynaptic neuron by NO from the postsynaptic neuron is a type of feedback loop and suggests a retrograde messenger role for NO (reviewed in Butler and Williams, 1993). As a retrograde messenger NO has been implicated in the potentiation of memory through the reinforcement of certain neural transmissions.

In response to cellular invasion by a pathogenic organism or to a tumor cell the immune system mounts two separate lines of defense. There is a humoral response which produces antibodies to the pathogen and culminates in complement activation. There is also a cellular response by cytotoxic T cells, natural killer cells and cytotoxic activated macrophages. These cells destroy diseased cells by ingestion or by the release of toxic
substances. Activation of macrophages occurs by binding to infected cells and is accompanied by the production of the inducible form of NOS. The inducible form of NOS (iNOS) is different from the constitutively expressed NOS in that it is active at basal intracellular calcium levels. Since iNOS is tightly bound to calmodulin it is fully active at basal calcium levels. Once expressed the iNOS will produce large amounts of NO for several hours or until the substrate supply is depleted or the enzyme is no longer active. This high localized concentration of NO is sufficient to destroy the bound infected cell. The cytotoxicity of NO is due to its inhibition of several important enzymes such as aconitase, an enzyme of the citric acid cycle, complex I and II of the mitochondrial electron transport chain and ribonucleotide reductase, the enzyme responsible for the reduction of ribonucleotides to the deoxyribonucleotides that are necessary for DNA synthesis. NO inhibits aconitase and complexes I and II by reacting with catalytically important iron-sulfur clusters. Three sites within the active site of ribonucleotide reductase are potentially susceptible to NO damage: a tyrosine radical, a nonheme iron, and several thiol groups.

1.4.3. Nitric Oxide Biosynthesis

As mentioned NO is synthesized intracellularly by the enzyme NOS. The biosynthesis of NO depends on the activity of NOS which is largely determined by the availability of L-arginine (Fig. 4). The transport of L-arginine is therefore also important in NO biosynthesis.
Fig. 4. L-Arginine-dependent NO production. System \( y^+ \) transports L-arginine intracellularly where it may be transformed to citrulline and NO by either particulate or cytosolic nitric oxide synthase (NOS). Not shown in this illustration is the requirement of nitric oxide synthase for NADPH, \( \text{Ca}^{2+} \)/calmodulin, and tetrahydrobiopterin. The cellular effects of NO are often mediated by an activation of guanylate cyclase (GC). Through the phosphorylation of specific proteins the intracellular elevation of cGMP leads to the resequestration of \( \text{Ca}^{2+} \) by \( \text{Ca}^{2+} \) stores.
1.4.3.1. Nitric Oxide Synthase (E.C. 1.14.13.39)

Nitric oxide synthase (NOS) catalyzes the five electron stepwise oxidation of L-arginine to L-citrulline and NO. NOS is the only known enzyme whose activity requires all of the following four prosthetic groups; FAD, FMN, tetrahydrobiopterin, and a heme. The enzyme has been recently reviewed by Marletta (1993) and others (Nathan, 1992; Lowenstein and Synder, 1992).

\[
\text{L-arginine} + 1.5 \text{NADPH} \rightarrow \text{L-citrulline} + \text{NO} + 1.5 \text{NADP}^+ 
\]

There is both a constitutive and an inducible form of mammalian NOS. The constitutive form has been identified in many cells including endothelial cells, neurons, and recently platelets. Evidence for a platelet NOS has been reported (Malinski et al., 1993; Radomski et al., 1990) and confirmed in our lab by the purification of the platelet constitutive isoform to homogeneity (Muruganandam and Mutus, 1994). The constitutive isoform is regulated by Ca\(^{2+}\)/calmodulin complexes. The inducible isoform has been found in macrophages, neutrophils and endothelial cells and is not regulated by Ca\(^{2+}\)/calmodulin complexes. The immune cells of rats and mice have been shown to express the inducible isoform in response to lipopolysaccharide, γ-interferon, interleukin 1β, and tumor necrosis factor α, but the agents responsible for this induction in human macrophages and other cells have not been determined. The inducible isoform in rat renal mesangial cells has been shown to be independently induced by interleukin 1β or a cAMP-dependent pathway (Kunz, 1994). The inducible isoform binds calmodulin tightly and consequently is active at basal Ca\(^{2+}\) levels. Both forms are homodimeric enzymes of approximately 130-160 kDa subunits (Marletta, 1993). The platelet isoform, however, was a dimer of approximately
80 kDa subunits (Muruganandam and Mutus, 1994). Most of the isoforms discovered have been cytosolic proteins but there are recent reports of particulate NOS which possess a N-terminal myristoylation that serves as a membrane anchor (Busconi and Michel, 1993).

The cDNA sequences of constitutive NOSs from rat cerebellum and bovine aortic endothelial cells as well as the inducible murine macrophage NOS have been reported (Bredt et al., 1991; Lamas et al., 1992; Nishida et al., 1992). By sequence analogy to other enzymes the NOS amino acid sequence is divided about equally into a reductase and an oxygenase domain (Marletta, 1993). The reductase domain contains the binding sites for NADPH, FAD and FMN while it is presumed that the oxygenase domain binds the heme, tetrahydrobiopterin and L-arginine. The binding site for the Ca$^{2+}$/calmodulin complex is between the reductase and oxygenase domains. The Ca$^{2+}$/calmodulin complex controls NOS activity by promoting electron transfer from the flavins of the reductase domain to the heme of the oxygenase domains (Marletta, 1993). The control of intramolecular electron transfer is a new role for calmodulin.

The complex catalytic mechanism of NOS has attracted much attention and is conserved among those isoforms analyzed. The reaction has been divided into a two step mechanism in which the first step involves a two electron oxidation of L-arginine to form N$^\cdot$-hydroxy-L-arginine. A three electron oxidation of the intermediate, N$^\cdot$-hydroxy-L-arginine, results in the production of L-citrulline and NO. The heme complex plays a central role in catalysis. In the resting state the NOS heme contains a five-coordinate ferric ion and is bound to the enzyme through the coordination of the heme iron to a
thiolate of a cysteine residue (Nelson and Strobel, 1988). The sixth iron ligand position is free to bind molecular oxygen when in its reduced ferrous form. The mechanism that has emerged from various studies (Marletta, 1993; Korth et al., 1994) is shown schematically in Fig. 5. An electrophilic perferryl species [FeO]^{3+} converts L-arginine to N^c-hydroxy-L-arginine and a nucleophilic peroxo-iron species [FeOOH]^+ converts N^c-hydroxy-L-arginine to L-citrulline and NO.

To convert L-arginine to NO, NOS requires 1.5 equivalents of NADPH which equals three electrons. Since NADPH transfers two electrons at a time it is conjectured that the dual flavin nature of the enzyme (FAD and FMN) enables it to store the fourth electron for the next catalytic cycle. Dual flavin enzymes can store up to four electrons.

The role of tetrahydrobiopterin in NOS activity remains unclear. As a result of the following studies, it appears that tetrahydrobiopterin may serve a structural rather than catalytic role. NOS activity is not dependent on tetrahydrobiopterin, but it is enhanced when bound to tetrahydrobiopterin in a 1:1 stoichiometry (Hevel and Marletta, 1992). The addition of tetrahydrobiopterin does not affect the initial rate of the reaction and continued catalysis in the absence of tetrahydrobiopterin results in the gradual irreversible inactivation of the enzyme (Giovanelli et al., 1991). Tetrahydrobiopterin may also have a role in the promotion of subunit assembly (Marletta, 1993). The finding that NOS that was partially depleted of tetrahydrobiopterin continued to oxidize NADPH, but transferred the electrons to molecular oxygen to produce superoxide and hydrogen peroxide, has suggested that tetrahydrobiopterin may function in the maintenance of the proper structural arrangement for the optimum electron transfer (Marletta, 1993).
Fig. 5. Reaction mechanism of nitric oxide synthase. The events are as described in the text. PPIX refers to protoporphyrin IX which when conjugated to iron forms the catalytically important heme group present in NOS. The symbol † is used here and by the program ISIS Draw® to represent a free radical.
1.4.3.2. L-Arginine Membrane Transport

Possible fates of influxed cellular L-arginine include incorporation into protein, involvement in the urea cycle, and as recently discovered, conversion to nitric oxide through the action of the complex enzyme nitric oxide synthase (NOS). As a precursor to NO, L-arginine can now be considered important in signal transduction processes. All aspects of L-arginine metabolism are therefore of renewed interest especially in cells such as platelets where NO has been shown to have a potent and measurable effect. The membrane protein systems responsible for L-arginine transport are particularly interesting due to their obvious importance to NOS substrate supply.

L-Arginine transport across the plasma membrane has been observed and characterized in many mammalian cells (reviewed in White, 1985). The membrane protein system responsible for L-arginine transport in most mammalian cells has been termed system $y^+$. The ordinary substrates for system $y^+$ are the cationic amino acids L-arginine, L-lysine and L-ornithine and are transported with approximately equal affinity (uptake $K_M \approx 100 \mu M$). The reported range of normal plasma L-arginine concentrations is from 23 to 86 $\mu M$ (Dickenson et al., 1965). There are several characteristics of system $y^+$ that can be used to identify its presence in cell membranes. System $y^+$ is Na$^+$-independent, saturable with increasing substrate concentration, readily inhibited by certain substrate analogs and other cationic amino acids, sensitive to membrane polarization, pH insensitive, and susceptible to trans-stimulation. The uptake of L-arginine has been found to be a reliable indicator of membrane potential in human fibroblasts (Bussolati et al., 1989).
The steady state kinetics of L-arginine transport by system y⁺ has been satisfactorily described by a iso uni uni reaction which is a reaction that involves only one substrate at either side of the plasma membrane and an isomerization step that moves the protein binding site from one face to another (White and Christensen, 1982a). The iso uni uni mechanism is depicted by the following scheme where S₁ and S₂ represent the extracellular and intracellular substrate concentrations, respectively (White and Christensen, 1982a). Y₁ and Y₂ correspond to the extracellular and intracellular substrate binding site of system y⁺.

\[
\begin{align*}
K_1 S_1 & \quad K_2 & \quad K_3 \\
K_4 S_2 & \quad K_5 & \quad K_6 \\
Y_1 & \quad Y_2 
\end{align*}
\]

The steady state rate equation for the above scheme is as follows where \( N_{1\rightarrow 2} \) is the net influx rate of substrate.

\[
N_{1\rightarrow 2} = \frac{V_{\text{max}}^1 [S_1]([S_1]-[S_2])/K_{c1}}{K_{M1}(1+[S_2]/K_{M2})+[S_1](1-[S_2]/K_{c2})}
\]

Cationic amino acids have been shown to accumulate in fibroblasts at intracellular:extracellular distribution ratios between 10 and 20 (White et al., 1982). To attain this asymmetry the influx rate \( (V_{\text{max}}^1 / K_{M1}^1) \) must be greater than the efflux rate \( (V_{\text{max}}^1 / K_{M1}^1) \). In fibroblasts and hepatoma cells the determination of the kinetic parameters \( (K_M \text{ and } V_{\text{max}}) \) or cationic amino acid efflux and influx were determined and the influx
$K_m/V_{max}$ ratio was in fact approximately 10 times greater than the efflux ratio (White and Christensen, 1982a). The energy required to sustain this distribution ratio may be partially attributed to the membrane potential (negative inside). However, it has been suggested on the basis of thermodynamic equilibrium calculations that the membrane potential required to account for the distribution ratio may not be attainable by most cells (White, 1985). The alternate energy source, if actually present, is unknown.

Normal liver does not express system $y^+$ activity (White and Christensen, 1982b). However, it has been shown in mouse liver that a distinct L-arginine transport system exists (Closs et al., 1993). Relative to system $y^+$, the murine liver L-arginine transport system is of lower affinity ($K_m = 2-5$ mM) and higher capacity since the $V_{max}$ was three to four fold greater. L-Arginine uptake by this low affinity system is only significant at substrate concentrations that exceed the normal plasma concentrations. The murine liver L-arginine transport system may function to remove excess plasma L-arginine.

Hepatic transformation with chemical carcinogens leads to a decreased urea production by the isolated perfused liver (Burke and Miller, 1960) which may be partially attributed to the decreased arginase activity in hepatoma cells as compared to normal liver (Knox, 1967). An additional event that occurs upon hepatic transformation is the expression of system $y^+$ activity (Vadgama and Christensen, 1983; White and Christensen, 1982b). Hepatoma cells such as HTC and Hep G2 express system $y^+$ (Goenn et al., 1992; White and Christensen, 1982). The loss of arginase and appearance of system $y^+$ has been suggested to be a potential critical stage in the successful transformation or
normal hepatocytes to hepatoma cells (liver cells such as Hep G2 cells do express system
\( y^+ \) activity) (White, 1985; White and Christensen, 1982).

A new cationic amino acid transport system that recognizes L-lysine and L-leucine
with equal affinity has been identified in human erythrocytes and named system \( y^+L \) (Deves
\textit{et al}., 1992). The ability of this system to transport L-arginine has been suggested but not
demonstrated. This transport system has been differentiated from system \( y^+ \) on the basis
of sensitivity to N-ethylmaleimide inhibition (Deves \textit{et al}., 1993). System \( y^+ \) is inhibited by
N-ethylmaleimide while system \( y^+L \) is not.

Interestingly, the identity of the ecotropic murine leukemia virus receptor is the
murine isoform of system \( y^+ \) (Kim \textit{et al}., 1991; Wang \textit{et al}., 1991). Upon binding of the
ecotropic murine leukemia virus the cells are infected, the activity of system \( y^+ \) is inhibited,
and the expression of system \( y^+ \) is down-modulated (Wang \textit{et al}., 1992). The ecotropic
murine leukemia virus does not bind to the system \( y^+ \) isoforms in non-murine cells. From
its cDNA the mouse system \( y^+ \) has been found to be comprised of 622 amino acids, 14
potential membrane spanning domains and a combined molecular weight of 67 kDa

There are only a few other studies that have produced any structural information
about system \( y^+ \). Radiation inactivation analysis has shown that system \( y^+ \) activity of the
rat kidney cortex corresponded to a 90 kDa protein (Beliveau \textit{et al}., 1990). System \( y^+ \)
activity from rabbit kidney cortex was associated with an average mRNA chain length of
1.8 to 2.4 kb (Bertran \textit{et al}., 1992). The difference in the reported molecular weights for
system $y^*$ obviously suggests some degree of species-dependent heterogeneity for system $y^*$.

The utility of L-arginine in NO biosynthesis was not realized at the time that most of the basic characteristics of system $y^*$ were revealed (i.e. prior to 1985). Current studies on L-arginine transport are aware of the importance of L-arginine in NO formation and consequently utilize experimental methods to assess the role of L-arginine transport in NO biosynthesis. Some cells known to produce NO and recently shown to display system $y^*$ activity include human endothelial cells, human polymorphonuclear leukocytes, human T lymphocytes, rat aorta vascular smooth muscle cells, porcine pulmonary artery endothelial cells, murine macrophages, and murine neurons (Bussolati et al., 1993; Low et al., 1993; Greene, et al., 1993; Sato et al., 1991; Stoll et al., 1993; Boyd and Crawford, 1992).

In many of these cells system $y^*$ activity has been shown to be enhanced upon cellular activation by mechanisms that involve increased protein synthesis or NO-dependent stimulation or both. It has been independently shown by several groups that system $y^*$ is induced in murine macrophages by lipopolysaccharide and $\gamma$-interferon (Baydoun et al., 1993; Bogle et al., 1992; Sato et al., 1992; Sato et al., 1991). Since dexamethasone induced system $y^*$ but inhibited NOS expression in murine macrophages it appears that different signalling pathways are responsible for the induction of each protein (Baydoun et al., 1993). However, the penultimate signalling pathway for the induction of both proteins may involve a protein kinase C mechanism since both proteins were induced by phorbol esters in murine macrophages (Horteland et al., 1993). Exposure of porcine pulmonary artery endothelial cells to E. coli endotoxin stimulated system $y^*$ activity
between two and five fold through increased protein synthesis (Lind et al., 1993). System y was also induced in activated human T lymphocytes (Boyd and Crawford, 1992).

1.4.4. Regulation of Nitric Oxide Biosynthesis

The regulation of cellular NO production from L-arginine is critical as high NO concentrations are cytotoxic (Sexton et al., 1994; Maragos et al., 1993) NO production is therefore bound to be tightly controlled. The improper regulation of NO metabolism is likely to lead to pathophysiological conditions. For example NO may account for the destruction of the pancreatic islet cells in type I diabetes (Kroencke et al., 1991). Several potential posttranslational regulatory mechanisms of NO production have been discovered (Nussler et al., 1994; Assreuy et al., 1993; Michel et al., 1993; Rengasamy and Johns, 1993; Riesco et al., 1993).

Constitutive NOS is a Ca$$^{2+}$$/calmodulin dependent enzyme that displays maximal activity when cells are activated in a manner that elevates intracellular calcium. During cellular activation through intracellular calcium elevation, a situation can be envisioned where a cellular mechanism is required to limit the overproduction of NO. A feed-back inhibition response on the enzyme, itself, or on the L-arginine transport protein could be an important cellular protection mechanism against NO toxicity.

NOS from murine macrophages has been shown to be irreversibly feedback inhibited by NO (Assreuy et al., 1993) while NOS from bovine cerebellum was shown to be reversibly inhibited by NO (Rengasamy and Johns, 1993). The phosphorylation of NOS has been shown but its in vivo consequences upon NOS activity have not been
clearly demonstrated (Nathan and Xie, 1994). However, the phosphorylation of NOS in endothelial cells is associated with the mobilization of NOS from the particulate fraction to the cytosolic fraction (Michel et al., 1993).

L-Arginine transport has been shown to be differentially modulated by NO. In endothelin-1 activated human polymorphonuclear leukocytes L-arginine transport appears to be feedback inhibited by NO in a cGMP-dependent manner (Riesco et al., 1993). Prior to this study L-arginine transport had not been examined in human polymorphonuclear leukocytes and the presence of system $y^+$ remains undetermined. Evidence has also been presented for the stimulation of L-arginine transport through system $y^+$ by NO in porcine aortic endothelial cells (Bogle et al., 1991). In these endothelial cells $N^G$-nitro-L-arginine, NOS inhibitor, inhibited L-arginine uptake while NO release was completely abolished. Since $N^G$-nitro-L-arginine is not an inhibitor of system $y^+$ the observed inhibition of L-arginine uptake is likely a consequence of decreased NO generation. They suggest that neither NO itself nor cGMP directly stimulates L-arginine uptake as sodium nitroprusside generates NO and raises cGMP levels but did not affect L-arginine uptake (Bogle et al., 1991). It is conceivable that both inhibition and stimulation of L-arginine transport may occur if the effects were dependent on different intracellular NO concentrations. The feed-back regulation of L-arginine transport by NO warrants further study.

In anucleated platelets no protein synthesis occurs except that attributable to the mitochondria and the urea cycle occurs mainly in the liver. Platelets do, however, contain a constitutively expressed isoform of NOS (Muruganandam and Mutus, 1994) and platelet-
derived NO has been shown to inhibit platelet activation (Malinski et al., 1993; Pronai et al., 1991; Radomski et al., 1990). Therefore, it is likely that much of the influxed L-arginine required by the platelet is utilized in the formation of NO. However, platelet L-arginine transport has not been studied in detail. There is an early report that surveyed the ability of platelets to take up a number of different amino acids (Zieve and Solomon, 1968). In that study, however, it was inconclusively stated that platelets are incapable of taking up L-arginine. More current studies have implicated the presence of a platelet L-arginine transport system (Radomski et al., 1990).

1.5 Aim of the Present Study

The aim of the first part of this study was to determine whether or not GSH metabolism is involved in the regulation of platelet function. As described in Section 1.3.3.2, the intracellular GSH:GSSG ratio may be a type of signal transduction mechanism, particularly if it can be altered in response to a hormone:receptor complex. Intuitively, the effect of GSH on platelet function may be observed either by reducing or elevating the intracellular GSH concentration. Intracellular GSH depletion can be accomplished with the use of thiol oxidizing or alkylation reagents. Many such reagents are not specific for GSH and consequently clear information on the cellular role of GSH in a particular process can not be attained. We attempted to characterize a new thiol reagent, a Mannich base of an α,β-unsaturated ketone, for its selective GSH-depleting properties using intact platelets. In our attempts to elevate intraplatelet GSH we discovered that platelets contain a specific GSH transport system. Having identified the
presence of the platelet GSH carrier we initiated its characterization with the aim of comparing it to other known GSH carriers. In the course of our studies on GSH metabolism and its interaction with some Mannich bases of $\alpha,\beta$-unsaturated ketones we made some new observations that were not restricted to platelets. These observations are presented in Chapter 3.

The second part of this study reflects our interest in platelet nitric oxide metabolism and the post-translational mechanisms for the regulation of nitric oxide production. In these studies we sought to characterize, for the first time, platelet L-arginine transport. Since L-arginine transport has been considered rate-limiting in the NOS-mediated production of NO, its regulation may be central to NO generation. We examined the platelet L-arginine transport system for feedback regulation by NO.

Since the reaction between NO and GSH to form S-nitrosothiol (GSNO) is of physiological significance, the investigation of some properties of GSNO was a natural extension of the preceding studies. Early in our investigations on the rates of GSNO decomposition we observed that the S-nitroso bond of GSNO was photolabile. Our subsequent aim was to determine if GSNO could be utilized as a new type of photochemotherapeutic agent.
Chapter 2

MATERIALS AND EQUIPMENT

2.1. MATERIALS

Citrated whole blood was provided by the hematology department at the Hotel Dieu hospital, Windsor, ON (519-973-4444 ext 143) and outdated platelet concentrates were obtained from the Alberta division of the Red Cross.

Distilled deionized water was prepared using a Barnstead Fi-Stream distiller (Sybron Corp. Dubuque, Iowa 52001) and a Milli-Q water purification system (Millipore Corp. Milford, MA 01757).

The following materials were purchased from the Sigma Chemical Co. (St. Louis, MO 63178): γ-glutamyl-p-nitroanilide, 1-chloro-2,4-dinitrobenzene, 5-bromo-4-chloro-3-indoyl phosphate, acivicin, acrylamide, apyrase type VIII, bovine erythrocyte glutathione peroxidase, bovine intestinal mucosa glutathione reductase, Chelex-100, Coomassie brilliant blue R-250, D-arginine, dithiothreitol, ethacrynic acid, goat anti-rabbit IgG-alkaline phosphatase conjugate, GSH, GSSG, horse liver GST, human hemoglobin, L-arginine, L-citrulline, L-lysine, L-ornithine, leupeptin, lubrol PX, N,N'-methylene-bis-acrylamide, N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES), NADPH, nitroblue tetrazolium, phenylmethanesulfonyl fluoride, placenta GST, polyvinylpyrolidone, procion red, Sephadex G-25, Sigma-Fluor (scintillation fluid), and yeast glutathione reductase.

[3H]-L-Arginine and [3H]-glutathione was obtained from New England Nuclear (Boston, MA 02118) and [14C]-inulin was from Amersham Canada (Oakville, ON L6L
N<sup>O</sup>-Monomethyl-L-arginine was obtained from Calbiochem (La Jolla, CA 92037). Cumene hydroperoxide and trans-4-phenyl-3-buten-2-one were obtained from Aldrich (Milwaukee, WI 53233). Monobromobimane was obtained from Molecular Probes Inc. (Eugene, OR 97402-0414) CM-Sepharose, Sepharose 4B was from Pharmacia Fine Chemicals (Uppsala, Sweden). Rat liver was obtained frozen from Pel-Freez® (Rogers, AR). CDDP and compounds 1, 2, and 3 were generous gifts of Dr. Dimmock. Ammonium persulfate, Ponceau S, SDS-PAGE molecular standards, and Tween-20 were obtained from Bio-Rad Laboratories (Richmond, CA 94804). RPMI 1640 medium and fetal bovine serum were from GIBCO (Grand Island, NY). HL-60 cells were obtained from the American Type Culture Collection (ATCC No. CCL 240, batch F-10175). NO gas was from Alphagaz (St. John’s NF). E. coli paste was from Worthington Biochemicals (Freehold, NJ 07728). Dow Corning 550 silicone oil and Dow Corning 200, 1.0 centistokes silicone oil were from Dow Corning (Midland, MI 48686-0994). Antibodies specific to alpha and mu class GST were obtained from Novacstra Laboratories (Newcastle upon Tyne, U.K.) and antibody specific to pi class GST was obtained from Crystal Chem. (Chicago, IL). The 0.45 μm nitrocellulose membranes were from Costar (Cambridge, MA 02139). The phenol reagent (Folin and Ciocalteu reagent) was from BDH. Electron microscopy grade epoxy (mordcast resin, nadic methyl anhydride, and dodecenyl succinic anhydride) was supplied by Ted Pella Inc. (Reading, CA 96049) while uranyl acetate and osmium tetroxide were purchased from J.B. EM services Inc. (Point Clare, PQ H9R 4S8).

All other reagents were of reagent grade or better.
2.2. EQUIPMENT

The following is a list of equipment used in the performance of the described experiments:

300 W quartz halogen lamp from a slide projector;

Beckman J-6B centrifuge (Palo Alto, CA 92634-3100);

Bio-Rad 1330 HPLC gradient system (Richmond, CA 94804);

Bio-Rad Econo System (Richmond, CA 94804);

Bio-Rad Mini Trans-Blot® electrophoretic transfer cell apparatus (Richmond, CA 94804);

Bio-Rad power supply model 500/200 (Richmond, CA 94804);

Chemiluminescent NOX analyzer (model 14 B/E Thermo Electron Corp., Franklin, MA);

CO2 regulated incubator (Autoflow, Nuare, Plymouth, MN 55447);

Coulter Counter T-890 or STKS (Couter Electronics, Hialeah, FL 33012);

EM 201 electron microscope (Philips);

French press;

He-Ne laser (1 mW, Melles Griot);

hemocytometer (American Hospital Supply Corp., McGraw Park, IL 60085);

Hitachi model F-3010 spectrofluorometer (Tokyo, Japan);

hyperion microplate reader III (Miami, FL 331186);

Kontes hand held homogenizer (Mandel Scientific Co., Guelph, ON N1H 6J3);

laminar flow hood (Nuare, Plymouth, MN 55447);

Millipore filtration apparatus (Millipore Corp. Milford, MA 01757);

Shimadzu CR3A chromatopac integrator (Kyoto, Japan);

Shimadzu RF540 flow-through spectrofluorometer (Kyoto, Japan);
Shimadzu UV-160 spectrophotometer (Kyoto, Japan) equipped with a thermostated cell holder;

Sorval Ultra pro 80 ultracentrifuge (Newton, CT 08475);

Sorval RC5 centrifuge (Newton, CT 08475);

Ultramicrotome MX6000-XL (RMC Inc.);

UV filter that eliminated wavelengths below 335 nm (Schott).
Chapter 3

METHODS

3.1. Platelet Isolation

Human blood was obtained in citrated (3.8 %) vacucontainers from patients prior to undergoing day surgery at a local hospital. The patients attested that they had not taken aspirin in the last 5 days. The blood was utilized the day of collection. Platelets were isolated by centrifugation similarly to that described by Muruganandam et al. (1993). Whole blood was first spun at 130 x g for 20 minutes. The platelet rich plasma was then centrifuged at 3300 x g for 20 minutes to pellet the platelets followed by 1 wash with wash buffer (10 mM HEPES, 137 mM NaCl, 3.7 mM KCl, 5.5 mM glucose at pH 6.8) at 1800 x g for 20 minutes. The platelet pellet was then resuspended in incubation buffer (137 mM NaCl, 3.7 mM KCl, 10 mM HEPES, 5.5 mM glucose at pH 7.4).

3.2. Measurement of Platelet Aggregation

Platelets destined for aggregation measurements were prepared differently than described above. To measure ADP-induced platelet aggregation the following procedure was followed. Immediately after the platelets were isolated from plasma they were resuspended in wash buffer and treated with 2.5 U/mL apyrase for 15 minutes at 37°C after which they were centrifuged. The washed platelet pellet was resuspended in suspension buffer (137 mM NaCl, 2.7 mM KCl, 3 mM NaH₂PO₄, 1 mM MgCl₂, 5.5 mM glucose, 0.35 % albumin, 3.5 mM HEPES, pH 7.4) that contained a low amount of apyrase (0.1 U/mL) to ensure ADP responsiveness. Aggregation was initiated following
the addition of 50 μg/mL fibrinogen and 2 mM CaCl₂ by a 5 μL aliquot of ADP (2-10 μM final) in suspension buffer that did not contain apyrase. Platelets prepared for the measurement of thrombin-induced aggregation were prepared in the same manner except that it was not necessary to include apyrase. Thrombin-induced aggregation was initiated by the addition of 0.2-0.5 U/mL agonist. Platelet aggregation was measured photometrically in a Shimadzu spectrophotometer (UV-160) equipped with a thermostatted cell holder capable of stirring the platelet suspension. Room lights were darkened so that the sample compartment lid could be opened without interfering with aggregation measurements.

3.3. Preparation of Platelet Plasma Membrane Vesicles (PPMV)

PPMV were prepared according to an established procedure using a glycerol loading procedure followed by hypotonic lysis (Barber and Jamieson, 1970; Harmon et al., 1992). Outdated platelet concentrates were obtained from the Red Cross and washed with platelet wash buffer as before (Section 3.1.) and resuspended in incubation buffer so that platelets from 4 units of concentrate are contained in 10 mL of incubation buffer. The platelet suspension was then layered onto 30 mL of a 0-40 % linear glycerol gradient containing loading buffer (86 mM Tris, 96.5 mM choline chloride, 86 mM glucose, 100 mM EGTA, 1.4 mM EDTA, pH 7.4) in 50 mL tubes and centrifuged for 30 minutes at 1500 x g followed by 30 minutes at 3300 x g all at 4°C in a Beckman J6B centrifuge equipped with a swing-out rotor. The glycerol was then aspirated away and the glycerol-loaded platelet pellet was then hypotonically lysed by the addition of 4-5 volumes of lysis buffer (loading buffer plus 50 μg/mL leupeptin and 2 mM PMSF) and vigorous dispersion
through a 18-gauge needle attached to a 25 mL syringe. After extensive syringing, the lysed platelet suspension was centrifuged at 3300 x g for 30 minutes. The lysed platelet supernatant was then layered onto a sucrose step gradient prepared in 35 mL Ultracrimp tubes (Sorval) by layering 10 mL of 33 % sucrose in lysis buffer over 66 % sucrose in lysis buffer and then centrifuged at 63 000 x g for 2 hours in a Sorval ultra pro-80 using a fixed angle rotor. The membranes layered on top of the 33 % sucrose layer and were obtained using a syringe attached to a 10 cm piece of plastic tubing. The membranes were then concentrated by another centrifugation at 63 000 x g (30 min.) and resuspended in 140 mM choline chloride, 10 mM HEPES, 0.2 mM PMSF, 50 μg/mL leupeptin, pH 7.4 and frozen at -80°C until use.

3.4. Electron Microscopy of Platelet Membranes

The integrity of the PPMV preparation was assessed by electron microscopy (Barber and Jamieson, 1970). PPMV (30 μg protein) was fixed with 1.25 % glutaraldehyde in buffer (200 mM sucrose, 50 mM sodium phosphate, pH 7.3) for 2.5 hours (200 μL final volume). Fixed PPMV was then washed once with 200 μL of the above buffer by centrifugation in a desk-top microcentrifuge before staining with 1 % OsO₄ for 1 hour. Following removal of the OsO₄ by centrifugation, the PPMV preparation was dehydrated by 5 minute successive incubations with increasing ethanol percentages (50, 70, 90, 100 %). While in 100 % ethanol the PPMV preparation was stained with 2 % uranyl acetate for 2 hours, after which it was washed with 100 % ethanol once and treated 3 more times to 10 minute 100 % ethanol dehydrations. The dehydrated
PPMV preparation was then treated with propylene oxide 3 times for 10 minutes each and exposed to propylene oxide:epoxy (3:1) for 3 hours, after which it was incubated overnight with propylene oxide:epoxy (1:3) on rotor. Epoxy (Ted Pella Inc.) was prepared according to package instructions. The PPMV preparation was then treated with 3 changes of 100% epoxy, the first treatment was for 4 hours, the second was overnight, and the third was for 4 hours. The PPMV was flat embedded in epoxy by overnight polymerization at 70°C. Ultrathin sections (90 nm) were prepared using an ultramicrotome and collected onto copper grids. The grids were then stained with 2% uranyl acetate for 15 minutes at 40°C followed by staining with lead citrate (3%) for 3 minutes. Finally, grids were viewed in an electron microscope and photographs were taken at magnifications from 7 K to 20 K.

3.5. HPLC Analysis of Intra-Platelet GSH

Platelet samples were homogenized in 25 mM Tris, 1 mM EDTA, pH 8.0 with a Kontes homogenizer (1 minute at 12,000 and 1 minute at 15,000 rpm) on ice and then centrifuged at 5000 x g for 10 min. 60 nmol DTT was then added followed by 244 nmol monobromobimane and a 30 min incubation in the dark. The reaction was arrested by the addition of 10 μL of acetic acid. Derivatized samples were filtered through 0.45 μM nylon membranes before HPLC injection. The glutathione:bimane adduct was measured by reverse phase (C18) HPLC with fluorescent detection (Muruganandam et al., 1992). HPLC separation of the bimane adducts of glutathione, cysteine and homocysteine was achieved isocratically using 10% acetonitrile, 0.1% trifluoroacetic acid, pH 3.9 with a
flow rate of 0.5 mL/min. The column was regenerated with 60 % acetonitrile, 0.1 % trifluoroacetic acid, pH 3.9. The quantification of total platelet GSH was preformed using standards prepared from authentic GSH. Measurements were preformed in triplicate.

3.6. Sulphhydryl Titrations (Ellman, 1958)

GSH and other thiol solutions were prepared fresh in distilled deionized H₂O and were quantitated using 1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) or DTNB in 100 mM Tris, 1 mM EDTA, pH 7.5 using an extinction coefficient for the nitrothiobenzoate anion of 13,600 L·(mol·cm)⁻¹ (Ellman, 1958).

3.7. Synthesis of N-Formyl Glutathione (Levey et al., 1993a)

To an ice cold solution of GSH (3 g in 30 mL of 99 % formic acid) 15 mL of acetic acid was added. The mixture was stirred 1 hour at room temperature after which the N-formylation was considered complete on the basis of a negative reaction with ninhydrin. The product was precipitated by the addition of 5 volumes of diethyl ether and an overnight incubation at 0°C. The ether was decanted and the precipitate was obtained by vacuum filtration followed by drying under a stream of helium followed by placement in an evacuated dessicator with P₂O₅ as the desicant. The product was recrystallized once by dissolving it in a minimum amount of H₂O followed by the addition of 2 volumes of ethanol and the addition of ethyl acetate to the point of turbidity. Diethyl ether (4 volumes) was then added and the product was collected on standing overnight at 0°C after which the product was dried as before. The melting point of the obtained product was
156-160°C which was in agreement with that of Levy et al. (1993a) who reported 159-161°C.

3.8. Synthesis of N-Acetyl Glutathione (Levy et al., 1993a)

Five grams of GSH was dissolved in a minimal amount of H₂O (~ 12 mL) and added with a burette over 1 hour to a mixture of 50 mL acetic acid and 50 mL acetic anhydride at room temperature. After the addition was complete the solution was chilled on ice and 25 mL acetic anhydride was added. Upon standing overnight at room temperature 6 volumes of diethyl ether was added. Prior to adding diethyl ether the reaction was judged complete on the basis of a negative reaction with ninhydrin (0.2 % in 95 % ethanol). The precipitated product was collected by vacuum filtration after standing overnight at 0°C. It was then dried under a stream of helium followed by placement in an evacuated dessicator with P₂O₅ as the desicant. The melting point of the obtained product was 135-138°C which was in agreement with that of Levy et al. (1993a) who reported 139-143°C.

3.9. Synthesis of Glutathione Monoethyl Ester (Anderson et al., 1985)

GSH (5 g) was mixed with 5 mL of HCl and 49 mL of ethanol and allowed to stand at 4°C for 8 hours after which the reaction was deemed complete by thin layer chromatography (TLC) with silica plates (Kodak) in n-propanol:acetic acid:water (10:1:5 v/v). Typical Rᶠ values for GSH and GSH monoethyl ester were 0.4 and 0.6, respectively. TLC plates were stained with ninhydrin which were in agreement with Anderson et al.
(1985). To the completed esterification reaction 500 mL of ice-cold diethyl ether was added and the product was allowed to settle for 4 hours. The ether was decanted. The product was obtained by vacuum filtration and dried under a stream of helium followed by placement in an evacuated desiccator with P₂O₅ as the desiccant.

3.10. Synthesis of Glutathione Diethyl Ester (Levy et al., 1993)

GSH (5 g) was added to an ice cold solution that contained 100 mL of ethanol and 5.6 mL of H₂SO₄. Once GSH dissolved it was allowed to stand at 4°C for 7 days. Cold diethyl ether was then added and precipitate was collected as a white syrup after 4 hours at 4°C. The syrup was obtained by vacuum filtration and dried under a stream of helium followed by placement in an evacuated desiccator with P₂O₅ as the desiccant. The dried syrup (powder) was then dissolved in a minimal amount of H₂O and the pH adjusted to 4.5 with 10 M NaOH. The solution was then applied to a Chelex-100 column (5 x 10 cm) and eluted with H₂O. The eluant was monitored by absorbance at 254 nm and by reaction with DTNB as described in Section 3.6. According to Levy et al. (1993) the first elution peak corresponds to GSH monoethyl ester and the second to the diethyl ester. The diethyl ester peak was pooled and lyophilized. The resulting dry solid was extracted 3 times with cold absolute ethanol and the combined extracts (~ 50 mL) were evaporated to dryness by rotary evaporation. Confirmation of the purified species as GSH diethyl ester was obtained by TLC as described above for GSH monoethyl ester. The obtained Rₜ value for GSH diethyl ester was 0.73 which was in agreement with Anderson et al. (1985).
3.11. Synthesis of S-(2,4-Dinitrophenyl) Glutathione (Vince et al., 1971)

GSH (0.6 g) was dissolved in 2 mL of H₂O by the addition of 0.16 g of Na₂CO₃. Ethanol (~ 20 mL) was then added to the point of turbidity, at which point an equimolar amount of 1-chloro-2,4-dinitrobenzene (CDNB) was slowly added as a fine powder. If undissolved starting material (CDNB) was evident and more ethanol (~ 10 mL) was added. The solution was stirred for 3 hours in the dark at room temperature. The pH was adjusted to ~ 3.5 by dropwise addition of 47% HI and the mixture was chilled on ice for 1 hour. The product was obtained by vacuum filtration and dried under a stream of helium followed by placement in an evacuated desicator with P₂O₅ as the desicant. The melting point of the product was in agreement with that of Vince et al. (1971) (194-195°C).

3.12. Preparation of Procion Red Sepharose 4B (Dean and Watson, 1979)

To 100 g (wet weight) of Sepharose 4B in 350 mL a solution of procion red (1 g in 100 mL water) was added and mixed on a shaker for 5 minutes. 50 mL of 20% NaCl was then added and after brief stirring the mixture was allowed to stand 30 minutes, at which point 2.5 mL of 5 M NaOH was added. The mixture was then incubated for 3 days at room temperature on a shaker. Using a sintered glass funnel the gel was then washed extensively with water, followed by 1 M NaCl, 4-8 M urea, and water again.

3.13. Purification of *E. coli* Glutathione Reductase

Glutathione reductase (GR) from *E. coli* was purified according to the method of Scrutton et al. (1987). *E. coli* paste (40 g) was washed twice with buffer A (20 mM
potassium phosphate, 1 mM EDTA, 1 mM DTT, 10 μM FAD) and centrifugation at 3300 x g for 20 minutes at 4°C. The pellet was resuspended in 20 mL of buffer A and disrupted with 2 passages through a French press at 4°C and 20 000 lb/in$^2$. The extract was clarified by centrifugation at 15 000 x g for 1 hour at 4°C. The enzyme was fractionated by additions of solid (NH$_4$)$_2$SO$_4$. The enzyme was in the 40-80 % (NH$_4$)$_2$SO$_4$ fraction, which was then dialyzed against 2 changes of 3 L of buffer B (5 mM sodium phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol). The dialyzed protein was then applied to a P rocion red column (2.5 x 6 cm) equilibrated with buffer B containing 0.1 M NaCl. After extensive washing with buffer B that contained 0.1 M NaCl GR was eluted with buffer B and 0.2 M NaCl. The final step of the purification involved ultrafiltration through an Amicon PM10 membrane. The protein was judged pure by SDS-PAGE with Coomassie blue staining.

3.14. Platelet Glutathione Reductase

Platelets were isolated from human whole blood as previously described (Section 3.1) and were suspended in 100 mM sodium phosphate buffer containing, 1 mM EDTA, pH 7.0 and frozen at -80°C until enough platelets were obtained (about 150 subjects). Pooled platelets were then thawed, homogenized by a Kontes hand-held homogenizer and centrifuged at 15 000 x g in a desk top centrifuge for 4 min. The glutathione reductase-containing supernatant was then used for kinetic assays without further purification.
3.15. Purification of Equine Liver Alpha Class Glutathione S-Transferase

Alpha class GST was obtained from a purified equine liver GST mixture (Sigma) according to a modification of the procedure of Li and Ishibashi (1990). GST (2.5 mg) was applied to a CM-Sepharose column (2 x 5 cm) equilibrated with buffer A (10 mM sodium phosphate buffer, 1 mM EDTA, pH 6.0). GST-α was eluted with a linear gradient up to 60 % buffer A containing 1 M sodium chloride (0.6 M sodium chloride final). Protein elution profiles were monitored by absorption at 280 nm. Purified alpha GST was assessed as being pure by SDS PAGE (Laemmli, 1970). Protein concentration was determined using the method of Bradford with BSA as the standard (Bradford, 1976).

3.16. Platelet GST

Whole human blood (approx. 5 mL per patient, freshly drawn) collected in sodium EDTA vacutainers was first centrifuged at 200 x g for 10 min to obtain platelet rich plasma which was then centrifuged at 4000 x g for 20 min to sediment platelets. The platelets from approximately 10 patients were pooled and then washed twice with 1 ml of 5 mM Hepes, 1 mM EDTA pH 7.4 and resuspended in 1 ml of the same buffer. The platelet suspension was then homogenized with a Kontes hand-held homogenizer and centrifuged at 15000 x g in a desk top airfuge (Eppendorf) for 20 min. The cytosolic fraction was then used as a crude source of GST.
3.17. Rat Liver GST

One rat liver (7.7 g wet weight) was homogenized in the presence of 15 ml of 10 mM sodium phosphate, 1 mM EDTA pH 7.0. The cytosolic fraction was isolated by centrifugation of the homogenate at 15 000 x g at 4°C for 60 minutes.

3.18. Isolation of Platelet Plasma Membrane L-Arginine Binding Proteins

PPMV (~10 mg) was dissolved in 5 mL of column buffer (10 mM Tris, 140 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 0.5 % lubrol PX at pH 7.4). The protein was then loaded onto a L-arginine agarose (12 atom spacer, Sigma) column (0.8 x 3.5 cm) and washed thoroughly (~50 mL) with column buffer. Bound protein was then eluted with column buffer containing 100 mM L-arginine. The bound protein was pooled and lyophilized. Protein purity and molecular weight was determined with the concentrated protein by SDS-PAGE by comparison to a broad range of SDS-PAGE molecular weight standards.

3.19. Cytosolic Protein Estimation

Protein concentrations were estimated using the Bio-Rad microassay according to the package directions with bovine serum albumin as the standard. The Bio-Rad microassay is a modification of the Bradford assay (Bradford, 1976).
3.20. Membrane Protein Estimation

Platelet plasma membrane protein was measured by a Lowry assay modified to detect protein in the presence of lipid (Markwell et al., 1978). Just prior to performing the assay reagent A (2 % NaCO₃, 0.4 % NaOH, 0.16 % sodium tartrate, and 1 % SDS) and reagent B (4 % CuSO₄·5H₂O) are mixed 100:1. Protein (100 μL) was added to 1 mL of the reagent A:reagent B mixture and incubated 15 minutes at room temperature, at which point 100 μL of diluted (1:1) phenol reagent (BDH) was added and the mixture was allowed to stand for 45 minutes at room temperature. The protein concentration was estimated by measuring the absorbance at 660 nm and using a bovine serum albumin protein standard curve.

3.21. Glutathione Reductase Assay

Glutathione reductase (GR) activity was determined by monitoring the decrease in NADPH absorbance at 340 nm and 30°C (Δε = 6220 M⁻¹cm⁻¹; Racker, 1955). In all cases the reaction mixtures contained GSSG, NADPH, and enzyme in 100 mM sodium phosphate buffer, 1mM EDTA at pH 7.0 with or without GSH in a final volume of 1 mL in a 1 cm path length cuvette. When GSH concentrations were varied the concentration of GSSG was 1 mM and NADPH was 100 μM which was added last to start the reaction. When NADPH concentrations were varied the concentration of GSSG was also 1 mM and the reaction was initiated by the addition of 10 μL of a NADPH solution. In order to vary GSSG substrate concentrations in the presence of GSH inhibitor, the contaminating GSSG in GSH was first reduced by a one hour preincubation of 5 mM GSH with enzyme and 30
μM NADPH after which the concentration of NADPH in the cuvette was adjusted to 100 μM and reaction initiated by 10 μL of a GSSG solution. Control reaction mixtures when GSSG was varied in the absence of GSH contained 30 μM NADP⁺ to account for any inhibition by the NADP⁺ formed during the incubation. All activity measurements were performed at least in triplicate.

3.22. Glutathione S-Transferase Assays

GST assays with substrates other than CDDP were performed according to published procedures. Except where noted, the assay buffer was 100 mM sodium phosphate, 1 mM EDTA pH 6.5 buffer and in all cases the reaction temperature was constant at 30°C. The reactions were initiated by the addition of 10 μl of electrophilic or peroxide substrate to make a final volume of 1 ml in a 1 cm path length cuvette. Nonenzymatic rates of GSH conjugation were subtracted from enzymatic rates. All activity determinations were done at least in triplicate and error calculated from the standard deviation.

CDNB. GST activity toward CDNB was measured according to Habig and Jakoby (1981) by monitoring the initial rate of absorbance increase at 340 nm (Δε = 9600 M⁻¹cm⁻¹). For typical activity measurements the initial CDNB and GSH concentrations were 1 mM and 5 mM, respectively. CDNB was dissolved in ethanol. The percentage of ethanol in the cuvette (1%) did not inhibit the enzyme.

Ethacrynic acid. GST activity toward ethacrynic acid was measured according to Habig and Jakoby (1981) by monitoring the initial rate of absorbance increase at 270 nm (Δε =
5000 M$^{-1}$cm$^1$). For typical activity measurements the initial ethacrynic acid and GSH concentrations were 0.2 mM and 0.25 mM, respectively. Ethacrynic acid was dissolved in water.

*trans*-4-Phenyl-3-buten-2-one. GST activity toward *trans*-4-phenyl-3-buten-2-one was measured according to Habig and Jakoby (1981) by monitoring the initial rate of absorbance decrease at 290 nm ($\Delta\varepsilon = 24\,800\, M^{-1} cm^{-1}$). For typical activity measurements the initial *trans*-4-phenyl-3-buten-2-one and GSH concentrations were 50 µM and 250 µM, respectively. *trans*-4-Phenyl-3-buten-2-one was dissolved in ethanol. The percentage of ethanol in the cuvette (1%) did not inhibit the enzyme.

*Cumene hydroperoxide*. GST peroxidase activity toward cumene hydroperoxide was measured according to Lawrence and Burke (1976). The reaction mixture contained 0.2 mM NADPH, 1 U/mL yeast GR, 1 mM GSH, and 1.5 mM cumene hydroperoxide in 100 mM sodium phosphate, 1 mM EDTA pH 7.0 buffer. Upon addition of cumene hydroperoxide the initial rate of NADPH oxidation was monitored by the decrease in absorbance at 340 nm ($\Delta\varepsilon = 6220\, M^{-1} cm^{-1}$). Cumene hydroperoxide was dissolved in ethanol. The percentage of ethanol in the cuvette (1%) did not inhibit the enzyme.

*CDDP*. The conditions for the measurement enzyme activity with CDDP were the same as in the above assays except that CDDP was dissolved in methanol and a 0.5 ml cuvette with a 0.2 cm pathlength was utilized due to the high extinction coefficient ($\varepsilon = 21\,000\, M^{-1} cm^{-1}$) of the compound at 307 nm. The rate of conjugation of CDDP to GSH was observed by the decrease in absorbance at 307 nm per minute. Typical CDDP activity measurements were performed in the presence of 500 µM GSH and 300 µM CDDP. The
determination of kinetic parameters (\(K_M\) and \(V_{\text{max}}\)) were performed with 1 mM GSH and 300 \(\mu\)M CDDP. The GST activity measurements with CDDP of the elution fractions shown in Fig. 13 were performed in the presence of 10 \(\mu\)M CDDP and 100 \(\mu\)M GSH in a 1 ml, 1 cm pathlength cuvette. Except where noted, CDDP was added last. The nonenzymatic rate of conjugation was subtracted from the total rate to yield the net enzymatic rate. The percentage of methanol in the cuvette was 1%.

**Monobromobimane.** GST activity towards monobromobimane was measured using the Hulbert and Yakubu (1983) spectrofluorometric method (\(\lambda_{\text{ex}} = 392\) nm and \(\lambda_{\text{em}} = 480\) nm). Typical activity measurements were obtained from the initial rate of fluorescence increase in the presence of 10 \(\mu\)M monobromobimane and 100 \(\mu\)M GSH. Monobromobimane was dissolved in acetonitrile which was maintained at 10 % in the cuvette, an amount that did not inhibit the enzyme.

### 3.23. \(\gamma\)-Glutamyl Transpeptidase Assay

\(\gamma\)-GT activity was measured according to Meister et al. (1981) using the artificial substrate \(\gamma\)-glutamyl-\(p\)-nitroanilide. Initial rates of \(\gamma\)-glutamyl-\(p\)-nitroanilide hydrolysis by \(\gamma\)-GT is measured by the absorbance increase at 410 nm. Typical activity measurements were obtained in the presence of 1 mM \(\gamma\)-glutamyl-\(p\)-nitroanilide and 20 mM glycylglycine in 100 mM Tris-HCl, pH 8.0 at 37°C. A 5 mM \(\gamma\)-glutamyl-\(p\)-nitroanilide solution was prepared by adding 80 mg of \(\gamma\)-glutamyl-\(p\)-nitroanilide to 20 mL of 1 M HCl and stirring until the compound dissolved. Water (30 mL) and 0.73 g or Tris base was then added and
the pH adjusted to 8.0. The solution was made up to 60 mL with water and stored frozen until use.

Platelet γ-GT was inactivated by a 1 hour incubation of intact platelets with 0.5 mM acivicin in 137 mM NaCl, 3.7 mM KCl, 10 mM HEPES, 5.5 mM glucose at pH 7.4 at room temperature. The platelets were then homogenized in 100 mM Tris, 0.1 % Triton X-100, pH 8.0 and centrifuged at 5000 x g for 10 min. γ-GT activity measurements were performed on the supernatant.

3.24. Glutathione Peroxidase Assay

GPX activity was monitored according to Flohe and Gunzler (1984). A typical reaction mixture initially contained in 1 mL; 1 mM GSH, 0.1 mM NADPH, 0.24 U yeast GR, 0.12 mM t-butyl hydroperoxide in 100 mM sodium phosphate, 1mM EDTA, pH 7.0 buffer. GSH, GR, and GPX were preincubated for 10 minutes which was followed by an additional 3 minute preincubation after NADPH was added. The reaction was initiated by the addition of 120 μM t-butyl hydroperoxide. The initial rate of absorbance decrease at 340 nm (NADPH oxidation) was used to monitor GPX.

3.25. SDS-PAGE (Laemmli, 1970)

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was performed using mini-gels (8 cm x 10 cm) with a home made system based on the Mighty Small™ minigel unit. The percent total polyacrylamide in the resolving gel varied from 8-15 % (29:1 acrylamide:bis-acrylamide) in the presence of 0.375 M Tris-HCl, pH 8.8. The
stacking gel always contained 5% total acrylamide (29:1 acrylamide:bis-acrylamide) and 0.125 M Tris-HCl, pH 6.8. The resolving gel and the stacking gel contained 0.1% SDS and polymerization was initiated with the addition of 0.1% ammonium persulfate and 5-10 µL of TEMED.

Protein samples were mixed 1:1 with sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH 6.5) and boiled for ~3 minutes. After loading samples the electrophoresis unit was run at constant voltage (100 V) for ~2 hours or until dye front reached the bottom of the gel. The gel was then stained with Coomassie blue (0.1% Coomassie R-250, 40% methanol, 10% acetic acid). Different SDS-PAGE molecular weight standards were used.

3.26. Immunoblot Analysis

Protein was transferred electrophoretically from 15% SDS-polyacrylamide gels to nitrocellulose membranes using a Bio-Rad Mini Trans-Blot® electrophoretic transfer cell apparatus as described by Towbin et al. (1979). Transfer was confirmed by performing a Ponceau S stain (0.2%) of the nitrocellulose membrane. After destaining the membranes with water, the membranes were blocked for 6 hours with 20 mL of blocking solution (50 mM Tris, 0.5 M NaCl, 2% polyvinylpyrrolidone, pH 7.4). The membranes were then incubated for 1 hour with a 1:1000 dilution of primary anti-GST rabbit antibody, followed by a 1 hour incubation with alkaline phosphatase conjugated anti-rabbit antibody. Color development was produced upon incubation with the alkaline phosphatase substrates using 5-bromo-4-chloro-3-indoxyl phosphate and nitroblue tetrazolium usually with 2-3 minutes.
When color development was sufficient the membranes were washed several times with distilled water.

3.27. Measurement of Glutathione Transport

*Intact platelets.* Before measuring uptake platelets were treated with 0.5 mM acivicin after which the volume was adjusted so that the final concentration of acivicin was 0.11 mM. The uptake of \(^{3}\text{H}\)-L-GSH (0.02 - 0.04 µCi/trial) by intact platelets was performed in incubation buffer (137 mM NaCl, 3.7 mM KCl, 10 mM HEPES, 5.5 mM glucose at pH 7.4) in the presence of 4 times molar excess of DTT at 37°C in a final volume of 50 µL. At the specified time the platelet suspension (~100 - ~200 \(\times\) 10^9 platelets/trial) was diluted with 500 µL of the incubation buffer and layered over a silicon oil mixture (0.83 mL of Dow Corning 550 + 0.17 mL Dow Corning 200, 1.0 centistokes per 1 mL, final density = 1.02 g/mL) and centrifuged for 30 sec at 9350 \(\times\) g in a swing out rotor microcentrifuge (Feinberg et al., 1974). The supernatant and most of the oil layer was aspirated away and the remaining platelet pellet was dissolved in 250 µL of 2 % SDS before transferring to scintillation fluid and radioactive counting. The results obtained were corrected for inter-platelet trapping of radiolabel using \(^{14}\text{C}\)-inulin to estimate the % trapping that occurred per number of platelets. Platelet counts were performed using a Coulter STKS electronic particle counter. All experiments were performed at least in quadruplicate and the results expressed are averages with the error bars represented by their standard deviation.

*Platelet plasma membrane vesicles.* The uptake of \(^{3}\text{H}\)-GSH into platelet plasma membrane vesicles (PPMV) was performed in incubation buffer in the presence of 4 times
molar excess of DTT at room temperature in a final volume of 50 μL. [3H]-GSH was added last. To stop [3H]-GSH uptake the membrane vesicles were diluted with 500 μL of incubation buffer and rapidly vacuum filtered onto nitrocellulose membranes (0.45 μm pore size) through a Millipore filtration apparatus. Dried filters were dissolved in scintillation fluid overnight before measuring radioactivity. When the concentration of [3H]-GSH was varied blanks were performed to account for an increase in the background. All experiments were performed at least in quadruplicate and the results expressed are averages with the error bars represented by their standard deviation.

3.28. Measurement of L-Arginine Uptake

The uptake of [3H]-L-arginine (0.02 - 0.04 μCi/trial) by intact platelets and PPMV was performed in the same manner as [3H]-GSH except that it was not necessary to preincubate the platelets with acivicin and DTT was omitted in the incubation mixture. When [3H]-L-arginine uptake was performed using PPMV the nitrocellulose membranes were presoaked in 100 mM L-arginine to minimize nonspecific [3H]-L-arginine binding.

3.29. Preparation of Oxyhemoglobin

Oxyhemoglobin was prepared by reducing human hemoglobin (30 mg dissolved in degassed, deionized, distilled H2O) with excess sodium dithionite (60 mg). Isolation of oxyhemoglobin was achieved by passage over a Sephadex G-25 column equilibrated and eluted with degassed PBS if the oxyhemoglobin was to be used in cell culture experiments.
or degassed, deionized, distilled H₂O if the oxyhemoglobin was intended for NO measurements. Oxyhemoglobin was either used fresh or stored frozen for up to 1 week.

3.30. Preparation of authentic NO solutions

Phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄) (PBS) was first deoxygenated by sonication under vacuum for 1 hour followed by purging with helium for 2 hours in a stirred septum-sealed flask. NO gas was passed through saturated KOH before being introduced into the deoxygenated PBS.

3.31. NO Measurement

NO solutions were measured by the oxyhemoglobin method (Doyle and Hoekstra, 1981) using an extinction coefficient of 83 441 L·cm⁻¹·mol⁻¹ at 405 nm (Muruganandam and Mutus, 1994). To 1 mL of a 10 μM oxyhemoglobin dissolved in deionized, distilled H₂O, 5 μL of a saturated NO solution (~1 mM) was added. The decrease in absorbance at 405 nm was used to quantitate NO.

3.32. Synthesis of S-Nitrosogluthathione (Hart, 1985)

To an ice cold GSH solution (1.5 g) in 8 mL of H₂O and 2.5 mL 2 M HCl 0.345 g of sodium nitrite was added. The mixture was stirred at 4°C for 40 minutes, treated with 10 mL of acetone and stirred for an additional 10 minutes. The pink precipitated product was obtained by vacuum filtration and washed successively with ice cold H₂O (5 x 1 mL) acetone (3 x 10 mL) and diethyl ether (3 x 10 mL). The product (GSNO) was dried by
placement in an evacuated dessicator with P₂O₅ as the desiccant. The reaction was performed in the dark. GSNO was quantified according to its absorbance at 545 nm (ε = 15.9 M⁻¹cm⁻¹).

3.33. S-Nitrosoglutathione Photolysis and NO Detection

Rates of NO evolution were directly measured in a sample chamber attached to a nitrogen gas flow-through system (Drury et al., 1992) equipped with an on-line chemiluminescence NOₓ analyzer in the presence and absence of light from various sources. The sample chamber, which was capable of purging aqueous solutions with nitrogen, was thermostated with a water jacket and contained two glass flat windows. The sample volume in the sample chamber was 25 mL. Light for the photolysis experiments was from overhead fluorescent room lights, a 300 W quartz halogen lamp, or a 1 mW He-Ne 543.5 nm laser. The light source was positioned 25 cm away from the glass window of the sample chamber. The NO evolution rates, f_NO, was calculated using the equation f_NO = f[NO], where f is the total flow rate (mL·min⁻¹) through the reactor. The concentration dependence on the rate of NO production by GSNO photolysis was determined using 543.5 nm laser light (1 mW).

3.34. Measurement of Quantum Yield of S-Nitrosoglutathione Photolysis

The quantum yield of S-nitrosoglutathione photolysis was measured by potassium ferrioxalate actinometry (Calvert and Pitts, 1966). An aliquot (25 mL) of a 0.15 M potassium ferrioxalate (K₃Fe(C₂O₄)₃) solution was placed in the sample chamber described
above (Section 3.33) and exposed to light from a 1 mW 543.5 nm laser for 6.5 hours, after which time a significant amount of K₃Fe(C₂O₄)₃ had been photolyzed. Two 10 mL aliquots were withdrawn and added to 2 mL of aqueous 0.1 % phenanthroline and 5 mL of a buffer containing 0.6 M NaO₂C₂H₃ and 0.36 M H₂SO₄. The mixture was made up to 25 mL with water and allowed to stand in the dark for 1 hour, after which the absorbance was measured at 510 nm. A standard was prepared using FeSO₄ in 0.1 M H₂SO₄ that had been standardized with potassium permanganate (KMnO₄). The concentration of the potassium permanganate solution was determined accurately using the primary standard sodium oxalate (Na₂C₂O₄). The number of Fe²⁺ ions liberated during photolysis (n_{Fe²⁺}) was then calculated using the following equation:

\[ n_{Fe^{2+}} = \frac{6.023 \times 10^{20} V_3 \log(I_0/I)}{V_2 l \varepsilon} = 7.3 \times 10^{17} \text{ ions} \]

where \( V_1 \) = sample volume irradiated (25 mL); \( V_2 \) = volume of aliquot (10 mL); \( V_3 \) = volume to which aliquot diluted (25 mL); \( \log(I_0/I) \) = absorbance at 510 nm; \( l \) = cuvette pathlength; and \( \varepsilon \) = extinction coefficient (slope of standard curve) which was calculated as 8505.5 M⁻¹ cm⁻¹.

The laser light intensity (I₀) was then calculated:

\[ I_0 = \frac{n_{Fe^{2+}}}{\Phi B (1 - 10^{-\varepsilon l \gamma})} = 1.1 \times 10^{15} \text{ quanta/s} \]

where \( \Phi B \) = quantum yield of potassium ferrioxalate solution at 546 nm (0.15, Calvert and Pitts, 1966); \( t \) = irradiation time (6.5 h or 23 400 s); \( \varepsilon \) = extinction coefficient of potassium ferrioxalate solution at 543.5 nm (0.13 M⁻¹ cm⁻¹); \( [A] \) = concentration of potassium ferrioxalate solution (0.15 M); \( l \) = length of cell (5 cm).
The quantum yield of GSNO photolysis ($\Phi_{NO}$) could then be obtained

$$\Phi_{NO} = \frac{\text{(molecules of NO released)}}{\text{(quanta absorbed by GSNO)}}$$

3.35. Nitrite Measurement

Nitrite was measured according to James and Glaven (1989) using the Greiss reaction with equal volumes of 1 % sulphanilamide in 2.5 % phosphoric acid and 0.1 % naphthylethylenediamine dihydrochloride in 2.5 % phosphoric acid. Nitrite was determined in GSNO solutions composed of RPMI 1640 media in the presence and absence of light from a 300 W quartz halogen lamp that was filtered by passage through a 3 cm solution of copper sulfate (100 g/L) and by the employment of a UV filter that eliminated wavelengths below 335 nm. The data presented here was obtained using a microplate reader at 540 nm. Nitrite concentrations were determined from a standard curve using sodium nitrite. Each measurement is the average of 4 to 7 readings with the error between measurements being expressed by their standard deviation.

3.36. Cell Culture and Cell Survival Assays

HL-60 cells were grown in suspension in RPMI 1640 medium supplemented with 20 % fetal bovine serum in a CO$_2$ regulated incubator at 37°C. Cells were transferred to two twelve well plates at a final concentration of 400 000/mL per well and treated with 25 µL of a GSNO solution so that the final concentration of GSNO in 1 mL final volume was either 0.1, 0.5, or 1.0 mM. The control received 25 µL of a 140 mM NaCl solution which was used to dissolve the GSNO. One plate was incubated in the dark while the other was
subjected to light from the same lamp used in the measurement of nitrite. The twelve well plate was positioned 36 cm from the filtered light source inside a laminar flow hood and exposed to the light for 2 hours. After 2 hours half of the cells were washed with PBS and resuspended in GSNO-free RPMI 1640 medium in new twelve well plates at a concentration of 200 000 cells/mL while the remaining cells were diluted to 200 000 cells/mL and maintained in the original twelve well plates with final GSNO concentrations equal to 0.05, 0.25 and 0.5 mM. At the specified time cells were mixed 1:1 with 0.4 % trypan blue and counted with a hemocytometer.

3.37. Data Analysis

The analysis of enzyme kinetic and membrane transport data was typically performed using the \( v \) versus \([S]\) form of the data together with a curve fit computer program (SigmaPlot). The enzyme kinetic parameters, \( K_M \) and \( V_{max} \), were obtained from a fit of the obtained \( v \) versus \([S]\) data to the Michaelis-Menten-Henri equation:

\[
\frac{v}{[S]} = \frac{V_{max}}{K_M + [S]} \quad \text{Eqn. 1}
\]

To obtain the \( K_M \) and \( V_{max} \) of a membrane transport process that contains an element of nonspecific diffusion the Michaelis-Menten-Henri equation was used together with a diffusion term:

\[
\frac{v}{[S]} = \frac{V_{max}}{K_M + [S]} + P \cdot s \quad \text{Eqn. 2}
\]

Where \( S \) is the extracellular substrate concentration, \( P \) is the rate of nonspecific diffusion and \( s \) is the extracellular substrate in moles.
To extract the IC\textsubscript{50} or LD\textsubscript{50} value from a set of data in the presence of increasing inhibitor or toxic agent the following equation describing simple saturation was utilized:

\[
\% \text{activity} = 100 - \frac{100[I]}{IC_{50} + [I]}
\]

Eqn. 3

where \% activity is calculated from the observed activity in the absence of inhibitor or toxic agent (I) and the IC\textsubscript{50} term could be replaced by LD\textsubscript{50}.

The following equations were utilized to describe the different types of enzyme inhibition encountered in the following studies (Segal, 1975):

\textit{competitive inhibition}

\[
\nu = \frac{V_{\text{max}}[S]}{K_M \left(1 + \frac{[I]}{K_I}\right) + [S]}
\]

Eqn. 4

\textit{uncompetitive inhibition}

\[
\nu = \frac{V_{\text{max}}[S]}{K_M + [S] \left(1 + \frac{[I]}{K_I}\right)}
\]

Eqn. 5

\textit{noncompetitive inhibition}

\[
\nu = \frac{V_{\text{max}}[S]}{K_M \left(1 + \frac{[I]}{K_I}\right) + [S] \left(1 + \frac{[I]}{K_I}\right)}
\]

Eqn. 6
\textit{hyperbolic mixed type inhibition}

\begin{equation}
    v = \frac{V_{\text{max}}[S]}{\alpha K_M \left( \frac{[I] + K_i}{\beta[I] + K_i} \right) + [S] \left( \frac{[I] + \alpha K_i}{\beta[I] + \alpha K_i} \right)}
\end{equation}

where $0 < \alpha < 1$, $0 < \beta < 1$. 

Eqn. 7
Chapter 4

RESULTS

4.1. Effect of GSH-Depleting Agents on Platelet Aggregation

Initially, we were interested in confirming whether or not GSH had a role in platelet function. We used the established GSH-depleting reagent 1-chloro-2,4-dinitrobenzene (CDNB) and a novel thiol reagent (CDDP) to investigate the effect of depleted GSH on platelet aggregation.

It was found that a 20 minute preincubation with 1 mM CDNB potently inhibited thrombin-induced platelet aggregation while a 20 minute preincubation with 0.1 mM CDNB accelerated the rate of aggregation (Fig. 6). ADP-induced platelet aggregation was also inhibited by preincubations with 1 mM CDNB or with the novel thiol reagent CDDP (1 mM) (Fig. 7). CDNB essentially inactivates platelet aggregation when preincubated for 60 minutes (Fig. 7A). CDDP did not affect the extent of aggregation as much as it inhibited the initial rate of aggregation (Fig. 7B). The greater inhibition of platelet aggregation by CDNB is likely not attributable to the reduction of [GSH]_p alone since both thiol reagents were found to deplete [GSH]_p by approximately the same extent (Fig. 8).

The ability of the compounds to inhibit the thiol sensitive enzyme, glutathione reductase (GR), was utilized as a potential index of their degrees of protein thiol reactivity. As shown in Fig. 9 CDNB inhibits bovine intestinal mucosa (BIM) GR to a much greater extent than does CDDP. At 100 μM CDNB inhibited GR in an apparent uncompetitive manner with a calculated K_i of 24.59 ± 0.03 μM. The observed GR inhibition pattern due to 1 mM CDNB resembles noncompetitive inhibition, with respect
Fig. 6. Effect of a CDNB preincubation on thrombin-induced platelet aggregation. Platelet aggregation was performed as described in the Methods Section 3.2. The platelets were preincubated in a volume of 100 μL with either 0.1 mM or 1 mM CDNB for 20 minutes in suspension buffer (137 mM NaCl, 2.7 mM KCl, 3 mM NaH₂PO₄, 1 mM MgCl₂, 5.5 mM glucose, 0.35 % albumin, 3.5 mM HEPES, pH 7.4) that did not contain CaCl₂ at room temperature. Ethanol was used to dissolve CDNB. In 100 μL the platelets were exposed to 5 % ethanol. The platelets were then diluted to 1.2 mL into a stirred cuvette with suspension buffer that contained 2 mM CaCl₂. Aggregation was initiated by the addition of 10 μL of a thrombin solution so that the final thrombin concentration was 0.5 U/mL. Data shown here is average of 2-3 separate trials.
Fig. 7. Effect of CDNB and CDDP on ADP-induced platelet aggregation. Platelet aggregation was performed as described in the Methods Section 3.2. The platelets were preincubated in a volume of 100 μL with either 1 mM CDNB (Panel A) or 1 mM CDDP (Panel B) for the indicated times in suspension buffer (137 mM NaCl, 2.7 mM KCl, 3 mM NaH₂PO₄, 1 mM MgCl₂, 5.5 mM glucose, 0.35 % albumin, 3.5 mM HEPES, pH 7.4) that contained 0.1 U/mL apyrase but no CaCl₂ at room temperature. CDNB was dissolved in ethanol and CDDP in methanol. In 100 μL the platelets were exposed to 5 % alcohol. The platelets were then diluted to 1.2 mL into a stirred cuvette with suspension buffer that contained 2 mM CaCl₂ and 50 μg/mL fibrinogen but no apyrase. Aggregation was initiated by the addition of 5 μL of a ADP solution so that the final ADP concentration was 8 μM. Data shown here is average of 2-3 separate trials.
Fig. 8. Reduction of intra-platelet GSH by CDNB or CDDP. Platelets were incubated with 1 mM CDNB (filled circles) or CDDP (filled triangles) in 100 µL of incubation buffer (137 mM NaCl, 3.7 mM KCl, 10 mM HEPES, 5.5 mM glucose at pH 7.4) for various times. CDNB was dissolved in ethanol and CDDP in methanol and the final alcohol concentration to which the platelets were exposed was 5%. At the indicated time platelets were pelleted and washed once in a desk top microcentrifuge. The platelet pellet was then resuspended in 500 µL of 25 mM Tris, 1 mM EDTA, pH 8.0 buffer before homogenization, monobromobimane derivatization and HPLC analysis as described in Section 3.5. Values shown are the averages of three separate experiments and the error bars represent their standard deviation.
Fig. 9. Effects of CDNB and CDDP on bovine intestinal mucosa GR activity. Double reciprocal plots were constructed to analyze the inhibition of GR by CDNB and CDDP. CDNB was dissolved in ethanol and CDDP in methanol and the final alcohol concentration to which the enzyme was exposed was 1%. **Panel A:** GR activity was measured as described in Section 3.21 with varied GSSG in the absence of inhibitor (circles) or the presence of 100 µM CDNB (triangles) or 1 mM CDNB (squares). NADPH was added last. **Panel B:** GR activity was measured in the absence of inhibitor (diamonds) or the presence of 40 µM CDDP (inverted triangles). Higher concentrations of CDDP was not tested due to the high extinction coefficient of CDDP. The enzyme was present at a concentration of 0.6 µg/mL. The observed initial rates were corrected for absorbance changes due to GSH conjugation to the inhibitors. Values shown are the averages of three separate experiments and the error bars represent their standard deviation.
to the enzyme already inhibited by 100 μM CDNB (Fig. 9A). At 50 μM CDDP only slightly inhibited the enzyme in an apparent noncompetitive manner (Fig. 9B). The effect of CDDP on GR was observed only at 40 μM due to the high extinction coefficient of CDDP (21 000 L·mol⁻¹·cm⁻¹ at 307 nm). At concentrations greater than 50 μM the decrease in absorbance at 340 nm (NADPH oxidation) is difficult to observe. The data obtained in Fig. 9 was adjusted for the rate of conjugation to GSH produced by GR from GSSG.

4.2. The Inhibition of Glutathione S-Transferase by Certain Mannich Bases of α,β-Unsaturated Ketones

The effect of CDDP and some related compounds (compounds 1, 2, and 3) on certain enzymes involved in GSH metabolism were tested. Compounds 1, 2 and 3 are all 3,5-bis-benzylidene-4-piperidones that vary only in the degree of N-piperdone substitution (structures: Section 1.3.4.3). It was subsequently found that glutathione reductase was slightly inhibited by all 4 compounds. The degree and type of GR inhibition exhibited by compounds 1, 2, and 3 was approximately the same as that previously shown for CDDP (Fig. 9) (Data not shown for compounds 1, 2, and 3). The enzyme γ-glutamyltranspeptidase (γ-GT) was not inhibited by either compound (data not shown). Due to limitations in the glutathione peroxidase (GPX) enzyme assay system, the effect of the compounds on GPX activity could not be tested.

Glutathione S-transferase (GST), however, was found to be inhibited by compounds 1, 2, and 3 but not by CDDP (Fig. 10). The observed IC₅₀ values for compounds 1, 2, and 3 were 36, 34, and 6.0 μM, respectively, and were obtained using
Fig. 10. The inhibition of GST by Mannich bases of α,β-unsaturated ketones. GST activity was determined in the presence of different inhibitor concentrations using the fluorescent monobromobimane assay as described in Section 3.22. Monobromobimane was added last. The inhibitors were dissolved in methanol and the final alcohol concentration to which the enzyme was exposed was 1%. The assays contained 1.8 μg/mL GST and the obtained values were corrected for inner filter effects from the inhibitor compounds. Values shown are the averages of three separate experiments and the error bars represent their standard deviation.
equine liver GST that had not been resolved into distinct classes. Compound 3, which had
the lowest IC$_{50}$, inhibited the enzyme to a maximum of approximately 44.6 ± 1.9 % of
control at 100 μM, the highest concentration of compound tested. In contrast,
compounds 1 and 2 at 100 μM inhibited the enzyme to 26.5 ± 12.6 and 25.3 ± 21.4 % of
control, respectively. From inspection of the IC$_{50}$ graphs (Fig. 10), it is evident that
compounds 1 and 2, unlike compound 3, are likely to completely inactivate the enzyme at
concentrations greater than 100 μM.

The inhibition of GST by compounds 1, 2, and 3 was further examined with the
use of double reciprocal plots (Fig. 11). Against the observed rates of GST-catalyzed
GSH conjugation to monobromobimane (mBB) an apparent noncompetitive inhibition
pattern is evident (Fig. 11). The K$_i$ values for compounds 1, 2, and 3 were 53.4 ± 2.6
μM, 7.5 ± 1.0 μM, and 22.6 ± 1.3 μM, respectively, as obtained from a fit of the v versus
[mBB] data to the equation describing noncompetitive (eqn. 6 in Section 3.37). It is
evident that the IC$_{50}$ values obtained in this instance are misleading since the compound
with the lowest value (compound 3) was not found to be the best inhibitor.

4.3. Glutathione Conjugation to CDDP is Selectively Catalyzed by Alpha Class
Glutathione S-Transferase

The conjugation of glutathione to various electrophiles occurs either
nonenzymatically or both nonenzymatically and enzymatically through catalysis by GST.
The isozymes of GST are selective towards different substrates as described in Section
1.3.4.1. The discovery of additional substrates that are specifically catalyzed by certain
isozymes or classes of GST is useful to the accurate identification of the enzyme class(es)
Fig. 11. Double reciprocal plots showing Equine Liver GST inhibition by 3,5-bisbenzylidene piperidones. The inhibition of GST by 50 μM of compound 1 (diamonds), compound 2 (triangles), and compound 3 (squares) was compared to the control (circles) in the presence of varied monobromobimane (mBB) concentrations using double reciprocal plots. GST activity was determined in the presence of different inhibitor concentrations using the fluorescent monobromobimane assay as described in Section 3.22. Monobromobimane was added last. The inhibitors were dissolved in methanol and the final alcohol concentration to which the enzyme was exposed was 1%. The control contained 1% methanol. The assays contained 1.8 μg/mL GST and the obtained values were corrected for inner filter effects from the inhibitor compounds. Values shown are the averages of three separate experiments.
present in a particular tissue.

As shown in Fig. 12A, the formation of the conjugate between CDDP and GSH (the DDP-GSH conjugate) is catalyzed by GST from equine liver not resolved into classes. The glutathione conjugation to a related compound, NC 1109 (structure: Section 1.3.4.2.), was also catalyzed by a mixture of GST isozymes. GST did not catalyze the glutathione conjugation to either of the 3,5-bis-benzylidene-4-piperdones (compounds 1, 2, and 3; data not shown).

To determine if CDDP was specific towards a single enzyme class, the equine liver GST mixture was separated by CM-Sepharose CL-6B at pH 6.0 as shown in the elution profile (Fig. 13). The enzyme fraction that bound to CM-Sepharose (fraction B) displayed high activity toward CDDP whereas the fraction that did not bind displayed low activity toward CDDP (Fig. 13). Confirmation that fraction B was the basic isoform, GST-α (pI 7.8-8.8), was obtained from immunoblot analysis with GST class specific antibodies (Fig. 14) and the use of GST class selective substrates (Table 2). Immunoblot analysis demonstrates that fraction B is composed entirely of alpha class GST (Fig. 14). Only the antibody specific for alpha class GST recognized the protein in fraction B.

Fraction B displayed relatively high activity towards cumene hydroperoxide, a substrate known to have highest activity toward the alpha isoform (Mannervik and Danielson, 1988). Fraction A is likely to be composed of mainly mu class GST since relatively high activity was observed towards the mu class selective substrate trans-4-phenyl-3-buten-2-one (TPBO). In addition, the pi isoform has been found to be either absent or present only in very low amounts in liver (Hayes et al., 1991; Di Ilio and
Fig. 12. Equine liver GST catalyzed conjugation of CDDP and NC 1109 to GSH. Panel A: The absorbance decrease of CDDP is shown in the absence (circles) or presence (squares) of 5 μg/mL GST. Panel B: The absorbance decrease of NC 1109 is shown in the absence (circles) or presence (squares) of 3 μg/mL GST. The assay procedure was conducted as described for CDDP GST activity measurement in Section 3.22.
Fig. 13. Separation of alpha and mu class equine liver GST by CM-Sepharose chromatography. Purified equine liver GST (2.5 mg) was applied to a CM-Sepharose column (2 x 5 cm) equilibrated with 10 mM sodium phosphate buffer, 1 mM EDTA, pH 6.0 buffer. The neutral protein fraction A was eluted within the first 10 tubes (1.5 mL/tube). The basic protein fraction B was then eluted with a linear NaCl gradient (0-0.6 M NaCl in 30 min; flow rate = 1.0 mL/min). Protein was monitored using absorbance at 280 nm (circles) and enzyme activity was detected using 10 μM CDDP and 100 μM GSH (bars).
Table 2. GST specific activities towards class selective substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Equine liver GST (Fraction A: mu class) (μmol/min/mg)</th>
<th>Equine liver GST (Fraction B: alpha class) (μmol/min/mg)</th>
<th>Placenta GST (pi class) (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB (nonspecific)</td>
<td>3.67 ± 0.31</td>
<td>31.3 ± 1.8</td>
<td>2.02 ± 0.26</td>
</tr>
<tr>
<td>Ethacrynic Acid (pi-specific)</td>
<td>0.676 ± 0.056</td>
<td>0.134 ± 0.042</td>
<td>15.6 ± 1.2</td>
</tr>
<tr>
<td>Cumene</td>
<td>0.597 ± 0.060</td>
<td>3.96 ± 0.32</td>
<td>0.047 ± 0.006</td>
</tr>
<tr>
<td>Hydroperoxide (alpha-specific)</td>
<td>0.531 ± 0.063</td>
<td>0.063 ± 0.008</td>
<td>0.098 ± 0.012</td>
</tr>
<tr>
<td>TPBO (mu-specific)</td>
<td>nil</td>
<td>8.10 ± 0.13</td>
<td>0.511 ± 0.022</td>
</tr>
<tr>
<td>CDDP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TPBO: *trans*-4-phenyl-3-buten-2-one
Fig. 14. Immunoblot analysis of equine liver GST fraction B. Panel A: Ponceau S stain on equine liver GST fraction B electrophoretically transferred from 15% polyacrylamide gels (2 µg protein/lane) to nitrocellulose. Panel B: Immunoblots of equine liver GST fraction B produced by removal of the Ponceau S stain and cutting the nitrocellulose sheet into strips. Strips 1-3 were incubated with anti-alpha GST, anti-mu GST, and anti-pi GST, respectively. GST was visualized using an alkaline phosphatase conjugated secondary antibody detection system. The following molecular mass standards were used: lysozyme (18.5 kDa), soybean trypsin inhibitor (27.5 kDa), carbonic anhydrase (32.5 kDa), and ovalbumin (49.5 kDa).
Federicic, 1990; Gupta et al., 1990; Jensson et al., 1985; Tu et al., 1983). Activity measurements with GST from placenta were performed because it is known to express only the pi isoform (Dao et al., 1982, 1984; Mannervik and Guthenberg, 1981; Polidoro et al., 1981) as demonstrated here by its relatively high activity towards the pi class selective substrate ethacrynic acid (Mannervik and Danielson, 1988).

Using the pi (placenta), mu (fraction A), and alpha (fraction B) isoforms it was shown that the conjugation of GSH to CDDP is preferentially catalyzed by alpha class GST (Table 2). A study of the selectivity of CDDP towards the three major classes of GST revealed that this reagent displays 15.9 times more activity towards the horse liver alpha isoform than the pi isoform from human placenta and that the horse liver mu isoform of GST did not display any detectable activity (Table 2). Specific activities obtained were ~0 (unable to detect) for the mu class GST, 0.51 ± 0.02 μmol/min/mg protein for the pi class GST, and 8.1 ± 0.13 μmol/min/mg protein for the alpha class GST. The specific activity of alpha class GST towards CDDP was approximately two-fold larger than that of cumene hydroperoxide (3.96± 0.32 μmol/min/mg protein), the common alpha class selective substrate.

The double reciprocal plots shown in Fig. 15 demonstrate a similarity in the $K_m$ between the purified equine liver alpha class GST and the crude rat liver GST. The $K_m$ and $V_{max}$ of CDDP for the equine liver GST-α class were determined to be 225.7 μM and 14.6 μmol/min/mg protein, respectively. The $K_m$ and $V_{max}$ of CDDP for the crude rat liver GST were determined to be 259.9 μM and 0.082 μmol/min/mg, respectively.
Fig. 15. Double reciprocal plots of CDDP concentration versus enzymatic rate. A) Equine liver alpha class GST. B) Crude Rat liver GST. The concentration of CDDP was varied from 50 to 300 µM in the presence of 1 mM GSH. The measurement of GST activity at each concentration was performed as described in Section 3.22. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
The human platelet exhibits no detectable GST activity towards the conjugation of CDDP to GSH which is in agreement with the finding the human platelet contains only the pi isoform (Rogerson et al., 1984; Federici et al., 1985; Loscalzo and Freedman, 1986) (data not shown).

4.4. Platelet Glutathione Transport

Previous studies on the role of \([\text{GSH}]_p\) in platelet function has been determined using thiol reagents to deplete \([\text{GSH}]_p\). As the above studies indicate (Section 4.2 and 4.3) many thiol reagents are likely to have effects other than the reduction of \([\text{GSH}]_p\). The effect of elevated \([\text{GSH}]_p\) on platelet function has not yet been determined despite the fact that it avoids the complications of using thiol reagents. In this study platelets were investigated for their ability to influx extracellular GSH so that we could observe the effects of elevated \([\text{GSH}]_p\) on platelet aggregation. Several cells, other than platelets, have been shown to possess an intact GSH transport system (described in Section 1.3.5.2).

Glutathione transport was observed in intact platelets and in platelet plasma membrane vesicles (PPMV). PPMV were prepared by loading the intact platelets with glycerol and then hypotonically lysing the platelets in glycerol-free buffer (Barber and Jamieson, 1970; Harmon et al., 1992). The vesicles prepared in this manner have been determined to be primarily right-side out (Barber and Jamieson, 1970). The integrity of the vesicle prepared was determined by visual inspection of electron micrographs (Fig. 16).
Fig. 16. Electron micrograph of platelet plasma membrane vesicles (PPMV). The integrity of the PPMV preparation was assessed by electron microscopy as described in Section 3.4. The final magnification was 77,000 x.
Since this is the first report of GSH uptake by platelets, various aspects of the mechanism of platelet GSH transport were characterized. Before initiating this characterization, however, it was necessary to determine whether or not the observed apparent GSH uptake was due to actual transport or to a binding to the membrane. If uptake is being observed it was also necessary to determine if GSH was being taken up intact or in a hydrolyzed form such as cysteinylglycine, the product of γ-glutamyl transpeptidase (γ-GT). Acivicin is a known irreversible inhibitor of γ-GT (Reed et al., 1980) and a concentration of 0.5 mM has been shown here to completely inactivate the enzyme after 60 minutes (Fig. 17A). The uptake of GSH by acivicin-treated platelets was not significantly altered from that observed in platelets not treated with acivicin (data not shown). The incubation of platelets with 0.5 mM [3H]-GSH was shown by a HPLC analysis of the cytosol to result in an increase in one radioactive peak which corresponded to GSH (Fig 17B). Disruption of the platelets by sonication following incubation with 160 μM [3H]-GSH and separation of the membrane and cytosolic fractions by centrifugation revealed that essentially all of the radioactivity was from the cytosol (Fig. 17C). The percentage of transporter substrate associated with the membrane may be estimated by an osmotic shrinking technique (Muré and Kinne, 1980; Sachs et al., 1980). GSH uptake in PPMV decreased with increasing sucrose concentration which is a method known to decrease intra-cellular volume (Fig. 17D). The amount of GSH that does bind to the platelet membrane is estimated by the y-intercept of Fig. 17D (i.e. 28 %).

In Fig. 18 the uptake of 250 μM GSH by intact platelets over time is shown to be Na⁺-independent. When potassium chloride was substituted for sodium chloride in the
Fig. 17. Evidence for platelet uptake of intact GSH. Panel A: Platelets were incubated for different times with 0.5 mM acivicin in GSH uptake incubation buffer at room temperature. At the indicated time platelets were homogenized and \( \gamma \)-glutamyl transpeptidase (\( \gamma \)-GT) activity was measured on the 5000 x g supernatant as described in Section 3.23. Panel B: The uptake of 500 \( \mu \)M GSH by intact acivicin-treated platelets was observed by monobromobimane derivatization of homogenized platelets followed by reverse-phase HPLC with fluorescent detection as described in Section 3.5. The data shown corresponds to the increase in the GSH elution peak which was the sole radioactive peak. The results are expressed as pmol GSH per mg total platelet protein. Panel C: The location of the radioactivity following uptake of 160 \( \mu \)M GSH by intact acivicin-treated platelets was observed by sonication of the platelets and separation of the platelet cytosol from the membrane fraction by centrifugation (16 000 x g for 30 min). Panel D: The effect of increasing sucrose concentrations on GSH uptake by platelet plasma membrane vesicles was observed. The intra-vesicular choline chloride concentration (137 mM) was divided by the sucrose concentration which was varied from 180 - 600 mM. Data shown here is average of 3 separate trials and the error bars represent their standard deviation.
Fig. 18. Na⁺-independence of platelet GSH uptake. The uptake of 250 μM GSH by intact acivicin-treated platelets was observed in GSH uptake incubation buffer (circles) or in GSH uptake incubation buffer with KCl substituted for NaCl (squares). GSH uptake was performed as described in Section 3.27. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
incubation buffer the amount of GSH uptake was not affected. In addition, the finding that a 30 minute preincubation of platelets with 1 mM ouabain, an inhibitor of the Na\(^+\)/K\(^+\) pump, did not inhibit platelet GSH uptake (data not shown) provides further evidence that platelet GSH uptake is independent of sodium cotransport.

It is apparent from Fig. 19 that GSH uptake by PPMV is concentration dependent but does not exhibit a clear saturation point. Incomplete saturation of substrate uptake is a common phenomenon and has been previously observed for GSH in other cells (Vincenzini et al., 1992). At concentrations greater than about 50 μM, however, the increase in the rate of uptake is decreased and consequently the GSH transport system of interest can be considered saturated. The increase in the rate of GSH uptake at high concentrations is due either to another carrier that transports GSH albeit with a much lower affinity or to an element of nonspecific diffusion. Comparable \(K_M\) and \(V_{\text{max}}\) values were obtained using either a fit to the Michaelis-Menten-Henri equation or a Lineweaver Burke plot of the data with the nonspecific diffusion rate subtracted. The kinetic constants, \(K_M\) and \(V_{\text{max}}\), were determined from a fit to the Michaelis-Menten-Henri equation with a diffusion term (eqn. 2 in Section 3.37) (Hofer, 1981) to be 18.2 ± 3.6 μM and 178 ± 27 pmol/min/mg protein, respectively. The diffusion term was estimated to be 7.91 x 10\(^{-4}\) min\(^{-1}\)(mg protein\(^{-1}\)).

The effect of potential inhibitors of PPMV GSH uptake is shown in Fig. 20. In general, only those compounds which resembled GSH were found to be inhibitors of PPMV GSH uptake. In terms of percent inhibition, monoethyl GSH (48.2 ± 3.2 %) was the most potent inhibitor tested followed by N-formyl GSH (41.4 ± 3.4 %), N-acetyl GSH
Fig. 19. Concentration dependence of platelet GSH uptake. The uptake of different concentrations of GSH by platelet plasma membrane vesicles was measured after a 30 second incubation at room temperature as described in Section 3.27. The values were adjusted for non-specific binding of the radioactive GSH to the nitrocellulose membranes. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
Fig. 20. Inhibition of platelet GSH uptake. The uptake of 25 μM GSH by platelet plasma membrane vesicles was measured at room temperature after 30 seconds in the presence or absence of 250 μM of a potential inhibitor as described in Section 3.27. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
(36 ± 4.3 %) and diester GSH (28.2 ± 5.0 %). The S-substituted GSH analogs S-dinitrophenyl glutathione and S-nitrosoglutathione were relatively poor inhibitors that all inhibited less than 20 %. Glycine, an amino acid component of GSH and the site of the radioactive label in [³H]-GSH, did not inhibit GSH uptake which suggests that GSH was not hydrolyzed to glycine. Methionine, an amino acid previously shown to inhibit GSH uptake in hepatocytes (Aw et al., 1986), also did not inhibit PPMV GSH uptake. The GSH amino acid component, cysteine and the GSH fragment, cysteinylglycine, both slightly inhibited GSH uptake by about the same extent (~20 %).

GSH uptake by intact platelets was found to be sensitive to membrane polarization (Fig. 21). Depolarization by preincubation with increasing concentrations of KCl (2.5 - 100 mM) resulted in an increase in GSH uptake. Uptake of GSH increases by approximately 66.5 ± 6.1 % following a preincubation with 50 mM KCl. Preloading cells with high extracellular concentrations of KCl is known to result in membrane depolarization (Bussolati et al., 1986). Incubation of cells with increasing concentrations of the membrane permeable thiocyanate anion from NaSCN results in hyperpolarization of cell membranes. NaSCN-induced hyperpolarization of the platelet membrane inhibited GSH uptake by about 29.5 ± 2.0 % at a concentration of 50 mM.

The uptake of GSH by intact platelets has also been found to be enhanced by a CDNB preincubation with the platelets (Fig. 22). When platelets were preincubated for 1 hour with varying CDNB concentrations GSH uptake was increased. The CDNB-mediated increase in GSH uptake was maximal at 80 µM where it was about 40 % greater than the control (Fig 22A). The kinetic parameters, $K_M$ and $V_{max}$, for platelet GSH uptake
Fig. 21. Effect of membrane potential on GSH uptake. The effect of a 1 hour preincubation at 37°C with different concentrations (10 - 100 mM) of KCl (circles) or NaSCN (squares) on the uptake of 100 μM GSH by intact acivicin-treated platelets after a 5 minute incubation was measured as described in Section 3.27. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
Fig. 22. Effect of a CDNB-mediated decrease in intra-platelet GSH on GSH uptake. **Panel A:** The uptake of 500 μM GSH by intact acivicin-treated platelets in the presence or absence of increasing concentrations of CDNB (20 - 160 μM) was measured as described in Section 3.27. Platelets were preincubated at room temperature with CDNB for 1 hour prior to adding radioactive GSH which was incubated for 2 minutes with platelets. **Panel B:** The concentration dependence of GSH uptake by intact acivicin-treated platelets in the presence (squares) or absence (circles) of a 20 minute preincubation with 100 μM CDNB was measured as described in Section 3.27. Platelets were preincubated at room temperature with CDNB for 1 hour prior to a 1 minute incubation with GSH. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
appear to be dependent on \([\text{GSH}]_i\) (Fig. 22B). In the absence of CDNB the apparent \(K_M\) and \(V_{\text{max}}\) were 137 \(\mu\text{M}\) and 42.2 pmol/min/10\(^9\) platelets, respectively. When \([\text{GSH}]_i\) was reduced by a preincubation with 100 \(\mu\text{M}\) CDNB the apparent \(K_M\) and \(V_{\text{max}}\) was found to be 31.7 \(\mu\text{M}\) and 31.3 nmol/min/10\(^9\) platelets, respectively.

When platelets were preincubated for 27 minutes with 1 mM GSH, the rate of ADP-induced aggregation was inhibited by about 50 % at the 300 second time point (Fig. 23A). The degree of inhibition reached a maximum after approximately a 20 minutes preincubation time with GSH (data not shown). The time-dependent GSH mediated inhibition of platelet aggregation is indicative of GSH uptake by the platelet. That the uptake of 1 mM GSH by intact platelets also saturated after about 20 minutes (Fig. 23B) strongly suggests that the observed inhibition of platelet aggregation by GSH corresponds to GSH uptake and not extra-platelet GSH. In the absence of a preincubation step, the inhibition of platelet aggregation by extra-platelet GSH can be observed to have an apparent IC\(_{50}\) value of 3.8 mM towards ADP-induced aggregation (Fig 23C). Low extra-platelet concentrations of GSH such as 1 mM exhibit minimal inhibition of platelet aggregation (< 16 %) and therefore cannot account for the time-dependent inhibition of GSH. Furthermore, when the effect of elevated \([\text{GSH}]_i\) on platelet aggregation was determined, platelets were preincubated with 1 mM in a final volume of 50 \(\mu\text{L}\) which was then diluted to 1 mL before measuring aggregation. A final GSH concentration of 50 \(\mu\text{M}\) is not expected to affect platelet aggregation.
Fig. 23. Effect of GSH uptake on platelet aggregation. Panel A: Platelet aggregation was measured using 8 μM ADP as described in the Section 3.2 with or without a preincubation with 1 mM GSH (7 or 26 min) at room temperature. Platelets were preincubated with 1 mM GSH in GSH uptake buffer in a volume of 50 μL. At the specified time the platelets were diluted with suspension buffer to a final volume of 1 mL. The concentration of GSH present during aggregation measurements was 50 μM. Data shown is representative of the result from a typical experiment. Panel B: The uptake of 1 mM GSH by intact platelets was measured as described in the Experimental Procedures section. Panel C: The effect of different concentrations of GSH (0.88 - 8.8 mM) on ADP-induced platelet aggregation was determined in the absence of a preincubation step. GSH was added just prior to adding ADP (8 μM). Results are expressed as a percentage of the extent of aggregation observed in the absence of GSH.
4.5. Inhibition of Glutathione Reductase by Reduced Glutathione

We have found that one potential \textit{in vivo} consequence of elevated intracellular [GSH] may be the inhibition of GR. In this study we describe the finding that GR is feedback inhibited by GSH. As is discussed in Section 1.3.3.3, this inhibition may effect the intracellular ratio of GSH:GSSG.

When the activity of glutathione reductases from human platelets, bovine intestinal mucosa, yeast and \textit{E. coli} was monitored as a function of GSH at constant GSSG (1 mM) and NADPH (100 \mu M) it was found that the reductases from all sources were inhibited by GSH but not to the same extent. The IC\textsubscript{50} values for the GRs from the various sources are summarized in Fig. 24. The lowest IC\textsubscript{50} (4.71 \pm 0.21 mM) was obtained with yeast GR while the \textit{E. coli} enzyme was the least sensitive to inhibition by GSH (IC\textsubscript{50} = 21.91 \pm 1.2 mM). The IC\textsubscript{50} values estimated for the bovine and human enzymes were 8.09 \pm 0.59 mM and 10.07 \pm 0.75 mM, respectively.

The manner in which GSH interacts with the various GRs was determined from the initial rates of GSSG reduction and NADPH oxidation in the presence and absence of a physiologically relevant concentration of GSH (5 mM; 10 mM in the case of \textit{E. coli}) (Fig. 25 and Fig. 26). With respect to the initial rate of NADPH oxidation the bovine, human and yeast GRs were inhibited in an apparent uncompetitive manner by GSH which is in agreement with the results of others (Chung \textit{et al.}, 1991; Worthington and Rosemeyer, 1976). In contrast, there was no discernible inhibition of \textit{E. coli} GR (Fig. 25, data only shown for \textit{E. coli} and bovine GR). The estimated K\textsubscript{i} values of GSH towards GR
Fig. 24. Estimated IC\textsubscript{50} values for GRs from various sources. Initial rates of GR catalyzed NADPH (100 \textmu M) oxidation (in the presence of 1 mM GSSG) monitored as a function of increasing GSH concentrations. The rate in the absence of GSH was defined as 100 \% activity. BIM, bovine intestinal mucosa. The IC\textsubscript{50} values were estimated from a fit of the data to a hyperbolic saturation function.
Fig. 25. Double reciprocal plots of GR-catalyzed NADPH oxidation in the presence and absence of GSH. Initial GR rates were monitored as a function of varied NADPH concentrations in the presence (triangles) and absence (circles) of GSH (5 mM with bovine intestinal mucosa, 10 mM with E. coli). The solid line represents the best fit of the data to the simple saturation equation or to the equation for uncompetitive inhibition. The unit of inverse velocity ($1/v$) is (mmol/min/mg)$^{-1}$. Panel A: Bovine intestinal mucosa GR (0.61 mg/mL) exhibited an apparent $K_M$ towards NADPH of 7.19 ± 0.57 μM and GSH inhibits with an apparent $K_i$ of 3.87 ± 0.36 mM. Panel B: E.coli GR (289 mg/mL) exhibited an apparent $K_M$ of 33.23 μM and the $K_i$ of GSH was not measurable.
Fig. 26. Double reciprocal plots of GR-catalyzed GSSG reduction in the presence and absence of GSH. Initial GR rates were monitored as a function of varied GSSG concentrations with 100 μM NADPH in the presence (triangles) and absence (circles) of 5 mM GSH. The solid line represents the best fit of the data to the simple saturation equation or to the appropriate inhibition equation (Section 3.37). Panel A: Crude human platelet GR (5.75 mg/mL) exhibited an apparent $K_M$ of $35.44 \pm 1.1$ μM towards GSSG and a GSH $K_i$ of $6.61 \pm 0.13$ mM. Panel B: Bovine intestinal mucosa GR (0.61 mg/mL) exhibited an apparent $K_M$ of $97.45 \pm 5.9$ μM towards GSSG and a GSH $K_i$ of $2.9 \pm 0.17$ mM ($\alpha = 0.45$, $\beta = 0.24$). Panel C: Yeast GR (1.39 mg/mL) exhibited an apparent $K_M$ of $68.7 \pm 5.4$ μM towards GSSG and a GSH of $K_i$ of $2.4 \pm 0.35$ mM ($\alpha = 0.24$, $\beta = 0.17$). Panel D: *E. coli* glutathione reductase (289 mg/mL) exhibited an apparent $K_M$ 56.70 ± 3.9 μM towards GSSG and a GSH $K_i$ of 12.11 ± 0.48 mM.
catalyzed NADPH oxidation for the bovine, human and yeast enzymes were 3.87 ± 0.36 mM, 12.92 ± 0.11 mM and 4.7 ± 1.8 mM respectively.

It was with the GR catalyzed reduction of GSSG that significant species dependent differences became evident (Fig. 26). GSH inhibited the human GR catalyzed reduction of GSSG in an apparent uncompetitive manner, with an estimated apparent Ki of 6.61 ± 0.13 mM (eqn. 5 in Section 3.37). In the case of the bovine and yeast enzymes the data for the inhibition of GSSG reduction was best accommodated by hyperbolic mixed inhibition (eqn. 7 in Section 3.37) (Ki = 2.92 ± 0.11 mM and 2.4 ± 0.35 mM, respectively). In contrast, the inhibition of the E. coli enzyme was best fitted by an equation describing competitive type inhibition (apparent Ki = 12.11 ± 0.48 mM) (Fig. 26) (eqn. 4 in Section 3.37). The competitive inhibition of the E. coli enzyme accounts for the high IC50 estimated for this enzyme since saturating concentrations of the substrates (GSSG, 1 mM; NADPH, 100 μM) were employed in the IC50 determinations (Fig. 24).

4.6. Platelet L-Arginine Transport

Recently, it has been established that NO is a potent inhibitor of platelet activation (Section 1.4.2). The finding that platelets contain an isoform of nitric oxide synthase (NOS) suggests that platelet-derived-NO may be important in the physiological regulation of platelet activation (Muruganandam and Mutus, 1994). L-Arginine is a precursor to the NOS-catalyzed formation of NO. The intracellular availability of L-arginine is therefore expected to influence the cellular production of NO. As shown in Fig. 27 L-arginine (1 mM) inhibits ADP-induced aggregation in an apparent time-dependent manner. It is
Fig. 27. Effect of L-arginine on ADP-induced platelet aggregation. Platelets were prepared as described in Section 3.2. The platelets were exposed to 1 mM L-arginine in suspension buffer (137 mM NaCl, 2.7 mM KCl, 3 mM NaH₂PO₄, 1 mM MgCl₂, 5.5 mM glucose, 0.35 % albumin, 3.5 mM HEPES, pH 7.4) that contained 2 mM CaCl₂ and 50 μg/mL fibrinogen but no apyrase. Aggregation was initiated by the addition of 5 μL of a ADP solution so that the final ADP concentration was 5 μM. The control was not exposed to L-arginine. Data shown here is average of 2-3 separate trials.
evident that the addition of L-arginine to the medium enhances the reversal of platelet aggregation without effecting the initial rate of aggregation.

As mentioned in Section 1.4.3.2 L-arginine transport has been characterized in several mammalian cells where system y\(^+\) is predominant. Despite the possible importance of platelet-derived-NO in the regulation of platelet activation the L-arginine transport system in platelets has not been characterized. In this study we initiated the characterization of the platelet L-arginine carrier using intact platelets and PPMV.

As shown in Fig. 28 the uptake of 100 \(\mu\text{M}\) L-arginine by intact platelets (Fig. 28A) and PPMV (Fig. 28B) approaches a maximum after approximately 30 minutes and occurs primarily in a \(\text{Na}^+\)-independent manner. The uptake of L-arginine in both intact platelets and PPMV is only slightly increased in the presence of 137 mM NaCl. The \(\text{Na}^+\)-independence of L-arginine uptake is a necessary characteristic for system y\(^+\) activity (White, 1985).

A portion of the observed uptake of L-arginine may be attributed merely to the binding of L-arginine to the membrane. The percentage of transporter substrate associated with the membrane may be estimated by an osmotic shrinking technique (Murer and Kinne, 1980; Sachs et al., 1980). L-Arginine uptake in PPMV decreased with increasing sucrose concentration which is a method known to decrease intra-cellular volume (Fig. 29). The intra-vesicular choline chloride concentration (140 mM) was divided by increasing sucrose concentrations to form the x-axis in Fig. 29. The amount of L-arginine that does bind to the platelet membrane corresponds to the y-intercept of Fig. 29 (i.e. 12.5 %).
Fig. 28. Na⁺-independence of L-arginine uptake. Uptake of 100 μM L-arginine in the presence (closed circles) and absence (closed triangles) of 137 mM NaCl by intact platelets (Panel A) at 37 °C and platelet plasma membrane vesicles at room temperature (96.5 μg) (Panel B). Choline chloride was iso-osmotically substituted for NaCl in its absence. Uptake was performed as described in Section 3.28. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
Fig. 29. Effect of osmolarity on L-arginine uptake. L-Arginine uptake (100 μM) was measured in platelet plasma membrane vesicles (57 μg) at room temperature in the presence of increasing sucrose concentrations (200 - 778 mM shown). The intra-vesicle choline concentration (140 mM) was divided by the concentration of sucrose to obtain the abscissa values. Uptake was performed as described in Section 3.28. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
It is apparent from Fig. 30 that L-arginine uptake in PPMV is concentration dependent but does not exhibit a clear saturation point. Incomplete saturation of amino acid uptake is a common phenomenon and has been previously observed for L-arginine in other cells (White, 1985). At concentrations greater than about 200 μM, however, the rate of uptake is decreased and consequently the L-arginine transport system of interest can be considered saturated. The increase in the rate of L-arginine uptake at high L-arginine concentrations is due either to another carrier that transports L-arginine albeit with a much lower affinity or to an element of nonspecific diffusion. Comparable $K_M$ and $V_{max}$ values were obtained using either a fit to the Michaelis-Menten-Henri equation or a Lineweaver Burke plot of the data with the nonspecific diffusion rate subtracted. The kinetic constants, $K_M$ and $V_{max}$, were determined from a fit to the Michaelis-Menten-Henri equation with a diffusion term (Hofer, 1981) (eqn. 2 in Section 3.37) to be $86.3 \pm 6.0$ μM and $21.9 \pm 9.7$ pmol/min/mg protein, respectively. The diffusion term was estimated to be $1.66 \times 10^{-3} \pm 0.28 \times 10^{-3}$ min$^{-1}$ (mg protein)$^{-1}$.

The presence of an L-arginine carrier of the system $y^+$ type in the platelet membrane was further demonstrated by the inhibition of L-arginine uptake in PPMV (Fig. 31A). L-arginine analogs and other cationic amino acids were found to inhibit L-arginine to different extents. [$^3$H]-L-arginine uptake is inhibited substantially by nonradioactive L-arginine ($86.9 \pm 11.9 \%$) but not by D-arginine. The L-arginine analogs homo-L-arginine and N$^\text{G}$-monomethyl-L-arginine, inhibited L-arginine uptake by $25.8 \pm 0.59$ and $36.9 \pm 0.1$ %, respectively. The two cationic amino acids lysine and ornithine inhibited L-arginine uptake by $34.3 \pm 0.33$ and $24.2 \pm 1.7$ %, respectively. Based on the relative degrees of
Fig. 30. Concentration dependence of L-arginine uptake. L-Arginine uptake values were determined at room temperature after a 30 s incubation with platelet plasma membrane vesicles (62 μg). The concentration of L-arginine was varied from 10 to 500 μM. Uptake was performed as described in Section 3.28. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
Fig. 31. Inhibition of L-arginine uptake. In *Panel A* L-arginine uptake values were determined at room temperature after 1 minute incubation of platelet plasma membrane vesicles (71 μg) with 50 μM L-arginine in the presence or absence of 5 mM inhibitor. The vesicles were added last to minimize any influence of trans-stimulation by the inhibitors. In *Panel B* the concentration of L-arginine was varied (20, 50 and 200 μM) in the absence (closed circles) or presence of 1 mM L-lysine (closed squares), L-ornithine (closed triangles) or N⁶-monomethyl-L-arginine (open diamonds). The L-arginine uptake values were determined in platelet plasma membrane vesicles (93 μg) after a 30 s incubation with L-arginine at room temperature. Uptake was performed as described in Section 3.28. *The difference between L-arginine uptake in the presence and absence of inhibitor was statistically significant (P < 0.05). Data shown here is average of 4 separate trials and the error bars represent their standard deviation.*
inhibition the platelet L-arginine transporter appears to possess a higher affinity for L-arginine substrate than lysine or ornithine. As expected histidine was not found to inhibit L-arginine uptake.

Citrulline, a product of the NOS catalyzed conversion of L-arginine to NO, was found not to be an inhibitor of L-arginine uptake in PPMV (Fig. 31A). The effect of citrulline on platelet L-arginine uptake was measured to determine if citrulline is transported by the L-arginine carrier. If citrulline and L-arginine do share the same carrier, the accuracy of L-arginine uptake by intact platelets may be compromised due to the NOS catalyzed conversion of [³H]-L-arginine to [³H]-citrulline.

The nature of L-arginine uptake inhibition in PPMV by N⁶-monomethyl-L-arginine, L-lysine and L-ornithine was investigated using a Lineweaver-Burke double reciprocal plot (Fig. 31B) from which a competitive inhibition pattern is evident. The apparent $K_i$ for each inhibitor, L-lysine, L-ornithine, and N⁶-monomethyl-L-arginine, as determined from a fit to the following equation describing competitive inhibition (eqn. 4 in Section 3.37) was 302, 338 and 395 μM, respectively.

When PPMV were preloaded with different substrates of the L-arginine carrier trans-stimulation of L-arginine uptake was observed (Fig. 32A). Trans-stimulation is indicative of a bi-directional carrier or anti-porter which has been reported to be the case with L-arginine transport in other cells (White, 1985). Trans-stimulation of L-arginine uptake was observed for L-arginine itself and those L-arginine analogs and amino acids that were found to inhibit L-arginine uptake in Fig. 31. Further evidence for the
Fig. 32. Evidence for bidirectionality of L-arginine transport. In Panel A the trans-stimulation of L-arginine uptake was observed when platelet plasma membrane vesicles (96 µg) were preloaded with 10 mM of amino acids or amino acid analogs for 60 minutes then washed once by centrifugation (16 000 x g for 30 minutes) before resuspension in incubation buffer and immediate measurement of 100 µM L-arginine. Uptake was performed as described in Section 3.28. In Panel B the efflux of L-arginine was measured from intact platelets preloaded with 100 µM L-arginine for 60 minutes. After 60 minutes the platelets were centrifuged (1750 x g for 2 minutes) and resuspended in L-arginine-free incubation buffer. Aliquots of the supernatant were assayed for radioactivity by scintillation counting. * The difference between L-arginine uptake in the presence and absence of inhibitor was statistically significant (P < 0.05). Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
bi-directionality of L-arginine transport is obtained by the efflux of L-arginine from intact platelets over time (Fig. 32B).

The uptake of L-arginine by PPMV increases slightly with increasing pH (Fig. 33). As the pH was varied from 9.0 to 5.0 L-arginine uptake decreased approximately 34 % (from 45.2 to 30.1 pmol L-arginine/min/mg protein).

L-arginine uptake by intact platelets was found to be sensitive to membrane polarization as depolarization by preincubation with increasing concentrations of KCl (2.5 - 140 mM) resulted in a decrease in L-arginine uptake (Fig. 34). Uptake of L-arginine decreases by approximately 14.3 ± 1.1 % on increasing extracellular KCl from 2.5 to 50 mM. Preloading cells with high extracellular concentrations of KCl are known to result in membrane depolarization (Bussolati et al., 1986). Incubation of cells with increasing concentrations of the membrane permeable thiocyanate anion from NaSCN results in hyperpolarization of cell membranes. NaSCN-induced hyperpolarization of the platelet membrane resulted in an increase in L-arginine uptake by about 16.5 ± 1.0 % of control. The uptake of L-arginine has been previously shown to be very sensitive to membrane potential and even provides a reliable indication of membrane potential in human fibroblasts (Bussolati et al., 1989).

Fig. 35 illustrates the results of an experiment designed to determine whether or not platelet L-arginine uptake is altered during the course of aggregation. Platelet L-arginine uptake was observed over time in the presence or absence of aggregating conditions (ADP and fibrinogen). As shown in Fig 35A there is a substantial increase in the amount of radioactively labelled L-arginine associated with the platelet at all points
Fig. 33. Dependence of L-arginine uptake on pH. L-Arginine (100 μM) uptake was measured in platelet plasma membrane vesicles (96 μg) for 2 minutes in different sodium phosphate buffers (20 mM) ranging in pH from 5.0 to 9.0. Uptake was performed as described in Section 3.28. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
Fig. 34. Dependence of L-arginine uptake on membrane potential. L-Arginine (100 μM) uptake was measured in intact platelets in the presence of increasing concentrations (2.5 - 140 mM) of either KCl (closed circles) or NaSCN (closed triangles). Intact platelets were preincubated with either different concentrations of KCl or NaSCN before incubation with 100 μM L-arginine for 2 minutes at 37 °C. The total salt concentration under each assay condition was maintained at 140 mM by the addition of varying amounts of NaCl. Uptake was performed as described in Section 3.28. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
Fig. 35. Effect of ADP-induced platelet aggregation on apparent L-arginine uptake. Platelets were prepared for ADP-induced aggregation as described in Section 3.2. Aggregation was initiated by the addition of 5 μL of a ADP solution so that the final ADP concentration was 5 μM in suspension buffer (137 mM NaCl, 2.7 mM KCl, 3 mM NaH₂PO₄, 1 mM MgCl₂, 5.5 mM glucose, 0.35 % albumin, 3.5 mM HEPES, pH 7.4) that 2 mM CaCl₂ and 50 μg/mL fibrinogen and no apyrase. Aggregation was observed in the absence (circles) or presence (squares) of 100 μM [³H]-L-arginine and [¹⁴C]-inulin. At the indicated time points after the initiation of aggregation the platelet suspension was centrifuged over a silicone oil mixture and the pellet treated as described in Section 3.28. Panel A: demonstrates the effect of ADP-induced platelet aggregation on apparent L-arginine uptake. The percent increase in apparent L-arginine uptake during aggregation is shown (triangles). Panel B: demonstrates the effect of platelet aggregation on the [¹⁴C]-inulin association with the platelet membrane. The percent increase in apparent [¹⁴C]-inulin membrane association during aggregation is shown (triangles). Data shown here is the average of 3 separate trials and the error bars represent their standard deviation.
during platelet aggregation. It is not known, however, if the increase in radioactivity (from [\textsuperscript{3}H]-L-arginine) during aggregation can be attributed to stimulated L-arginine uptake or to enhanced membrane binding.

Inulin is a large oligosaccharide that was used in the transport assays to estimate substrate trapping by the platelets. As shown in Fig. 35B the amount of radioactive inulin ([\textsuperscript{14}C]-inulin) associated with the isolated platelet pellet was dramatically increased during platelet aggregation. The increase in radioactivity from inulin is not indicative of a membrane transport event as the amount associated with the aggregating platelets did not increase during aggregation. The increase in inulin radioactivity must be due to increased binding to the membrane of the activated platelets. With both L-arginine and inulin the increase in radioactivity associated with the activated platelets was maximal at the earliest time point (30 seconds) and decayed thereafter (Fig. 35 A and B).

L-arginine uptake by both intact platelets and PPMV is sensitive to NO as shown in Fig. 36A. At low NO concentrations (0.068 - 1.7 \textmu M) there is an apparent increase in L-arginine uptake by intact platelets by 22.1 \pm 0.8 \% at 0.68 \textmu M. The effect of NO at low concentrations was abolished when stricter precautions were taken to deoxygenate the platelet incubation buffer. When the incubation buffer was sonicated under vacuum for 30 minutes followed by purging with helium for 2 hours the stimulatory effect of low NO concentrations on L-arginine uptake was not observed (Fig. 36A). In the presence of oxygen, NO is rapidly oxidized to NO\textsuperscript{2-} which is then slowly oxidized to NO\textsuperscript{3-} (Stamler et al., 1992). The uptake of L-arginine by PPMV was not stimulated by low NO concentrations when the buffer was not deoxygenated (Fig. 36A). At 68 \textmu M NO L-
**Fig. 36. Effect of NO, NO$_2^-$ and NO$_3^-$ on L-arginine uptake.** In *Panel A* the effect of increasing concentrations of NO (0.068 - 68 μM) on the uptake of 100 μM L-arginine after 5 minutes at 37 °C by intact platelets was determined in non-deoxygenated (closed circles) and in fully deoxygenated (closed triangles) incubation buffer (137 mM NaCl, 3.7 mM KCl, 10 mM HEPES, 5.5 mM glucose at pH 7.4). The effect of NO on the uptake of 100 μM L-arginine after 5 minutes by platelet plasma membrane vesicles (95 μg) was also determined (open inverted triangles). In *Panel B* the effect of different concentrations of NO$_2^-$ (closed squares) and NO$_3^-$ (closed diamonds) (0.5 μM to 10 mM) on the uptake of 100 μM L-arginine after 5 minutes by intact platelets was determined. Experiments from both panels were conducted by a 5 minute preincubation of NO, NO$_2^-$ or NO$_3^-$ before incubation with L-arginine. Uptake was performed as described in Section 3.28. Data shown here is average of 4 separate trials and the error bars represent their standard deviation.
arginine uptake by intact platelets and PPMV is inhibited to similar but not the same extents with percent inhibition values of $44.1 \pm 3.1 \%$ and $30.3 \pm 2.0 \%$, respectively. The effect of higher NO concentrations on L-arginine uptake could not be determined because of solubility limitations with NO stock solutions.

Since low NO concentrations stimulated L-arginine uptake only in the presence of dissolved oxygen higher oxidation states of NO such as $NO_2^-$ and $NO_3^-$ may be involved in this stimulation. To test this hypothesis, the effect of $NO_2^-$ and $NO_3^-$ on L-arginine uptake in intact platelets was determined (Fig. 36B). It is evident from Fig. 36B that the addition of low concentrations (0.5 - 5.0 μM) of $NO_2^-$ but not $NO_3^-$ resulted in a stimulation of L-arginine uptake. At the lowest concentration of $NO_2^-$ tested maximum stimulation was observed at $13.3 \pm 1.0 \%$. Low concentrations of $NO_2^-$ did not stimulate L-arginine uptake by PPMV (data not shown).

L-Arginine binding proteins were isolated from platelet plasma membranes using an L-arginine agarose affinity column. SDS PAGE analysis of the eluted protein revealed two major bands: one at approximately 35.5 kDa and one at approximately 140.5 kDa (Fig. 37).

4.7. Photolysis of S-Nitrosoglutathione: Potential Photochemotherapeutic Applications

Since NO forms an adduct with GSH that is present physiologically, we became interested in using S-nitrosoglutathione (GSNO) as a NO delivery system. We initiated our studies by examining the decomposition rates of GSNO to liberate NO. Very early in our investigation we made a surprising discovery.
Fig. 37. 8% SDS-PAGE of protein eluted from L-arginine agarose. Protein in lanes 2, 3 and 4 were not reduced with β-mercaptoethanol before loading while protein in lanes 5, 6 and 7 were pretreated with β-mercaptoethanol. Lanes 2 and 7 correspond to the total protein in platelet plasma membrane vesicles (15 µg). Protein in lanes 3 and 6 correspond to the protein eluted from the L-arginine agarose column (35 µg). The protein that bound to the L-arginine agarose column and was eluted by 100 mM L-arginine is shown in lanes 4 and 5 (10 µg). Lanes 1 and 8 show the molecular weight standards used (myosin 205 000 Da, β-galactosidase 116 500 Da, bovine serum albumin 80 000 Da, ovalbumin 49 500 Da, carbonic anhydrase 32 000 Da, soybean trypsin inhibitor 27 500 Da, lysozyme 18 500 Da, aprotinin 6 500 Da).
Unexpectedly, when GSNO decomposition was observed at room temperature and pH 7.4 with a gas flow-through system (Drury et al., 1992) in the presence of overhead fluorescent room lights, the rate of NO production was enhanced approximately 2 fold over the dark rate (Fig. 38). It has already been shown that irradiation of S-nitrosothiols with UV light (Hg-arc lamp) causes photolysis of the S-NO bond and release of NO (Stamler et al., 1992). Since room lights emit mainly visible light (>350 nm) we examined this phenomenon further. GSNO exhibits absorption maxima at 340 nm and 545 nm with extinction coefficients of 922 and 15.9 cm·mM⁻¹, respectively (Hart, 1985). Irradiation of GSNO at either of its absorption maxima results in the release of NO.

Figure 38 is a representative experiment that shows the enhanced rate of NO production over the dark rate for a 500 μM GSNO solution dissolved in 10 mM HEPES, 140 mM KCl at pH 7.4 and 25°C in the presence and absence of overhead fluorescent room lighting and a quartz halogen lamp. Each light source was exposed to the sample for approximately 3 minutes. When the sample is exposed to light an increase in NO production from GSNO decomposition is observed and when the light is turned off the rate of NO production returns to that of the dark rate. The observed photolysis was not due to photolysis of nitrite as a 34 mM solution of sodium nitrite did not generate any NO when exposed to light (data not shown).

Net NO evolution rates (light rate minus dark rate) and their dependence on GSNO concentration are presented in Fig. 39. The data shown in Fig. 39 was obtained using low pH and low temperature (pH 5.0 and 5°C) in order to minimize NO loss by non-photolytic means and thereby provide an increase in the observed rate of NO production.
Fig. 38. GSNO photolysis. Rates of NO evolution, $f_{NO}$, from a 500 µM GSNO solution dissolved in 10 mM HEPES, 140 mM KCl, at pH 7.4 and 25°C are shown in the presence and absence of overhead fluorescent room lights (a) and an unfiltered 300 W quartz halogen lamp (b). NO was measured in a thermostated sample chamber with a nitrogen gas flow-through system equipped with an on-line chemiluminescence NO$_x$ analyzer as described by Drury et al. (1992).
Fig. 39. Demonstration of the concentration dependence of the net rates of GSNO photolysis. The net rate, $f_{NO}$, is the difference between the rate observed in the presence of 543.5 nm laser light and the rate observed in the dark. (Inset: plot of $f_{NO}$ vs mol of GSNO yields a slope equal to $k_{obs}$ in units of min$^{-1}$.) Measurements shown are the averages of at least duplicate trials and the error represents their standard deviation. These studies were performed in 10 mM piperazine, 140 mM KCl and 0.5 mM EDTA at pH 5.0 and 5°C.
by photolysis with 543.5 nm laser light (1 mW). The laser light intensity was measured by potassium ferrioxalate actinometry to be $1.01 \times 10^{15}$ quanta/sec. The quantum yield of NO generation by 543.5 nm laser light was determined to be $0.056 \pm 0.002$. This quantum yield was essentially invariant over the range of GSNO tested (0.25 - 3.5 mM). The plot of the NO production rate versus GSNO concentration was best approximated by a first order process ($k_{obs} = 4.9 \times 10^{-7} \pm 0.3 \times 10^{-7} s^{-1}$) (Fig. 39, inset).

Photolysis of GSNO in the medium used to grow the cells (RPMI 1640) is presented in Fig. 40 where the extent of photolysis is measured by the formation of nitrite from the NO produced. As shown in Fig. 40, the extent of NO release from a 100 μM solution of GSNO over 3 hours increases substantially when exposed to light. After 1.5 and 3 hours of GSNO exposure to light 3.0 and 3.6 nmol of nitrite were formed per 20 nmol GSNO which corresponds to 15 and 18%, respectively, of the total NO that can be released from GSNO. For a 500 μM and a 1000 μM GSNO solution, 108.3 μM and 222.4 μM NO$_2^-$, respectively, was detected after 2 hours of exposure to light (data not shown). When nitrite was determined for a 100 μM GSNO solution dissolved in 10 mM HEPES, 140 mM NaCl, pH 7.4 the amount of NO$_2^-$ measured after 1 hour was greater than that measured for solutions dissolved in RPMI 1640 media (20.7 μM versus 10.1 μM, respectively).

In order to assess the cytotoxic potential of the photochemical release of NO from GSNO, HL-60 cells in culture were exposed to varying concentrations of GSNO in the presence and absence of light from a 300 W quartz halogen lamp filtered with a 3 cm copper sulfate solution and a 335 nm cut-off filter (Fig. 41). Cells treated with GSNO and
Fig. 40. Nitrite production from GSNO photolysis. GSNO (100 μM) was dissolved in RPMI 1640 medium and either exposed to light from a 300 W quartz halogen lamp that was filtered by passage through a 3 cm solution of copper sulfate (100 g/L) and by the employment of a UV filter that eliminated wavelengths below 335 nm (circles) or kept in the dark (inverted triangles). At different incubations times nitrite was measured as described in the materials and methods section.
Fig. 41. GSNO photo-induced cytotoxicity. Aliquots of 400 000 HL-60 cells/mL were treated with different concentrations of GSNO (0, 0.1, 0.5, and 1.0 mM) in the dark (filled bars) or in the presence of filtered light (open bars) for 2 hours. After 2 hours 200 000 cells were removed and washed with PBS and resuspended in 1 mL of GSNO-free media (Panel A). The remaining HL-60 cells were diluted one half with RPMI 1640 medium so that the final cell concentration was 200 000 cells/mL and the final GSNO concentrations were 0.05, 0.25, and 0.5 mM (Panel B). Panel A displays the effect of a brief GSNO exposure while Panel B displays the effect of a continuous GSNO exposure on HL-60 cell growth. Viable cell counts were performed at different growth times. Values are the average of triplicate experiments.
exposed to light were found to exhibit substantially more cell death especially at 0.5 and 1.0 mM GSNO as compared to the controls. When the cells were exposed to GSNO for 2 hours (Fig. 41A) there was a more pronounced difference in the extent of cell death between the light and dark conditions than when the cells were continuously exposed to GSNO (Fig. 41B). Table 3 presents the apparent LD$_{50}$ values of GSNO cytotoxicity obtained for the data presented in figure 4 by a fit to a simple saturation function. The cells exposed to light and GSNO for 2 hours exhibited maximal sensitivity to GSNO at 12 and 24 hour growth points. A decrease in sensitivity was noticed at 38 and 70 hour growth points (Table 3). In the absence of GSNO, light from the quartz halogen lamp was cytotoxic. Exposure of the cells to the light alone killed on average approximately 40% of the cells.

Coincubation of the cells with oxyhemoglobin results in a diminished effect of GSNO when the cells were continuously exposed to GSNO for 24 hours (Fig. 42). Oxyhemoglobin is oxidized to methemoglobin by NO which is concomitantly oxidized to NO$_3^-$ (Doyle and Hoogstra, 1981). Presumably, the addition of oxyhemoglobin competes successfully over the cellular constituents for the binding of NO resulting in the prevention of the cytotoxic effects of GSNO.

To determine whether or not the protective effect of oxyhemoglobin could be attributed to an inner filter effect a 2 mm thick solution of oxyhemoglobin (125 μM) in optically clear plastic was placed above a 200 μM solution of GSNO in RPMI 1640 media. The oxyhemoglobin filter inhibited photolysis of GSNO after two hours by the filtered quartz halogen lamp by about 25 % (45.8 μM NO$_2^-$ in the control versus 34.4 μM NO$_2^-$ with the oxyhemoglobin filter). Despite the optical filtering properties of
Table 3. Apparent LD<sub>50</sub> values of GSNO cytotoxicity

<table>
<thead>
<tr>
<th>time (h)</th>
<th>Two Hour GSNO Exposure</th>
<th></th>
<th>Continuous GSNO Exposure</th>
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<tr>
<td></td>
<td>dark</td>
<td>light</td>
<td>dark</td>
<td>light</td>
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<tr>
<td></td>
<td>LD&lt;sub&gt;50&lt;/sub&gt; (mM)</td>
<td>LD&lt;sub&gt;50&lt;/sub&gt; (mM)</td>
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<td>LD&lt;sub&gt;50&lt;/sub&gt; (mM)</td>
</tr>
<tr>
<td>12</td>
<td>1.9 ± 1.3</td>
<td>0.44 ± 0.22</td>
<td>1.81 ± 0.72</td>
<td>1.01 ± 0.30</td>
</tr>
<tr>
<td>24</td>
<td>1.7 ± 0.7</td>
<td>0.23 ± 0.012</td>
<td>0.41 ± 0.0081</td>
<td>0.44 ± 0.017</td>
</tr>
<tr>
<td>38</td>
<td>1.9 ± 0.2</td>
<td>0.95 ± 0.18</td>
<td>0.12 ± 0.0030</td>
<td>0.17 ± 0.015</td>
</tr>
<tr>
<td>70</td>
<td>1.1 ± 0.2</td>
<td>0.53 ± 0.16</td>
<td>0.080 ± 0.015</td>
<td>0.097 ± 0.016</td>
</tr>
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Fig. 42. Protection from GSNO cytotoxicity by oxyhemoglobin treatment. The ability of 125 μM oxyhemoglobin to protect against HL-60 cell cytotoxicity induced by 200 μM GSNO was assessed in the presence (open bars) and absence (filled bars) of a 2 hour exposure to light. Viable cell counts were performed after 24 hours of cell growth. Data shown here is average of 3 separate experiments and the error bars represent their standard deviation.
oxyhemoglobin, a significant amount of NO is produced from GSNO photolysis in the were completely protected from the cytotoxic effect of GSNO. Thus the observed effect of oxyhemoglobin can be attributed to its NO-scavenging properties and to a minor extent to an inner filter effect. That oxyhemoglobin protects the cells from GSNO strongly suggests that it is the NO released from GSNO that is acting as the cytotoxic agent.
Chapter 5

DISCUSSION

The physiological regulation of platelet function is imperative. The main platelet function is to prevent the loss of blood from severed blood vessels. In the absence of functional mechanisms to antagonize platelet activation, platelets are expected to hyperaggregate which leads to thrombus formation. Circulating platelet aggregates or thrombi in the vasculature induce myocardial and cerebral infarctions when they become lodged in capillaries. The complete elucidation of the mechanisms regulating platelet activation may lead to a fuller understanding of the etiology of vascular infarctions and possibly suggest new approaches for treatment.

Out of necessity, the balance of previous investigations on platelet function favor mechanisms that promote activation as opposed to regulatory mechanisms. The determination of the cellular events governing the regulation of platelet activation is likely to be dependent upon prior knowledge of platelet activation mechanisms. As described in Section 1.2.2.10, basic aspects of the mechanisms leading to platelet activation have been revealed. In general, events that cause an elevation of the intracellular [calcium] induce platelet activation.

Platelet activation is known to be antagonized by agents such as prostacyclin (PGI₂), prostaglandin D₂ (PGD₂) and NO which mediate their effects by the intracellular elevation of cyclic nucleotides (PGI₂ and PGD₂ elevate cAMP while NO elevates cGMP). It is not likely, however, that these platelet antagonists are completely responsible for the physiological regulation of platelet activation. The production of PGI₂ and PGD₂ is
dependent upon cell stimulation (Section 1.2.2.6) and consequently may not regulate the initiation of platelet activation. PGI₂ is produced from endothelial cell arachidonic acid metabolism while PGD₂ is a product of the platelet arachidonic acid metabolism. Platelet arachidonic acid metabolism is a late event in platelet activation and its induction leads to irreversibly aggregation. PGD₂ is therefore not likely to influence the onset of irreversible platelet activation in the platelet where it is produced. Endothelium-derived-NO and PGI₂ that are destined for platelets must diffuse through the blood. Diffusion is unlikely to affect the potency of PGI₂ since platelets contain a PGI₂ receptor but it will reduce the efficacy and immediacy of endothelium-derived NO as an inhibitor of platelet activation since there are many NO targets in blood, other than platelets. Consequently, the primary role of PGD₂, PGI₂, and endothelium-derived NO may be to prevent the over-accumulation of aggregated platelets once platelet activation has been initiated. Platelet-derived NO may elicit a more immediate and localized platelet response. As described in a following paragraph, it is conceivable that platelet-derived NO may be important in reversing platelet activation.

Some aspect of a platelet regulatory mechanism(s) must be capable of preventing the initiation of activation due to false stimuli. False stimuli may be either poor agonists such as viruses, bacteria, tumor cells, or immune complexes (Packham, 1994) or they may be represented by low circulating concentrations of an actual platelet agonist such as ADP. Understandably, full platelet activation is only desired when a blood vessel is damaged. Platelets must therefore possess a means of reversing the activation process when an agonist is encountered that is not associated with a ruptured blood vessel. A threshold
response which most false stimuli could not overcome would fulfill this assumed requirement of platelet function. The regulation of platelet activation by intracellular levels of glutathione (GSH) may represent a type of threshold response regulatory mechanism.

Reduced [GSH]_i has been suggested to enhance agonist-induced platelet activation through increased platelet arachidonic acid metabolism (Bosia et al., 1985; Hofman et al., 1980; Matsuda et al., 1979). Intracellular GSH therefore appears to regulate platelet activation. The suggested mechanism for the apparent regulation of platelet activation by [GSH]_i involves the inhibition of the production of arachidonic acid metabolites (Bosia et al., 1985; Hofman et al., 1980; Matsuda et al., 1979). As described in Section 1.3.8, [GSH]_i indirectly inhibits the production of the potent platelet agonist thromboxane A_2 (TxA_2). TxA_2 production or arachidonic acid metabolism is a component of the release reaction which is a late event in platelet activation sequence. The induction of the release reaction, which is also comprised of the secretion of granular contents, is associated with the onset of irreversible platelet aggregation. Certain agonists, such as low [ADP] in the presence of physiological [Ca^{2+}], are not capable of promoting the release reaction and consequently exhibit only reversible platelet activation (Packham, 1994). Apparently, the onset of the platelet release reaction is regulated by an intra-platelet mechanism(s). The inhibition of TxA_2 production by [GSH]_i is a likely regulatory mechanism which may participate in the reversal of platelet activation.

Prior to this study the role of [GSH]_i had only been investigated using thiol reagents as GSH depleting agents. Many of the commonly used GSH-depleting agents are
not entirely specific towards GSH and consequently their effects on platelet aggregation may not be attributed solely to the depletion of [GSH]_{ip}. The development of more specific GSH-depleting agents would benefit investigations into the role of GSH in platelet activation and cell function in general. An alternate way of assessing the role of [GSH]_{ip} in platelet function, that has not been previously utilized, involves the elevation of [GSH]_{ip}. The effect of elevated [GSH]_{ip} on platelet function should be opposite to that of reduced [GSH]_{ip} if, in fact, [GSH]_{ip} does have a role in the regulation of platelet activation. An advantage of using elevated [GSH]_{ip} as opposed to reduced [GSH]_{ip} to assess the role of [GSH]_{ip} in platelet function is that nonspecific effects of GSH-depleting agents can be avoided. The effect of elevated [GSH]_{ip} on platelet function had not been previously shown possibly because it was not known that platelets were capable of taking up intact GSH. In our investigation into the role of [GSH]_{ip} in platelet function both of the above approaches were utilized. A novel thiol reagent (CDDP) was characterized with respect to its ability to act as a specific [GSH]_{ip} depleting agent. The specificity of CDDP towards GSH was assessed by its ability to inhibit the enzymes glutathione reductase (GR) and glutathione S-transferase (GST). In the course of this study we made some general discoveries concerning the GSH metabolic enzymes GR and GST. Our efforts to assess the effects of elevated [GSH]_{ip} on platelet function led to the identification of a specific transport system for intact GSH in the platelet membrane. Since platelet GSH transport had not been previously shown we initiated its characterization.
[GSH]_p may represent an important regulatory mechanism for the onset of part of the platelet release reaction and subsequent irreversible platelet aggregation. The inhibition of the platelet release reaction alone is not sufficient to induce the reversal of platelet activation. In order for the reversal of platelet activation to occur there should also be a pathway(s) whose product(s) deactivate platelets. One such candidate pathway is the platelet L-arginine/NO pathway. NO is a potent inhibitor of platelet aggregation that is produced by the platelets during platelet activation. When platelets are activated the Ca^{2+}/CaM-dependent NOS enzyme is also activated. The NO produced within the platelet may antagonize the continuation of platelet activation possibly leading, in some cases, to the reversal of platelet aggregation. The mechanism whereby platelets transiently fill the gaps that form between endothelial cells in stressed capillaries (Section 1.2.2) is not known but could conceivably involve platelet-derived NO.

Presumably, there should be a feed-back response for limiting the production of NO and thereby allowing platelet activation to proceed when an actual agonist is encountered in sufficiently high concentrations to signify a ruptured blood vessel. The feed-back inhibition of NO production may occur by inhibition of NOS which has been shown in murine macrophages (Assreuy et al., 1993). L-Arginine uptake has been shown to be affected by NO in macrophages (Bogle et al., 1992), polymorphonuclear leukocytes (Riesco and coworkers, 1993), and endothelial cells (Bogle et al., 1992). Prior to this study L-arginine transport in platelets had not been characterized. We initiated the characterization of the platelet L-arginine transport system with the ultimate intention of checking for feed-back effects by NO.
5.1. Effect of GSH-Depleting Agents on Platelet Aggregation

Evidence for a role of GSH in the regulation of platelet activation can be extracted from the finding that when \([\text{GSH}]_{\text{ip}}\) was lowered by a preincubation with 0.1 mM CDNB the rate of ADP-induced platelet aggregation was enhanced (Fig. 6). This result is in agreement with that of Bosia et al. (1985) who reported that the reduction of \([\text{GSH}]_{\text{ip}}\) by CDNB causes platelet aggregation at subthreshold concentrations of arachidonic acid. Both our results (Fig. 6) and previous results (Bosia et al., 1985) may be explained by the regulation of cyclooxygenase activity. Cyclooxygenase is a key enzyme in the conversion of arachidonic acid to TxA2 that is activated by trace amounts of peroxides (Lands, 1984). By diminishing the intracellular levels of peroxides, GSH may be an important regulator of TxA2 production and hence platelet activation.

When \([\text{GSH}]_{\text{ip}}\) was more substantially lowered by a preincubation with either 1 mM CDNB or 1 mM CDDP ADP-induced platelet aggregation was inhibited but not to the same extents (Fig. 6). To assess the actual effect of depleted \([\text{GSH}]_{\text{ip}}\) on platelet aggregation it is useful to compare results obtained using different GSH depleting agents. The goal is to arrive upon a thiol reagent that is specific towards GSH. Depletion of \([\text{GSH}]_{\text{ip}}\) with CDNB essentially inactivated ADP-induced platelet aggregation while CDDP only inhibited the rate of aggregation (Fig. 6). Since both thiol reagents deplete \([\text{GSH}]_{\text{ip}}\) by about the same extent (Fig. 7) the difference in the degree to which they inhibit platelet aggregation is unlikely to be attributable to a GSH dependent process. Presumably, a CDNB concentration of 1 mM is capable of inhibiting platelet aggregation by a means distinct from its conjugation to GSH such as the inhibition of one or more thiol
sensitive proteins that are critical for aggregation to proceed. The inhibition of platelet aggregation by GSH depletion with CDDP may better approximate the true importance of [GSH]$_{p}$ in the ability of platelets to aggregate in response to ADP.

As an index of their relative abilities to react with protein thiols we compared the ability of CDNB and CDDP to inhibit the enzymes GST and GR. GST is known to possess a regulatory thiol (Caccuri et al., 1992) and GR contains catalytically important cysteine thiols (Section 1.3.3.1). CDNB has been previously shown to inhibit the enzymes GST (Caccuri et al., 1992) and GR (Bilzer et al., 1984). We confirmed that CDNB is an inhibitor of GR (Fig. 8A), since Hill et al. (1989b) reported contradictory findings to the original report (Bilzer et al., 1984). CDDP is a poor inhibitor of GR (Fig. 8B) and does not inhibit GST (Fig. 9D).

In our experiments we have found that the inhibition of GR by 100 μM CDNB is best approximated by an uncompetitive inhibition pattern with respect to GSSG as the varied substrate (Fig 8A). Uncompetitive with respect to GSSG suggests that CDNB reacts with the ES complex where ES is a mixed disulfide between GR and glutathione (ES = GR-S-SG). This is not unexpected since in the GR catalytic cycle NADPH binds GR first and reduces an active site disulfide to yield the reduced enzyme (EH$_2$) which awaits the second substrate, GSSG. GSSG interacts with Cys-58 (human erythrocyte enzyme) at the active site through disulfide exchange. Normally a second disulfide exchange reaction occurs between Cys-63 and the Cys-58-S-SG mixed disulfide to liberate a second GSH molecule. One way that CDNB (100 μM) may uncompetitively inhibit GR with respect to GSSG is by alkylation of the active site Cys-63, thereby preventing the
production of the second molecule of GSH (Fig. 42). The existence of an inactivated GR with Cys-58 locked into an irreversible mixed disulfide with GSH and Cys-63 alkylated by CDNB has not been shown. At high concentrations (1 mM) CDNB potently inhibits GR in a manner which can be approximated by a mixed type inhibition pattern with respect to GSSG as the varied substrate (Fig. 8A). When the inhibition pattern at 1 mM CDNB is compared to that of 100 μM CDNB an apparent noncompetitive inhibition pattern is evident. Presumably, while 100 μM CDNB inhibits GR only by alkylating an active site thiol, 1 mM CDNB inhibits GR by interacting with an active site thiol and another less reactive site on the protein. This less reactive site is likely another cysteine such as Cys-2 (human erythrocyte). Though CDDP is a poor GR inhibitor it can be seen that it does not inhibit in an apparent uncompetitive manner (Fig. 8B). It is difficult to determine the inhibition pattern of CDDP but it appears that it may be approximated by a noncompetitive inhibition mechanism. The presence of noncompetitive GR inhibition by CDDP may suggest that it is incapable of interacting with an active site thiol but is able to react with the same postulated second alkylation site as high [CDNB].

The near inactivation of platelet aggregation by 1 mM CDNB may, at least partially, be attributed to the inhibition of GR. When GR is inhibited the [GSSG], which normally only transiently accumulates during platelet activation (Burch and Burch, 1990), should be sustained for longer periods of time. Elevated intracellular [GSSG] may inhibit platelet aggregation by thiol exchange with redox sensitive proteins or enzymes that are important in the activation process. As described in Section 1.3.3.2, protein disulfide
exchange with small molecular weight disulfides such as GSSG alters the activities of many proteins and enzymes.

5.2. The Inhibition of Glutathione S-Transferase by Certain Mannich Bases of $\alpha,\beta$-Unsaturated Ketones

Since we had access to a number of analogs (including CDDP) that contained the $\alpha,\beta$-unsaturated ketone moiety we proceeded to examine them for their ability to inhibit selected enzymes of GSH metabolism. Compounds 1, 2 and 3 are 3,5-bis-benzylidene-4-piperidonones that vary only in their degree of N-piperdone substitution (for structures see Section 1.3.4.3). It was subsequently found that GR was only slightly inhibited by all 4 compounds (compounds 1, 2, 3 and CDDP) and $\gamma$-glutamyltranspeptidase ($\gamma$-GT) was not inhibited by either compound (data not shown). GST, however, was inhibited by compounds 1, 2 and 3 but not by CDDP (Fig. 9). Recall from Section 1.3.4.1 that GST represents a family of isozymes which catalyze the conjugation of GSH to various electrophiles.

As described in Section 1.3.4.3, the inhibition of GST has potential applications in combination chemotherapy, particularly if the selective inhibition of certain isozymes can be achieved. The preliminary results reported here were performed using equine liver GST that had not been resolved into classes. It is therefore not known if these compounds display class selectivity in their inhibition of GST. It is evident that while the compounds examined do not inhibit GST to the same extent (Fig. 9), they all exhibit apparent noncompetitive inhibition (Fig. 10). Noncompetitive inhibition is due to inhibitor
interaction at a site away from the active site. One possibility that remains to be proven is that these compounds are capable of alkylating a regulatory thiol on GST. In the human placenta enzyme a regulatory cysteine thiol has been identified at position 47 by its reaction with CDNB (Caccuri et al., 1992). The alkylation of Cys-47 was protected by a GSH analog which suggests that the thiol may be near the GSH binding site (G site).

The differences in the extent of GST inhibition by the 3,5-bis-benzylidene-4-piperdones may be attributed to charge differences on the nitrogen atom of the piperdone ring. The net charge of these compounds is due to the charge on the piperdone nitrogen. Compound 2 was the most potent inhibitor and contains a relatively uncharged tertiary piperdone amine with a methyl substitution. Compound 1, the next most potent inhibitor, contains an unsubstituted piperdone secondary amine which is more readily protonated. Generally, secondary amines have higher pKₐ values than tertiary amines which enables secondary amines to be more positively charged at a near neutral pH. The relatively poor inhibitor, compound 3, contains a positively charged quaternary amine with two methyl group substitutions. The observed order of GST inhibition by the 3,5-bis-benzylidene-4-piperdones examined suggests that their interaction site on the protein is positively charged.

The presence of a positively charged amino acid environment surrounding the regulatory thiol may signify a binding site for slightly negatively charged cystine. Cystine has previously been shown to inactivate the human placenta enzyme by interacting with a regulatory thiol (Nishihara et al., 1991). That the enzyme was protected against cystine inactivation by GSH analogs suggests that the thiol is near the G-site. The activity of the
resulting GST- mixed disulfide was restored by the actions of GSH and a thioltransferase (Terada et al., 1993). It is likely that the 3,5-bis-benzylidene-4-piperdones interact at a physiologically important regulatory thiol on the enzymes. These preliminary results suggest that neutral or negatively charged analogs may be more potent inhibitors.

5.3. Glutathione Conjugation to CDDP is Selectively Catalyzed by Alpha Class Glutathione S-Transferase

Interestingly, it was found that while CDDP was the only Mannich base of α,β-unsaturated ketone tested that did not inhibit GST, it was also the only one capable of being an equine liver GST substrate. A compound that resembles CDDP, called NC 1109 (structure Section 1.3.4.2), was also found to be an equine liver GST substrate. Despite the presence of the same thiol-reactive moiety, CDDP and the 3,5-bis-benzylidene-4-piperdones (compounds 1-3) are not very similar in structure. Some undetermined aspect(s) of the 3,5-bis-benzylidene-4-piperdone structure must prevent them from behaving as equine liver GST substrates. GSH conjugation to CDDP was initially found to be catalyzed by equine liver GST that had not resolved into separate classes (Fig. 11).

The liver is composed mainly of alpha and mu class GSTs (Hayes et al., 1991; Di Ilio and Federicic, 1990; Gupta et al., 1990; Jensson et al., 1985; Tu et al., 1983). Platelet GST, which is composed exclusively of pi class GST (Loscalzo and Freedman, 1986; Federici et al., 1985), did not catalyze GSH conjugation to CDDP (data not shown). It therefore appeared that CDDP may be a class selective GST substrate. The discovery of class selective GST substrates is useful to the more accurate identification of the enzyme
class(es) present in a particular tissue. A study was undertaken to determine the GST substrate class selectivity, if any, of CDDP.

Equine liver alpha and mu class GSTs were separated by CM-sepharose ion exchange owing to their different pIs. When the pH of the equine liver GST mixture was 6.0 the elution of mu class GST (pKa ~ 6) was only slightly retarded (fraction A), while alpha class GST, whose pKa ranges from 8-9, bound to the positively charged CM-Sepharose resin (fraction B). CDDP conjugation to GSH was subsequently shown to be mainly catalyzed by fraction B. Confirmation of the identity of Fraction B as alpha class GST was obtained from activity measurements with known GST class selective substrates and from immunoblot analysis with GST class specific antibodies. Placenta and platelets have been shown to express only the pi isoform (Loscalzo and Freedman, 1986; Federici et al., 1985; Rogerson et al., 1984; Mannervik and Guthenberg, 1981). The pi class GST from platelet and placenta (data only shown for placenta) as well as the mu class GST from equine liver (CM Sepharose fraction A) displayed very low activity towards GSH conjugation to CDDP relative to that of alpha class GST from equine liver. Based on the preceding observation we conclude that CDDP is an alpha class selective substrate. CDDP may, therefore, find use in screening different tissues for the expression of alpha class GST.

Cumene hydroperoxide, the commonly employed alpha class selective GST substrate detects the GSH peroxidase activity of alpha class GST. An advantage of CDDP over cumene hydroperoxide is that it is not subject to interference by the presence of selenium-dependent GSH peroxidase when measurements are performed on crude
samples (Lawrence and Burk, 1976). CDDP is the first example of an alpha class selective GST specific substrate that monitors GSH conjugation activity rather than GSH peroxidase activity.

Possible uses of novel substrates such as CDDP for measuring GST activity can be summarized as follows: 1) they may provide more information on tissue-specific distributions of GST; 2) they may lead to the discovery of new forms of GST; 3) they may lead to the discovery of new endogenous substrates; and 4) they may be used in conjunction with their analogs to probe the amino acids in the active site of GST.

5.4. Platelet Glutathione Transport

Previous investigations into the role of $[\text{GSH}]_p$ in the regulation of platelet activation have utilized thiol reagents as GSH depleting agents. As previously discussed, with GSH depleting agents it is often difficult to ensure that the observed effect is due to diminished $[\text{GSH}]_p$ and not the thiol reagent itself. An alternative means of determining whether or not $[\text{GSH}]_p$ is involved in the regulation of platelet activation could involve the elevation of $[\text{GSH}]_p$. Platelet GSH uptake is an obvious means of elevating $[\text{GSH}]_p$, but has not been previously shown. It may be advantageous for platelets in the maintenance of normal function and in the regulation of platelet activation to possess a high affinity GSH transport system capable of influxing the typically low concentrations of GSH available in plasma (2-10 μM: Flagg et al., 1993; Svardal et al., 1990; Lash and Jones, 1985). The uptake of GSH by platelets from the plasma may be important in maintaining a $[\text{GSH}]_p$ capable of preventing platelet hyperaggregation.
In addition to measuring the effects of elevated \([\text{GSH}]_\text{p}\) on platelet aggregation we proceeded to characterized platelet GSH transport. The kinetic characterization of the platelet GSH transport system was performed using platelet plasma membrane vesicles (PPMV). The use of vesicles prevents the further cellular metabolism of transported substrate and enables manipulation of the intra-vesicular milieu. The properties of GSH transport observed in PPMV are not expected to be influenced by cellular events and are consequently more indicative of the characteristics of the platelet GSH carrier protein. GSH transport in intact platelets, however, more closely approximates the \textit{in vivo} situation. The comparison of GSH transport in PPMV to that observed in intact platelets may reveal the presence of cellular regulatory events.

The presence of a specific intact GSH transport system in the platelet membrane was verified in several ways. GSH uptake in the presence of acivicin, a \(\gamma\)-GT inactivating agent, must be via intact GSH transport as confirmed by HPLC analysis (Fig. 16). Further evidence for the presence of a specific GSH transport can be extracted from the finding that platelet GSH uptake was inhibited substantially only by GSH analogs. It appears, therefore, that the platelet GSH transport system is very specific towards the recognition of the GSH molecule. GSH analogs that inhibited platelet GSH uptake were monoethyl ester GSH, diethyl ester GSH, N-formyl GSH, and N-acetyl GSH. The monoethyl ester GSH derivative has been previously shown to inhibit intestinal GSH uptake (Vincenzini \textit{et al.}, 1992). The other GSH analogs had not been previously examined as inhibitors of GSH transport. No apparent inhibition of platelet GSH uptake by the S-substituted GSH
analogs, S-dinitrophenyl glutathione (GS-DNP) and S-nitrosoglutathione (GSNO) was evident which is different from that of other cells (Vincenzini et al., 1992).

As described in Section 1.3.5.2, intact GSH transport has been previously studied in several other cells (Table 1). In general, few characteristics are conserved among GSH transport systems from different cells. Despite the apparent heterogeneity of GSH transport systems northern blot analysis using cloned rat liver canicular GSH transporter DNA detected complementary mRNA in kidney, intestine, lung and brain (Yi et al., 1994). Apparently, at least some GSH transport systems are very similar at the amino acid level.

GSH transport systems from some cells are dependent on sodium cotransport while others are not. The apparent sodium-independence of GSH transport in platelets likens the platelet GSH transport system to that of the kidney brush-border (Lash and Jones, 1984) or the intestinal brush-border (Vincenzini et al., 1992).

The platelet GSH transport system was found to be dependent on membrane potential. The majority of evidence suggests that dependence on membrane potential may be a general characteristic of all the GSH transport systems that have been examined. The manner in which membrane potential effects GSH transport, however, is not the same. Since GSH is a negatively charged molecule at physiological pH values, it is expected that membrane hyperpolarization (more negative inside) should inhibit GSH uptake while membrane depolarization (more positive inside) should enhance uptake. These expectations were fulfilled by the GSH transport in platelets and other sodium-independent systems. The sodium-dependent kidney basolateral membrane GSH transport system displays the opposite behavior with respect to a dependence on membrane
potential. Across the kidney basolateral membrane GSH transport is enhanced by membrane hyperpolarization and inhibited by membrane depolarization.

The concentration dependence of platelet GSH uptake is similar to that of other cells in that it contains an element of nonspecific diffusion at high [GSH]. The nonspecific diffusion of GSH may be attributed to the ability of GSH to serve as a low affinity substrate for other transport systems. Alternatively, GSH may simply diffuse across the membrane, possibly as a metal salt, when present at sufficiently high concentrations. The kinetic parameters, $K_M$ and $V_{max}$, can still be obtained from the data by either a subtraction of the nondiffusion rate or a fit to the Michaelis-Menten-Henri equation with an added term to account for the diffusion. The $K_M$ for GSH uptake by PPMV is between 10 and 20 times lower than that of membranes from liver (Inoue et al., 1983) and kidney (Inoue and Morino, 1985), but is similar to that observed in basolateral membranes from intestinal cells (Vincenzini et al., 1992). The low $K_M$ suggests that the platelet GSH transport system may be capable of taking up GSH from the plasma in vivo.

The uptake of GSH by intact platelets was enhanced by the depletion of $[\text{GSH}]_p$ by pretreatment with 100 μM CDNB. The apparent $K_M$ for the uptake of GSH by intact platelets decreased from 137 μM to 31.7 μM following CDNB treatment. A decrease in $[\text{GSH}]_p$ by CDNB may enhance platelet GSH uptake by the oxidation of a putative thiol on the GSH carrier protein. The platelet GSH carrier protein appears to be sensitive to the intra-platelet thiol status. Further support for the postulated redox control of platelet GSH transport may be extracted from the finding that cysteine and cysteinylglycine inhibited GSH uptake in PPMV. Cysteine is transported by a specific amino acid transport
system and consequently would not be expected to competitively inhibit GSH uptake. If there is a specific transport system for cysteinylglycine, it has not been identified and reported. Cysteine and cysteinylglycine may inhibit platelet GSH uptake by reducing a redox sensitive thiol on the GSH carrier protein.

A recent report is in agreement with our finding of the apparent redox regulation of GSH transport (Lu et al., 1993). The hepatic GSH transport system was shown to be sensitive to intracellular thiol-disulfide effects (Lu et al., 1993). In that system it was found that cystine inhibited GSH efflux through the GSH carrier. DTT was reported to reverse the effects of cystine and to stimulate efflux to levels 4-5 times above the control (Lu et al., 1993). The combination of our GSH transport results in platelets and those of Lu et al. (1993) for hepatocytes may lead to a generalized theory for the regulation of GSH transport by the intracellular thiol status. In platelets GSH uptake is stimulated by low [GSH]o, and inhibited by reducing agents (cysteine and cysteinylglycine) while the opposite behavior is reported for hepatocyte GSH efflux (Lu et al., 1993). It, therefore, appears that high intracellular reducing conditions favors GSH efflux while a more oxidative intracellular environment favors the influx component of the GSH transport system. On the basis of our data and others (Lu et al., 1993) we suggest that GSH transport systems in general may be under thiol redox control.

Thiol redox regulation of GSH transport may prevent the detection of a GSH transport system in certain tissues. If the GSH transport system is particularly sensitive to thiol redox control and/or the intracellular [GSH] is high, GSH transport may be completely inactivated. In such a situation the cell would be thought not to contain a
GSH transport system when in fact it did, albeit inactivated. Erythrocytes require high intracellular [GSH] to maintain their shape and have been reported not to exhibit specific intact GSH transport. Due to the importance of GSH in erythrocyte function it would be to their advantage to possess a GSH transport system capable of influxing the low [GSH] available in plasma. Perhaps the reexamination of tissues previously thought not to express a GSH transport system is in order.

The $K_M$ for GSH uptake in $[\text{GSH}]_p$-reduced platelets (i.e CDNB-treated platelets) is shifted closer to the $K_M$ observed in vesicles which are devoid of GSH. GSH uptake in vesicles represents an estimation of GSH uptake in platelets that are completely depleted of their thiol contents. The plasma GSH concentration is also much closer to the $K_M$ of $[\text{GSH}]_p$-reduced platelets. It is likely that in vivo platelets loaded with a full complement of GSH may not take up an appreciable amount of GSH. Under physiological conditions where $[\text{GSH}]_p$ is decreased, such as during platelet aggregation (Burch and Burch, 1990), platelet GSH uptake may be stimulated to the extent where it may take up plasma GSH. During platelet aggregation it has been shown that there is a transitory increase in the amount of GSSG in the platelet but little or no change in the amount of total GSH (GSH + GSSG) (Burch and Burch, 1990). The decrease in $[\text{GSH}]_p$ may activate GSH uptake but not result in an increase in the amount of total GSH if some GSSG is concomitantly effluxed from the platelet. The cellular efflux of GSSG during conditions of oxidative stress has been demonstrated using human erythrocytes (Masuda et al., 1993).

Our results on the effect of elevated $[\text{GSH}]_p$ on ADP-induced platelet aggregation suggests a regulatory role for GSH in platelet activation. Platelet aggregation was
inhibited by elevated $[\text{GSH}]_p$ and enhanced by a slight reduction of $[\text{GSH}]_p$ (Section 4.1). These results are in agreement with the proposed participation of $[\text{GSH}]_p$ in the regulation of cyclooxygenase activity (Lands, 1984). When $[\text{GSH}]_p$ is elevated the intracellular [peroxide] is reduced which prevents the peroxide-induced activation of cyclooxygenase. The prevention of cyclooxygenase activation will inhibit further arachidonic acid metabolism such as the production of the potent platelet agonist $\text{TxA}_2$.

The inhibition of ADP-induced platelet aggregation by exogenous GSH was found to have an approximate IC$_{50}$ of 3.8 mM which was not in agreement with that of Thomas et al. (1986b) who reported a value of 0.61 mM. The reason for this discrepancy is unclear but may be attributed to the fact that Thomas et al. (1986b) preincubated the platelets with GSH for 2 minutes before measuring aggregation which may have permitted enough GSH to enter the platelet to enhance the apparent inhibitory effect of exogenous GSH. In our experiment we added GSH just prior to adding agonist (i.e. < 30 s).

In conclusion, this is the first report demonstrating carrier-mediated intact GSH uptake by human platelets. The platelet GSH transport system does not share all the properties of any single GSH transport system from other cells. Consequently, the platelet GSH transport system represents a new addition to the heterogeneous family of GSH transport systems. Our results and those of others (Lu et al., 1993) suggest that one general property of GSH transport systems may be regulation by the intracellular thiol redox status. As discussed above, the inhibition of platelet aggregation by elevated $[\text{GSH}]_p$ is in agreement with the previously postulated indirect role of $[\text{GSH}]_p$ in the regulation of $\text{TxA}_2$ production.
5.5. Inhibition of Glutathione Reductase by Reduced Glutathione

One consequence of elevated intracellular [GSH] is the potentiation of the feed-back inhibition of GR by GSH (Sexton and Mutus, 1992; Chung et al., 1991). When the intracellular [GSH] is elevated, the *in vivo* inhibition of GR by GSH may lead to either an unchanged or a reduced GSH:GSSG ratio. The tendency of elevated intracellular GSH to increase the GSH:GSSG ratio may be circumvented by the GSH inhibition of GR which may be an important physiological mechanism of maintaining a constant GSH:GSSG ratio. However, when intracellular GSH is elevated in cells that are under oxidative stress, the inhibited GR will not be capable of reducing the increased GSSG production and the GSH:GSSG ratio may actually decrease. The potential role of decreased intracellular GSH:GSSG ratio in the modulation of enzyme activity has been discussed (Section 1.3.3.2). The feed-back inhibition of GR by GSH adds a new dimension to the proposed concept of redox control over cellular events.

Since platelets elicit a measurable aggregation response when challenged with agonist they represent an ideal cellular system in which to measure the global effects of alterations in intracellular GSH:GSSG ratio. The inhibitory effects of elevated [GSH] on platelet aggregation may, in part, be associated with a decreased GSH:GSSG ratio. A decreased GSH:GSSG ratio may be expected during platelet aggregation for two reasons. First, platelet GR has been shown in this study to be inhibited by GSH. Second, GSSG is reportedly elevated during platelet aggregation (Burch and Burch, 1990). Since the GPX-mediated increase in intracellular levels of GSSG will not be reduced as efficiently by GR when [GSH] is elevated, the intracellular GSH:GSSG ratio should be decreased.
Elevated intracellular levels of GSSG have been previously associated with the inhibition of platelet aggregation (Caruso et al., 1984). Diamide, a GSH oxidizing reagent, inhibits platelet aggregation and in some cases reverses platelet aggregation (Caruso et al., 1984).

While our initial manuscript was being prepared for publication, a report appeared in the literature describing the inhibition of rat liver GR by GSH (Chung et al., 1991). The present study expands upon the findings of Chung et al. (1991) by indicating that GRs from a variety of sources are differentially susceptible to feed-back inhibition by GSH. Of the enzymes tested (human platelet, bovine intestinal mucosa, yeast, and E. coli), the extent of inhibition by GSH was greatest with yeast GR and weakest with E. coli GR. GSH does not appear to interact at a common site with the enzymes studied as the inhibition patterns were dissimilar. Species-dependent differences in the regulation of GR have not been previously reported.

There are small differences in the primary structure of GR from the human erythrocyte and E. coli that may influence the overall tertiary and quaternary structure as well as enzyme catalysis. The two enzymes differ with respect to the total number of residues (478, in human erythrocyte (Krauth-Siegel et al., 1982); 450, in E. coli (Greer and Perham, 1986). In addition, GR from human erythrocytes and other eucaryotic sources contain cysteines at positions 2 and 90 which are absent in E. coli (Greer and Perham, 1986). The native homodimeric structure of eucaryotic GR is thought to be stabilized by an intersubunit disulfide bridge between Cys-90 thiols of different subunits. No such bridge is possible in the E. coli enzyme as the residue at position 90 is a threonine.
With respect to NADPH as the varied substrate, the enzyme from human platelets, bovine intestinal mucosa, and yeast were inhibited by GSH in an apparent uncompetitive manner. In contrast, *E. coli* GR was not inhibited, when NADPH was varied in the presence of saturating GSSG concentrations. Uncompetitive inhibition requires the interaction of the inhibitor with the ES complex where S is the varied substrate. Uncompetitive inhibition of eucaryotic GR by GSH reflects the interaction of GSH with the GR:NADPH complex or the NADPH-reduced enzyme. NADPH binds GR before GSSG to reduce a catalytically important active site disulfide before dissociating from the enzyme as NADP⁺. One explanation for the uncompetitive inhibition of eucaryotic GR may be that the GR:NADPH complex or the reduced enzyme undergoes a conformational change to make the Cys90-Cys90' intersubunit disulfide more accessible to disulfide exchange with GSH. The resulting GS-S-GR mixed disulfide would likely destabilize the subunit interactions and since the catalytic site is located at the subunit interface, enzyme catalysis may be inhibited. Since *E. coli* does not contain an intersubunit disulfide no inhibition with respect to NADPH is expected; nor was it observed. The hypothesis that the intersubunit disulfide may have a role in the regulation of enzyme activity was first proposed from a crystallographic analysis of human erythrocyte GR by Thieme et al (1981).

When GSSG was the varied GR substrate more species-dependent differences in the manner that GSH inhibits GR were evident. In the case of the human platelet GR, GSH inhibited GSSG reduction in an apparent uncompetitive manner suggesting that GSH combines with the ES complex. The ES complex in this case refers to the mixed disulfide
that is transiently formed during catalysis between reduced enzyme and GSSG. GSH appears to interact at multiple sites on the yeast and the bovine enzymes since observed inhibition patterns were best approximated by an equation describing hyperbolic mixed type inhibition. With *E. coli* GR the slight inhibition of GSSG reduction by GSH was of the competitive type. These results, coupled with the report (Chung *et al.*, 1991) that rat liver GR is inhibited in a non-linear, noncompetitive manner by GSH, suggest that there is a great deal of heterogeneity among GRs from different sources with respect to regulation by GSH.

A plausible explanation for the apparent uncompetitive inhibition of human platelet GR by GSH with respect to varied GSSG may involve disulfide exchange between GSH and the mixed disulfide GR-S-SG intermediate. This hypothetical situation can be envisioned as a futile cycle with no net production of GSH and no further consumption of NADPH. This reversible mechanism could be very effective in the *in vivo* regulation of GR activity. An alternate explanation for the apparent uncompetitive inhibition of human platelet GR by GSH may involve the preferential reduction of the Cys90-Cys90' intersubunit disulfide in the mixed disulfide GR-S-SG intermediate possibly through a conformational change that makes the intersubunit disulfide more accessible to attack by GSH.

GSH was a relatively poor inhibitor of *E. coli* GR but when GSSG was the varied substrate, inhibition was discernable at high concentrations of GSH (10 mM). The inhibition of *E. coli* GR by GSH was best approximated by an apparent competitive
inhibition pattern with respect to varied GSSG. Competitive inhibition of GR by GSH can be simply explained by a competition of GSH for the GSSG binding site on the enzyme.

The hyperbolic mixed type inhibition of bovine intestinal mucosa and yeast enzymes GRs by GSH with respect to varied GSSG may be due to the combination of both uncompetitive and competitive inhibition patterns. In GR from these sources GSH may interact with the enzyme in the manner described above for human platelet GR as well as the manner described for *E. coli* GR.

Feedback inhibition as a means of regulatory control is not unknown in GSH metabolism as the first step of GSH biosynthesis, catalyzed by the enzyme, γ-glutamylcysteine synthetase, is feedback inhibited by GSH (Seelig and Meister, 1985). The results of the present study and also that of Chung *et al.* (1991) indicate that GR is also feedback inhibited by GSH. It has also been suggested that intracellular GSSG could regulate intracellular enzymes via redox control (Ziegler, 1985). A physiological consequence of the finding that glutathione reductase is inhibited by GSH would be to decrease the intracellular GSH:GSSG ratio which lends further support to the concept of redox regulation of intracellular enzymes by GSSG.

In summary, the results presented here have confirmed that GRs from various sources are inhibited by physiologically relevant concentrations GSH. Furthermore, the studies have detected kinetic heterogeneity among GRs from different sources with respect to inhibition by GSH. It seems likely that this inhibition will occur *in vivo* and will significantly elevate intracellular GSSG levels.
5.6. Platelet L-Arginine Transport

Platelet-derived NO is very likely to be important in the regulation of platelet activation. Only recently has the presence of a platelet isoform of NOS been shown by purification of the enzyme to homogeneity (Muruganandam and Mutus, 1994). We have shown here that preincubation of platelets with the NOS substrate, L-arginine inhibits platelet aggregation. This result is in agreement with that of Radomski et al. (1990) who showed that L-arginine inhibited collagen-induced aggregation. The time-dependent manner of the inhibition of platelet aggregation by L-arginine is indicative of L-arginine transport. The platelet production of NO is therefore expected to antagonize platelet activation.

The fact that extracellular L-arginine inhibits platelet aggregation in a NO-dependent manner suggests that platelet NO production is limited by L-arginine availability. It therefore appears that platelet NO production is dependent on extracellular L-arginine which makes L-arginine transport the rate determining step. This would not be the first instance where L-arginine uptake by system Y was found to be a rate determining step. L-Arginine uptake across the hepatocyte membrane has been shown to be the rate-limiting step in the urea cycle (White and Christensen, 1982). However, the low platelet NOS $K_M$ towards L-arginine (0.18 $\mu$M: Muruganandam and Mutus, 1994) does not favor L-arginine transport as being the rate-determining step of platelet NO synthesis. The intra-platelet [L-arginine] has not been determined but in other cells it is normally around 0.8 mM (Baydoun et al., 1990; Mitchell et al., 1990). Due to the low NOS $K_M$ for L-arginine it is not clear how the intra-platelet [L-arginine] may be rate limiting towards NO
production. It may be that during platelet activation intra-platelet L-arginine is consumed by NOS. Malinski et al. (1993) have electrochemically measured NO release from activated platelets to be $4 \times 10^{-18} \text{ mol/platelet}$. Since the average total platelet volume is about 8 fl. (Sharpe and Trinick, 1993) an underestimation of the intra-platelet L-arginine concentration required for NO biosynthesis is approximately 0.6 mM. This value likely underestimates the platelet L-arginine requirement for two reasons: 1) the total platelet volume used is greater than the intracellular volume; and 2) the amount of NO released from the platelets is not expected to reflect the total amount of NO produced due to the presence of several intra-platelet NO targets, such as thiols (including GSH) and hemes. It is therefore very likely that the resting levels of intra-platelet L-arginine is consumed during platelet activation. The further production of NO by the platelet is then dependent upon influxed L-arginine. In such a scenario L-arginine transport is the rate-determining step in platelet NO synthesis only after activation has been initiated.

A thorough understanding of the L-arginine transport system in platelets may lead to a description of how the production of NO is regulated during platelet activation. As described in Section 1.4.3.2, L-arginine transport in many mammalian cells is mediated by a cationic amino acid transport system, called system $y^+$, which also transports L-lysine and L-ornithine with approximately equal affinity. Based on the apparent importance of L-arginine transport in the regulation of platelet activation the L-arginine carrier protein system was characterized to determine if it shared properties associated with L-arginine transport in other mammalian cells. Prior to the initiation of our study platelet L-arginine transport had not been examined in detail.
Diagnostically, platelet L-arginine uptake was found to be primarily Na\(^+\)-independent. The slight increase in L-arginine uptake in the presence of Na\(^+\) may be due to cross reactivity with other amino acid uptake systems. The competitive inhibition of L-arginine uptake by the cationic amino acids L-lysine and L-ornithine as well as by L-arginine analogue N\(^\text{C}\)-monomethyl-L-arginine was consistent with that of system y\(^+\) from other cells. The degree of inhibition, however, was less than that observed in other cells. That nonradioactive L-arginine essentially diminished L-arginine uptake suggests that the platelet L-arginine carrier appears to preferentially transport L-arginine.

Evidence for the bidirectionality of the platelet L-arginine carrier was obtained from the finding that PPMV L-arginine uptake was trans-stimulated and from L-arginine efflux from preloaded intact platelets. That efflux reached a maximum after about 5 minutes and was thereafter diminished suggests that the effluxed L-arginine was again taken up by the platelet. The platelet L-arginine carrier therefore appears to display a preference for the influx rather than efflux of L-arginine.

Further evidence for a system y\(^+\) type L-arginine transporter was shown by the sensitivity of platelet L-arginine uptake to KCl or NaSCN-induced changes in membrane polarization. As expected for a positively charged molecule, the uptake of L-arginine was enhanced by membrane hyperpolarization (more negative inside) with NaSCN and inhibited by membrane depolarization (more positive inside) with KCl.

The only difference observed from that of system y\(^+\) from most other cells is that platelet L-arginine uptake was found to be slightly pH dependent. This result is different from that of L-arginine uptake reported in human fibroblasts and hepatoma cells where the
uptake was shown to be pH insensitive (White et al., 1982; White and Christensen, 1982). The platelet L-arginine carrier therefore appears to have at least one unique characteristic which suggests the possibility of some degree of heterogeneity in L-arginine carriers from different cells.

The $K_M$ of L-arginine uptake by PPMV was found to be well within the range reported for system $y^+$ from other cells (25 - 200 μM: White, 1985). The $K_M$ was determined using PPMV and not intact platelets mainly because PPMV do not contain L-arginine and are therefore not likely to produce results complicated by trans-stimulation effects or L-arginine degradation. The estimated $K_M$ was close to the reported range of normal plasma L-arginine concentrations (23 - 86 μM: Dickenson et al., 1965).

An experiment designed to determine whether or not L-arginine uptake is altered during ADP-induced platelet aggregation yielded some interesting, though not easily explained, results. The observed increase in L-arginine uptake during aggregation may be due to either an actual increase in influxed L-arginine or to an increase in the amount of L-arginine that binds to the membrane. If L-arginine influx is enhanced during platelet activation, a physiological platelet regulatory mechanism may be at work where the onset of platelet aggregation is antagonized. Further experimentation is required to explain the fate of extracellular L-arginine during platelet activation, because it cannot be ruled out that L-arginine merely binds with an increased affinity to the membranes of activated platelets. An early event in platelet activation, which is associated with the onset of platelet shape change, is the exposure of negatively charged procoagulant lipids on the extracellular surface of the platelet membrane (Mann et al., 1992; Ehrman et al., 1978). It
may be that the positively charged L-arginine binds with an increased affinity to the negatively charged procoagulant platelet membrane.

Inulin is a large oligosaccharide that was used in the transport assays to estimate substrate trapping by the platelets. It was thought that the inclusion of inulin (\(^{14}\)C-inulin) in a dual radioactive label experiment with L-arginine (\(^{3}\)H-L-arginine) would demonstrate whether or not the increased \(^{3}\)H-L-arginine detected in isolated activated platelets was due to influxed L-arginine. The amount of inulin associated with the platelet pellet increased to a maximum of about 2500 % over the control (no ADP). For reasons unknown, the increase in \(^{14}\)C-inulin radioactivity was much greater than that of \(^{3}\)H-L-arginine. Perhaps inulin binds to a receptor that is exposed during platelet activation such as the fibrinogen receptor. It is also difficult to speculate why the observed increase in \(^{3}\)H-L-arginine and \(^{14}\)C-inulin radioactivity was maximal at the earliest measured time point of platelet aggregation and decayed thereafter.

In order for platelet activation to lead to aggregation the regulation of platelet NO production must be tightly controlled. When a weak agonist (\(i.e., not associated with a ruptured vessel\)) is encountered, the NO produced during the initial stages of platelet activation may inhibit aggregation. Platelet aggregation in response to a strong agonist (\(i.e., likely associated with a ruptured vessel\)) may proceed via the inhibition of NO production. The NO feed-back inhibition of either NOS and/or L-arginine transport would allow strong agonist-induced platelet aggregation to occur. Despite the importance of platelet-derived NO in platelet function, the regulation of platelet NO production has not been examined to date. As discussed above, the mechanism by which the transport of
L-arginine into the platelet occurs may be an important and possibly even rate-determining step towards the production of platelet-derived NO.

There is currently much interest in determining the mechanisms that govern the production of NO from L-arginine. Platelet NOS is a Ca$^{2+}$/calmodulin dependent enzyme and is consequently expected to display maximal activity when intraplatelet [Ca$^{2+}$]$_i$ is elevated. Intraplatelet [Ca$^{2+}$]$_i$ is elevated when platelets are activated by a platelet agonist. Increased [Ca$^{2+}$]$_i$ is necessary for platelet aggregation to occur and apparently for the maximal production of NO by NOS. An apparent paradox is evident in that an increased [Ca$^{2+}$]$_i$ appears to both stimulate and inhibit platelet activation. For platelet aggregation to proceed there should be a mechanism for regulating the production of NO and/or its effectors. A situation may be envisioned where at the low [Ca$^{2+}$]$_i$ evoked by weak agonists the NO produced by the platelet would prevent full aggregation. In this hypothetical scenario strong agonists which maximally elevate [Ca$^{2+}$]$_i$ would activate NOS to the point where the intraplatelet concentration of NO would inhibit its own production. There are many potential mechanisms for the regulation of cellular NO production and evidence for several have been reported (Nussler et al., 1994; Assreuy et al., 1993; Michel et al., 1993; Rengasamy and Johns, 1993; Riesco et al., 1993; Bogle et al., 1992).

NOS from murine macrophages has been shown to be irreversible feedback inhibited by NO (Assreuy et al., 1993) while NOS from bovine cerebellum was shown to be reversibly inhibited by NO (Rengasamy and Johns, 1993). Many effects of NO on L-arginine uptake have been reported. Activation of macrophages by lipopolysaccharide induces NO production and an increase in L-arginine uptake due to an increase in the
expression of the system $\gamma^+$ carrier (Bogle et al., 1992). Riesco and coworkers (1993) report that L-arginine uptake in human polymorphonuclear leukocytes is stimulated by endothelin-1 and feedback inhibited by NO through the actions of elevated intracellular cGMP. Bogle et al. (1991) have found that bradykinin stimulates L-arginine uptake in vascular endothelial cells through the actions of NO. They suggest that neither NO itself nor cGMP directly stimulates L-arginine uptake as sodium nitroprusside generates NO and raises cGMP levels but did not affect L-arginine uptake.

We have shown here that the platelet L-arginine transport system was targeted by NO. The effect of exogenous NO on platelet L-arginine uptake was found to depend on the concentration of NO and whether or not the assay was performed under deoxygenated conditions. Low concentrations of NO were found to stimulate L-arginine uptake only when precautions were not taken to exclude oxygen from the incubation medium. The stimulatory effect of NO on platelet L-arginine uptake may, therefore, be attributed to higher oxidation states of NO. The aqueous rate of NO oxidation to $\text{NO}_2^-$ is very rapid in the presence of dissolved oxygen (Ford et al., 1993). NO oxidation to $\text{NO}_2^-$ is expected to occur in the oxygen rich environment of a cell. To the best of our knowledge there are no examples of $\text{NO}_2^-$-specific effects on cell or protein function. Nevertheless, our results indicate a small, but possibly significant, increase in L-arginine uptake due to the presence of $\text{NO}_2^-$ but not $\text{NO}_3^-$. 

One possibility is that an increased intracellular $\text{NO}_2^-$ concentration may hyperpolarize the membrane which would stimulate L-arginine uptake. The inability of $\text{NO}_3^-$ to stimulate L-arginine uptake, however, suggests that the stimulatory effect of $\text{NO}_2^-$
may not be due to membrane hyperpolarization. Since the NO/NO$_2^-$-induced stimulation of L-arginine uptake was not evident in PPMV, an intracellular process such as a cGMP-dependent mechanism dependent mechanism may be involved. The actual stimulation mechanism for L-arginine uptake remains to be determined.

Under deoxygenated conditions and at higher concentrations NO was found to inhibit platelet L-arginine uptake. This inhibition could be a physiological mechanism of feedback regulating the platelet NO production. The inhibition of L-arginine transport by NO may occur during platelet activation. Malinski et al. (1993) reported that at least 4 x 10$^{-18}$ mol/platelet NO is produced during platelet activation. As NO is produced by the platelet a portion will diffuse out of the platelet thereby preventing the accumulation of otherwise toxic, high intracellular NO concentrations. If NO did accumulate inside the platelet the intracellular concentration would likely exceed 0.6 mM (as calculated above). It seems reasonable that during platelet activation the intracellular NO concentration, at any given time, may be sufficient to significantly inhibit L-arginine transport (i.e. > 50 μM).

Though reasonable, it is still uncertain whether or not the inhibition of L-arginine uptake by NO would occur in vivo. The approximate IC$_{50}$ of exogenous NO on platelet L-arginine uptake is high with transport being maximally inhibited by only about 44 %. The [NO] that was actually present during platelet L-arginine transport was likely significantly less than that added initially due to the highly reactive nature of NO. Consequently, in vivo platelet L-arginine transport may be inhibited by lower [NO]. Rengasamy and Johns (1993) have suggested that the high concentrations of NO required
to inhibit bovine cerebellum NOS in vitro may be significantly less in vivo due to the high localized concentration of NO surrounding NOS, the source of NO. If NOS is close in proximity to the L-arginine carrier as is believed (Bogle et al., 1991), the L-arginine carrier would also be exposed to a high localized concentration of NO during platelet activation. The concentration of endogenous NO required to inhibit L-arginine uptake may therefore be much less due to NO channeling to the L-arginine carrier and the high rate of NO addition to thiols or amines.

In contrast to the report by Riesco et al. (1993) with polymorphonuclear leukocytes the inhibition of L-arginine uptake in platelets by NO does not appear to be mediated through a cGMP dependent mechanism. Platelet L-arginine uptake was inhibited by NO in intact platelets and in PPMV. Since vesicles obviously do not contain cGMP synthesis and transducing capabilities the inhibitory effect of NO on platelet L-arginine appears to be due to a direct effect on the system y⁺ carrier. This direct effect could most likely be exhibited through thiol or amine nitrosation.

Two L-arginine binding proteins were isolated by L-arginine affinity chromatography with approximate molecular weights of 35.5 and 140.5 kDa. Neither protein contains a disulfide as identical SDS-PAGE patterns were obtained under reducing and nonreducing conditions. It is very likely, given the high affinity for L-arginine of these proteins, that one or possibly both of these proteins correspond to the platelet system y⁺. The exact identity of the L-arginine carrier protein remains to be established. Limiting the L-arginine carrier protein to one of two (or both) proteins is a significant finding, however, especially since the system y⁺ protein has been identified in few mammalian cells.
The mouse system $y^+$ protein has been found to be the ecotropic murine retrovirus receptor (Kim et al., 1991; Wang et al., 1991). From its cDNA the mouse system $y^+$ has been found to be comprised of 622 amino acids, 14 potential membrane spanning domains and a combined molecular weight of 67 kDa (Albritton et al., 1989). Radiation inactivation analysis has shown that system $y^+$ activity of the rat kidney cortex corresponded to a 90 kDa protein (Beliveau et al., 1990). System $y^+$ activity from rabbit kidney cortex was associated with an average mRNA chain length of 1.8 to 2.4 kb (Bertran et al., 1992). The difference in the reported molecular weights for system $y^+$ suggests some degree of species-dependent heterogeneity for system $y^+$.

To summarize, platelet L-arginine uptake has been found to be mediated by system $y^+$. The platelet L-arginine transport system appears to be differentially feed-back regulated by NO. Low [NO] in the presence of oxygen leads to the stimulation of L-arginine uptake while high [NO] inhibits L-arginine uptake. Two L-arginine-binding platelet membrane proteins were isolated by affinity chromatography. Whether or not either or both of these proteins are associated with platelet L-arginine transport remains to be determined.

5.7. Photolysis of S-Nitrosoglutathione: Potential Photochemotherapeutic Applications

Our interests in GSH and NO metabolisms led to an investigation of their reaction product, S-nitrosoglutathione (GSNO) (Section 1.4.1). GSNO may function physiologically as a scavenger or storage pool of NO. GSNO decomposes readily under physiological conditions to release NO and may, therefore, represent a means of
prolonging the effects of NO. Early in our investigation on the decomposition rates of GSNO we discovered that GSNO can be photolyzed to release NO (Fig. 37).

Nitric oxide has been shown to be important immunologically in the action of cytotoxic activated macrophages upon tumor cells (Li et al., 1991; Keller et al., 1990; Thompson et al., 1990; Stuehr and Nathan, 1989). The cytotoxic effects of NO in the immune system has created interest in existing compounds and in generating compounds which release NO and thereby inhibit tumor cell growth (Maragos et al., 1993). As a cytotoxic agent NO inhibits key metabolic pathways to block growth or kill cells outright. We have shown here that with HL-60 cells there is not a massive instantaneous cell death from GSNO treatment and photolysis as evidenced by no significant increase in the extent of trypan blue staining after treatment. Rather, the effects of GSNO are more long-term which is consistent with the inhibition of some aspect(s) of cellular metabolism (Fig. 40).

The site of action on susceptible cells appears to have been traced to enzymes that contain catalytically active non-heme iron coordinated to sulfur atoms (Hibbs et al., 1988). NO has also been shown to react with aconitase to inhibit mitochondrial respiration (Hibbs et al., 1988, to inhibit protein synthesis (Curran et al., 1991), and to cause the release of iron from ferritin (Reif and Simmons, 1990). Upon introduction of NO to these enzymes, the Fe-S clusters are destroyed with the concomitant loss of iron. Recently NO has also been implicated in the destruction of essential tyrosine free radicals in the active site of ribonucleotide reductase (Lepoivre et al., 1991), a necessary enzyme for DNA synthesis.

Due to the short lifetime of NO in biological solutions of ~4 s or less (Stamler et al., 1992) NO-donors offer the possibility to function as anti-tumor agents with localized
effects. The problem would be to produce NO at the desired site. Compounds that release NO photolytically, especially with visible light, would be appealing photochemotherapeutic agents especially if their spontaneous decomposition rate to NO were negligible. The tissue of interest could be preferentially irradiated.

HL-60 cells that were exposed to GSNO for only 2 hours exhibited a significant difference in the LD_{50} values between the light and dark conditions (Fig. 40A). In contrast, the photoinduced cytotoxic effect was negligible when cells were continuously exposed to GSNO (Fig 40B). This is not surprising as GSNO decomposes in the absence of light. Clearly then, GSNO is not an entirely suitable photochemotherapeutic agent due to its dark decomposition rate as judged by the continued cytotoxicity over several days of exposure. However, GSNO is a useful model compound for providing \textit{in vitro} evidence for the anti-tumor potential of S-nitrosothiols.

To rule out the possibility that GSNO decomposition and photodecomposition was an artifact of purging a solution of GSNO with nitrogen gas, nitrite, a stable oxidation product of NO, was measured as a function of the time of photoirradiation in an unpurged GSNO solution. Under the conditions employed nitrite formation was observed in the presence of visible light but very little was detected in the dark (Fig. 39). The low rate of dark GSNO decomposition, as measured by NO\textsubscript{2}\textsuperscript{-}, may be partly due to the large dependence on the concentration of NO in the reaction with O\textsubscript{2} to form NO\textsubscript{2}\textsuperscript{-} (Ford \textit{et al.}, 1993). At low concentrations of NO it is possible that very little NO\textsubscript{2}\textsuperscript{-} is formed.

Photolysis of GSNO in a buffered solution yields substantially more NO (measured as NO\textsubscript{2}\textsuperscript{-}) than when in the media. This difference is most likely due to the presence of
other targets for NO in the media and due to the eventual formation of NO$_3^-$ which will compete with the cells for available NO. The concentration of NO detected in the media therefore represents the approximate amount exposed to the cells.

The HL-60 cells were more sensitive to GSNO under conditions of continuous exposure than 2 hour pulsed exposure. This was not unexpected as high concentrations of NO, such as that formed by GSNO photolysis, have a shorter lifetime in solution than low NO concentrations such as that obtained by dark GSNO decomposition (Ford et al., 1993). Others have also found that compounds that release NO slowly are more cytotoxic than those that release NO rapidly (Maragos et al., 1993). Cells exposed to NO only during GSNO photolysis (2 hours) were also able to recover to some degree after 38 hours of treatment. Presumably, some aspect(s) of the cytotoxic effect of NO is reversible. The cytotoxicity of light from the filtered quartz halogen lamp is most likely attributable to the effects of UV-A light on cells (Smith, 1989; Rahn, 1979).

As a consequence of our finding that GSNO is photolyzed to release nitric oxide, we suggest that precautions be taken to conduct experiments in the absence of light when attempting to ascertain the physiological effects of intact GSNO. It is interesting to note that none of the reports on GSNO, of which we are aware, state that the experiment(s) were performed in the dark.

In conclusion, the preliminary evidence presented here of the cytotoxic effect of GSNO irradiation suggests that GSNO, and potentially related compounds, possess properties that make them ideal candidates for visible light phototherapy.
Chapter 6

CONCLUSIONS

Our investigations into platelet GSH and L-arginine/NO metabolism suggests that intra-platelet GSH \([\text{[GSH]}_p]\) and platelet-derived NO exhibit regulatory roles in platelet activation. The identification of new regulatory mechanisms of platelet activation may find application in the etiology and treatment of pathological vascular conditions such as cerebral or myocardial infarctions. The antagonization of platelet activation by the established anti-aggregation agents, endothelium-derived NO and prostacyclin (PGI\(_2\)), is unlikely to regulate the initiation of platelet activation. Since endothelium-derived NO and PGI\(_2\) are produced by activated endothelial cells, under basal conditions they are not expected to influence the onset of platelet aggregation in the primary platelets. Primary platelets refer to those platelets that are activated first in the platelet aggregation cascade. Endothelium-derived NO and PGI\(_2\) are more likely to modulate the aggregation of additional platelets once the aggregation cascade is underway. In contrast, \([\text{[GSH]}_p]\) and platelet-derived NO may represent a means of regulating the onset of irreversible platelet aggregation by the platelet, itself. Under certain situations it may be advantageous for platelets, themselves, to be able to regulate their own activation. In the absence of the internal platelet regulation of platelet activation endothelium-derived antagonists may arrest aggregation only after it has begun. The resulting, presumably small, circulating platelet aggregates may lead to cerebral or myocardial infarctions. Internal platelet regulatory mechanisms of platelet activation may also be employed when platelets reversibly aggregate to prevent the loss of blood from stressed capillaries (Section 1.2.2).
The regulation of platelet activation by [GSH]_p appears to occur via two distinct mechanisms. One way that [GSH]_p modulates platelet activation has been previously suggested to be through the regulation of the production of the potent platelet release reaction agonist thromboxane A₂ (TxA₂) (Thomas et al., 1986b). [GSH]_p is believed to inhibit TxA₂ biosynthesis by preventing the peroxide-induced activation of cyclooxygenase, an important enzyme in one of the transformation steps of arachidonic acid to TxA₂. Our data on the effects of either a slight reduction or elevation in [GSH]_p on platelet aggregation are in agreement with this hypothesis. The effects of elevated [GSH]_p on platelet function had not been previously examined possibly because it was not known that platelets were capable of taking up intact GSH.

We discovered that platelets contain a specific, high affinity GSH transport system that is capable of influxing the low levels of GSH present in the plasma. Since the platelet GSH transport system was found not to share all the properties of any single previously characterized GSH transport, we conclude that it must represent a distinct isoform. Platelet GSH transport, and possibly GSH transport systems in general, appear to be under intracellular thiol redox control. We recommend the reexamination of tissues previously thought not to express a GSH transport system since thiol redox regulation may prevent the detection of a GSH transport system.

One potential in vivo consequence of elevated [GSH]_p is the feed-back inhibition of glutathione reductase (GR). We demonstrated that the GSH inhibition GR was subject to species-dependent differences with respect to the observed inhibition mechanisms. These differences are novel examples of heterogeneity in an otherwise conserved enzyme.
Depending on the cellular situation the inhibition of GR by elevated GSH may lead to either an unchanged or a reduced GSH:GSSG ratio. If the cell is currently under oxidative stress so that GSSG is elevated the inhibited GR will not be able to prevent GSSG accumulation and the reduction of the intracellular GSH:GSSG ratio. Platelets are under oxidative stress during platelet activation so the GSH:GSSG ratio may be lowered when [GSH]_p is elevated. Alterations in the intracellular ratio of GSH:GSSG has been shown to affect the activity of redox-sensitive proteins and enzymes (Gilbert, 1990; Ziegler, 1985).

The second way in which [GSH]_p regulates platelet activation may involve the alteration of the activity of redox-sensitive proteins that are critical to the onset of aggregation. When [GSH]_p was more substantially depleted with high concentrations (1 mM) of CDNB or the novel GSH-depleting thiol reagent 1-(4-Chlorophenyl)-4,4-dimethyl-5-diethylamino-1-penten-3-one hydrobromide (CDDP) platelet aggregation was inhibited. Both thiol reagents depleted [GSH]_p by approximately the same extent but CDNB inhibited aggregation much more than CDDP. CDDP may therefore be a more specific GSH-depleting agent than CDNB. In fact it was shown that CDNB is a more potent inhibitor of GR. The CDNB inhibition of GR may account for the near inactivation of platelet aggregation by CDNB since elevated intra-platelet GSSG has been previously shown to inhibit platelet aggregation (Caruso et al., 1984).

In the course of this part of the study it was observed that CDDP is an alpha class selective GST substrate and that certain analogs of CDDP are GST inhibitors. As a alpha class selective substrate CDDP may find use in the assessment of GST isozyme tissue
distribution. The GST inhibitors are 3,5-bis (benzylidene)-4-piperidones that varied only in the degree of N-piperdone substitution (from secondary to quaternary). Increased inhibition of GST by these compounds appears to have been associated with the lack of a positively charged piperdone nitrogen.

Since extracellular L-arginine was shown to inhibit platelet aggregation it appears that platelet-derived NO regulates the onset of aggregation. This result and various reports in the literature suggests that L-arginine uptake becomes the rate-determining step towards NO production during platelet activation once internal L-arginine has been consumed (Malinski et al., 1993; Radomski et al., 1990). We characterized platelet L-arginine uptake and showed for the first time that it is mediated by the cationic amino acid transporter, system y'. Platelet L-arginine transport was feed-back regulated in vitro in a dual concentration-dependent manner by NO. Low NO concentrations appeared to stimulate L-arginine uptake by intact platelets while at higher concentrations uptake was inhibited. Our data suggests that the stimulation of L-arginine transport may be mediated by the oxidation of NO together with an intracellular event(s) while NO inhibition may be attributed to a direct nitrosylation of the L-arginine carrier protein.

S-nitrosoglutathione (GSNO), the product of the reaction between GSH and NO was found to be photolysed by visible light. The release of NO by GSNO photolysis resulted in an enhanced NO-dependent cytotoxicity towards HL-60 cells. GSNO, or related compounds, may therefore find use as photochemotherapeutic agents.
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PUBLICATIONS


ABSTRACTS


