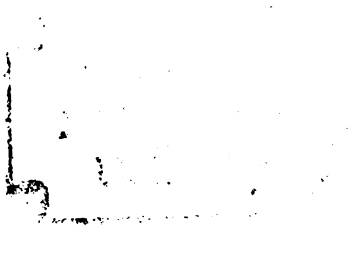

This is to certify that the

thesis entitled

SUBCELLULAR LOCALIZATION OF PGH SYNTHASE

presented by

Thomas Edmund Rollins

has been accepted towards fulfillment
of the requirements for

M.S. degree in Biochemistry

William L. Smith

Major professor

Date April 9, 1981

SUBCELLULAR LOCALIZATION OF PGH SYNTHASE

By

Thomas Edmund Rollins

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1981

ABSTRACT

SUBCELLULAR LOCALIZATION OF PGH SYNTHASE

By

Thomas Edmund Rollins

There were two phases to these studies. In the first phase the subcellular location of PGH synthase in Swiss mouse 3T3 cells was determined by electron microscopic immunocytochemistry using specific anti-PGH synthase IgG in the peroxidase anti-peroxidase staining procedure. Electron dense deposits were found associated with the endoplasmic reticulum and nuclear membrane in cells stained using immune but not preimmune IgG.

In the second phase the transverse orientation of PGH synthase in the microsomal membranes of sheep vesicular glands was deduced on the basis of the susceptibility of the synthase to protease digestion and the ability of the enzyme to interact with specific monoclonal antibodies. Protease treatment of intact sheep vesicular gland microsomes caused the destruction of cyclooxygenase activity. When the active site of PGH synthase was labelled with [^{3}H]-acetylsalicylic acid and the microsomes incubated with protease, 90% of the tritium label was cleaved from the membrane. Three monoclonal antibodies which interact with different determinants in the pure PGH synthase were found to interact with the PGH synthase present in intact microsomes. The antigenic reactivity of the microsomal enzyme was unaffected by protease digestion.

My experiments indicate (a) that the PGH synthase is localized on the cytoplasmic surface of the endoplasmic reticulum and nuclear membranes, (b) that an active site fragment of PGH synthase can be cleaved from the membrane by treatment with proteinase K and (c) that three antigenic sites on the PGH synthase are resistant to protease digestion.

To Grae

ACKNOWLEDGEMENTS

I would like to express my appreciation of the financial support and professional guidance given me by my major professor, Dr. William Smith. His ability to patiently guide me through my tenure here has made my stay enjoyable. I am grateful for the stimulating discussions and constructive criticism from my lab friends Frank Grenier, Arlyn Garcia Perez, Dave DeWitt, Jeff Day and Martha Kabalin.

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ABBREVIATIONS

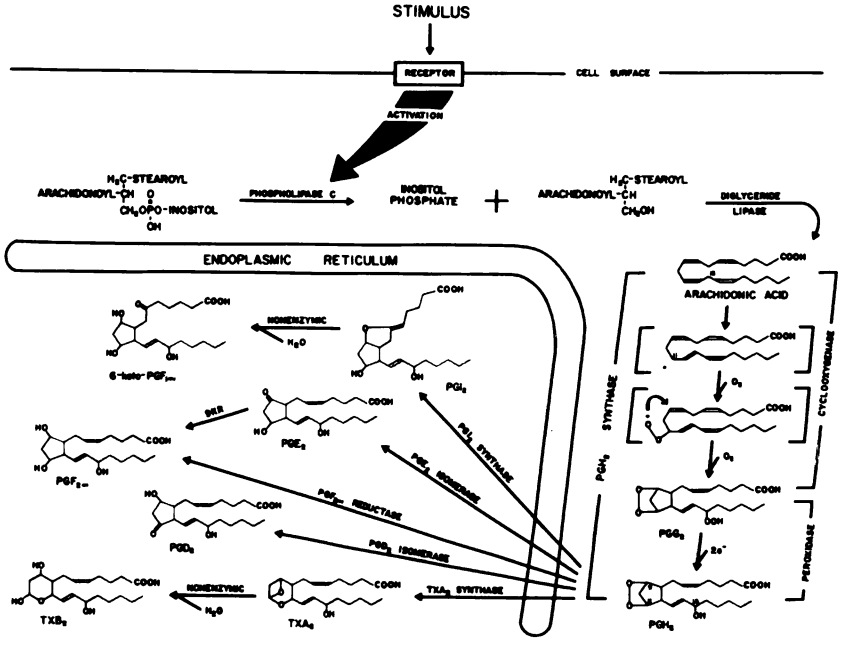
PG	prostaglandin
PGH	prostaglandin endoperoxide
20:4	arachidonic acid
BSA	bovine serum albumin
DDC	diethyl dithiocarbamate
PI	phosphatidyl inositol

INTRODUCTION

The study of prostaglandin metabolism has expanded tremendously since the early 1960's. Competitive and noncompetitive inhibitors have been developed for the major prostaglandin (PG) biosynthetic enzymes and new, more specific inhibitors are being sought. A new class of compounds, the leukotrienes, which are related biosynthetically to the prostaglandins, have recently been identified. Still we know relatively little about the properties of the enzymes involved in prostaglandin synthesis or how biosynthesis is regulated in the cell. The studies presented in this thesis have focused on determining the subcellular location of the PGH synthase.

PGH synthase is the first enzyme of prostaglandin biosynthesis. The enzyme contains both cyclooxygenase and peroxidase activities (1-4). The cyclooxygenase catalyzes the oxygenation of fatty acids, such as arachidonic acid, leading to the formation of the cyclic endoperoxide, PGG_2 (5), Figure 1. The peroxidase activity uses PGG_2 as a substrate reducing the hydroperoxy group, at position 15, to the hydroxyl group of PGH_2 (3,4). The prostaglandin endoperoxide, PGH_2, is then converted by other enzymes in the pathway to the corresponding prostaglandins and thromboxanes. The cyclooxygenase activity has a limited substrate specificity while the peroxidase catalyzes the reduction of a variety of structurally diverse hydroperoxides, Figure 1.

Figure 1. Prostaglandin biosynthetic pathway.


STIMULUS
RECEPTOR
CELL SURFACE
ACTIVATION
PHOSPHOLIPASE C
INOSITOL PHOSPHATE
DIGLYCERIDE LIPASE
ENDOPLASMIC RETICULUM
ARACHIDONIC ACID
CYCLOOXYGENASE
PEROXIDASE
PGH SYNTHASE
6-keto-PGF1α
PGI2
PGE2
PGF2α
PGD2
TXB2
TXA2
PGH2
PGI2 SYNTHASE
PGE2 ISOMERASE
PGF2α REDUCTASE
PGD2 ISOMERASE
TXA2 SYNTHASE
NONENZYMIC
9αR

PGH synthase is a membrane-bound, heme-containing glycoprotein with a subunit molecular weight of about 72,000 (1,2,4). The enzyme exists as a dimer with identical subunits. Each subunit binds one heme molecule which functions in both the oxygenase and peroxidase reactions. Mn^{++}-heme can substitute for Fe^{++} heme in the oxygenase but not the peroxidase reaction (6). These findings are consistent with observations which indicate that specific inhibitors of PGH synthase block only the cyclooxygenase activity without affecting the peroxidase activity (1,7).

Acetylsalicylic acid is a specific, irreversible inhibitor of PGH synthase and acts by acetylating an active site serine hydroxyl group (1). In this work I will demonstrate that after labelling the enzyme in sheep vesicular gland microsomes with [^{3}H]acetylsalicylic acid that most of the label is solubilized by proteinase K treatment.

There are essentially two approaches which can be used to determine the subcellular location of an enzyme. The first is to separate cellular organelles by differential centrifugation and then determine with which fraction the activity is associated (8,9). Contamination of one fraction of organelles with another fraction is a common problem in trying to localize enzymatic activity using this approach. A second approach is to use antibodies directed against specific antigens to localize cellular proteins and enzymes (10-18). In this thesis, I will show the specificity of IgG against anti-PGH synthase and demonstrate its use in localizing the PGH synthase by electron microscopy. The advantages of the immunocytochemical approach are that it is not necessary to separate cellular organelles to elucidate the location of a specific component and that if the antibody is monospecific there is little question of cross contamination.

Monoclonal antibodies are specific for single antigenic determinants (19). To generate monoclonal antibodies, splenic lymphocytes from immunized mice are fused with myeloma cells to produce long-lived hydridoma cell lines. These lines produce large amounts of monospecific antibodies against the antigen of interest. Monoclonal antibodies can be used to identify the presence of a specific cell type or cell membrane characteristic (20), or to localize specific antigens intra- and extracellularly. Monoclonal antibodies may also be used in immunoaffinity chromatography to purify RNA or proteins. In this thesis I will describe the use of four monoclonal antibodies directed against the PGH synthase in localizing and quantitating the enzyme.

The studies in this thesis address three questions regarding the PGH synthase. (a) Where in the cell is the PGH synthase located? (b) On which side of the membrane is the enzyme located? and (c) What are the spatial relationships between the active site and the antigenic determinants on the PGH synthase molecule.

LITERATURE REVIEW

Prostaglandin Synthesis

Prostaglandins are a complex group of oxygenated fatty acids which have been detected in nearly all mammalian tissues. Prostaglandins are not stored in tissues but are synthesized in response to stimuli that cause the release of free fatty acids. A variety of factors can induce fatty acid release including inflammatory stimuli (21,22), calcium ionophores (23), tumor promoters (24), hormones (25), and mechanical agitation (26). The fatty acid released in response to specific stimuli (e.g. hormones) is generally 5,8,11,14-eicosatetraenoic acid, arachidonic acid. Free arachidonic acid acts as a substrate for PGH synthase, the first enzyme in the prostaglandin biosynthetic pathway. This enzyme converts arachidonic acid to the endoperoxide PGH_2, which is then converted to a variety of biologically active products, the nature of which is determined by the enzyme content of the tissue in question. For example, PGI_2 is found to be synthesized by endothelial cells while thromboxane A_2 is synthesized by platelets. The pathways for formation of various prostaglandin derivatives appears in Figure 1. One can visualize prostaglandin production as occurring in three steps. The first is stimulus and release of arachidonic acid. The second is the conversion of arachidonic acid to PGG_2 and PGH_2 via the PGH synthase reaction. And the third phase is the conversion of PGH_2 to the biologically active

compounds. The release of arachidonate is believed to be the major control point for prostaglandin synthesis.

Two mechanisms exist for the release of free arachidonate from phosphoglycerides. The first mechanism involves release of arachidonate from its normal esterified form at the 2-position of cellular phosphoglycerides through the action of a phosholipase A_2. It is believed that a stimulus acts upon the cell surface to produce a second messenger which stimulates the action of the phospholipase A_2 (27). It has been shown that activation of phospholipase A_2 in response to a toxin results in a selective release of arachidonic acid in 3T3 mouse fibroblasts (28). A second mechanism has been proposed which involves thromboxane synthesis by platelets; the importance of this mechanism has not been established in other cell types (29,30). In this alternate pathway two enzymes work sequentially to release free arachidonate from phosphatidylinositol. The first enzyme, phospholipase C, cleaves phosphatidylinositol specifically to yield the diglyceride and inositol phosphate. The next enzyme, diglyceride lipase, releases the fatty acid from the 2-position (29). In platelets it has been shown that nearly 90% of phosphatidyl inositol exists as 1-stearoyl 2-arachidonoyl phosphatidylinositol. Therefore, the sequential action of these two enzymes results in a selective release of arachidonic acid by platelets (30). It has been shown recently that platelets stimulated with thrombin selectively release arachidonate as the major free fatty acid. Following release of 20:4, phosphatidyl inositol is resynthesized. This newly formed phosphatidylinositol has a fatty acid composition different from the parent molecule but then undergoes a deacylation-reacylation process which results in the unique 1-stearoyl 2-arachidonoyl species (30). How phospholipase C is

stimulated by thrombin or other agents is unresolved, but mobilization of intracellular Ca^{++} is apparently involved (31,32).

The conversion of arachidonic acid to PGH_2

The PGH synthase which contains two enzymatic activities converts arachidonic acid to PGG_2 via a cyclooxygenase (33) and then to PGH_2 by a nonspecific peroxidase. This enzyme has been purified to electrophoretic homogeneity (3). Most of the information to date comes from studies of the sheep vesicular gland enzyme, although the rabbit kidney enzyme has similar kinetic and antigenic properties (34).

The first step in the synthesis of PGH_2 involves a stereospecific protium abstraction from C_{13} of arachidonic acid (Figure 1). The abstraction is followed by rearrangement of a double bond between C_{11} and C_{12} to yield a carbon centered radical at C_{11} (34,35). Molecular oxygen then adds to the radical to yield an 11 hydroperoxy radical. This radical attacks C_9 and a series of rearrangements occur leading to the formation of a cyclopentane ring encompassing C_8 through C_{12} and a new carbon radical at C_{15}. A second molecule of oxygen reacts with the radical at C_{15} to yield a 15-hydroperoxy radical. The H atom on the hydroperoxyl group is initially from the protium abstraction at C_{13}. The peroxidase activity along with a reducing agent acts upon this hydroperoxyl group at C_{15} of PGG_2 to yield the PGH_2 (36). PGG_2 and PGH_2 are short lived intermediates but may function *in vivo* since both have biological activity (37,38).

Metabolic regulation of PGH synthase

Synthesis, degradation, feedback inhibition and availability of

substrates are a few ways the PGH synthase is regulated. Availability of arachidonic acid is the rate limiting step. Heme is required for both the cyclooxygenase and peroxidase reactions. Therefore, the rate of formation of PGH_2 is directly dependent upon how effectively PGH synthase competes for heme with other proteins.

After synthesizing a limited number of endoperoxides, the enzyme itself becomes irreversibly inactivated (33). This will eventually lead to a decreased rate of production of prostaglandins unless a cell synthesizes new PGH synthase. The irreversible inactivation probably stems from the generation of free radicals during the formation of PGG_2 and PGH_2; the oxidant generated may cause protein degradation. The resynthesis of PGH synthase is affected by various hormones and other stimuli (34). This self-catalyzed destruction of PGH synthase may be one mechanism by which PGH synthase levels are regulated.

Pharmacological regulation of PGH synthase

Nonsteroidal anti-inflammatory drugs selectively inhibit the PGH synthase. The action of nonsteroidal anti-inflammatory drugs on the cyclooxygenase necessarily regulates the synthesis of all subsequent compounds. The initial abstraction of the hydrogen atom from C_{13} of arachidonic acid is apparently inhibited by these drugs. Two distinct classes of inhibitors exist. The first class includes ibuprofen, flufenamic and mefenamic aicd which are relatively weak, reversible inhibitors. Indomethacin, flurbiprofen and meclofenamic acid are more potent irreversible compounds as compared to the first class (35). All irreversible inhibitors cause a time-dependent inactivation of cyclooxygenase activity. Phenolic compounds reduce the inhibitory capacity of irreversible

inhibitors but enhance inhibition by the reversible inhibitors (35). Effective removal of the reversible inhibitors by dilution, metabolism, or increases in fatty acid substrate concentrations restore cyclooxygenase activity. To recover PGH synthase activity following exposure to irreversible inhibitors requires new synthesis of the enzyme. The mechanism of irreversible cyclooxygenase inhibition has not been resolved. Indomethacin and flurbiprofen, although irreversible inhibitors, cause no covalent modification of the cyclooxygenase. These agents must cause structural changes in the cyclooxygenase which may or may not be reversible *in vivo*. Acetylsalicylic acid, another irreversible inhibitor does acetylate an internal serine hydroxyl on the enzyme (1). This covalent binding, however, does not strictly parallel enzyme inactivation (37).

Conversion of PGH_2 to other prostaglandins

$PGF_{2\alpha}$, PGD_2, PGE_2, TxA_2, and PGI_2 are the major physiologically active prostaglandins. All of these products with the exception of $PGF_{2\alpha}$ can be derived from PGH_2 in a single enzymatic step. PGD_2 is synthesized via a PGH-PGD isomerase (38,39), PGI_2 by prostacyclin synthase (40), thromboxane A_2 by TxA_2 synthase and PGE_2 via a PGH-PGE isomerase. It is not clear whether $PGF_{2\alpha}$ is synthesized directly by reduction of PGH_2 or indirectly from PGE_2 through a 9-keto PGE_2 reductase (41).

All these enzymes are membrane bound with the exception of the PGH-PGD_2 isomerase and the 9-keto PGE_2 reductase. These membrane-bound enzymes probably occur in close association with the PGH synthase (42, 43). Only the PGH synthase and the PGH-PGD isomerase have been purified to electrophoretic homogeniety (3,38).

The PGH-PGD isomerase has a molecular weight of approximately 85,000. It is a cytosolic enzyme and is stabilized by thiol compounds, but thiols are not required for enzymatic activity (38,39).

Distinct cell types seem to synthesize only one major type of prostaglandin. For example, human platelets appear to form mainly TxA_2 (44), bovine endothelial cells produce prostacyclin, PGI_2 (45), and rabbit kidney collecting tubule cells produce mainly PGE_2 (56). The reason why differentiated cells produce a single prostaglandin is not known. Although different stimuli can cause prostaglandin release by one given cell type, changing the stimulus does not affect the type of prostaglandin formed. For example, endothelial cells stimulated by thrombin, trypsin, or Ca^{++} ionophore release only PGI_2. This suggests that endothelial cells have only PGI_2 synthase and lack other enzymes that use PGH_2 as a substrate. Apparently, cells are programmed during development to produce specific prostaglandin biosynthetic enzymes. In any given cell the specific activity of enzymes which use PGH_2 as a substrate are substantially higher than the specific activity of PGH_2 synthase. Therefore, most of the PGH_2 synthesized is probably transformed enzymatically to a specific product in differentiated cells.

Prostaglandin catabolism

Prostaglandins are catabolized rapidly *in vivo*. TxA_2 is hydrolyzed nonenzymatically to TxB_2 with a $t_{1/2}$ = 30 sec at 37°. This hydrolysis is probably the major mechanism for inactivation of TxA_2. TxB_2 has no known biological activity.

There are Type I and Type II 15-hydroxy prostaglandin dehydrogenases which oxidize the hydroxyl group at position 15 of PGE_2, PGD_2,

$PGF_{2\alpha}$ and PGI_2 thereby converting these prostaglandins to inactive 15-keto forms. Type I utilizes NAD^+ and type II utilizes $NADP^+$ as the hydride acceptor (47). The type I dehydrogenase has been partially purified from swine kidney cortex while type II enzyme has been purified to homogeneity from swine kidney medulla. The 9-keto prostaglandin reductase is associated with the type II dehydrogenase (48).

Another enzyme of prostaglandin catabolism is the 15-keto prostaglandin $D^{\Delta 13}$ reductase which reduces the double bond between C_{13} and C_{14}. This enzyme has been purified from bovine lung (49). There also exists a 9-hydroxy prostaglandin dehydrogenase which oxidizes the hydroxy group at position 9 (50).

Mechanism of action of prostaglandins

All prostaglandins are probably effective within or near the cells in which they are synthesized. PGI_2 is the only known prostaglandin that may act as a circulating hormone although this concept has been disputed (51). Prostaglandins act on different cell types to stimulate the action of adenylate cyclase to release cAMP (75). It is not clear, however, if prostaglandins can sometimes cause a direct effect or always act via a secondary messenger.

One physiological model that has been proposed involves prostaglandin synthesis as one of the participants in the first line of defense in hemostasis. Platelets from the bloodstream and endothelial cells which line the vasculature release thromboxane A_2 and PGI_2, respectively, in response to blood vessel damage. Thromboxane A_2 has a half life of approximately 30 seconds at 37° and is the most potent of the eicosanoids in contracting aortic tissue and triggering platelet aggregation. In

contrast, PGI_2 is a vasodilator and prevents platelet aggregation. In response to a damaged vessel wall, blood platelets release a variety of products one of which is PGH_2. PGH_2 is thought to migrate to nearby endothelial cells which then convert PGH_2 to PGI_2. PGI_2 increases adenylate cyclase activity in platelets raising intracellular cAMP levels; this, in turn, prevents further TxA_2 production by inhibiting arachidonic acid release. This interplay between TxA_2 and PGI_2 permits platelets to aggregate at injured sites on arterial walls, without occluding the vessel. In this situation one sees two different prostaglandins from two distinct cell sources affecting a biological response. In many biochemical mechanisms it is not the effect of one prostaglandin which controls a response but the ratio of two compounds involved in the process.

Summary

Prostaglandins are "local" hormones which affect cells near their site of synthesis. The overall synthesis is controlled primarily at the level of release of arachidonic acid. Individual cell types capable of prostaglandin synthesis produce only one major type of prostaglandin in response to different stimuli. Degradation of prostaglandins leads to inactive biological compounds. Two types of nonsteroidal anti-inflammatory drugs now exist for the cyclooxygenase and new inhibitors of PGI_2 synthase and TxA_2 synthase are being developed. These new inhibitors should help elucidate the biochemical mechanisms underlying the actions of these two hormones.

Membranes as functional boundaries

The phospholipid bilayer is thought to be the basic component of which virtually all biological membranes are made. A number of theories exist to describe how proteins are associated with and assembled into the membrane. It is clear that there is an intimate relation between the membrane and the protein in terms of structure and function. This section of the review will discuss (a) how proteins are situated in the endoplasmic reticulum, (b) the interrelationships of membrane lipids and proteins and (c) current theories of how proteins are assembled into biological membranes.

Eukaryotic cells contain a variety of membranous organelles which may have arisen from prokaryotic ancestors (52). These membranes apparently exist to compartmentalize functions and are thereby play an important role in metabolic control (63). For example, the nuclear membrane of eucaryotic cells separates the nucleus from the cytoplasm and thus transcription from translation. Intracellular membranes also contain proteins of the electron transport mechanism and enzymes of steroid and phospholipid metabolism. Other membrane proteins may act to control electrochemical gradients across intracellular membranes (108).

Components

Biological membranes are composed of glycerophospholipids and cholesterol derivatives arranged in bilayers with polar head groups at the two surfaces. The lipid comprising the membrane seems to have no special structural relation to the membrane proteins (53). Two general types of membrane proteins occur. The peripheral proteins are hydrophilic but contain small nonpolar regions which bind to the membrane. These

proteins can be dissociated by treatment of membranes with high concentrations of salt. In contrast, integral membrane proteins are more tightly associated with the membrane sometimes spanning the bilayer. This high degree of association is due mainly to the large number of hydrophobic sequences found in these proteins. Most lipids diffuse rather rapidly in the plane of the bilayer (54), while the movement of proteins is less rapid (55). There is little evidence that either phospholipids or proteins rotate transversely (flip-flop) in the membrane (56-58).

Using purified specific phospholipases it has been found that phospholipid species are distributed assymetrically in the transverse plane of the membrane of microsomes. It is interesting to note here that in the rat liver endoplasmic reticulum most of the phosphatidyl inositol, PI, which is a prostaglandin precursor in platelets is found on the luminal side of the endoplasmic reticulum (62). In platelets, a specific phospholipase C, utilizes PI along with a diglyceride lipase to release 20:4 for PGH synthase. Phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol are all synthesized on the cytoplasmic surface of the endoplasmic reticulum (63). The phospholipids are then integrated into the membrane of the endoplasmic reticulum.

The endoplasmic reticulum

Endoplasmic reticulum is an intracellular network of tubules, vesicles, and lamellae (63). The functions of this organelle include protein synthesis and transport, synthesis of phospholipids, cholesterol and triglycerides, and metabolism of xenobiotics. In a cell devoted to protein synthesis, 19% of the total protein, 48% of the total phospholipid

and 58% of the total RNA is associated with the endoplasmic reticulum. The membrane itself is approximately 60-70% protein and 30-40% phospholipid by weight (64,65). At least 34 polypeptides have been identified with the endoplasmic reticulum of rat liver (66). Phospholipid composition varies with cell type. For example, platelets contain relatively large amounts of phosphatidyl inositol while rat hepatocytes contain very little.

The endoplasmic reticulum is easily and extensively disrupted by gentle homogenization. This disruption causes the endoplasmic reticulum to be pinched off into spheres of membrane called microsomes. These microsomes are formed with their cytoplasmic side out, luminal side in (67). Most proteins are located on only one side of the microsomal sphere although there are a few examples of proteins which traverse the bilayer. It is relatively easy to study proteins present on the cytoplasmic side of the microsomes. Microsomes are impermeable to exogenous proteins and even small charged molecules.

Topology in the transverse plane

To ascertain the transverse distribution of an enzyme in the endoplasmic reticulum one can determine whether that protein, as it exists in intact microsomes, is accessible to proteases, antibodies and other probes to which the membrane is impermeable (61). This impermeability should never be assumed, but tested with each probe to be used.

Microsomes have been shown to be impermeable to uncharged molecules greater than 600 molecular weight and to charged substances with molecular weights as low as 90. Treatment of intact microsomes with proteases does not destroy the permeability of the membrane (68). All microsomal

enzymes that have been investigated have been found to have their catalytic activity associated with only one side of the membrane. This suggests that all proteins are arranged in the membrane with specific orientations.

Binding of membrane proteins

The membrane-bound protein which has been investigated most extensively is cytochrome b_5. It is an integral membrane protein of the endoplasmic reticulum (69-72). In studies involving protease treatment of rat liver microsomes, the cytochrome b_5 has been found to have a catalytically active, hydrophilic head and a noncatalytically active hydrophobic tail. Detergent-solubilized cytochrme b_5 has a molecular weight of 16,700 while a protease-derived cytochrome b_5 has a molecular weight of 11,000. The difference in size is due to a relatively hydrophobic amino terminus having 44 amino acid residues (73). The hydrophobic segment of the cytochrome b_5 is thought to anchor the protein in the endoplasmic reticulum. The other portion of the molecule extends into the cytoplasm where it can interact with substrates. NADH-cytochrome b_5 reductase (70) and NADPH cytochrome C reductase also bind to the endoplasmic reticulum in a manner similar to that found for cytochrome b_5 (59).

Topology in the lateral plane and lipid-protein interrelationships

Techniques of subfractionation (59), freeze fracture electron microscopy (60), and reconstitution of isolated components have been used to study enzyme topology in the lateral plane of the membrane bilayer.

These techniques allow one to investigate specific associations between membrane proteins and the phospholipids surrounding them.

Prior to 1978 phospholipids were thought to be relatively inert, serving as a support matrix for membrane-bound protein. Hirata and Axelrod showed that phosphatidyl ethanolamine is methylated on the inside of the membrane of erythrocyte ghosts by a methyl transferase I and transported to the outside of the membrane by methyl transferase II, which also methylates CH_3-phosphatidyl ethanolamine to dimethyl-PE and finally to phosphatidyl choline. They proposed that a specific interaction of phospholipid and protein is essential for these processes to occur (74).

Assembly of proteins into membrane

After mRNA is transcribed in the nucleus, it is processed and moves to the cytoplasm to associate with ribosomes for translation into a unique protein. Two theories have been proposed which try to explain the process of translation of mRNA into membrane bound proteins. Wickner's theory is known as the membrane trigger hypothesis (76), whereas Blobel and Milstein's theory is called the signal hypothesis (77).

The Membrane Trigger Hypothesis

Using this hypothesis Wickner emphasizes the ability of a membrane lipid bilayer to trigger the folding of a polypeptide into a conformation that spans the bilayer or is at least associated with it. Information encoded in the N-terminal region of the nascent peptide is thought to activate the protein for membrane assembly by altering the folding pathway. Membrane binding to the nascent protein triggers the protein to expose hydrophobic residues to the bilayer. This may or may not occur

before the protein is completely synthesized. Finally the N-terminal sequence is removed proteolytically.

The Signal Hypothesis

In the model proposed by Blobel and Milstein, a protein destined to be membrane bound has a hydrophobic N-terminal signal sequence that causes it and the ribosome to which it is bound to bind to a specific protein transport channel. The force of polypeptide chain elongation drives the chain through the bilayer. Once the N-terminal sequence has traversed the bilayer it is then cleaved proteolytically leaving a membrane-bound peptide.

Differences between the two models

The membrane trigger hypothesis differs significantly from the signal hypothesis. The trigger hypothesis does not require a protein transport channel or concommittant protein synthesis and transport or even that the ribosomes be bound to the endoplasmic reticulum during protein synthesis. However, according to Blobel's model, protein is synthesized and integrated into the membrane at the same time.

The signal hypothesis has been useful in describing the synthesis of secretory proteins (78-80). However, the model appears to be of limited value in explaining the synthesis of membrane-bound proteins. It has been shown in the case of the M13 coat protein of bacteriophage that translocation of a peptide does not have to occur during protein synthesis (81). Also some proteins are pleiotropic, spanning the bilayer several times, as is the case for bacteriorhodopsin (82). It is hard to

imagine a protein transport channel which allows a protein to cross the bilayer several times.

The membrane trigger hypothesis states that protein transport channels do not exist but that interaction of the nascent sequence with the bilayer is required for folding the protein into a conformation that easily binds the membrane.

The way to resolve the differences between the two models is to determine if protein transport channels exist. This can be done genetically by selecting various temperature selective mutants whose membranes do not effectively transport or bind nascent proteins. One should then be able to genetically map the deleted gene responsible for the protein channel (if indeed it exists), to prove the existence of the protein responsible for transporting nascent peptides through the membrane.

MATERIALS AND METHODS

Reagents

Flurbiprofen was obtained from the UPJOHN Company. Flufenamic acid was obtained from Aldrich. Acetylsalicylic acid, trypsin, bovine serum albumin (99% fatty acid free), polyoxyethylene sorbitan monolaurate (Tween 20), sodium diethyldithiocarbamate, mannose-6-phosphate, 2-mercaptoethanol, 3,3'-diaminobenzidine and lysine were obtained from Sigma Chemical Co., St. Louis, MO. Proteinase K was purchased from E.M. Biochemicals. Collagenase was purchased from Worthington Biochemicals and thermolysin was obtained from Boehringer-Manheim. Arachidonic acid was obtained from Nu-Check prep. Goat anti-rabbit whole serum, goat anti-rabbit IgG, and peroxidase anti-peroxidase (rabbit) were purchased from Miles Research Products. Paraformaldehyde, Araldite 502, dodecenyl succinic anhydride, 2,4,6-tri-(dimethylaminomethyl)phenol, osmium tetroxide, Epon 812, and propylene oxide were from Electron Microscopy Sciences. Dulbecco's modified Eagle media, fetal calf serum, antibiotic-antimycotic (100x), and glutamine were obtained from Grand Island Biological Co. Protein A-Sepharose was from Pharmacia Fine Chemicals. Other chemicals were reagent grade obtained from common commercial sources.

Microsomal preparation

Sheep vesicular glands and rat liver were obtained from freshly slaughtered animals, frozen as whole tissue on dry ice and stored at

-80°C. No appreciable loss in cyclooxygenase activity occurred over time. Both sheep vesicular gland and rat liver were handled in the same way. Preparation of microsomes was performed at 4°. Tissue was weighed and then homogenized in 0.1 M tris-chloride, pH 7.4, containing 20 mM diethyldithiocarbamate at a tissue to buffer ratio of 1:10 (w/v). Tissues were homogenized using a Polyton PCV 1 for 3 minutes at full speed. An initial centrifugation was performed at 12,000 x g for 15 minutes. The resulting supernatant was decanted and centrifuged at 200,000 x g for 35 minutes. The pellet was resuspended to a concentration of 10 mg protein/ml in starting buffer. Protein concentrations were determined using a Coomassie blue assay (83).

Cyclooxygenase assay

Cyclooxygenase activity was measured at 37° on a YSI oxygen monitor with a voltage offset control described by Smith and Lands (84). Aliquots of a suspension of sheep vesicular gland microsomes were added to oxygen electrode chambers containing 300 nmoles of arachidonic acid, 3 μmoles of phenol and 2 nmoles of bovine hemoglobin in a final volume of 3 ml of 0.1 M tris-chloride, pH 8.0. Negative controls were performed by measuring the rates of oxygen uptake in the presence of two specific inhibitors, Flurbiprofen, or flufenamic acid (85), (10^{-4} M). Further confirmation that oxygen uptake was a direct measure of PGH synthase comes from the observation of the self-catalyzed destruction phenomenon characteristic of the enzyme (1). One unit of cyclooxygenase is defined as that amount of enzyme that catalyzes the uptake of 1 nmoles of O_2 per minute per ml of assay solution at 37°.

Antisera

Rabbit preimmune and rabbit anti-cyclooxygenase sera were prepared as described previously (87). Rabbit IgG was isolated from both immune and preimmune sera using column chromatography on Protein A-Sepharose (88). Serum (5 ml) was applied to a column (1.1 x 4 cm) equilibrated at 4°C with 0.1 M sodium phosphate, pH 7.0. The column was washed with 20 volumes of equilibration buffer. The IgG fraction (20 to 35 mg) was then eluted with 1 M acetic acid, and the eluant was neutralized with concentrated NH_4OH. Isolated IgG was dialyzed overnight at 4° against 100 volumes of 0.1 M sodium phosphate, pH 7.0, diluted to a concentration of 2 mg/ml, and stored at this concentration in 0.1 sodium phosphate, pH 7.0, at -20°C. The protein concentration was based on an $\varepsilon^{1\%}_{280} = 1.45$ for IgG (86). Prior to use for immunocytochemistry, IgG was routinely diluted with 0.1 M sodium phosphate, pH 7.2, containing (0.9% NaCl phosphate-buffered saline) to a concentration of 5 to 50 µg/ml. Ouchterlony double diffusion analyses were performed in petri dishes containing 1.5% Bacto-agar.

Cell Culture

Swiss mouse 3T3 fibroblasts (ATCC CCL 92) obtained from American Type Culture Collection were grown in Dulbecco's modified Eagle media at 37° under a water-saturated 10% CO_2 atmosphere. Sterile transfers were performed following detachment of cells from flasks with 1% trypsin (GIBCO, 1/250) and 0.02% EDTA in phosphate-buffered saline solution, pH 7.2, and washing in Dulbecco's modified Eagle media containing 10% fetal calf serum. Cells used for staining were routinely grown on glass microscope slides or in polystyrene culture dishes (35 x 100 mm).

Immunocytochemistry

Mouse 3T3 cells were seeded at a concentration of 10^6 cells/150 mm^2 and grown in culture dishes for 24 h. Cells were then rinsed with phosphate-buffered saline to remove excess media and fixed for 4 h at 24°C in a freshly prepared solution of 10 mM $NaIO_4$, 75 mM lysine, 2% paraformaldehyde, and 37 mM sodium phosphate, pH 7.4 (89), containing 0 to 0.1% Tween 20. Fixed cells were subjected to three 15 min washes in phosphate-buffered saline pH 7.0, and then overlayed with IgG isolated from either rabbit anti-cyclooxygenase or preimmune sera at a final concentration of IgG of 5 to 50 µg/ml. All washes and dilutions of antisera were performed with 0.1 M sodium phosphate, pH 7.2, containing 0.9% NaCl. After incubation for 2.5 h at 24°, the cells were washed four times, 15 min each time. The cells were then incubated for 2 h at 24° with goat anti-rabbit IgG (1:10 dilution) and subsequently washed for 15 min four times. Peroxidase rabbit anti-peroxidase-soluble complex (1:50 dilution) was added to the cells which were then incubated for 2 h at 24°C and washed. Washed cells were incubated for 10 min with a solution containing 0.3 mM 3,3'-diaminobenzidine and 0.3 mM H_2O_2 in 0.05 M tris-chloride, pH 8.0 (90).

The stained cells were subjected to four 15-min washes and then post-fixed in 1% OsO_4 for 2 h at 24°. The cells were washed and then dehydrated by sequential exposure to 50%, 70%, 80%, 90% and 100% (three times) solutions of ethanol. After the third treatment with 100% ethanol, the cells were removed from culture dishes by scraping with a rubber policeman, transferred to 4-dram screw top vials, and collected by centrifugation at 500 x g for 2 min. Cell pellets were resuspended by agitation with propylene oxide and then mixed with an equivalent volume of

Epon-Araldite resin (Epon 812:Araldite 502:dodecenyl succinic anhydride, 25:20:60, v/v/v) and agitated for 4 h at 24°. Extra resin was then added to provide a ratio of resin to propylene oxide of 2 and the mixture agitated overnight. The cells were then collected by centrifugation and resuspended in Epon-Araldite resin to which had been added 0.024 volume of 2,4,6-tri-(dimethylaminomethyl)phenol. The samples were placed in Beem capsules and incubated for 72 h at 60°. The capsules were sectioned and sections examined and photographed using a Phillips model 201 transmission electron microscope. The sections were not counterstained with heavy metals. Kodak EM4463 film was used for photography. Light photomicroscopy of cells cultured, fixed, and stained on glass slides was performed with a Leitz Orthoplan microscope using Kodak TriX Pan film (ASA 28).

Precipitation of microsomal cyclooxygenase with Staphylococcus aureus-antibody complexes

Microsomes were prepared as described above. Attenuated Staphylococcus aureus (Cowen I strain) were prepared essentially as described by Kessler (109) and stored as 10% suspensions at -80°. Immediately prior to use S. aureus cells were washed twice in 0.1 M tris-chloride, pH 7.4 containing 5% BSA and 1% Tween 20, centrifuged at 500 x g for 5 minutes after each wash and resuspended in 0.1 M tris-chloride, pH 7.4. The cells were centrifuged again and resuspended in 0.1 M tris-chloride, pH 7.4. Anti-PGH synthase monoclonal IgG molecules secreted by clones cyo-1, cyo-5, cyo-7 or control 2c-3 IgG were linked noncovalently through the F_c region of the molecule to the F_c binding Protein A which is present on S. aureus cell surface. The antibody S. aureus complexes were

then mixed with either intact or detergent-solbuilized (1% Tween 20) microsomes prepared from sheep vesicular gland, and the mixture centrifuged to pellet S. aureus cells. Both the supernatant and pellet then were assayed for cyclooxygenase activity. The rationale underlying these experiments is that anti-PGH synthase IgG molecules will interact with antigenic sites exposed to the outside but not on the inside of microsomal spheres and that microsomes formed during homogenization of the endoplasmic reticulum are formed such that the cytoplasmic surfaces faces outward (91). Thus, precipitation of the cyclooxygenase activity of intact microsomes only can occur if antigenic sites on the enzyme face the cytoplasmic side of the endoplasmic reticulum. Experiments performed with solubilized enzyme serve as positive controls.

Protease digestion of PGH synthase from sheep vesicular gland microsomes

In these experiments, I determined if the cyclooxygenase as it exists in intact microsomes, was affected by digestion with various proteases. Microsomes were prepared in the usual manner. Cyclooxygenase activity was determined as described above. Proteinase K, collagenase, thermolysin, trypsin and a control, bovine serum albumin (BSA), were dissolved at a concentration of 1 mg/ml in 0.1 M tris-chloride, pH 8.0, containing 1 mM $CaCl_2$. A sample (50 µg) of each of these solutions was added to 1.0 ml aliquots sheep vesicular gland microsomes at 24° and incubated for 45 minutes. Cyclooxygenase activity was determined at zero and 45 min. After 45 min, preparations were cooled to 4° and subjected to centrifugation at 50,000 x g for 75 min. The resulting pellet was resuspended in 1.0 ml of 0.1 M tri-chloride, pH 7.4, containing 20 mM

diethyldithiocarbamate (DDC) and both supernatant and pellet fractions were assayed for cyclooxygenase activity.

Protease digestion of sheep vesicular gland microsomes labelled with [^{3}H]acetyl salicylic acid

[^{3}H]Acetylsalicylic acid, labelled in the acetyl moiety (50 Ci/mole) (92), was added at a concentration of 0.1 mM to 0.1 ml of sheep vesicular gland microsomes (8-12 mg/ml protein), and allowed to incubate 15 minutes at room temperature. The microsomes were then cooled to 4° and unlabelled acetylsalicylic acid added in excess (1 mM) to prevent further incorporation of radiolabel. The mixture was centrifuged at 50,000 x g for 75 minutes to precipitate the microsomes. The supernatant was discarded and the pellet resuspended in 0.1 ml of 0.1 M tris-chloride, pH 7.4, containing 20 mM DDC, centrifuged again as before and resuspended in its original volume. Proteinase K (100 µg) was added to 0.5 ml of sheep vesicular gland microsomes (10 mg/ml), which had been prelabeled with [^{3}H]acetylsalicylic acid. The digestion was stopped at 0,5,10,45 and 90 minutes following addition of protease by cooling the samples to 4°. Cyclooxygenase activity was determined in a parallel sample pretreated without acetylsalicylic acid. As expected, the aspirin-acetylated microsomes showed no cyclooxygenase activity (1). Samples from each time point from protease digestion were centrifuged at 50,000 x g for 75 min. The pellet was carefully separated from the supernatant and resuspended in its original volume. All pellets and supernatants were placed in liquid scintillation vials along with 7.0 ml of Bray's solution, vortexed and counted on a Searle Analytical liquid scintillation counter. [^{3}H]Acetyl salicylic acid was checked for purity by thin layer

chromatography. Selectivity of aspirin labelling of microsomes was assessed by determining if Flurbiprofen, flufenamic acid, or salicylic acid (10^{-4} M) prevented the incorporation of tritium into the microsomés.

Immunoradiometric assay of PGH synthase

Hybridoma line cyo-3 secretes an IgG_1 which interacts with PGH synthase but does not bind S. aureus cells. This IgG_1 was isolated from culture media, labelled with ^{125}I-Bolton-Hunter reagent (93) and used in an immunoradiometric assay for quantitating PGH synthase as follows (Figure 2): fixed amounts of ^{125}I-IgG_1 were incubated with fixed amounts of a precipitating complex prepared by affixing IgG_2 secreted by either cyo-1, cyo-5, cyo-7 or 2c-3 to attenuated S. aureus cells. Antigen (PGH synthase) was added at various concentrations to generate a standard curve. The amount of precipitated (cell bound) ^{125}I was determined by gamma counting in a Beckman Biogamma. A linear relation between precipitated ^{125}I and added sheep vesicular gland microsomes exists over a range of 0.005-0.005 units (0.17-1.7 ng), when using cyo-5 or cyo-1 S. aureus complexes.

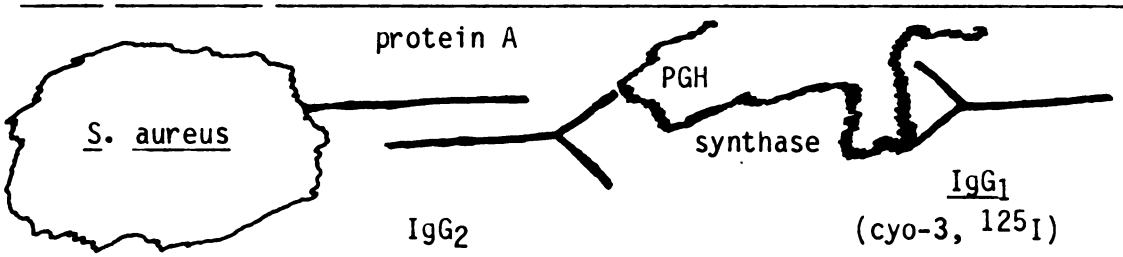


Figure 2. Immunoradiometric assay for quantitating PGH synthase

An experiment to determine if the antigenic activities of PGH synthase were destroyed by protease digestion was performed. Samples of microsomes (1.0 ml) prepared from sheep vesicular gland were treated as follows (a) proteinase K (50 µg) at 4° (b) BSA (50 µg) at 25° or (c) proteinase K (50 µg) at 25°. Cyclooxygenase activity was determined at 0, 45, 90 and 360 min. All samples were then diluted based on the original cyclooxygenase activity for measurement of antigenicity using the immunoradiometric assay. S. aureus anti-PGH synthase antibody complexes were prepared as described above. Solubilized microsomes (1% Tween 20 v/v) from all 3 samples were diluted into 100 µl 0.1 M tris-chloride, pH 7.4, 1% Tween 20 to contain the equivalent of 0.0-0.05 units of cyclooxygenase activity. S. aureus-IgG_2 complex, (0.01 ml; sufficient to bind 3 units of cyclooxygenase), was then added, followed by 0.01 ml of ^{125}I labelled IgG_1 (cyo-3), containing 30,000 cpm of ^{125}I. The assay samples were incubated overnight at 4°. Pellets were collected by centrifugation, washed once using 0.2 ml of assay buffer and recentrifuged. The supernatants were removed by aspiration and tubes containing the pellets were inserted into vials and counted using a Beckman Biogamma gamma counter.

A second experiment was performed in the same manner as the previous experiment except that after treatment of microsomes for 6 h the samples were centrifuged to prepare microsomal and supernatant fractions. The centrifugation was at 50,000 x g for 75 minutes at 4° and supernatant and microsomes carefully separated. The samples were diluted as described above and the immunoradiometric assay was performed on both fractions.

Determining microsomal integrity by the mannose-6-phosphatase assay

I found that sheep vesicular gland microsomes had no detectable mannose-6 phosphatase activity. An alternative procedure outlined in Figure 3 was used to determine the permeability of the microsomes. Rat liver (2 g), was mixed with sheep vesicular gland (2 g) prior to preparing microsomes. Mannose-6-phosphatase activity of the rat liver microsomes was used as marker for the luminal surface of the membrane. Mixed rat liver-sheep vesicular gland microsomes were diluted to 10 mg protein per ml based on Coomassie blue protein assays. Proteinase K (50 µg) was added to 1 ml of the mixed microsome preparation and incubated as in the previous experiment. In a control sample BSA (50 µg) substituted for the protease. The incubation was allowed to proceed for 6 h at 24° and then cooled to 4°. Mannose-6-phosphatase and cyclooxygenase activities were determined in each sample. Mannose-6-phosphatase was assayed as follows. The protease- and albumin-treated samples were each split into two fractions; one was solubilized with 1% sodium taurocholate and the other sample was left untreated (Fig. 3). Mannose-6-phosphatase was assayed at 24° by adding 50 µl of a microsomal preparation to 1.0 ml of 0.1 M tris-chloride, pH 6.6 containing 2 mM mannose-6-phosphate and 10 mM β-mercaptoethanol. The reactions were allowed to proceed for 0,5,10,15 and 20 minutes and then stopped by addition of 0.25 ml of 10% trichloroacetic acid. After the assay was complete, 0.25 ml of 5 N H_2SO_4, 0.5 ml of ammonium molybdate and 2.5 mg of reducing reagent (sodium sulfite, sodium bisulfite, 1-amino-2-napthol-4-sulfonic acid, 1.2/1.2/0.2, W/W/W)) were added and vortexed after each addition in a modified Fiske-Subbarow method (94). Absorbance was recorded on a Hitachi double beam spectrophotometer at 650 nm after ten min.

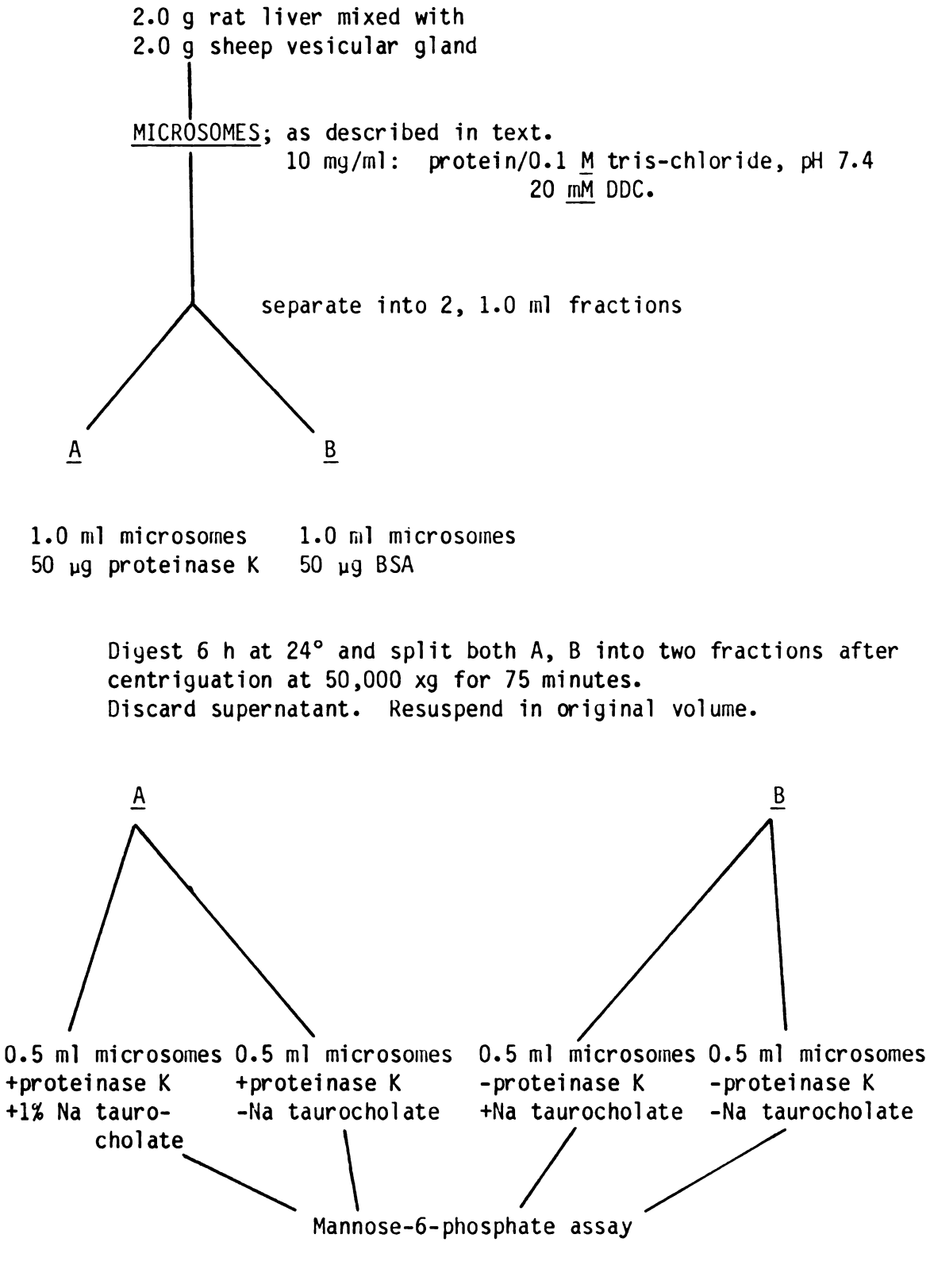


Figure 3. Procedure for monitoring integrity of microsomal membranes.

RESULTS

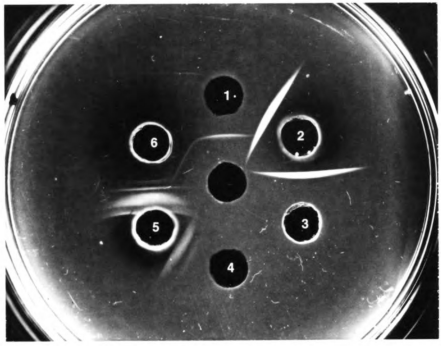
Specificity of Anti-PGH synthase IgG

We demonstrated previously that rabbit anti-PGH synthase serum is monospecific for the PGH synthase of sheep vesicular gland and that preimmune serum does not react with the enzyme (87). Ouchterlony double diffusion analysis (Figure 4) shows that both anti-PGH synthase serum and IgG isolated from the serum by chromatography on Protein A-Sepharose give single lines of precipitation with the purified sheep vesicular gland PGH synthase; furthermore, the reaction of identity between the two lines indicates that both immune serum and immune IgG interact with the same set of antigenic determinants on the enzyme. As expected, no immunoprecipitation lines were formed between the well containing the PGH synthase and wells containing either rabbit preimmune IgG or immune IgG which had been adsorbed with PGH synthase (Figure 4). Since the preparation of PGH synthase used to adsorb the anti-PGH synthase IgG is homogeneous by sodium dodecyl sulfate gel electrophoretic criteria (3), adsorption of the immune IgG with the purified enzyme will remove only those IgG molecules which interact specifically with the PGH synthase. Therefore, preimmune IgG and enzyme-adsorbed immune IgG provide appropriate negative controls for immunocytochemical staining with anti-PGH synthase IgG.

The purity of the anti-PGH synthase IgG was assessed by double diffusion analysis by comparing the reactions of both anti-PGH synthase serum and IgG with goat anti-rabbit whole serum and with goat anti-rabbit IgG

Figure 4. Ouchterlony double diffusion analysis of the interaction of anti-PGH synthase serum and anti-PGH synthase IgG with sheep vesicular gland PGH synthase. Center well, purified PGH synthase (5 µg (3)); well 1, IgG purified from rabbit anti-PGH synthase serum by Protein A-Sepharose chromatography (138 µg); well 2, goat anti-rabbit IgG (1:1 dilution); well 3, anti-PGH synthase IgG (138 µg) mixed with purified PGH synthase (2 µg); well 4, partially purified IgG from rabbit preimmune serum (50 µg); well 5, goat anti-rabbit whole serum (1:1 dilution); well 6, rabbit anti-PGH synthase serum (1:1 dilution). All dilutions were with 0.1 M sodium phosphate, pH 7.0. The initial volume in all wells was 25 µl.

Figure 5. Ouchterlony double diffusion analysis of the purity of IgG isolated from rabbit preimmune and anti-PGH synthase sera. Center well, goat anti-rabbit whole serum (1:4 dilution); well 1, IgG isolated from rabbit anti-PGH synthase serum (50 µg); well 2, rabbit anti-PGH synthase serum (1:4 dilution); wells 3 and 6, goat anti-rabbit IgG (1:4 dilution); well 4, IgG isolated from rabbit preimmune serum (50 µg); well 5, rabbit preimmune serum. All dilutions were with 0.1 M sodium phosphate, pH 7.0. The initial volume in all wells was 25 µl.


1
2
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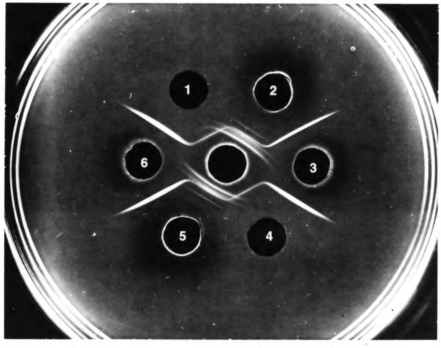
1
2
3
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6

(Figure 5). The anti-PGH synthase IgG gives a single line of immunoprecipitation with both goat anti-rabbit whole serum and anti-rabbit IgG with a reaction of identity present at the intersection of the lines. In contrast, multiple lines of precipitation are apparent between the well containing anti-PGH synthase serum and that containing goat anti-rabbit whole serum. Thus, the only serum proteins present in the anti-PGH synthase IgG preparation are IgG molecules. Analogous results were obtained in testing the purity of the IgG isolated from preimmune serum (Figure 5).

Immunocytochemistry

Swiss mouse 3T3 cells grown on glass slides and then quick frozen in isopentane (-70°C) and air-dried could be stained for cyclooxygenase antigenicity with IgG (6.20 to 50 μg of IgG/ml) isolated from rabbit anti-PGH synthase serum using the peroxidase-anti-peroxidase procedure developed by Sternberger and co-workers (95). Cells processed in this manner were used initially to determine what fixation conditions could be employed to retain PGH synthase antigenicity for subsequent electron microscopy. Cells incubated for as long as 4 h at 24°C using the periodate/lysine/paraformaldehyde fixative developed by McLean and Nakane (89) stained positively for the PGH synthase. Although fixation with glutaraldehyde provided better retention of cellular ultrastructure, treatment of 3T3 cells with 0.05% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2, for 5 min at 4° completely abolished PGH synthase immunoreactivity. Therefore, the periodate/lysine/paraformaldehyde fixative was used for all subsequent immunocytochemistry.

In order to stain unfrozen cells, it was necessary to include Tween 20 at concentrations of 0.03 to 0.1% in the fixation solution. This treatment permits penetration of immunolabeling reagents without causing extensive solubilization of integral membrane proteins (96-98). Tween 20 has no major effect on the catalytic or immunochemical properties of the PGH synthase (2,3). Figure 6 shows a photomicrograph of 3T3 cells which were subjected to immunocytochemical staining after fixation in periodate/lysine/paraformaldehyde solutions containing 0.05% Tween 20. PGH synthase antigenicity is apparent as dark rings around nuclei and can also be seen somewhat diffusely in the cytoplasm. Significantly, no staining of the plasma membrane was observed. When IgG isolated from the preimune serum was substituted for the immune IgG at equivalent concentrations, no staining was observed, nor did staining occur with immune IgG which had been preincubated with homogeneous (3) PGH synthase (100 μg of IgG/15 μg of PGH synthase for 30 min at 24°). These latter two results confirm that the staining noted in Figure 6 is actually due to the selective interaction of anti-PGH synthase IgG with the enzyme. Our studies by light microscopy coupled with the results of previous subcellular localization studies which employed differential centrifugation techniques suggested that the PGH synthase is associated with the endoplasmic reticulum and the nuclear membrane.

To verify these interpretations, we extended our work to the ultrastructural level. Mouse 3T3 cells cultured in petri dishes were fixed in solutions containing 0.05% Tween 20 and stained using immune IgG, preimmune IgG, or immune IgG adsorbed with PGH synthase as described above and then processed for elecron microscopy. In those 3T3 cells stained with IgG isolated from anti-PGH synthase serum (Figure 7A), electron-dense

Figure 6. **Light photomicrograph of mouse 3T3 fibroblasts stained using anti-PGH synthase IgG as described in the text.** NM, nuclear membrane. Bar line represents 42 μM.

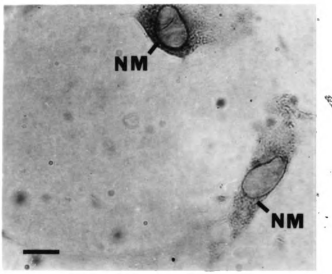
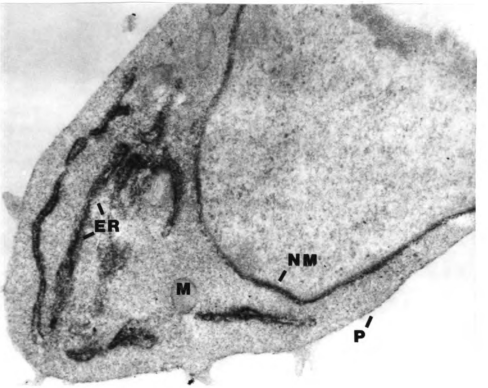
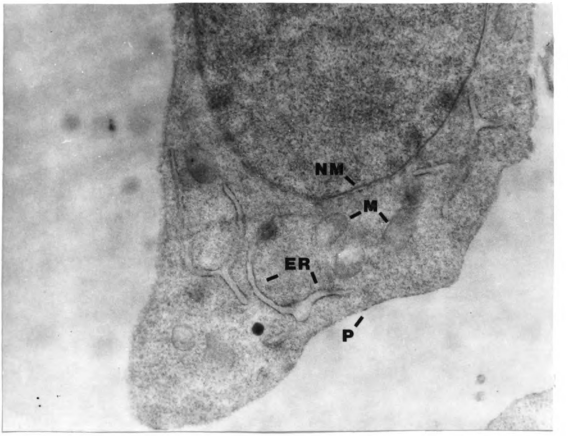

NM
NM

Figure 7. **Electron micrograph of a mouse 3T3 fibroblast stained using anti-PGH synthase IgG (A) and rabbit preimmune IgG (B) as described in the text.** NM, nuclear membrane; ER, endoplasmic reticulum; M, mitochondria; P, plasma membrane; N, nucleus. Bar line represents 1 μM.

A



B



staining was found throughout the endoplasmic reticulum and on the nuclear membrane in 87% of 464 cells examined in five separate experiments. In contrast, electron-dense staining occurred only on limited areas of the endoplasmic reticulum of 11% of 382 cells examined after staining with preimmune IgG or immune IgG adsorbed with purified PGH synthase (Figure 7B). No PGH synthase-positive staining was associated with mitochondria, in agreement with the results of studies which have employed centrifugal techniques to localize PGH synthase. The diffuse distribution of PGH synthase antigen over the entire endoplasmic reticulum is similar to that seen with other reticular enzymes, including NADPH cytochrome c reductase and glucose-6-phosphatase (63).

Careful examination of 3T3 cells stained using anti-cyclooxygenase IgG failed to reveal any electron-dense staining associated with the plasma membrane. The lack of plasma membrane staining is not due to the existence of an unusual nonantigenic enzyme form because the cyclooxygenase activity solubilized from 3T3 cell microsomes could be precipitated quantitatively by anti-PGH synthase serum. It is also doubtful that any PGH synthase was solubilized from the plasma membrane selectively during fixation since even cells that were quick frozen and stained without prior fixation exhibited no cell surface staining under the light microscope. Thus, our results indicate that the enzyme is not distributed uniformly over the cell surface. It also seems unlikely that the PGH synthase is concentrated in discrete pockets on the plasma membrane as has been observed for low density lipoprotein receptors on human fibroblasts (99).

Precipitation of microsomal PGH synthase with S. aureus-antibody complexes

Three different monoclonal antibodies against the PGH synthase (cyo-1, cyo-5 and cyo-7) and one control antibody (2c-3) were affixed to S. aureus cells and these complexes mixed with intact and solubilized microsomes prepared from sheep vesicular gland (Figure 8). When intact microsomes prepared from sheep vesicular gland were mixed with S. aureus cells complexed to one of the anti-PGH synthase antibodies, complete precipitation of cyclooxygenase activity occurred (i.e. all the enzyme activity was found in the S. aureus pellet). No appreciable precipitation resulted when a nonimmune monoclonal mouse IgG_2 (2c-3) was used. As expected, similar results were obtained with control, detergent solubilized microsomes. Antibodies secreted by cyo-1, -5 and -7 interact with different antigenic sites on the PGH synthase, thus our results indicate that at least 3 antigenic determinants on the sheep vesicular gland cyclooxygenase are situated on the outer surface of microsomal spheres and thus on the cytoplasmic side of the endoplasmic reticulum.

Protease inactivation of PGH synthase from sheep vesicular gland microsomes

Four proteases were tested for their ability to degrade microsomal PGH synthase during a 45 min incubation (Table 1). Thermolysin, trypsin, and proteinase K caused significant losses in cyclooxygenase activity. The greatest degree of inactivation was observed with proteinase K which destroyed 45% of the starting activity.

A second experiment was performed to determine if any cyclooxygenase activity could be solubilized by protease treatment of the microsomes.

Figure 8. **Immunoprecipitation of microsomal sheep vesicular gland PGH synthase by mouse monoclonal antibody-S. aureus complexes.** The experiment was performed as described in the text. Pellets (P) and supernatants (S) were obtained by centrifuging mixtures of various S. aureus-antibody complexes and microsomal or solubilized preparations of PGH synthase at 500 x g. Samples were then assayed for cyclooxygenase activity.

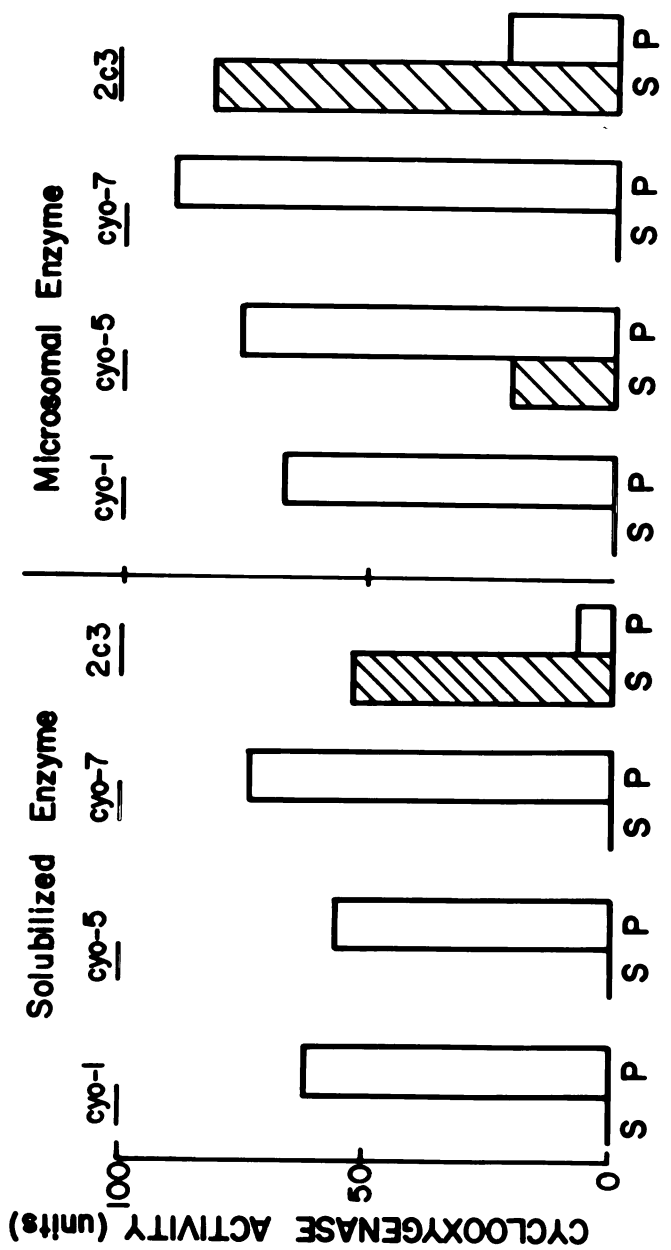

CYCLOOXYGENASE ACTIVITY (units)
100
50
0
Solubilized Enzyme
Microsomal Enzyme
cyo-1
cyo-5
cyo-7
2c3
S P

TABLE 1

Effect of protease digestion on the PGH synthase

of sheep vesicular gland microsomes

Sample	Cyclooxygenase activity before digestion (units x 10^{-3}/ml)	Cyclooxygenase activity after digestion (units x 10^{-3}/ml)	Loss of activity (%)
1. 1.0 ml sheep vesicular gland microsomes plus 100 µg BSA	13.4	13.4	0.0
2. 1.0 ml sheep vesicular gland microsomes plus 100 µg collagenase	13.4	12.4	7.4
3. 1.0 ml sheep vesicular gland microsomes plus 100 µg thermolysin	13.4	10.8	19.4
4. 1.0 ml sheep vesicular gland microsomes plus 100 µg trypsin	13.4	8.6	35.8
5. 1.0 ml sheep vesicular gland microsomes plus 100 µg proteinase K	13.4	7.6	44.3

The original microsomal protein concentration was 11 mg/ml. Samples were incubated for 45 min at 25°, and the reactions were stopped by placing the samples in 4°.

Following exposure of microsomes to different proteases, samples were centrifuged to isolate microsomes and then the supernatant and the resuspended pellet assayed for cyclooxygenase activity (Figure 9). It can be seen that after thermolysin, trypsin or proteinase K treatment up to 20% of the residual activity appears in the supernatant. This suggests that fragments of the PGH synthase possessing catalytic activity can be cleaved from the membrane.

Solubilization of a [^{3}H]acetyl-labeled fragment by protease treatment of aspirin labelled microsomes

This experiment was performed to determine if the acetylated active site containing peptide of the cyclooxygenase could be released from intact microsomes by protease digestion. Microsomes were labeled by treatment with [^{3}H]acetylsalicylic acid, incubated for various times with Proteinase K, and the amount of tritium released from the microsomal membrane was measured. The experiment was performed four times with similar results. One such experiment is depicted in Figure 10. In an average of four trials 72%±5% of the [^{3}H] label was lost within 10 minutes of adding proteinase K to the microsomes. The radioactivity released into the supernatant was nondialyzeable indicating that the release is not due to a simple esterase-catalyzed cleavage of acetyl groups. In control samples in which albumin was substituted for proteinase K, no release of tritium occurred. This experiment indicates that a peptide containing the active site serine residue of the PGH synthase is released into the supernatant following exposure of microsomes to proteinase K.

Figure 9. **Cyclooxygenase activities in microsomal and pellet fractions following treatment of intact sheep vesicular gland microsomes with proteases.** Sheep vesicular gland microsomes (containing 11.0 mg protein/1.0 ml 0.1 M tris-Cl, pH 7.4) were incubated for 45 min at 24° with various proteases (100 µg). Following incubation with proteases, microsomal pellet and supernatant fractions were prepared and assayed for cyclooxygenase activity as described in the text.

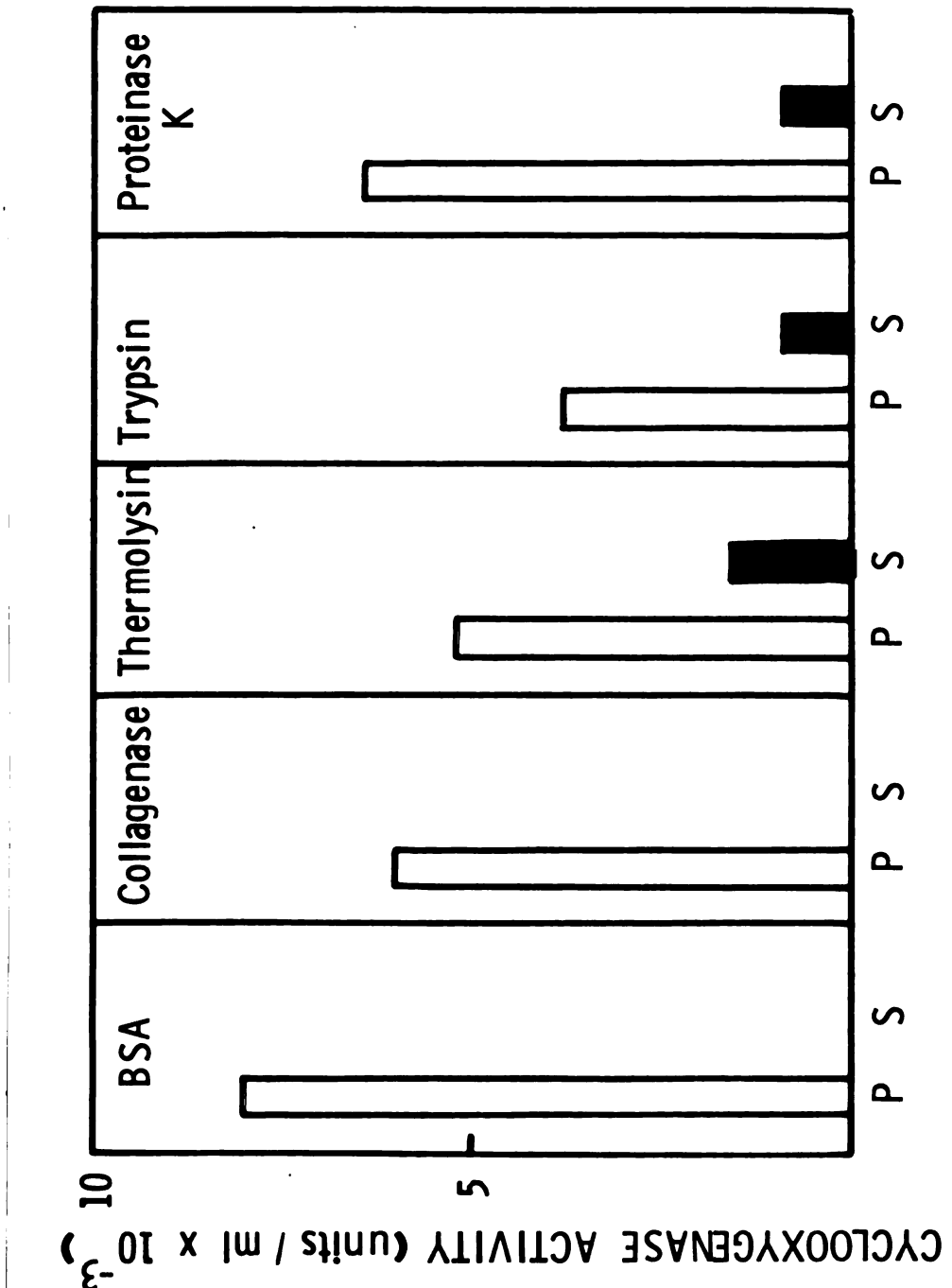
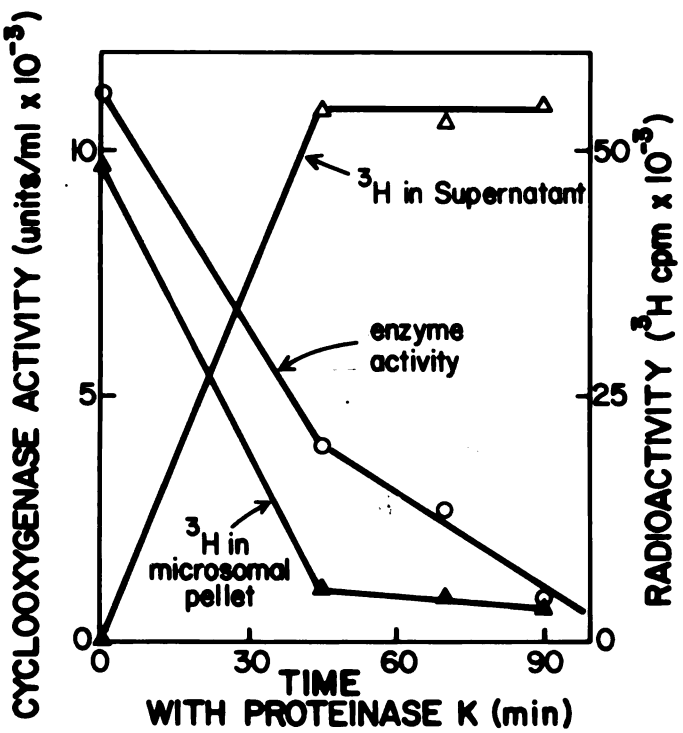

CYCLOOXYGENASE ACTIVITY (units / ml x 10^{-3})
10
5
BSA
Collagenase
Thermolysin
Trypsin
Proteinase K
P S
P S
P S
P S
P S

Figure 10. **Release of [^{3}H]-label from acetylated sheep vesicular gland microsomes treated with Proteinase K.** Microsomes were labelled with [^{3}H]acetylsalicylic acid and then treated with proteinase K for various times. (A) Samples were then centrifuged and a microsomal pellet and supernatant assayed for ^{3}H. (B) A control sample lacking the inhibitor was treated in the same way and assayed for cyclooxygenase activity.


CYCLOOXYGENASE ACTIVITY (units/ml x 10⁻³)
RADIOACTIVITY (³H cpm x 10⁻³)
10
5
0
50
25
0
0
30
60
90
TIME
WITH PROTEINASE K (min)
³H in Supernatant
enzyme activity
³H in microsomal pellet

Sensitivity of PGH synthase antigenic determinants to protease digestion

Intact sheep vesicular gland microsomes were treated with protease to destroy cyclooxygenase activity. The antigenic reactivities of protease-treated and BSA-treated samples were quantitated using an immunoradiometric assay as described in Methods. No loss of binding occurred following protease treatment of microsomes when assayed using any one of the IgG_2-*S. aureus* complexes as precipitating agents (Figure 11). These results suggest that essentially all the antigenic activity is retained in the protease-digested samples.

Since the cyclooxygenase active site is solubilized by protease treatment, it was of interest to determine if any of the antigenic determinants were also solubilized under similar conditions. Intact microsomes were first subjected to protease digestion and then microsomal and supernatant fractions were prepared. The antigenic activities associated with each fraction were measured using the immunoradiometric assay (Figures 12 and 13). Comparison of the slopes (cpm ^{125}I-bound in pellet/unit of cyclooxygenase activity) indicates that with all antibodies tested all of the antigenic activity associated with binding to *cyo*-5 and *cyo*-7 (i.e. *cyo*-3) remained associated with the membrane. However a portion of the *cyo*-1 site (20%) was solubilized. Thus, it is evident that a majority of the antigenic determinants are not solubilized by protease and are somehow protected from digestion.

Determination of microsomal integrity

In preliminary experiments I found that sheep vesicular gland microsomes had no glucose-6-phosphatase activity. Therefore, to use this enzyme as a marker for the luminal surface of the endoplasmic reticulum,

Figure 11. Immunoradiometric assay of protease digested sheep vesicular gland microsomes. Sheep vesicular gland microsomes (13-15 mg protein/1.0 ml 0.1 M tris-Cl, pH 7.4) were treated for 6 h at 4° with 100 µg proteinase K (A); sheep vesicular gland microsomes treated for 6 h at 25° with 100 µg BSA (B); and sheep vesicular gland microsomes treated for 6 h at 25° with 100 µg proteinase K (C). The three slopes represented in each graph correspond to (Δ) S. aureus-cyo-1 binding, (O) S. aureus-cyo-5 binding, and (●) S. aureus-2c-3 binding.

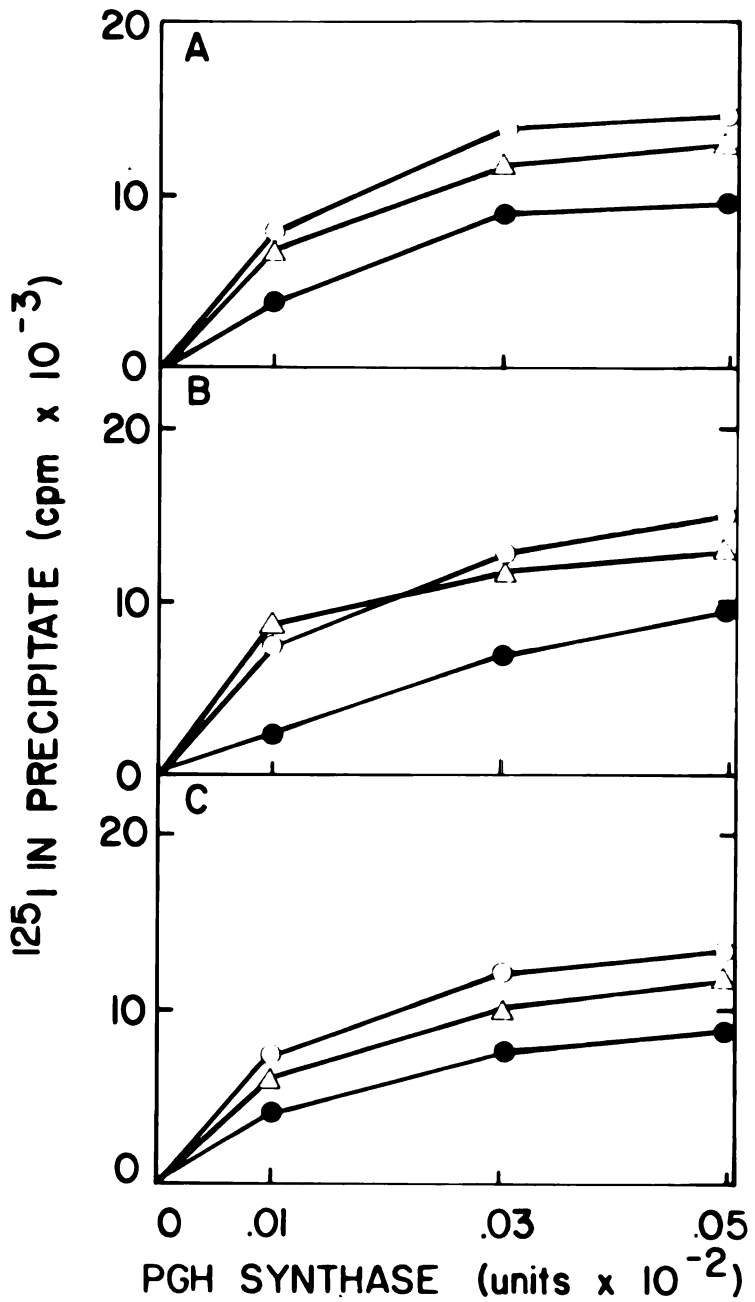

A
B
C
20
10
0
0
.01
.03
.05
^{125}I IN PRECIPITATE (cpm x 10^{-3})
PGH SYNTHASE (units x 10^{-2})

Figure 12. **Immunoradiometric assay of microsomal fraction of protease digested sheep vesicular gland microsomes.** (A) Sheep vesicular gland microsomes treated for 6 h at 25°C with 100 µg BSA; (B) sheep vesicular gland microsomes were treated for 6 h at 25°C with 100 µg proteinase K. The four slopes represented in each graph correspond to (▲) S. aureus-cyo-1 complex, (O) S. aureus-cyo-5 binding, (●) S. aureus-2c-3 binding, and (Δ) S. aureus-cyo-7 binding.

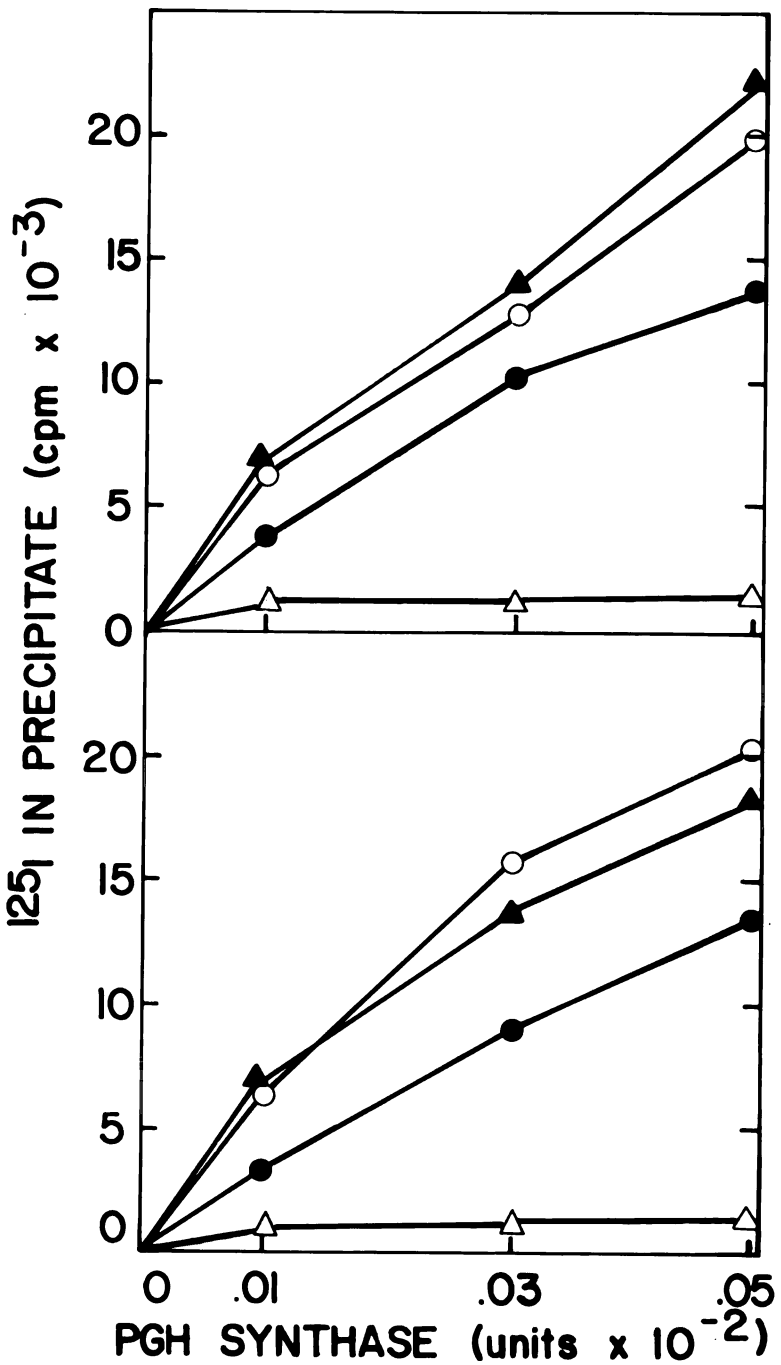
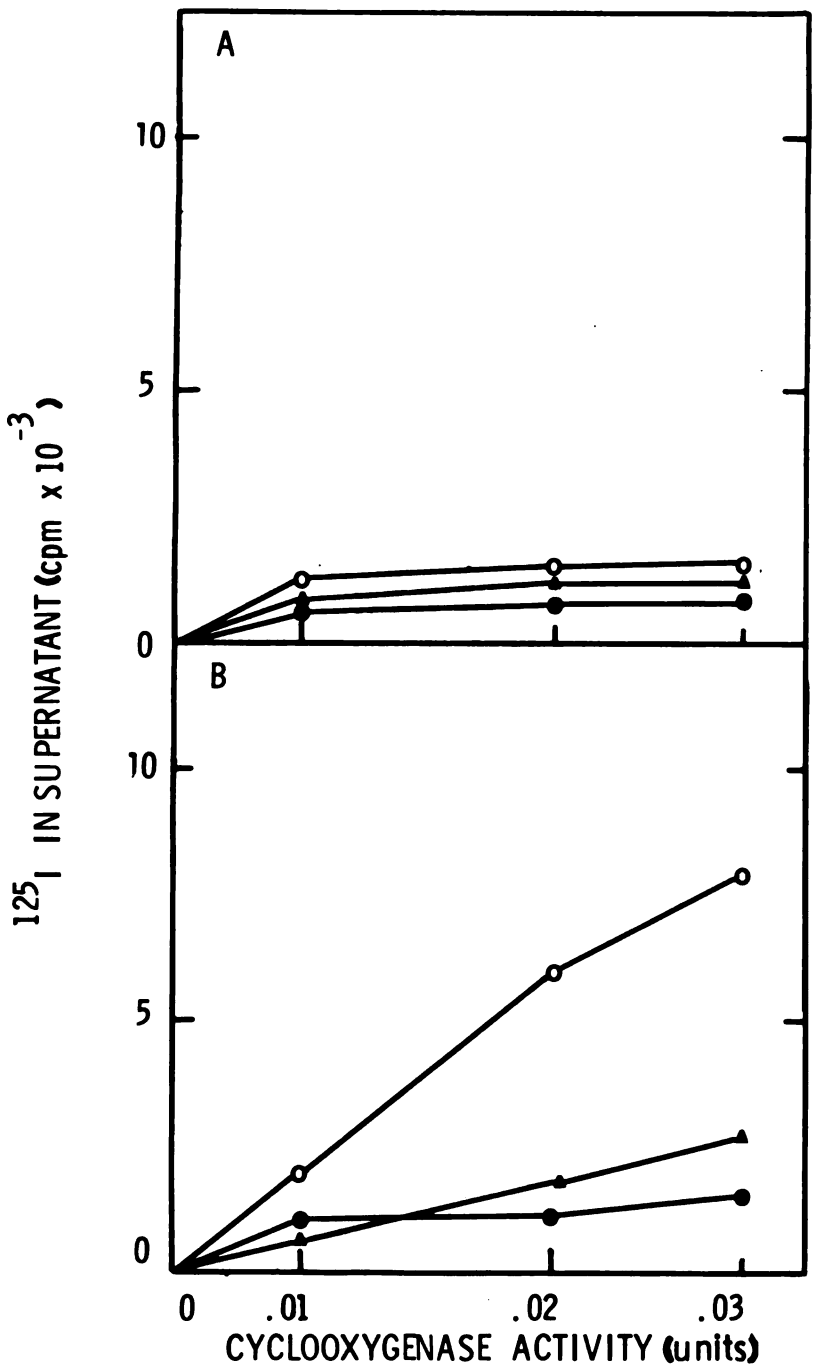

^{125}I IN PRECIPITATE (cpm x 10^{-3})
20
15
10
5
0
20
15
10
5
0
0
.01
.03
.05
PGH SYNTHASE (units x 10^{-2})

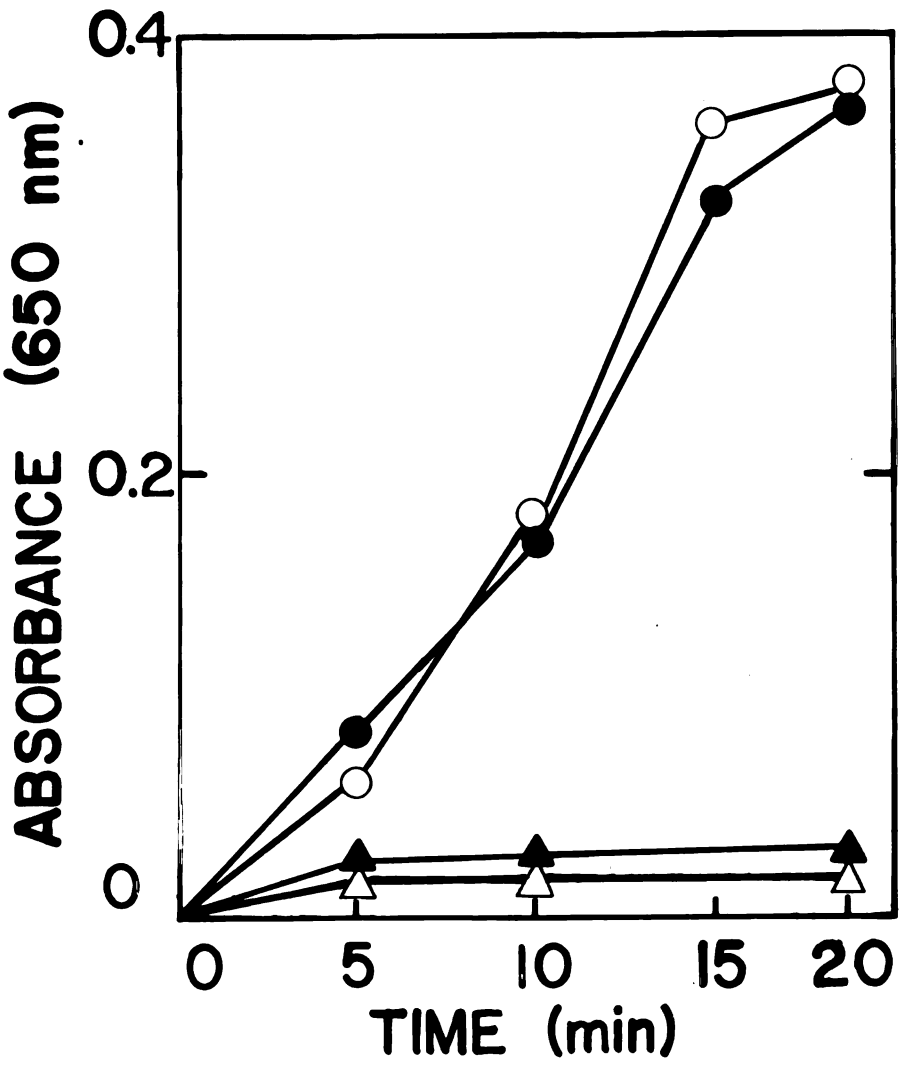
Figure 13. **Immunoradiometric assay of supernatant fraction of protease digested sheep vesicular gland microsomes.** (A) Sheep vesicular gland microsomes treated for 6 h at 25°C with 100 µg BSA; (B) sheep vesicular gland microsomes were treated for 6 h at 25°C with 100 µg proteinase K. The three slopes represented in each graph correspond to (O) S. aureus-cyo-1 binding, (Δ) S. aureus-cyo-5 binding and (●) S. aureus-2c-3 binding.


A
B
10
5
0
10
5
0
0
.01
.02
.03
^{125}I IN SUPERNATANT (cpm x 10^{-3})
CYCLOOXYGENASE ACTIVITY (units)

we prepared mixed microsomes from sheep vesicular and rat liver. Glucose-6-phosphatase activity of rat liver is actually a two component system consisting of a glucose-6-phosphate-specific carrier which mediates the transport of glucose-6-phosphate from the cytosol to the lumen of endoplasmic reticulum and a nonspecific phosphohydrolase localized on the luminal surface of the microsomal membrane (100). Mannose-6-phosphate is not a substrate for the carrier but is hydrolyzed by the phosphohydrolase component of this system. Thus, hydrolysis of mannose-6-phosphate occurs in the presence of disrupted but not intact microsomes (100). I used the latency of the mannose-6-phosphate phosphohydrolase to test for microsomal integrity. Mixed microsomes were incubated with either proteinase K or BSA, and then either subjected to solubilization with 1% sodium taurocholate or not solubilized (Figure 3). All four samples were assayed for mannose-6-phosphatase activity (Figure 14). There was no detectable mannose-6-phosphate hydrolysis by unsolubilized microsomes whether or not the microsomes were treated with proteinase K. However, equivalent amounts of mannose-6-phosphatase activity were present in solubilized microsomes indicating that the rat liver microsomes are intact and right side out. When solubilized microsomes were treated at 24° with proteinase K, the mannose-6-phosphatase activity disappears. Cyclooxygenase activity was also measured in this experiment before and after proteinase K treatment. Approximately 88% of the cyclooxygenase activity is lost after 360 min incubation. Parallel samples were run with BSA and only 10-12% of the cyclooxygenase activity is lost over 360 min at 24°.

In another experiment, the permeability of microsomes pretreated with aspirin was tested using mannose-6-phosphatase activity as a marker. Mixed microsomes were preincubated with aspirin, added to proteinase K or

Figure 14. Latency of mannose-6-phosphatase activity in mixed rat liver-sheep vesicular gland microsomes. Mannose-6-phosphatase activity was measured as described in the text. Four samples of the mixed microsomes were tested with (O) 50 μg proteinase K plus 1% Na taurocholate, (Δ) 50 μg proteinase K alone, (●) 50 μg BSA plus 1% Na taurocholate and (▲) 100 μg BSA.


0.4
0.2
0
ABSORBANCE (650 nm)
0
5
10
15
20
TIME (min)

BSA, and then either subjected to solubilization with 1% Na taurochloate or not solubilized. As before, there was no detectable mannose-6-phosphate hydrolysis by unsolubilized microsomes but activity was noted with solubilized microsomes. These results suggest that aspirin does not permeabilize sheep vesicular gland microsomes.

DISCUSSION

There were two phases to these studies. In the first phase the subcellular location of PGH synthase in Swiss Mouse 3T3 was determined by electron microscopic immunocytochemistry. In the second phase the transverse orientation of PGH synthase in the microsomal membranes of sheep vesicular glands was deduced on the basis of susceptibility of the synthase to protease digestion and ability of the enzyme to interact with specific monoclonal antibodies.

In a first set of experiments Swiss mouse 3T3 fibroblasts were grown in tissue culture, fixed with lysine-paraformaldehyde-periodate solutions containing 0 to 0.1% Tween 20, and then stained for cyclooxygenase antigenicity using rabbit anti-cyclooxygenase IgG in the peroxidase anti-peroxidase procedure. When examined by light microscopy, those cells fixed in the presence of 0.03 to 0.1% Tween 20 exhibited staining throughout the cytoplasm and around the nucleus but not on the cell surface. No staining occurred when either preimmune IgG or anti-cyclooxygenase IgG adsorbed with purified enzyme was substituted for the immune IgG. Electron microscopic examination of cells treated with fixative containing 0.05% Tween 20 and then stained for cyclooxygenase antigenicity revealed electron-dense deposits on the endoplasmic reticulum and nuclear membrane but not the mitochondrial or plasma membranes. No specific staining was seen in cells treated with control sera. Our results establish that conversion of arachidonic acid to the prostaglandin endoperoxide precursor

of PGE_2 actually takes place on the endoplasmic reticulum and the nuclear envelope.

In previous immunocytofluorescence studies of other cyclooxygenase-containing cells in kidney, uterus, and stomach, fluorescence has been found to be distributed diffusely throughout the cytoplasm, often in association with intense perinuclear staining (87,88,102,102), the same pattern seen in mouse 3T3 cells.

Exposure of 3T3 cells to agents such as thrombin, angiotensin II, and bradykinin causes a rapid hydrolysis of arachidonate from phospholipid stores and subsequent formation of PGE_2. One would anticipate that the coupling of arachidonate release with oxygenation would be most efficient if the phospholipid precursors were located near the cyclooxygenase on the endoplasmic reticulum or the nuclear membrane. If so, selective induction of prostaglandin synthesis by agents acting at the cell surface must involve the transmission of a signal necessary to activate lipases which cleave phospholipids of intracellular membranes.

The significance of having the cyclooxygenase present on the nuclear envelope is unclear. This may be a simple consequence of the continuity of the endoplasmic reticulum and nuclear membrane. In fact, a number of enzymes other than the cyclooxygenase, including cytochrome P-450 (99), NADPH-cytochrome c reductase (103,104), and UDP-glucocuronyltransferase (105), are associated with both the endoplasmic reticulum and nuclear membrane. Alternatively there may be a specific relationship between prostaglandins formed on the nuclear membrane and nuclear events. In this regard it should be noted that Rao and co-workers (106) have recently found a prostaglandin-binding protein on the nuclear membrane of luteal cells. Our results also suggest that cooxygenation of xenobiotics on

the nuclear membrane by the peroxidase associated with the cyclooxygenase to form mutagenic compounds (103) may have some biological importance.

In a second set of experiments intact microsomes were prepared from sheep vesicular glands, and the resuspended microsomes subjected to proteinase K digestion. A time dependent loss of cyclooxygenase activity occurred. Samples were then centrifuged to reisolate the microsomal pellet and a supernatant fraction and the cyclooxygenase activity measured in both fractions. Between 10 and 20% of the starting activity was released into the supernatant within five minutes and this activity decreased following longer exposure to proteinase K. In an additional experiment, microsomal PGH synthase was prelabelled by treatment with [^{3}H]acetylsalicyclic acid and the disappearance of tritium label from the microsomal pellet was measured after exposure to proteinase K. Approximately 70-75% of the tritium label was released into the supernatant within five minutes. Protease digested microsomes were tested for membrane integrity by including rat liver microsomes as an internal control and assaying for latent mannose-6-phosphate activity before and after protease digestion.

Microsomes form right side out relative to their orientation in the endoplasmic reticulum (63). Sheep vesicular gland microsomes were not fragmented or digested by treatment with proteinase K as shown by the latent mannose-6-phosphatase activity. Therefore, unsolubilized, intact microsomes are impermeable to proteinase K. Proteinase K causes a loss of cyclooxygenase activity in intact microsomes while no loss of activity occurs with BSA incubation. These results suggest that the PGH synthase lies on the outer surface of the microsomal membrane and therefore the outer surface of the endoplasmic reticulum. Release of cyclooxygenase

activity into the supernatant was due to release of an active site peptide fragment of the cyclooxygenase. Release of the holoenzyme did not occur because antigenic determinants of the PGH synthase are still bound to the microsomal membrane after proteinase K digestion. Evidence that active site release of the PGH synthase comes from following the loss of tritium active site of the PGH synthase in microsomal membranes. This tritium loss into the supernatant occurs within 10 minutes upon addition of proteinase K. These experiments indicate that the active site of the PGH synthase is the most accessible site on the enzyme for protease cleavage. The active site of many proteins bound to the endoplasmic reticulum face the cytosol (63). An active site should be available to interact with substrates and my data suggest that the active site of the PGH synthase lies exposed to the cytoplasm to interact with free arachidonic acid.

Finally, a series of experiments were performed to determine the location of antigenic sites on the PGH synthase and their susceptibility to degradation by protease. Monoclonal antibodies (cyo-7, cyo-5, cyo-1) against the PGH synthase and one control antibody (2c-3) were bound to attenuated Staphylococcus aureus cells. Each of these cell-antibody complexes was tested for its ability to precipitate PGH synthase of intact microsomes. Cyclooxygenase activity was precipitated by Staphylococcus aureus-anti-cyclooxygenase complexes but not by the S. aureus-2c-3 complex.

When intact, unsolbulized microsomes were added to Staphylococcus aureus-anti-PGH synthase complexes all cyclooxygenase activity was precipitated in the pellet. This demonstrates that all the antigenic sites of the PGH synthase present in microsomal membranes are located on the

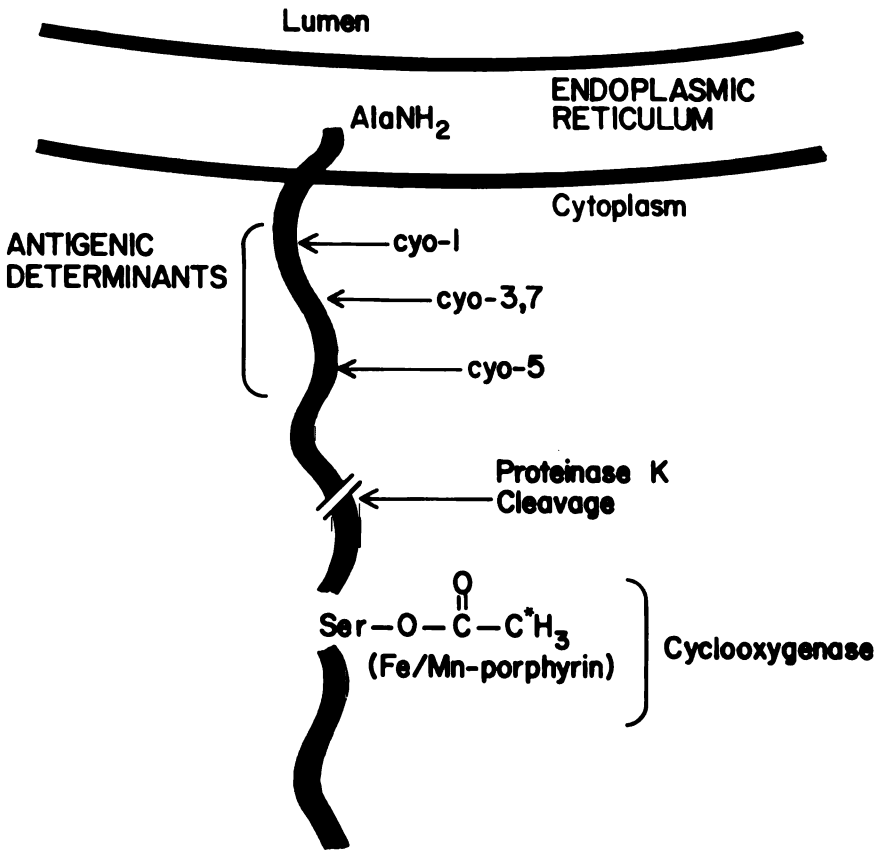
outside of the microsomal membrane and therefore lie on the cytoplasmic surface of the endoplasmic reticulum. The control complex Staphylococcus aureus-anti-2c-3 did not precipitate any of the cyclooxygenase activity which demonstrates the specificity on the Staphylococcus aureus-anti-PGH synthase complexes. These experiments show (a) the specificity of the anti-PGH synthase monoclonal antibodies and (b) further evidence that the PGH synthase is located on the cytoplasmic surface of the endoplasmic reticulum.

Utilizing an immunoradiometric assay to assay for the binding of antibodies secreted by cyo-1, cyo-3, and cyo-5, it was found that the antigenic reactivity remained following extensive protease digestion.

Staphylococcus aureus-anti-cyo-1, -anti-cyo-3, -anti-cyo-5 showed no loss of binding activity before and after protease digestion. This finding suggests that the antigenic sites are not digested by proteinase K. To further define where these antigenic determinants are (they could have been solubilized like the active site), the preparation was recentrifuged and an immunoradiometric assay performed on supernatant and microsomal fractions. All antigenic reactivity remained associated with the microsomal membrane fraction (except for a small portion associated with cyo-1) suggesting that no antigenic sites were solubilized by proteinase K. I have found that major portions of the enzyme are left on the microsome after digestion.

A model describing these results appears in Figure 15. The PGH synthase has its hydrophobic N-terminus bound to the endoplasmic reticulum which allows a major portion of the enzyme access to the cytoplasm. The active site is known to be easily released upon proteinase K digestion and is the most exposed site on the protein. The antigenic binding sites

Figure 15. Possible orientation of PGH synthase on the cytoplasmic surface of the endoplasmic reticulum based on protease digestion of active site enzyme and antigenic binding.


Lumen
ENDOPLASMIC RETICULUM
AlaNH2
Cytoplasm
ANTIGENIC DETERMINANTS
cyo-1
cyo-3,7
cyo-5
Proteinase K Cleavage
Ser—O—C—C*H3
(Fe/Mn-porphyrin)
Cyclooxygenase

are much more protected from proteinase K digestion. The cyo-1 binding site is the next least protected showing small losses of binding upon proteinase K digestion. Cyo-3 and cyo-5 are completely protected, not showing any antigenic binding loss.

It was thought that prostaglandins were produced and released from the plasma membrane of mammalian cells. From the studies on 3T3 cells and other cell types no PGH synthase exists on the plasma membrane. This implies that a message must first interact with the plasma membrane to stimulate a second message which either (a) stimulates phospholipases on the inside of the plasma membrane to release arachidonic acid or (b) send the secondary message to the endoplasmic reticulum membranes where the phospholipases can release arachidonic acid to the cytosolic active site of the PGH synthase.

Most membrane bound enzymes and proteins have been found on the cytoplasmic surface of the endoplasmic reticulum. Cytochrome b_5 (73) and NADPH-cytochrome c reductase (70) are examples with hydrophobic N-termini bound to phosopholipid membrane while a major portion of the protein extends into the cytoplasm to interact with substrates. The PGH synthase N-terminus has recently been sequenced and is highly hydrophobic (1).

If phospholipase action controls the release of arachidonic acid one economical way for the phospholipases to work would be to feed on the endoplasmic reticulum phospholipid stores. It is interesting to note that in most cell types phosphatidyl inositol (PI) lies on the luminal surface of the endoplasmic reticulum (63). If PI is cleaved by successive action of phospholipase c and diglyceride lipase in platelets it is reasonable to think that they may be associated with the luminal surface of the endoplasmic reticulum and use PI as a substrate. The arachidonic

acid released may be transported through the membrane much in the same way as the methyl transferase system as described by Hirata and Axelrod (74) to be dumped at the site of PGH synthase.

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