THE PURIFICATION, QUANTITATION AND IMMUNOFLUORESCENT LOCALIZATION OF THE PGI_2 FORMING ENZYMES, PGH SYNTHASE AND PGI_2 SYNTHASE, WITH MONOCLONAL ANTIBODIES

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A DISSERTATION

Submitted to

Michigan State University

in partial fulfuillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1982

ABSTRACT

THE PURIFICATION, QUANTITATION AND IMMUNOFLUORESCENT LOCALIZATION OF THE PGI_2 FORMING ENZYMES, PGH SYNTHASE AND PGI_2 SYNTHASE, WITH MONOCLONAL ANTIBODIES

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Four cloned hybridoma cell lines (designated cyo-1,3,5, and 7) have been isolated which secrete antibodies against the PGH synthase enzyme. The antibodies, when coupled with Protein-A bearing Staphlococcus aureus cells, precipitate PGH synthase activity and a single protein with a monomer molecular weight identical to the purified PGH synthase. Two of the antibodies which bind different antigenic sites on the PGH synthase have been used in a immunoradiometric assay for the enzyme which is 10^2-10^4 times more

sensitive than the most sensitive polarographic assay for PGH synthase.

Two cloned hybridoma cell lines (designated isn-1 and isn-3) which secrete antibodies against the PGI_2 synthase have also been isolated. These antibodies precipitate PGI_2 synthase activity and a single protein with a monomer molecular weight of 52,000 daltons. Active PGI_2 synthase was purified using an IgG_1 (isn-3) Affigel-10 immunoaffinity column and found to contain a protoporphyrin IX heme prosthetic group. The purified enzyme is inactivated by PGH_2 and 13-hydroperoxy-linoleic acid. The PGI_2 synthase heme spectrum is bleached during inactivation. Azo Analogue I, a substrate analogue of PGH_2 prevents inactivation and bleaching of the heme spectrum caused by PGH_2 and 13-hydroperoxy-linoleic acid, but only at concentrations which inhibit PGI_2 synthase activity. An immunoradiometric assay, analogous to the PGH synthase assay, has also been developed for the PGI_2 synthase.

Using the two immunoradiometric assays, the concentrations of PGH synthase and PGI_2 synthase in the cell layers of bovine aorta were determined. Smooth muscle and endothelial cells contain roughly equal amounts of PGI_2 synthase; endothelial cells, however, contain 20 fold more PGH synthase than smooth muscle cells. Immunofluorescent staining with the isn antibodies indicates that PGI_2 synthase is also present in most types of extra-vascular smooth muscle.

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ABBREVIATIONS

The abbreviations used are: PGH_2, 15-hydroxy-9α,11α-peroxido-prosta-5,13-dienoic acid; PGI_2, 9-deoxy-6,9α-epoxy-11α,15-dihydroxy-prota-5,13-dienoic acid; $PGF_{2\alpha}$, 9α,11α,15-trihydroxy-prosta-5,13-dienoic aicd; 6-keto-$PGF_{1\alpha}$, 9α,11α,15-trihydroxy-6-keto-prosta-13 enoic acid; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle medium; HEPES, N-2-hydroxy-ethylpeperazine-N'-2-ethanesulfonic acid; MES, 2-(N-morpholino)ethane-sulfonic acid; 13-HPLA, 13-hydroperoxy-9 *cis*, 11 *trans*-octadecadienocid acid.

LITERATURE REVIEW

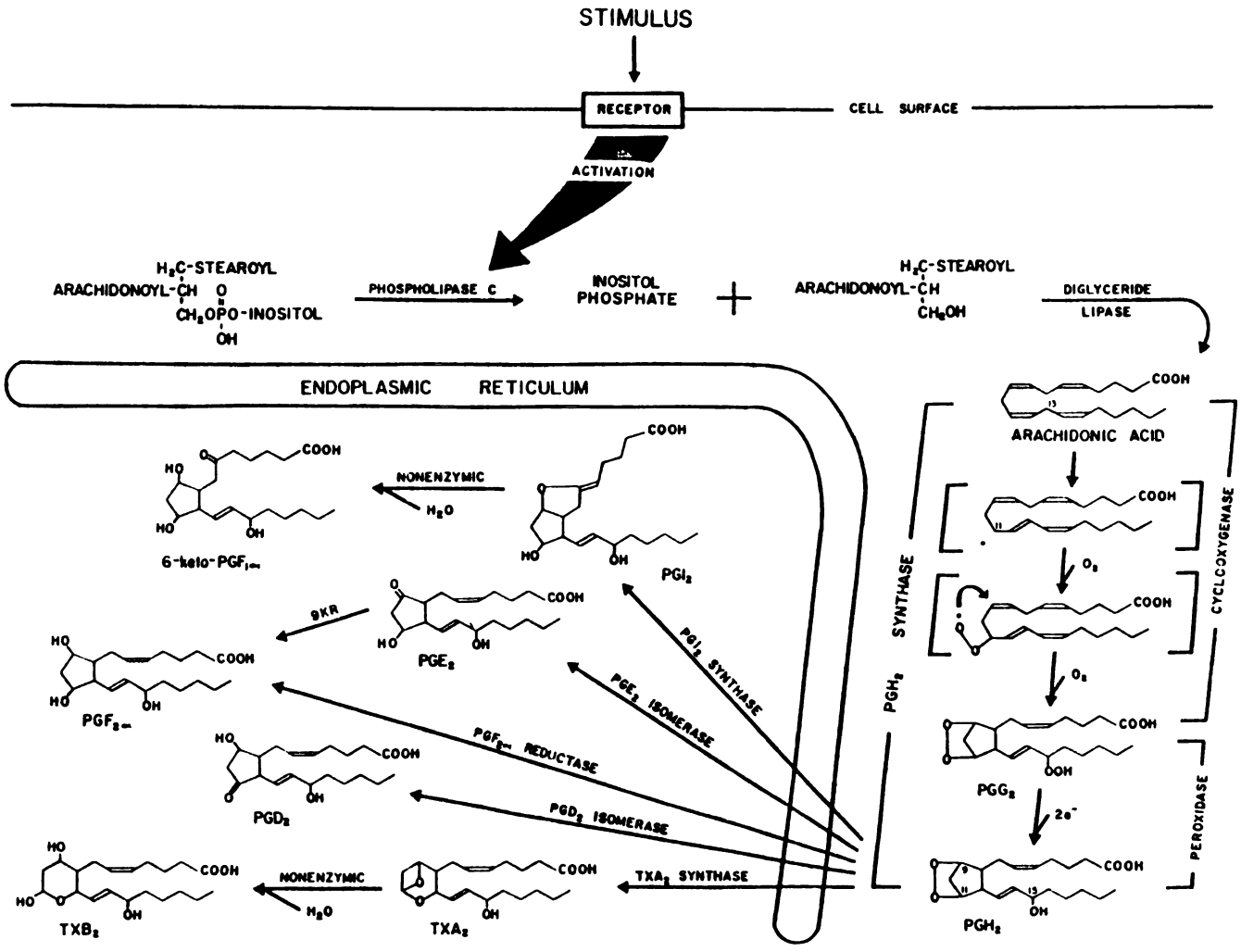
Introduction

In late 1976 Vane and coworkers discovered an enzymatic product of the action of pig aorta microsomes on protaglandin endoperoxides having pharmacological effects different from those of other known prostaglandins (Moncada et al. 1976). This new compound, which they designated PGX, was inactivated by boiling for 15 sec, was more potent at inhibiting platelet aggregation than any other known substance, and caused relaxation of rabbit mesenteric and coeliac arteries. This compound was subsequently identified (Johnson et al., 1976) as 9-deoxy-6,9 α epoxy-Δ5-$PGF_{1\alpha}$ (Figure 1) and was given the trivial name prostacyclin, now abbreviated PGI_2.

Biosynthesis of PGI_2

Prostacyclin is formed from arachidonic acid by the pathway illustrated in Figure 1. The major control point in the synthesis of PGI_2 and other prostaglandins is the hydrolysis of arachidonic acid from phospholipids. Release of arachidonic acid in cells that form PGI_2 can be elicited by a variety of hormonal and proteolytic stimuli including trypsin, thrombin (Weksler et al. 1978), bradykinin, angiotension II (Needleman et al., 1978; Hong, 1980) and platelet derived growth factor (Coughlin et al., 1980). Nonphysiological agents such as the calcium ionophore, A23187, or nitroglycerin can also cause

Figure 1. An overview of prostaglandin biosynthesis.


STIMULUS
RECEPTOR
CELL SURFACE
ACTIVATION
PHOSPHOLIPASE C
INOSITOL PHOSPHATE
DIGLYCERIDE LIPASE
ENDOPLASMIC RETICULUM
ARACHIDONIC ACID
NONENZYMIC
6-keto-PGF1α
PGI2
PGE2
PGF2α
PGD2
TXB2
TXA2
PGH2
PGG2
PGI2 SYNTHASE
PGE2 ISOMERASE
PGF2α REDUCTASE
PGD2 ISOMERASE
TXA2 SYNTHASE
SYNTHASE
CYCLOOXYGENASE
PEROXIDASE

fatty acid release and prostacyclin production (Ingerman et al. 1981; Weksler et al. 1978; Levine et al. 1981).

Once arachidonate is released from its normal esterified form, it is quickly converted to the prostaglandin endoperoxide, PGH_2. PGH_2 is synthesized by a single enzyme PGH synthase (also called cyclooxygenase or prostaglandin synthetase) which catalyzes two separate reactions; first, bis oxygenation of arachidonic acid to form PGG_2 (15-hydroperoxy-9α,11α-peroxidoprosta-5,13 dienoic acid), and second, reduction of the 15-hydroperoxy group to an alcohol yielding PGH_2, (Hamberg and Samuelsson, 1974). The PGH synthase, which has been purified to homogeneity by several groups (Hemler et al. 1976; VanderOuderaa et al. 1977), is a membrane bond enzyme of subunit molecular weight 72,000. The enzyme has one protoporphyin heme prosthetic group per subunit. This heme is essential for both cyclooxygenase and peroxidase activity (Titus et al., 1982; Roth et al., 1981). The enzyme requires peroxides for activity and undergoes a self-inactivation in vitro (Hemler et al., 1979; Smith and Lands, 1972) and probably in vivo (Harada et al., 1980). The PGH synthase of Swiss moused 3T3 fibroblasts is located on the cytoplasmic surface of the endoplasmic reticulum (Rollins and Smith, 1980; DeWitt et al., 1981). PGH synthase is widely known as a major site of action of aspirin and other non-steroidal anti-inflamatory drugs (Vane, 1971; Roth et al., 1975).

PGH_2 can disproportionate nonenzymatically yielding PGD_2, PGE_2, $PGF_{2\alpha}$ or can be converted enzymatically to the same compounds and PGI_2 or TxB_2. Homogeneous cell populations, derived from differentiated cells produce one prostaglandin predominately and

therefore probably contain only one type of PGH_2 metabolizing enzyme (Smith, 1981).

The PGI_2-forming enzyme, PGI_2 synthase, was first found in cells of the vasculature including both endothelial and smooth muscle cells. In fact, PGI_2 is the major prostaglandin produced by vascular tissue from all mammalian species examined to date (Bunting et al., 1976; Dusting et al., 1977). PGI_2 is also synthesized by the rat stomach (Greglewski et al., 1976). This latter discovery was actually preceded by an observation made five years earlier by Pace-Asciak and Wolfe (1971) that rat stomach homogenates synthesize 6-keto-$PGF_{1\alpha}$ from arachidonate: 6-keto-$PGF_{1\alpha}$ is now known to be the stable acid hydrolysis product of PGI_2. More recent work by Sun et al. (1977) indicates that PGI_2 is formed by all major tissues and organs including corpus luteum, uterus, stomach, small intestine, and lung.

PGI_2 is unstable in most biological systems. In fact an identifying test for PGI_2 is the lability of its biological activity; original reports (Moncada et al., 1976, Gryglewski et al., 1976) state that incubation of PGI_2 at 100° for 15 sec or at 37° for 10 min completely destroys the anti-aggregatory activity of this compound. When the structure of PGI_2 was determined, it became clear that the rapid loss of PGI_2 activity is the result of an acid catalyzed hydrolysis of the labile vinyl ether group yielding 6-keto $PGF_{1\alpha}$ (Johnson et al., 1976). Prostacyclin has been reported to have a half life of 2-3 min in buffer at neutral pH, but in blood the reported half-lives have varied from 2 to 15 min. Some authors have observed that albumin stabilizes the PGI_2 (Phifer et al., 1981). For convenience in pharmacological studies, PGI_2 solutions are usually

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buffered above pH 10. Under these conditions the half-life increases to days or weeks depending on storage conditions (Johnson et al., 1976).

PGI_2 Synthase

There are relatively few published reports on the characteristics of PGI_2 synthase, the enzyme that converts PGH_2 to PGI_2. One important observation made in early studies in Vane's laboratory was that PGI_2 formation was inhibited by low concentrations (IC_{50} = 0.50 µg/ml) of 15-hydroperoxy-arachidonic acid (Gryglewski et al., 1976). Later, Salmon et al. (1978) found that a variety of 18 and 20 carbon polyunsaturated fatty acid hydroperoxides inhibited PGI_2 synthase; hydroperoxides tested had IC_{50} values of approximately 1 µM and the inhibition was time dependent. If substrate, PGH_2, and hydroperoxy acid were added simultaneously little or no inhibition occurred, but PGI_2 synthase activity decreased with increasing preincubation times with hydroperoxy acids. Cofactors such as glutathione were found not to enhance enzyme activity. A saturating concentration of PGH_2 was reported to be 10 µM. The enzymatic production of PGI_2 from PGH_2 did not follow standard Michaelis-Menten kinetics. After an initial burst of PGI_2 synthesis, which was linear for slightly less than one minute (at 1.4 µM PGH_2), PGI_2 production abruptly halted. Watanabe et al. (1979) obtained similar results at higher substrate concentrations (PGH_2 = 40 µM). These authors reported that a given amount of enzyme catalyzed production of a finite amount of PGI_2, then stopped. No further PGI_2 formation occurred upon addition of fresh PGH_2 to the reaction mixture, but addition of fresh microsomes caused the synthesis of a

predictable amount of PGI_2. Watanabe et al. concluded that PGI_2 synthase activity halted not due to substrate availability or product inhibition, but rather due to inactivation of the PGI_2 synthase during catalysis.

Only relatively simple attempts at purifying the PGI_2 synthase have been published. The protocols involve solubilizing PGI_2 synthase activity from aorta microsomes with Triton X-100 and chromatographing the enzyme on DEAE-cellulose. A stepwise salt gradient is used to elute the PGI_2 synthase (Wlodawer and Hammarstrom, 1979; 1980). No specific activities have been reported for the partially purified enzyme, but in our hands this method yielded only a 10 fold purification with 20% of the starting activity. The most significant purification (4 fold) occurred at the solubilization step. Attempts to purify thromboxane A_2 synthase and PGH-PGE isomerase, which, like PGI_2 synthase, are microsomal enzyme have also met with only limited success (Hall et al., 1981; Yoshimoto et al., 1977; Wlodawer and Hammerstrom, 1978b; Moonen et al., 1982).

Effects of PGI_2 on Platelet Aggregation

Prostacyclin has been found to have two important effects on the cardiovascular system: (a) inhibition of platelet aggregation and (b) vasodilation. Both effects appear to be mediated via activation of adenylate cyclase. PGI_2 added to platelets causes an increase in intracellular cAMP levels which inhibits aggregation (Tateson et al., 1977; Gorman et al., 1977). PGI_2 added to vascular strips also increases cAMP concentrations apparently causing vasodilation (Miller et al., 1979). The platelet adenylate cyclase activation system operates through high affinity PGI_2 receptors which also bind PGE_1

(Shafer et al., 1979). The stable hydrolysis product of PGI_2, 6-keto-$PGF_{1\alpha}$, is 1000 times less active than prostacyclin in stimulating platelet adenylate cyclase.

It is important to note that thromboxane A_2 (TxA_2) formed by platelets (Needleman et al., 1976) opposes the effect of PGI_2 on adenylate cyclase; TxA_2 prevents the increase in cAMP caused by PGI_2 in platelets (Gorman et al. 1978). Not surprising, TxA_2 causes vasoconstriction and platelet aggregation (Hamburg et al. 1975). Thus, two prostanoid derivatives, both synthesized from the same substrate, PGH_2, have opposing effects.

As an inhibitor of platelet aggregation, PGI_2 is the most powerful pharmacological agent known, being 30 times more potent than the next most active prostaglandin, PGE_1 (Moncada and Vane, 1977b), and 1000 times more active than adenosine (Born, 1962). Prostacyclin, when tested in vivo, has been shown to inhibit formation of artificially induced thrombi in hamster cheek pouch (Higgs et al., 1977) and in the rabbit (Ubatuba et al., 1977). Prostacyclin has also been shown to disaggregate preformed platelet clumps in vitro (Moncada et al., 1976b; Gryglewski et al. 1978 a,b).

Effects of Prostacyclin in the Vasculature

Two physiological functions of prostacyclin have been postulated (Moncada and Vane, 1977). The first is that PGI_2 helps maintain vascular integrity by working to balance the effects of TxA_2, which is both a vasoconstrictor and a thrombogenic agent. Normally platelets do not adhere to healthy vascular endothelim. However when a vascular injury occurs and the endothelum is damaged, platelets bind the subendothelium and begin to aggregate. Aggregating platelets secrete

from their granules vasoactive and platelet-active substances including ADP, serotonin and calcium. Platelets also release TxA_2 which causes vasoconstriction and promotes further aggregation. With traumatic vascular injuries involving the subendothelium, a thrombus forms thereby preventing blood loss. When the injury to the vessel is minor, only one cell layer of platelets adheres. Further aggregation is presumeably inhibited by PGI_2 secreted by the vessel wall.

The second postulated function of PGI_2 is to regulate vascular smooth muscle tone. It has been postulated that PGI_2 secreted into the blood may have systemic hemodynamic effects. Infusion of PGI_2 decreases blood pressure in man (O'Grady *et al.*, 1980) and in pig (Clement *et al.*, 1980).

Is Prostacyclin a Circulating Hormone?

While pharmacological doses of prostacyclin can inhibit platelet aggregation *in vitro* and *in vivo* and infusions of PGI_2 will reduce blood pressure and cause other hemodynamic changes, it is difficult to establish the actual physiological importance of these phenomenon. One concept that has been given considerable experimental attention is that prostacyclin is a circulating hormone.

Gryglewski *et al.*, (1978) and Moncada *et al.*, (1978) proposed that PGI_2 is continuously secreted into the blood at concentrations sufficient to modulate blood pressure and platelet aggregation. There were several observations supporting this hypothesis. One is that prostacyclin has a relatively long biological half life ($T_{1/2}$ = 2-5 min) and is not metabolized by passage through the lung (Dusting *et al.*, 1978). Thromboxane A_2 is hydrolyzed too rapidly to have any effect distant from its synthesis ($T_{1/2}$ = 30 sec) and PGE_2,

PGD_2 and $PGF_{2\alpha}$ are catabolized by a single passage through the lungs. Although PGI_2 is catabolized by the same type of dehydrogenase that oxidizes PGE, PGD and PGF_α, PGI_2 is apparently not transported into
those cells in the lung containing 15-hydroxyprostaglandin dehydrogenase activity. In other experiments suggesting that PGI_2 is a circulating hormone, Gryglewski et al. (1978) and Moncada et al. (1978) obtained results indicating that PGI_2 was continuously released into the circulation by the lungs. In these experiments, blood from anesthesized animals was perfused over a collagen strip and returned intravenously to the animal. After a short period of time, platelets began to aggregate on the collagen strip and the increase in weight due to the growing thrombus could be recorded. These researchers found that by adding PGI_2 to the perfusing blood they could cause disaggregation and a decrease in thrombus weight; moreover, less PGI_2 was needed when added to arterial blood than was needed when added to venous blood to cause the same degree of disaggregation (i.e. weight change). Therefore, these investigators hypothesized that the difference in the disaggregation potential between the two blood sources was due to secretion of PGI_2 into the arterial blood by the lungs. They estimated that 100-200 pg of PGI_2 was secreted per ml of blood.

More recent studies indicate that PGI_2 is not a circulating hormone. Two different types of experiments have provided good evidence that there is not enough circulating PGI_2 to have any anti-thrombogenic or hemodynamic effects. The first type of experiment has involved passive immunization with anti-PGI_2 antibodies. Animals

injected with 6-keto-$PGF_{1\alpha}$ conjugated to a protein carrier will produce antibodies that bind PGI_2. These antibodies block PGI_2-mediated inhibition of platelet aggregation in vitro (Smith et al., 1978). Moreover, the simultaneous infusion of PGI_2 and anti-PGI_2 serum into cats completely inhibits the blood pressure lowering observed when PGI_2 is infused alone. However, infusion of anti-PGI_2 serum by itself has no effects. This latter result suggests that the level of circulating PGI_2 is normally too low to have important hemodynamic effects. In related experiments, Steer et al. (1980) have shown that infusion of anti-PGI_2 serum does not affect platelet cAMP levels although this antiserum does block the increases in platelet cAMP caused by infusions of PGI_2.

Direct measurements of PGI_2 concentrations in blood are consistent with the results of the passive immunization experiments. Quantitation of circulating PGI_2 in human blood by measurement of circulating 6-keto-$PGI_{1\alpha}$ using electron capture gas chromatography has indicated that PGF_2 is present at concentrations of less than 20 pg/ml (0.06 nM) (Christ-Hazelhof and Nugteren, 1981). The lowest level of PGI_2 that will cause detectable physiological effects is 0.1 nM (Steers et al., 1980).

The rate of secretion of PGI_2 into the circulation of man has also been estimated by Fitzgerald et al. (1981) who measured the rate of excretion of PGI_2 metabolites into the urine. Volunteers were infused at various rates with PGI_2 and excretion of PGI_2 metabolites was measured by stable isotope ratio gas chromatography mass spectroscopy. Extrapolation of the plotted excretion rates at the various PGI_2 infusion rates back to zero infusion resulted in an

estimation of an endogenous production of 0.08-0.10 ng PGI_2/kg/min. These authors reported that infusions of 2-4 ng/kg/min were required to cause any inhibition of platelet function in man, and concluded that PGI_2 is not a "circulating hormone" in man.

Thus, while most studies indicate that PGI_2 is not a circulating effector under normal resting conditions, there is evidence that biologically significant concentrations of PGI_2 may occur in certain pathological states. As measured by electron capture gas chromatography, the PGI_2 level in one human subject rose from less than 20 pg/ml to more than 200 pg/ml during a severe staphlococcus infection. PGI_2 levels in rabbits have been shown increase from 50 pg/ml to above 1000 pg/ml after endotoxin shock (Christ-Hazelhoff and Nugteren, 1981). Analysis of PGI_2 levels by radioimmunoassay of 6-keto-$PGF_{1\alpha}$ in serum has indicated that during increased ventilation accompanying anesthesia, PGI_2 concentrations rose from 17 to 190 pg/ml (Edlund et al., 1981).

Although PGI_2 is normally not released into the circulation at concentrations high enough to have systemic hemodynamic or platelet saving effects, there is still a strong possibility that PGI_2 modulates hemostasis, at localized sites in the vasculature. If PGI_2 is formed only during thombus formation or in response to adherence of platelets to vessel walls, then increases in PGI_2 concentrations will occur only at sites of stimulation. Such increases would help to disaggregate platelets and cause vasodilation at the injured sites; however, the systemic concentration of PGI_2 would not increase substantially.

Another possibility is that most of the PGI_2 formed, whether by the endothelial cell layer or by smooth muscle, remains in the cell of synthesis or acts only on closely neighboring cells. There is considerable evidence (see Chapter 5) that smooth muscle has a substantial capacity for forming PGI_2. However, only endothelial cells and not smooth muscle cells release PGI_2 into the vascular lumen (Eldor et al., 1981). It seems likely that the synthetic capacity of smooth muscle is important. We suspect that there are two distinct PGI_2 synthesizing systems in the vasculature, one in smooth muscle and an other in endothelial cells. PGI_2 formed by smooth muscle is probably important in modulating vascular tone while PGI_2 formed by the endothelium probably acts to regulate platelet function. The fact that PGI_2 is synthesized in virtually all smooth muscle (see Chapter 5) is even more evidence of the importance of PGI_2 in the intrinsic regulation of smooth muscle tone.

Platelet-Vessel Wall Interactions

Although the level of circulating PGI_2 is insufficient to inhibit platelet aggregation systemically, there is evidence that PGI_2 synthesized by vessel walls does inhibit thrombus formation on a local level. It has been shown using cultured endothelial cells that the production of PGI_2 is essential to inhibit thrombin-induced platelet adhesion to endothelial cells (Czervionke et al., 1979). Addition of thrombin and ^{51}Cr-labeled platelets to endothelial cell monolayers stimulates the production of 100 nM PGI_2. Under these conditions adherence of only 4% of ^{51}Cr-labeled platelets is observed; however, when thrombin and labeled platelets are added to aspirin-treated endothelial cells less than 3 nM PGI_2 was produced

and 44% of the platelets adhered. It is assumed that thrombin occurs naturally in response to any thrombus-forming stimuli *in vivo*; in those experiments in which no thrombin was added, there was no platelet adherence to either aspirin-treated or untreated endothelial cells.

More convincing evidence for the effects of PGI_2 formed endogenously on the regulation of thrombus formation comes from experiments on the *in vivo* effects of aspirin on adhesion of platelets to vessel walls of rabbits (Wu *et al.*, 1981). Aspirin was given at two doses to rabbits, a low dose which inhibits only platelet PGH_2 production, and a high dose, which inhibits PGH_2 synthesis by both platelets and vessel wall (Burch *et al.* 1978; Baenziger *et al.* 1979). ^{111}In-labeled platelets were injected and the rabbits were then sacrificed. The degree of thrombus formation was measured in sections of abdominal aorta from which the endothelium had previously been removed by balloon catheterization. Rabbits treated with low dose aspirin had significantly fewer adherent platelets when compared to either controls or animals receiving high-dose aspirin; moreover, the high-dose aspirin rabbits had significantly greater adherence of platelets than the controls. This study suggests that PGI_2 produced *in vivo* does inhibit platelet adherence to vessel walls.

Platelet-Vessel Wall Substrate Interactions

Vane and coworkers showed that microsomes from pig aorta converted only the endoperoxides, PGH_2 or PGG_2, and not arachidonic acid to PGI_2; even when using fresh tissue, they were able to show only a few percent conversion of arachidonic acid to PGI_2, compared to 60-80% conversion of PGH_2 (Moncada *et al.*, 1976b; Gryglewski *et al.*, 1976; Bunting *et al.*, 1976). These observations suggested that the PGH_2

synthesized and released by platelets was used by vessel walls to form PGI_2 which, in turn, inhibited further platelet aggregation. Such a self regulating hemostatic mechanism would assure that as long as a vascular injury was small further systemic aggregation would be averted. The donation of PGH_2 from platelets to vessel walls is called the "steal hypothesis" indicating that vessels steal endoperoxide. There is continuing controversy over the validity and importance of this mechanism.

Two investigations by Needleman et al. (1978; 1979) have indicated that exogenous PGH_2 is not used by the vasculature to produce PGI_2. In the first report Needleman and his colleagues found that in perfused rabbit heart arachidonate, but not PGH_2, could be used to produce PGI_2. They concluded that since heart could not use exogenous PGH_2 to form PGI_2 vessels probably do not use PGH_2 to form PGI_2. In a second report Needleman et al. (1979) found that when aortic microsomes pretreated with a PGH synthase inhibitor were mixed with platelets, PGI_2 was produced by the microsomes only when the conversion of PGH_2 to TxA_2 in the platelets was inhibited. Experiments involving the incubation of intact, aspirin-treated aorta with platelets yielded the same results. Thus, Needleman et al. (1979) concluded that endoperoxides are released by platelet cells only when they cannot be converted to TXA_2.

Aiken et al. (1981) obtained similar results in studies on the partially obstructed circumflex coronary artery in anesthesized dogs. A plastic constrictor was used that obstructed blood flow through the circumflex artery by approximately 34%. Blood flow was monitored using an electromagnetic flow probe. In control dogs 34% obstruction caused

a rapid formation of platelet aggregates as measured by decreased blood flow. By lightly tapping the arteries the aggregate could be dislodged and the flow resumed. Injection of a selective thromboxane synthase inhibitor (E)-2-methyl-3-[4-(3-pyridinyl methyl) phenyl]-2-propenoic acid (MPPA) prevented formation of the obstructing platelet aggregates. In MPPD-treated dogs, topical application of cyclooxygenase inhibitors to the artery at the site of obstruction had no effect on aggregate formation; however, when a PGI_2 synthase inhibitor was applied to the artery, thrombus formation occurred and flow decreased. These results indicate that _in vivo_, platelets endoperoxides originating from aggregating platelets can be used by vessel walls to form PGI_2 when platelet thromboxane synthesis is inhibited. However, in control animals not treated with effectors, thrombus formation does occur. This indicates that under the conditions used to occlude the circumflex artery production of PGI_2 from all sources of PGH_2 was insufficient to inhibit platelet aggregation.

Experiments conducted by mixing platelets and endothelial cells suggest that platelets may share endoperoxides (Marcus _et al._, 1980). When platelets and cultured, aspirin-treated endothelial cells were mixed together in a ratio of 50:1 (a ratio more closely approximating approaching that which occurs _in vivo_ than used in previous experiments) platelet aggregation induced by ionophore 23187, thrombin, or collagen was inhibited and PGI_2 was synthesized from arachidonic acid derived from labeled platelet phospholipids. When platelets were mixed with untreated endothelial cells and then stimulated, up to twice as much PGI_2 was produced as when endothelial cells alone were stimulated. At least in this case, when platelets and endothelial

cells are placed in intimate contact and when the ratio of platelets is adjusted to near physiological levels, platelet endoperoxides are shared. Marcus estimated that the total ratio of platelets to endothelial cells in man is near 1:1.

From the above discussion, I draw four major conclusions regarding the biological actions of PGI_2.

1) Prostacyclin is a physiologically important regulator of platelet vessel wall interactions and platelet aggegation.
2) Prostacyclin is not a circulating hormone, but rather, is a local effector, that is synthesized and secreted into the blood in response to specific stimuli, such as platelet aggregation.
3) Vascular smooth muscle cells synthesize PGI_2, probably for intrinsic regulation of vascular tone.
4) Prostacyclin production by vessel walls may be augmented by PGH_2 supplied by platelets.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST PGH SYNTHASE: AN IMMUNORADIOMETRIC ASSAY

In this chapter the isolation of hybridoma cell lines secreting immunoglobulins specific for PGH synthase is described. The physical characteristics, as well as the antigenic and species specificities of the antibodies secreted are reported. Also, an immunoradiometric assay, which employs two antibodies in tandem to measure PGH synthase enzyme concentrations is described.

METHODS

Materials

Trypticase soy broth soybean-casein digest media was from BBL Microbiology Systems, Cockeyville, MD. The following reagents were obtained from Sigma Chemical Company: hypoxanthine, penicillin, streptomycin sulfate, aminopterin, thymidine, 6-thioguanine, sodium diethyldithiocarbamate, acetylsalicylic acid, bovine hemoglobin, mannose-6-phosphate and Tween 20. Fetal calf serum was from KC Biologicals, Inc. Seaplaque agarose was from Marine Colloids Inc., Rockland, Maine. Freund's adjuvants, Hank's balanced salt solution and Dulbecco's modified Eagle medium (DMEM) containing D-glucose (4.5 g/l) and L-glutamine (200 mM) were purchased from Grand Island Biological Co. NCTC 109 medium was from Microbiological Associates, Bethesda, MD. Normal horse serum was from Flow Laboratories. Fluorescein isothicyanate (FITC)-labeled rabbit anti-mouse IgG, rabbit anti-mouse IgG, IgG_2, IgG_{2a} and IgG_{2b} were obtained from Miles laboratories. Protein A-Sepharose was purchased from Pharmacia Fine Chemicals. Arachidonic acid was obtained from Nu-Chek Preps, Elysian, MN. Bolton and Hunter's reagent (1400 Ci/mmole) was from Amersham.

Cell lines, animals, tissues

Female C57Bl mice (4-6 weeks old) were from Jackson Laboratories and female albino Swiss mice (4-6 weeks old) were from Spartan Animal Services, Michigan State University. Sheep vesicular glands and bovine

seminal vesicles were obtained from a local abattoir. Unless fresh tissue was required tissue samples were stored at -80°. Outdated human platelet concentrates donated by the Lansing Regional Red Cross Blood Distribution Center, were washed with Hank's balanced salt solution containing 1% EDTA, pH 7.4, prior to preparation of microsomes. Female Sprague-Dawley rats (150-200 g), female New Zealand white rabbits (2 kg) and virgin female guinea pigs (600-800 g) were obtained from local animal suppliers. SP2/0-Ag14, an 8-azaguanine-resistant myeloma strain was obtained from the Cell Distribution Center of the Salk Institute, LaJolla, CA. Swiss mouse 3T3 fibroblasts were from the American Type Culture Collection. Rats and guinea pigs were killed by decapitation, mice by cervical dislocation and rabbits by intravenous injection of 5% phenobarbitol.

Preparation of microsomes

All steps were performed at 4°. Rabbit or guinea pig kidney, rat small intestine, bovine seminal vesicles, sheep vesicular glands or human platelets were homogenized in 5-10 volumes of 0.1 M Tris-chloride, pH 8.0, containing 20 M diethyldithiocarbamate and 1 M phenol. Platelets were homogenized in a glass homogenizer with sonication in a Cole-Parmer ultrasonic cleaner. Other tissues were homogenized using a Polytron homogenizer. The homogenates were centrifuged at 10,000 x g for 10 min and the resulting supernatants centrifuged at 200,000 x g for 35 min. The resulting microsomal pellets were resuspended by homogenization in starting buffer to a protein concentration of 1-10 mg/ml as determined by the Coomassie Blue protein assay procedure (Bradford, 1976). If cyclooxygenase activity

was to be solubilized, Tween 20 was added to the resuspended microsomes to give a final concentration of 1.0% (w/v) detergent.

Cyclooxygenase assays

Cyclooxygenase activities were measured polarographically at 37° using a Yellow Springs Instrument Company Model 53 Oxygen Monitor essentially as described by Smith and Lands (1972). Reactions were initiated by the addition of enzyme to a reaction mixture composed of 3 ml of 0.1 M Tris-chloride, pH 8.0, containing 0.1 M arachidonic acid, 1 M phenol and 10 M bovine hemoglobin. One unit of cyclooxygenase activity is defined as that amount of enzyme which catalyzes the consumption of 1 nmole oxygen per min per ml of assay solution at 37°.

Myeloma-spleen cell fusions

Fusions were performed by modification of a method of Galfre et al. (1977). Four to six week old female C57/Bl mice or Swiss albino mice were immunized (i.p.) at two week intervals with 20 µg of PGH synthase purified from sheep vesicular gland (Hemler et al., 1976) and suspended in complete Freund's adjuvant. Three days after the third inoculation the mice were killed by cervical dislocation and their spleens removed under sterile conditions. The spleens were placed in 5 ml of Dulbecco's modified Eagle media (DMEM) containing 20 mM HEPES, pH 7.6, cut into pieces (1 cm^3) and then teased apart with scissors to release the lymphocytes. After vortexing the mixture, the large tissue fragments were allowed to settle briefly. The supernatant containing the lymphocytes was then centrifuged at 1000 x g for 5 min. Red blood cells in the pellet were removed by hypotonic lysis with 5.0 ml of 0.2% saline for 30 sec followed by 5.0 ml of 1.6% saline for 30 sec. Finally, 10 ml of DMEM containing 20 mM HEPES, pH 7.6 was added, and

the remaining spleen cells were then collected by centrifugation and resuspended in DMEM containing 20 mM HEPES, pH 7.6.

The mouse myeloma strain SP2/0-Ag14 was grown in DMEM containing 10% fetal bovine serum and 100 mg/l each of penicillin and streptomycin at 37° under a water-saturated 10% CO_2 atmosphere. SP2 myeloma cells ($1\text{-}5 \times 10^6$), which had been washed and resuspended in DMEM plus 20 M HEPES pH 7.6, were mixed with $1\text{-}5 \times 10^7$ of the isolated splenic lymphocytes. The cell mixture was collected by centrifugation at 1000 x g for 5 min in a sterile glass centrifuge tube. After removing the supernatant, the fusion was begun by gently shaking the cell pellet, largely intact, with a solution containing 35% polyethylene glycol 1000 (Baker) and 5% dimethylsulfoxide in DMEM for 1 min. During the ensuing 3 min the fusion solution was diluted with 3 ml of serum-free DMEM; then, over a period of 6 minutes, the fusion mixture was diluted further with 12 ml of DMEM containing 20% fetal bovine serum. Finally, the cells were collected by centrifugation, resuspended in 48 ml of HT media (DMEM containing 10% (v/v) fetal bovine serum, 10% (v/v) horse serum, 10% (v/v) NCTC 109 medium, 2 M glutamine, 100 M hypoxanthine, 16 M thymidine, 3 M glycine, 100 mg/l penicillin and 100 mg/l streptomycin) and dispensed into 2-24 well Costar 3524 cluster tissue-culture plates. After 24 hours, an additional 1 ml of HAT media (HT media plus 1 M aminopterin) was added to each well. Half of the media was replaced with fresh HAT media 2 and 4 days thereafter; 14-21 days after the cell fusion, when the media from those wells with growing hybridomas began to acidify (turn yellow), aliquots of media were removed to test for the presence of anti-PGH synthase antibody.

Selection of hybridomas producing antibody to PGH synthase

The Protein-A bearing Cowen I strain of Staphylococcus aureus, kindly provided by Dr. Ronald Patterson, was grown in Trypticase® soy broth soybean-casein digest media and attenuated as described by Kessler (1976). Formaldehyde-fixed, heat-killed S. aureus cells were stored at -80° as a 10% cell suspension in 10 mM HEPES, pH 7.5 containing 150 M NaCl. Prior to the immunoprecipitation assay, 1.0 ml aliquots of the cell suspensions were defrosted and washed by sequential centrifugation and resuspension as follows: (a) twice with 1 ml of 0.1 M Tris-chloride, pH 8.0 containing 5% bovine serum, (b) twice with 1 ml of 0.1 M Tris-chloride, pH 8.0 containing 1% Tween 20 (w/v), (c) once with 1 ml of 0.1 M Tris-chloride pH 8.0 containing 1% Tween 20 (w/v) and 80 µg of rabbit anti-mouse IgG and (d) twice with 1 ml of 0.1 M Tris-chloride, pH 8.0 containing 1% Tween 20 (w/v). Finally the cells were resuspended in 1 ml of 0.1 M Tris-chloride, pH 8.0 containing 1% Tween 20 (w/v).

For immunoprecipitation assays, 0.1 ml of the rabbit anti-mouse IgG-S. aureus suspension was mixed with 0.1 ml of medium removed from each well with growing hybridomas. The sample was vortexed and then centrifuged for 2 min at 500 x g on a desk-top centrifuge. A volume of solubilized sheep vesicular gland microsomes (ca. 5 µl) containing 25 units of cyclooxygenase activity was added to the S. aureus pellet along with 0.1 M Tris-chloride, pH 8.0 containing 20 mM diethyldithiocarbamate and 1 mM phenol to give a final volume of 0.1 ml. The mixture was vortexed and then centrifuged to pellet the cells. The supernatant was removed and the resulting pellet was resuspended in 0.1 ml of starting buffer. Both the supernatant and pellet were assayed for cyclooxygenase activity. Precipitation of cyclooxygenase

activity was taken as evidence that at least some of the hybridoma cells present in the test well were producing anti-PGH synthase antibodies.

Cells from wells yielding positive responses in the immunoprecipitation test were cloned in soft agar using Swiss mouse 3T3 cells as a feeder layer. Cells from individual clones were cultured and the media retested for anti-PGH synthase activity as described above. A few positive clones from each positive well were cultured, frozen and stored in liquid N_2.

Immunofluorescence staining for PGH synthase

Media from hybridoma clones secreting anti-PGH synthase antibodies were used in an indirect immunofluorescence procedure to detect the PGH synthase antigen in kidney sections. Sections (10 μM) from rabbit, rat, mouse and guinea pig kidney were cut on a Tissue-Tek cryotome and stained for cyclooxygenase antigenicity using sequential incubations with media from hybridoma cells (1:5 dilutions with 0.1 M sodium phosphate, pH 7.0) and then fluorescein-isothiocyanate (FITC)-labeled rabbit anti-mouse IgG (1:20 dilution in 0.1 M sodium phosphate, pH 7.0) essentially as described previously for rabbit anti-cyclooxygenase serum (Smith and Wilkin, 1977; Smith and Bell, 1978). Interaction of antibody with the PGH synthase was indicated by the appearance of fluoresence in renal medullary collecting tubules and interstitial cells in experimental but not control samples (Smith and Bell, 1978). Media from hybridoma-containing cultures which failed to cause immunoprecipitation of sheep vesicular gland PGH synthase were used as control media. A Leitz Orthoplan microscope was used to visualize fluorescent staining.

Preparation of IgG-free fetal calf serum and cell culture media

Fetal calf serum (100 ml) was adjusted to pH 8.2 and applied to a Protein A-Sepharose CL-4B column (1 x 5 cm) equilibrated with 0.1 M sodium phosphate, pH 8.0. The eluant was collected and bovine IgG absorbed to the column was then removed by washing with 2-3 column volumes of 0.1 M sodium citrate, pH 3.5. The column was reequilibrated with 0.1 M sodium phosphate, pH 8.0, and the entire procedure repeated. After three passages of fetal calf serum through the column, no detectable absorbance at 280 nm was found to elute with 0.1 M sodium citrate, pH 3.5. Media used for isolation of mouse IgG was standard HT media containing 20% IgG-free fetal calf serum and no horse serum.

Purification of mouse IgG_1 from hybridoma culture media

Medium from IgG (cyo-3) producing hybridoma cultures (free of bovine IgG) was adjusted to pH 8.2 and applied to a Protein A-Sepharose CL-4B column (1 x 5 cm). Absorbed material was eluted stepwise using 0.1 M buffers of pH 8.0 (sodium phosphate), pH 6.0, pH 4.5, and pH 3.5 (sodium citrate) (Ey et al., 1978). Anti-PGH synthase activity in each fraction was monitored by measuring the ability of the fraction, when mixed with rabbit anti-mouse IgG-S. aureus complexes, to precipitate cyclooxygenase activity. IgG_1 secreted by hybridoma line cyo-3 was eluted at pH 6.0. Fractions containing IgG_1 were pooled, dialyzed overnight against 0.125 M sodium borate, pH 8.4, and stored at -80°. In one experiment in which cyo-3 were grown to confluency, 9 mg of IgG_1 (as determined by the absorbance at 280 nm (ϵ = 1.4 $(mg/ml)^{-1}$ (Freedman et al., 1968)) was isolated from 120 ml of media; 1 µg of isolated IgG_1 when bound to 0.1 ml of the rabbit

anti-mouse IgG-S. aureus cell suspension was able to bind 18 units (ca. 0.6 µg) of cyclooxygenase activity.

Radioiodination of IgG_1 (cyo-3) and sheep vesicular gland microsomes

Both IgG_1 (cyo-3) and sheep vesicular gland microsomes were radioiodinated essentially as described by Bolton and Hunter (1973). For routine iodinations, an aliquot containing 0.4 mCi of Bolton and Hunter's reagent was evaporated under a gentle stream of dry N_2 in a 6 x 50 mm test tube. IgG_1 (40 µg, cyo-3) or solubilized sheep vesicular gland microsomes (400 µg of protein) in 0.01-0.05 ml of 0.125 M sodium borate, pH 8.4 was added and the sample incubated at 4° for 15 min with frequent agitation. Unreacted iodinating reagent was destroyed by the addition of 0.5 ml of 0.2 M glycine to the reaction buffer followed by a 10 min incubation at 4°. Products were separated by chromatography on a column of Biogel P-30 (0.5 x 7 cm) eluting with 0.05 M sodium phosphate, pH 7.4 containing 2.5 mg/ml gelatin and 0.02% NaN_3. Fractions eluting at the void volume were pooled and stored at -80° in small aliquots (0.2 ml) containing 12-20 µCi. The percentage of radiolabel incorporated into IgG_1 or microsomal protein using this procedure ranged from 60-80%. Most (ca. 80%) of the ^{125}I present in IgG_1 coelectrophoresed with the heavy chain on SDS-gel electrophoresis. Prior to use of ^{125}I-IgG_1 for immunoradiometric assays, samples were thawed and diluted in 0.05 M sodium phosphate, pH 7.4 containing 2.5 mg/ml gelatin.

Immunoradiometric assay of PGH synthase

A 10% (w/v) suspension of attenuated S. aureus cells was washed by centrifugation once in 0.1 M Tris-chloride, pH 7.4 containing 5% (w/v) BSA and 1% Tween-20, and then twice in 0.1 M Tris-chloride, pH 7.4 with

1% Tween-20 (the assay buffer) followed by resuspension to 10% (w/v) S. aureus in the assay buffer. Equal volumes of washed S. aureus suspensions and media from various IgG_2-producing hybridoma clones (cyo-1,5,7 or 2c3) were mixed, allowed to stand 15 min at 24° and then centrifuged. Pellets were resuspended and washed twice in the assay buffer and finally resuspended to 10% (w/v) S. aureus concentration.

Solubilized microsomes were diluted into 0.1 ml of assay buffer to contain the equivalent of 0.0-0.5 units of cyclooxygenase activity; 10 ml of S. aureus-IgG_2 complex preparation (sufficient to bind 3 units of cyclooxygenase activity) was then added. Finally, 0.01 ml of ^{125}I-IgG_1 (cyo-3), containing 50,000 ^{125}I cpm originally (this amount was not altered to compensate for the decay of the ^{125}I) was then added. Assay mixtures were incubated at 4° overnight. Pellets were collected by centrifugation, washed once in 0.2 ml of assay buffer and recentrifuged. The supernatants were removed by aspiration and the tubes containing the pellets were inserted into vials and counted using a Beckman Biogamma δ-counter.

Immunoprecipitation of ^{125}I-Labeled Sheep Vesicular Gland Microsomes

S. aureus cells washed as described above were further treated for the radio-immunoprecipitation as follows: First, 100 µg of rabbit anti-mouse IgG was added to each ml of a washed 10% (w/v) suspension of S. aureus cells in 0.1 M Tris-chloride, pH 8.0 containing 1% Tween 20 (w/v), 2 mM diethyldithocarbamate, 5 mM EDTA and 1 mM phenol. This latter buffer was used throughout the procedure. The cells were incubated with the rabbit anti-mouse IgG for 30 min and then collected by centrifugation. The supernatant was decanted, the pellet was washed

once by suspension in the same volume of buffer and the cells again collected by centrifugation. This latter S. aureus cell pellet was resuspended in the same volume of buffer and IgG from each of the following mouse hybridoma clones were added to separate aliqots of the S. aureus-rabbit anti-mouse cells: cyo-1 (IgG_{2b}), cyo-3 (igG_1), cyo-5 (IgG_2b), cyo-7 (IgG_{2b}) (all anti-PGH synthase antibodies), isn-1 (IgG_1), tsn-4 (IgG_1) and the 2c3 (IgG_{2b}) (all antibodies that do not precipitate PGH synthase activity). The final mouse IgG concentration in each case was 100 µg per l of S. aureus suspension. The antibodies and cells were incubated overnight at 4° to maximize antibody binding. Each mouse IgG-rabbit anti-mouse IgG-S. aureus complex was the collected by centrifugation, washed once and resuspended to 10% (w/v) in buffer.

To 10 µl of each of the S. aureus-conjugated antibody suspensions was added 2.74 µg of iodinated sheep vesicular gland protein (5.5 µCi) along with 100 µl of buffer; the mixture was allowed to stand for 30 min. The S. aureus cells were then collected by centrifugation and the supernatants removed by aspiration. The S. aureus pellets were subsequently washed three times with 100 µl of buffer. After the final wash, the supernatant was removed and 30 µl of reducing buffer (0.125 M Tris-chloride, pH 6.8, containing 4% sodium dodecyl sulfate, 20% glycerol, 0.002% bromophenol blue and 5% 2-mercaptoethanol) was added to each pellet. After heating at 100° for 5 min the S. aureus cells were again pelleted by centrifugation, and the supernatants were applied to a 0.75 mm thick 10% polyacrylamide slab gel containing 0.5% SDS and subjected to electrophoresis according to the method of Laemmli (1970). After electrophoresis, the gel was fixed in 10%

trichloroacetic acid - 20% methanol for 1 hr, stained with 50% methanol - 0.25% Coomassie Blue R-250 for 1 hr, destained in 7.5% acetic acid and dried on a Biorad Model 224 Gel Slab Dryer between two sheets of Biorad Dialysis membrane. The dried gel was then exposed to Kodak XAR-5 X-ray film for twelve hours prior to developing the film.

Ouchterlony double-diffusion and immunoprecipitation analyses

Double diffusion analyses were performed in 1.5 % Bacto-agar containing 0.02% NaN_3. Media (0.04 ml) from hybridoma cultures and rabbit anti-mouse IgG_{2a}, IgG_{2b} or IgG_1 serum (0.04 l) were placed in adjacent wells and allowed to diffuse at 24° for 16-24 hr prior to photography. Immunoprecipitation of IgG produced by mouse hybridomas was performed by incubating 0.005 ml of rabbit anti-mouse IgG and various amounts (0.01-0.1 ml) of culture media with enough 0.1 M Tris-chloride, pH 7.4, containing 0.1% Tween 20 to make a final volume of 0.5 ml. The samples were incubated overnight at 4°, collected by centrifugation at 2000 x g for 15 min and washed once with starting buffer.

RESULTS

Isolation of hybridoma cell lines secreting anti-PGH synthase IgG

Two separate fusions of SP2/0-Ag14 plasmacytomas with splenic lymphocytes from mice immunized with purified PGH synthase were performed. In the first fusion, lymphocytes were obtained from a C57Bl mouse; in the second, lymphocytes were obtained from an outbred strain of laboratory white mice. Media from 48 different wells containing hybridomas obtained growing in the first fusion were tested for anti-PGH synthase activity by mixing the media with rabbit anti-mouse IgG-*S. aureus* complexes, and then testing the resulting complexes for their abilities to precipitate solubilized cyclooxygenase activity; one hybridoma (*cyo*-1) was ultimately cloned from the first fusion. Three additional clones (*cyo*-3, *cyo*-5 and *cyo*-7) secreting anti-PGH synthase antibody were prepared from hybridomas obtained in the second fusion after screening media from 24 different hybridoma-containing wells. An additional hybridoma (*2c3*) was obtained from the first fusion. IgG secreted by *2c3* does not interact with the PGH synthase, and thus, *2c3* was used as a negative control in subsequent immunochemical tests.

Specificity of Monoclonal antibodies against PGH synthase

We performed the following experiments to determine if the IgG secreted by *cyo*-1, *cyo*-3, *cyo*-5 and *cyo*-7 interacted directly and selectively with the PGH synthase. A mixture of ^{125}-I-labeled sheep vesicular gland microsomal proteins were incubated with various

IgG-S. aureus complexes and the ^{125}I-labeled, precipitated proteins were examined by autoradiography following SDS-polyacrylamide gel electrophoresis (Figure 2). At least twenty-five easily identifiable proteins were present in the mixture of ^{125}I-labeled microsomal proteins. The mobilities and relative abundances of these proteins correspond to those detected by Coomassie Blue staining (not shown). The major radiolabeled protein precipitated in each case by the four different anti-PGH synthase antibodies (secreted by hybridoma lines cyo-1, cyo-3, cyo-5 and cyo-7) complexed to S. aureus cells electrophoresed with the same mobility as ^{125}I-labeled PGH synthase (ca. 70,000 daltons); in contrast, little or no radioactivity electrophoresing with this mobility was pecipitated by the three different control antibodies (secreted by hybridoma lines isn-1, tsn-4 and 2c3). A number of ^{125}I-labeled proteins with mobilities greater than the PGH synthase were precipitated by complexes of S. aureus cells involving both anti-PGH synthase and control antibodies. However, with the clear exception of ^{125}I-labeled PGH synthase, the ^{125}I-labeled proteins precipitated by the anti-PGH synthase antibodies were precipitated by control antibodies. These results indicate that the only obvious difference between immunoprecipitation with anti-PGH synthase and with control antibodies is in the precipitation of the PGH synthase itself.

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

Monoclonal antibodies secreted by hybridoma lines cyo-1, cyo-5, cyo-7 or 2c3 were affixed to S. aureus cells. These four different complexes were then mixed with either intact or solubilized microsomes

Figure 2. Specificity of monoclonal antibodies to PGH synthase. Sheep vesicular gland microsomes were solubolized and then iodinated. S. aureus cells were complexed with IgG secreted by cyo-1, cyo-3, cyo-5, cyo-7, or two control mouse hybrid lines, isn-3 and tsn-4, or were left unconjugated. S. aureus cells were incubated with ^{125}I-labeled microsomes from sheep vesicular glands (Lane a). The immunoprecipitants obtained were analyzed by SDS-polyacrylamide gel electrophoresis: IgG_{2b} (cyo-1)-S. aureus (Lane b); IgG (cyo-3)-S. aureus (Lane c); IgG_{2b} (cyo-5)-S. aureus (Lane d); IgG_{2b} (cyo-7)-S. aureus (Lane e); IgG_1 (isn-1)-S. aureus (Lane f); IgG_1-(tsn-4) S. aureus (Lane g); unconjugated S. aureus (Lane h); ^{125}I-labeled partially purified PGH synthase (lane i).

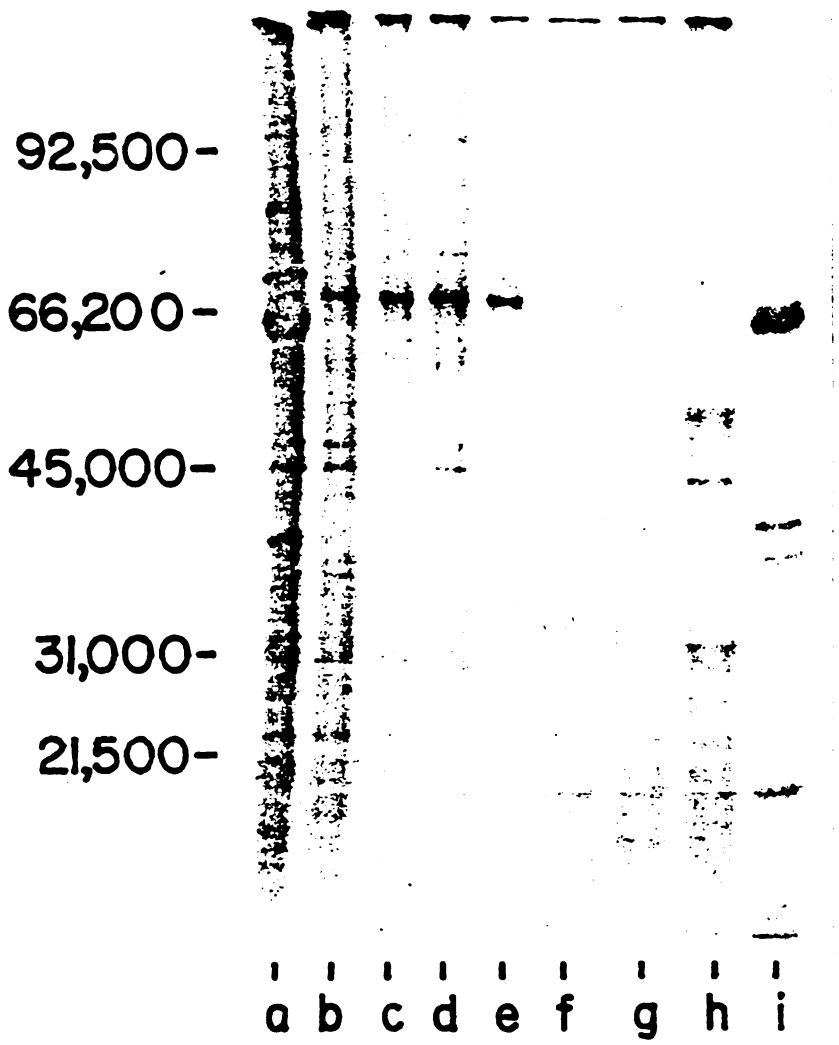

92,500-
66,200-
45,000-
31,000-
21,500-
a b c d e f g h i

Figure 3. Precipitation of PGH synthase from intact and solubilized sheep vesicular gland microsomes using monoclonal antibody-S. aureus complexes. Precipitating complexes of attenuated S. aureus cells with the indicated monoclonal antibodies were prepared as described in the text and then incubated with intact or solubilized (1% Tween 20) microsomes containing 100 units of cyclooxygenase activity. The samples were incubated 2-3 min, and a pellet (P) and supernatant (S) fraction was prepared by centrifugation and assayed for cyclooxygenase activity.

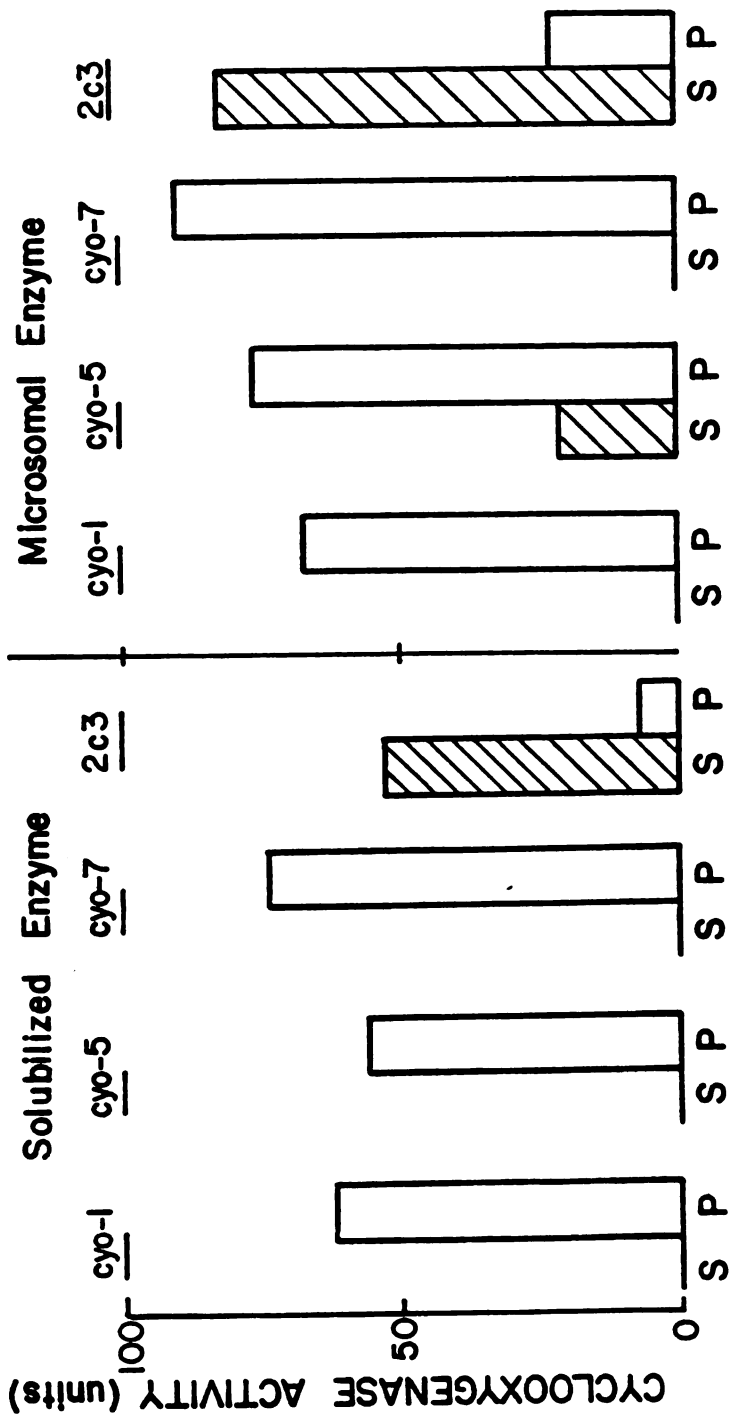

CYCLOOXYGENASE ACTIVITY (units)
100
50
0
Solubilized Enzyme
Microsomal Enzyme
cyo-1
cyo-5
cyo-7
2c3
S P

Table 1. Analysis of monoclonal antibodies against PGH_2 synthase

Antibodies produced by hybridoma line:	[a]Subclass	[b]Reactivity with PGH synthase from
cyo-1	IgG_{2b}	Positive: sheep, bovine human, rat Negative: guinea pig, rabbit, mouse, dog
cyo-3	IgG_1	Positive: sheep, bovine, human, guinea pig, rabbit Negative: rat, mouse, dog
cyo-5	IgG_{2b}	Positive: sheep, bovine, human, guinea pig, rabbit Negative: rat, mouse, dog
cyo-7	IgG_{2b}	Positive: sheep, bovine, human, guinea pig, rabbit Negative: rat, mouse, dog
2c3	IgG_2	negative with all species

[a]determined on the basis of Ouchterlony double-diffusion analyses with rabbit anti-mouse IgG_1, IgG_{2a} and IgG_{2b} antisera and elution profiles from Protein-A Sepharose (Ey et al., 1978) as described in the text.

[b]determined by immunoprecipitation of solubilized cyclooxygenase activity from sheep vesicular gland, rat small intestine, rabbit renal medulla, guinea pig renal medulla, bovine seminal vesicle and human platelet microsomes and/or by immunofluorescent staining using rat, dog, mouse, guinea pig and rabbit kidneys (Smith and Wilken 1977; Smith and Bell, 1978) as described in the text.

prepared from sheep vesicular gland, the samples centrifuged to pellet the S. aureus cells and cyclooxygenase activity measured in both the resuspended cell pellet and the supernatant (Figure 3). When intact microsomes prepared from sheep vesicular gland were mixed with S. aureus cells complexed to one of the anti-PGH synthase antibodies, 75-100% of the cyclooxygenase activity was found in the S. aureus cell pellet; only a small amount of activity was precipitated when the non-immune monoclonal mouse IgG_2 (2c3) was used. Although mixing the various monoclonal antibodies with the enzyme alone had no appreciable effect on cyclooxygenase activity (i.e. the antibodies are not directed against the active site), the recovery of S. aureus-bound cyclooxygenase activity averaged only about 70%. Similar results were obtained with control, detergent-solubilized microsomes (Figure 3) suggesting that the PGH synthase is equally reactive antigenetically in both solubilized and membrane-bound forms. Since IgG_2 molecules secreted by cyo-1, cyo-5 and cyo-7 interact with different antigenic sites on the PGH synthase (see below), our results indicate that at least three antigenic determinants on the sheep vesicular and cyclooxygenase are situated on the outer surface of microsomal spheres and thus on the cytoplasmic side of the endoplasmic reticulum (DeWitt, 1981).

Characterization of IgG subclass and Species Specificities of Anti-PGH Synthase Antibodies

In our initial screen for hybridomas secreting anti-PGH synthase activity, we used a S. aureus-rabbit anti-mouse IgG complex to precipitate mouse IgG from the culture media. Thus, it was unclear what subclass of mouse antibody was secreted by the different lines.

Single lines of immunoprecipitation were obtained when media from cyo-1, cyo-5 or cyo-7 were tested by Ouchterlony double-diffusion analysis against both rabbit anti-mouse IgG_{2a} and rabbit anti-mouse IgG_{2b} sera; however, media from these latter clones failed to react with rabbit anti-mouse IgG_1 serum. In contrast, media from cyo-3 gave a single line of precipitation with rabbit anti-mouse IgG_1 serum but was unreactive with either anti-IgG_{2a} or anti-IgG_{2b} sera. These results suggested that cyo-1,5 and 7 produce IgG_2 molecules and that cyo-3 produces an IgG_1. Media from cyo-1, cyo-3 and cyo-5 were examined further by observing the behavior of these different anti-PGH synthase monoclonal antibodies upon column chromatography on Protein A-Sepharose (Ey et al., 1978). As expected, IgG_1 secreted by cyo-3 was eluted from Protein A-Sepharose at pH 6.0 Ey et al., 1978). Anti-PGH synthase activities from cyo-1 and cyo-5 culture media were eluted between pH 3.0 and 3.5 and not between pH 4.0 and 4.5 . Thus, the IgG molecules produced by cyo-1 and cyo-5 are of the IgG_{2b} subclass (Ey et al., 1978). The results of the subclass analyses of different hybridoma lines are summarized in Table I.

Also summarized in Table I are data indicating the reactivities of different anti-PGH synthase immunoglobulins with synthases from different animals. The pattern of species cross-reactivities was the same for IgGs secreted by cyo-3, cyo-5 and cyo-7 which was, in turn, different from that of IgG produced by cyo-1. Thus IgG_{26} (cyo-1) reacts with a determinant different from the determinant(s) which interact with IgGs produced by other cyo-3, cyo-5 and cyo-7.

We developed an immunoradiometric assay for quantitating PGH synthase. The results of this assay (see below) indicate that IgGs

secreted by cyo-3 and cyo-7 interact with the same determinant but that cyo-5 produces an antibody which reacts with yet another antigenic site; thus, different hybridoma strains produce antibodies against three distinct antigenic sites on the PGH synthase.

Immunoradiometric Assay for PGH Synthase

The immunoadiometric assay for the PGH synthase is pictured schematically in Figure 4. IgG_1 secreted by cyo-3 was isolated from culture media and then labeled with ^{125}I-Bolton-Hunter reagent. Fixed amounts of this ^{125}I-IgG_1 (cyo-3) were incubated with (a) fixed amount of a precipitating complex prepared by affixing IgG_2 from cyo-1, cyo-5, cyo-7 or 2c3 to attenuated S. aureus cells and (b) varying levels of PGH synthase. The amount of cell-bound (precipitated) ^{125}I was then determined (Figure 5). A positive, linear relationship between precipitated ^{125}I and purified PGH synthase existed over the range of 0.0045-0.045 cyclooxygenase units (0.15-1.5 ng; slope = 765,000 cpm/unit) when using the IgG_{2O} (cyo-1)-or IgG_{2b}(cyo-5)-S. aureus cells as precipitating complexes; however, minimal ^{125}I above control levels was bound using the IgG_2(cyo-7)-S. aureus complex. Virtually identical slopes (i.e. cpm/unit) were obtained using both the purified enzyme and solubilized sheep vesicular gland microsomes (Figure 6). The observation that binding of ^{125}I-IgG_1 (cyo-3) cannot occur to enzyme that is bound to S. aureus cells via the IgG_2 secreted by cyo-7 suggested that cyo-3 and cyo-7 secrete immunoglobulins directed against the same site. In fact, IgGs produced by cyo-3 and cyo-7 apparently do bind the same or, at least, overlapping sites. Adsorption of intact sheep vesicular gland microsomes with an excess of IgG_2 secreted by cyo-7

Figure 4. Illustration of the interactions involved in the immunoradiometric assay for PGH synthase.

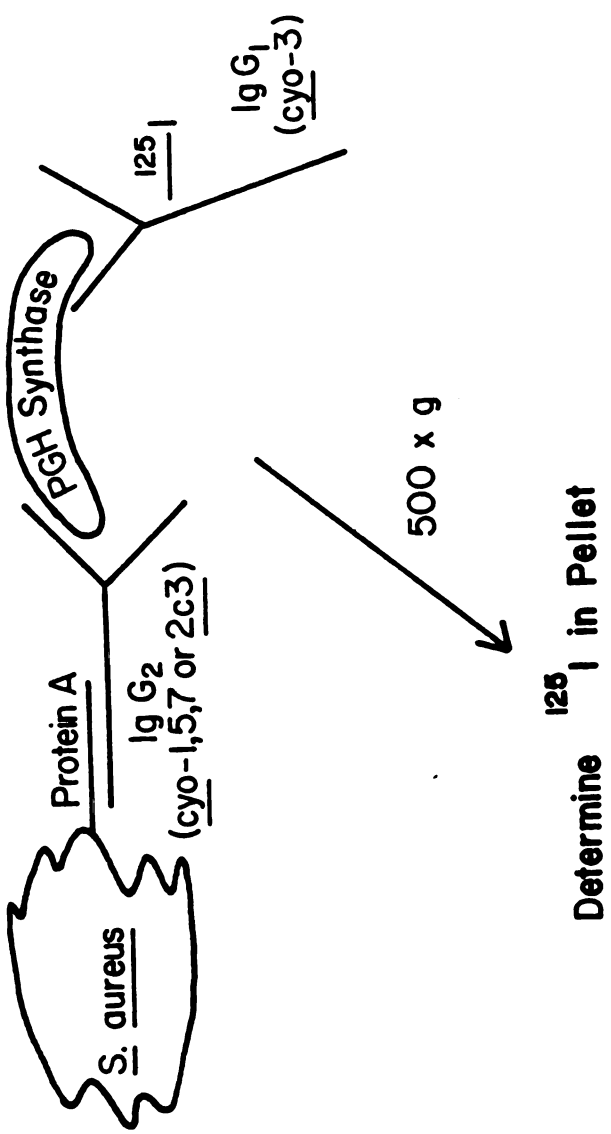
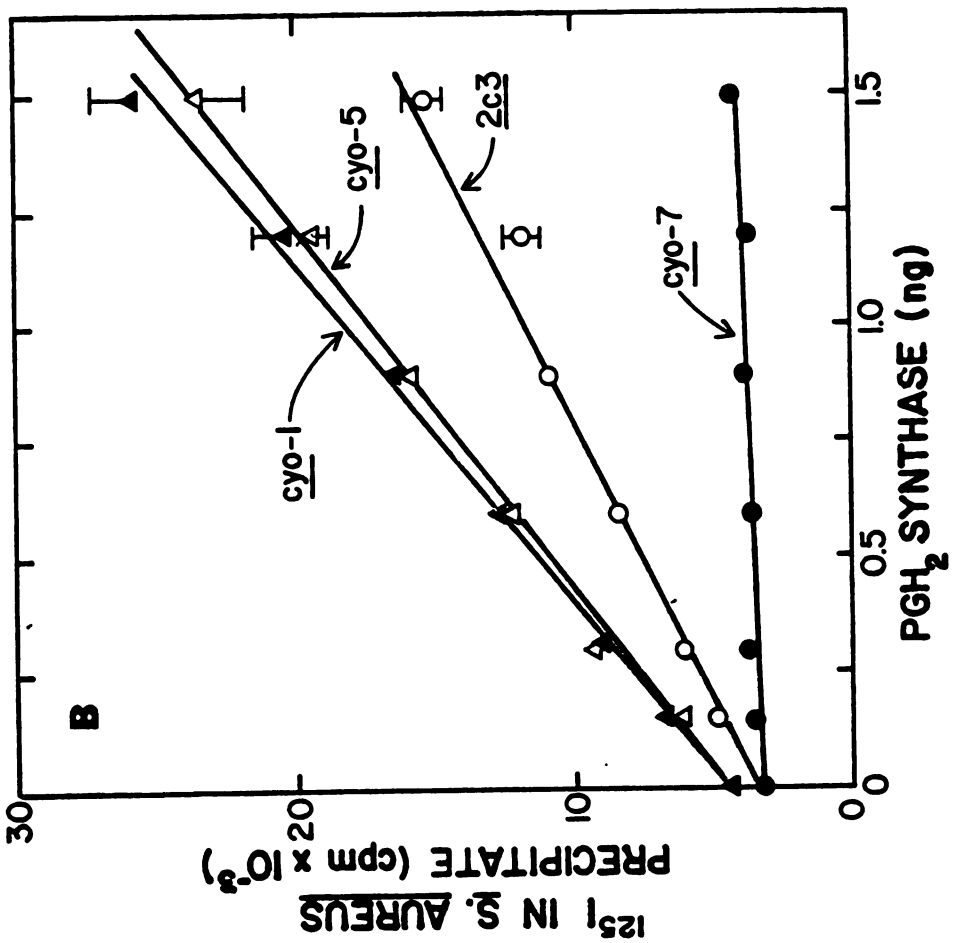

S. aureus
Protein A
Ig G2
(cyo-1,5,7 or 2c3)
PGH Synthase
125 I
Ig G1
(cyo-3)
500 x g
Determine 125 I in Pellet

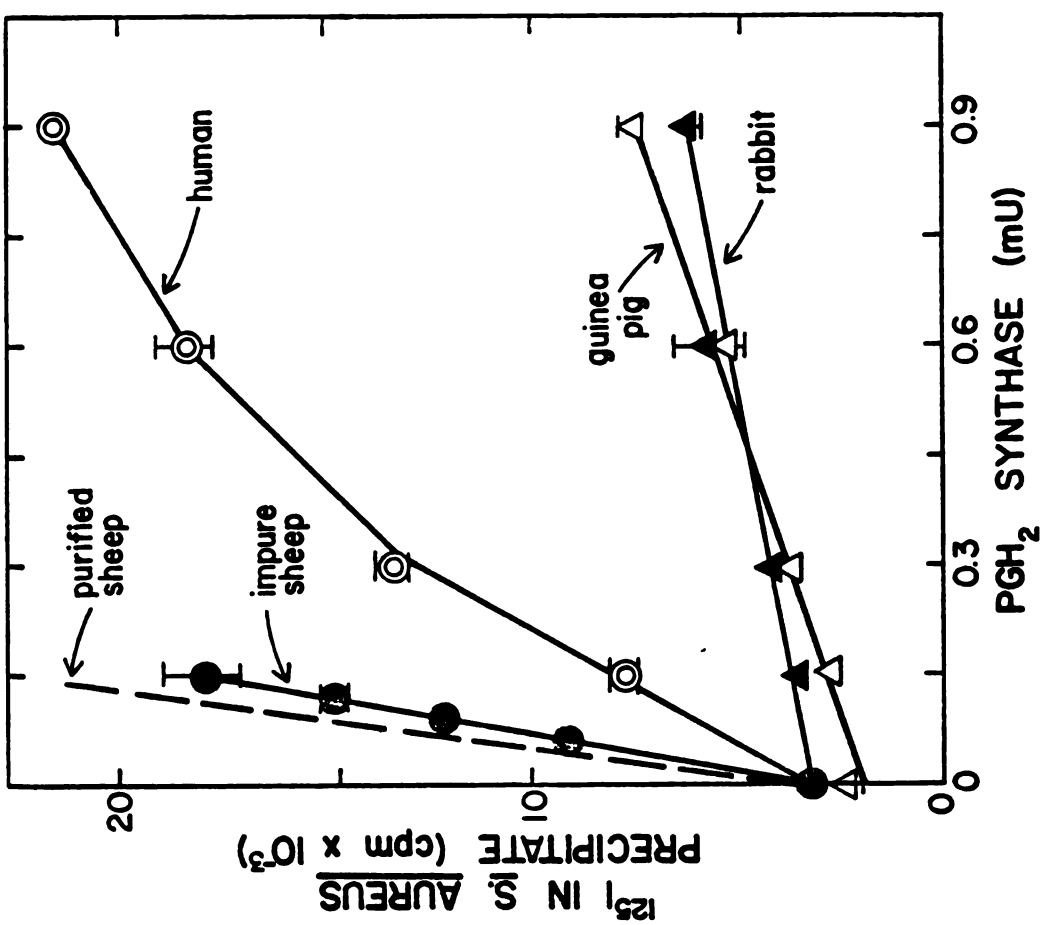
Figure 5. Immunoradiometric assay using purified sheep vesicular gland PGH synthase. IgG_{1b}-*S. aureus* complexes were prepared as described in the text with ^{125}I-IgG_1 (*cyo*-3) and incubated overnight at 4° with various amounts of the purified PGH synthase. The cell pellets were collected, washed and cell-bound radioactivity quantitated. *S. aureus* precipitating complexes were with IgG_2 secreted by: cyo-1, ▲——▲; cyo-5, △——△; cyo-7, ●——●; or 2c3, ○——○.


B
cyo-1
cyo-5
2c3
cyo-7
0
10
20
30
0
0.5
1.0
1.5
PGH2 SYNTHASE (ng)
125I IN S. AUREUS
PRECIPITATE (cpm x 10-3)

prevents subsequent binding of ^{125}I-IgG$_1$ (cyo-3); in contrast, adsorption of microsomes with IgG$_{2b}$ molecules secreted by cyo-1 and cyo-5 did not interfere with ^{125}I-IgG$_1$ (cyo-3) binding. Curiously, substantial precipitation of ^{125}I-IgG$_1$ occurs with the IgG$_2$(2c3)-S. aureus control complex (Figure 5). This latter result also occurred when S. aureus cells alone were substituted for the IgG$_2$(2c3)-S. aureus complex indicating that small amounts of the PGH synthase are bound to S. aureus cells in the absence of an intervening antibody. However, the fact that no binding of ^{125}I-IgG$_1$(cyo-3) occurs with enzyme bound to the IgG$_2$(cyo-7)-S. aureus complex suggests that the enzyme preferentially binds via antibody directed against it when that antibody is present on the S. aureus cells.

As shown in Figure 6 the purified PGH synthase and the enzyme from detergent solubilized sheep vesicular gland microsomes behave similarly when using IgG$_2$(cyo-5)-S. aureus precipitating complexes. The PGH synthases from solubilized microsomes prepared from guinea pig and rabbit kidneys are considerably less reactive on a per unit basis than the sheep enzyme; whether these differences represent species differences in antigen-antibody interactions or are a reflection of species differences in PGH synthase turnover numbers is unknown. However, as illustrated in detail in Figure 6 for the rabbit kidney, the human platelet and guinea pig kidney enzyme, the immunoradiometric assay is useful for quantitating small amounts of cyclooxygenase from a variety of tissues. For purposes of day to day standardization, we have found it useful to include pure PGH synthase as an external standard when using solubilized microsomal preparations.

Figure 6. Comparison of the reactivities of PGH synthases from different species in the immunoradiometric assay. IgG_{2b} (cyo-5)-S. aureus precipitating complexes were used in all the experiments. Control using IgG_{2b} (cyo-7)-S. aureus cells gave a slope of zero. Experiments were performed as described in the text and the legend to Figure 5 with solubilized microsomes prepared from: sheep vesicular glands, ●——●; human platelets, O——O; guinea pig renal medulla, Δ——Δ; and rabbit renal medulla, ▲——▲. A dotted line indicating the slope obtained with the purified sheep enzyme is also shown.


^{125}I IN S. AUREUS
PRECIPITATE (cpm x 10^{-3})
20
10
0
0
0.3
0.6
0.9
PGH_2 SYNTHASE (mU)
human
rabbit
guinea pig
purified sheep
impure sheep

In an attempt to optimize the radiometric assay, a number of factors were investigated to determine their influence on the reactivity of both the purified and solubilized microsomal sheep PGH synthase in the assay. These results are summarized in Table II.

Table 2. Factors influencing immunoradiometric assay for synthase from detergent solubilized sheep vesicular gland microsomes

No Effect on PGH Synthase Reactivity	Prevents Reactivity of PGH Synthase
1. Inactivation of enzyme with aspirin	1. Heating of enzyme at 60° 10 min.
2. Self-catalyzed destruction of enzyme	2. > 0.01% SDS in assay buffer
3. 0.1 M or 1 M NaCl in assay buffer	3. > 2 M urea in assay buffer
4. 0, 0.1, 1% Tween 20 in assay buffer	
5. Allowing enzyme to stand 48 h at 4°	

DISCUSSION

PGH synthase catalyzes the formation of the prostaglandin endoperoxide, PGH_2, from arachidonic acid and oxygen. Fluctuations in tissue concentrations of PGH synthase have been shown to occur in rat Graafian follicles in response to luteinizing hormone (Clark et al., 1978), in the ovine (Huslig et al., 1979) and guinea pig uterus (Poyser, 1979) during the estrous cycle and in the hydronephrotic rabbit kidney in response to perfusion ex vivo (Morrison et al. 1978). The diminished production of PGI_2 by arteries of rabbits subjected to high fat diets (Dembinska-Kiec et al., 1977) and the increased PGE_2 formation by kidneys of rabbits fed low salt diets (Stahl et al., 1979) could also be due to alterations in the levels of PGH synthase. These observations from several laboratories indicate that changes in PGH synthase levels can play a role in regulating the rates of prostaglandin formation in both normal and pathological situations. There are a number of methods for assaying PGH synthase enzyme activity including the polarographic assay, the use of radioactive fatty acid substrates and measurement of labeled products and the use of unlabeled fatty acids and measurement of products by radioimmunoassays. There does, however, exist a need for methods to quantitate changes in enzyme protein levels. In this chapter, we have described the preparation of four different monoclonal antibodies against the PGH synthase and the use of iodinated monoclonal antibodies for quantitating PGH synthase

protein concentrations in tissue extracts using an immunoradiometric assay. This immunoradiometric assay is 10^2-10^4 times as sensitive as the most accurate and sensitive polarographic assay for enzyme activity and should be useful for further studies on the regulation of PGH synthase synthesis.

CHAPTER III

MONOCLONAL ANTIBODIES AGAINST PGI_2 SYNTHASE: AN IMMUNORADIOMETRIC ASSAY FOR QUANTITATING THE ENZYME

In this chapter I describe the preparation of two hybridoma lines producing monoclonal antibodies against two different antigenic sites on the PGI_2 synthase enzyme molecule. The hybridoma lines were derived from mice immunized with a partially purified preparation of PGI_2 synthase from bovine aorta. Using these antibodies I have developed an immunoradiometric assay with which to quantitate PGI_2 synthase protein concentrations. This assay is 50-100 times more sensitive than conventional radiochromatographic enzyme activity assays.

METHODS

Materials

Triton-X-100 was from Calbiochem. DEAE-cellulose (DE-52) was from Whatman. Thin layer chromatography plates (Silica Gel 60, 0.25 mm) were from Analtech. $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ standards were purchased from Upjohn Diagnostics. Cellophane gel backing and molecular weight standards for polyacrylamide gel electrophoresis was obtained from BioRad Laboratories, Inc. All other materials were obtained from the sources listed in chapter II.

Animals and Tissues

Female ICR Swiss white mice, 4-6 weeks old used for immunizations and for isolation of splenocytes were obtained from Harlan Laboratories. Bovine aorta was obtained fresh at slaughter from Michigan State University Meats Laboratory and stored at -80°.

Preparation of [^{3}H]PGH_2

[^{3}H]PGH_2 was synthesized from [5,6,8,9,11,12,14,15^3-H-(N)] arachidonic acid (100 Ci/mmole; diluted as necessary to a specific activity of 10-20 Ci/mole (ca. 10,000-20,000 cpm/nmole) with unlabeled arachidonic acid) using a modification of the procedure of Hamberg *et al*. (1974); 85 ml of O_2 saturated 0.1 *M* sodium phosphate, pH 7.4 containing 500 μM phenol was mixed with 15 ml of freshly prepared sheep vesicular gland microsomes (10 mg protein/ml) and the sample equilibrated to 25°. [^{3}H] arachidonic acid (1-2.5 mg) in 0.4 ml of

ethanol was added, and the sample was incubated for 2 min and then acidified to pH 3.0 with 6 M HCl. The sample was extracted sequentially with 2-300 ml portions of ether cooled to 0° in 500 ml separatory funnels. The combined ether extracts were cooled to -78° and the ice was removed by filtration through glass wool. The resulting ether layer was evaporated to dryness on a rotary evaporator at 4° and the residue was dissolved in 20 ml of hexane/ether (8/2; v/v). Silicic acid chromatography was performed at 4° as described by Hamberg et al. (1974). [^{3}H]PGH_2, which was eluted with hexane/ether (4/6; v/v), was evaporated to dryness, redissolved in anhydrous acetone and stored in 100 µl (1,000 nmole) aliquots at -80°. Purity of [^{3}H]PGH_2 was routinely >90% when tested by thin-layer chromatography on Silica Gel G chromatography plates in ether/acetic acid (100/0.2; v/v; 4°); PGH_2 migrates just slightly ahead of PGB_2 in this system.

Enzyme Activity Assay of PGI_2 synthase

Aliquots of [^{3}H]PGH_2 (50 nmoles in 5 µl of acetone) were pipetted into individual samples of enzyme in 0.1 ml of Tris Buffer (0.1 M tris-chloride), pH 8.0, containing 0.5% Triton X-100 and 10^{-4} M Flurbiprofen). Incubations were performed at 24° for one minute and the reactions stopped by adding 50 µl of a freshly prepared solution of $FeCl_2$ (6 mg/ml). The protein was removed by addition of 0.3 ml of acetone and centrifugation at 1,500 g for 5 min (Salmon and Flower, 1982). The supernatants were then transferred to new tubes, acidified with 50 µl of 0.2 M HCl and 1 ml of $CHCl_3$ was added. After mixing thoroughly, the tubes were centrifuged to separate the phases. The aqueous layer was aspirated and the organic layer dried under N_2.

The residue was redissolved in 75 µl $CHCl_3$ and spotted on Silica Gel 60 250 µM thin layer chromatography plates which were then developed twice in the organic phase of ethyl acetate:trimethylpentane:acetic acid:H_2O (110:50:20:100; v/v/v/v). The region corresponding to standard 6-keto-$PGF_{1\alpha}$, as well as the rest of each vertical lane, was scraped into scintillation vials and counted. PGI_2 formation was calculated as the fraction of total radioactivity chromatographing with the 6-keto-$PGF_{1\alpha}$ standard multiplied by the total substrate (5 nmoles) added to each assay mixture.

PGI_2 synthase does not exhibit standard Michaelis-Menten kinetics. The assay is performed as a matter of convenience for one minute, although at the 50 µM substrate concentration, the PGI_2 synthase is always inactivated before one minute (Watanabe 1979). Thus, the rates reported below are not initial rates. Nevertheless, these rates are valid for comparing enzyme activities when the assays are run at concentrations of PGI_2 synthase that are linearly related to the amount of PGI_2 formation per min. One unit of activity is defined as the amount of enzyme that will catalyze the formation of 1 nmole of PGI_2 per min under the standard assay conditions.

PGI_2 Synthase Purification

Bovine aorta obtained fresh at slaughter was frozen immediately on dry ice, then stored at -80°. The abdominal region of the aorta beginning 15-20 cm from the heart is easiest to homogenize. Aorta was frozen in liquid nitrogen, shattered into small pieces (ca. 2 cm^3) with a hammer and homogenized in 2-3 volumes of ice cold 0.1 M Tris-chloride, pH 8.0, containing 10^{-4} M Flurbiprofen with a Polytron (Brinkman) homogenizer. Care was taken to maintain the buffer

temperature below 5° during homogenization. The homogenate was centrifuged at 10,000 x g for 10 min, and the resulting supernatant was centrifuged for 35 min at 200,000 x g to collect the microsomal pellet. When stored at -80° the pellets retain their PGI_2 synthase activity for 2-3 months.

Further purification of PGI_2 synthase was performed using a modification of the method of Wlodawer and Hammarstrom (1979). Microsomal pellets (0.5 g) from 25-30 g of tissue were resuspended with a glass homogenizer in 10 ml of Tris Buffer. This homogenate was centrifuged at 200,000 x g for 35 min. The washed pellet was resuspended in 10 ml of 10 mM sodium phosphate, pH 7.4 containing 0.5% Triton-X-100 and again centrifuged at 200,000 x g for 35 min. The supernatant was removed and the solubilized PGI_2 synthase was applied to a DE-52 cellulose column (2 x 8 cm) equilibrated with 10 mM sodium phosphate, pH 7.4 containing 0.1% Triton-X-100; the column was then washed with 60 ml of the equilibration buffer. PGI_2 synthase was eluted with 0.2 M sodium phosphate, pH 7.4, containing 0.1% Triton-X-100. The specific activity of the PGI_2 synthase at this step ranged from 100-225 with an average of 150 units/mg protein/min. This represents a purification of approximately 10 fold. A summary of a typical purification is presented in Table 3.

Immunization Protocol

PGI_2 synthase purified through DE-52 chromatography was used to immunize outbred 4-6 week old female ICR Swiss white mice. Approximately 250 µg of protein (average specific activity of 150 units/mg protein) in 0.2 ml of 0.2 M phosphate, pH 7.4 containing 0.1% Triton-X-100 was emulsified by sonication with 200 µl of complete

TABLE 3. Partial purification of PGI_2 synthase

Purification Step	Protein concentration (mg/ml)	Specific Activity nmoles of 6-keto-PGF1α/ min/mg protein	% Recovery of Activity	Fold Purification
1) 10,000 x g supernatant of homogenized aorta	4.5	12	100%	1
2) Microsomal suspension (0.1 M tris-chloride, pH 8.0)	3.9	23	45%	2
3) 200,00 x g supernatant of solubilized microsomes	1.05	94	41%	7.8
4) Eluant from DE-52 column	.73	110	24%	9.2

Freunds adjuvant and injected intraperitoneally. Two and four weeks later the mice were again inoculated using PGI_2 synthase (ca. 40 units) emulsified in incomplete Freunds adjuvant. Three days after the second booster, mice were killed by cervical dislocation. Spleens were removed aseptically and blood was collected to test for anti-PGI_2 synthase activity. Spleen cells from all mice were fused, but only those hybridomas from mice found to have anti-PGI_2 synthase activity in their serum were screened for anti-PGI_2 synthase activity.

Fusion of Mouse Spleen Cells

Spleen cells (1-5 x 10^7) from mice inoculated with PGI_2 synthase were fused with 1-5 x 10^6 HGPRT-negative SP2/0-Ag14 mouse myeloma cells as described in the preceding chapter (Chapter II) with the following modifications. After fusion, cells were suspended in 90 ml of complete HT medium and distributed into six 96 well tissue culture plates. After 24 hr, 150 µl of complete HAT medium (complete HT medium containing 1 µM aminopterin) was added to each well. Two and four days thereafter, 150 µl of medium was removed from each well and replaced with 150 µl of fresh complete HAT medium. When the medium in a well with growing hybridomas began to turn yellow (12-15 days after fusion), 200 µl of the spent medium was removed to test for anti-PGI_2 synthase antibody. This medium was replaced with 200 µl of complete HT medium.

Selection for Hybridoma Cells Producing Antibodies to PGI_2 Synthase

Staphylococcus aureus cells conjugated with rabbit anti-mouse IgG (Miles) can bind and precipitate all subclasses of mouse IgGs. When mixed with medium containing mouse anti-PGI_2 synthase antibody, the newly-formed S. aureus-rabbit anti-mouse IgG-mouse IgG complex will

precipitate solubilized PGI_2 synthase. This precipitate can be assayed for PGI_2 synthase activity. Immunoglobulin classes other than IgG are not detected by this method.

S. aureus (Cowen Strain I) was grown and attenuated by the method of Kessler as described in Chapter II. The rabbit anti-mouse IgG-S. aureus complexes were prepared by washing, collecting (by centrifugation) and resuspending 5 ml of a 10% S. aureus cell suspension (w/v) as follows: (a) twice with 5 ml of Tris Buffer containing 5% (w/v) bovine serum albumin, (b) once with 5 ml of Tris Buffer containing 250 µl of rabbit anti-mouse IgG (ca. 2.5 mg IgG/ml) and (c) once with 5 ml of Tris Buffer. Finally, the rabbit anti- mouse IgG-S. aureus was resuspended in 5 ml of Tris Buffer.

To assay for the presence of anti-PGI_2 synthase binding activity, either 50 µl of serum from mice immunized with PGI_2 synthase preparations or 200 µl of media from wells containing growing hybridomas (after the medium turns yellow) were mixed with 0.1 ml of the rabbit anti-mouse IgG-S. aureus suspension. The mixture was vortexed and centrifuged at 1,500 x g on a desk top centrifuge, and the supernatant was removed by aspiration. After resuspending the cell pellet in 0.5 ml of Tris Buffer, solubilized PGI_2 synthase (ca. 15 units) was added. The PGI_2 synthase used in this screening assay was obtained from detergent-solubilized microsomes prior to chromatography on DE-52. The mixture was vortexed briefly and again the S. aureus cells were pelleted by centrifugation at 1,500 x g for 5 min. The supernatant was removed by aspiration and the pellet was resuspended a second time in 1 ml of Tris Buffer. PGI_2 synthase activity was then assayed as described above.

Preparation of ^{125}I-labeled protein

Iodinated n-succinimidyl 3-(4-hydroxyphenyl)propionate, Bolton Hunter reagent, was synthesized from $Na^{125}I$ (100 mCi/ml) and the succinimydyl ester by the method of Bolton (1976). Microsomes were solubilized for iodination in 0.1 M sodium borate, pH 8.0, containing 0.5% Triton-X-100 and then were centrifuged at 48,000 x g to remove insoluble material. Solubilized protein (20 µl; 2 mg protein/ml) was iodinated with 250 µCi of Bolton Hunter reagent by the method of Bolton and Hunter (1973). After iodination, 0.5 ml of 0.2 M glycine was added to the iodination reaction mixture and ^{125}I-labeled protein was separated from unconjugated ester by chromatography on a BioGel P-30 column (1 x 6 cm) equilibrated with 0.05 M sodium phosphate, pH 7.4, containing 2.5 mg gelatin per ml and 0.02% sodium azide.

Immunoprecipitation of ^{125}I-Labeled Protein

One ml aliquots of a 10% suspension of S. aureus previously conjugated to rabbit anti-mouse IgG were incubated with 250 µg of purified mouse IgG produced by the following hybridoma cell lines; isn-1, isn-3, cyo-3 (anti-PGH_2 antibody) and tsn-4 (non-specific IgG_1). After a 12 hr incubation the S. aureus were collected by centrifugation and washed and resuspended in Tris Buffer. For immunoprecipitation, 0.3 ml containing 22 µCi of the ^{125}I-labeled aortic microsomes were added to 0.10 ml of each of the different S. aureus conjugated antibodies as well as to S. aureus conjugated solely to rabbit anti-mouse IgG antibody and to unconjugated S. aureus. To prevent non-specific binding of ^{125}I-labeled proteins 0.10 ml of Tris Buffer containing 1% BSA was added to each tube and the mixture was allowed to react for 30 min at 24°. The S. aureus were

precipitated by centrifugation and the cell pellets were washed with 0.2 ml of Tris Buffer. Next, the pellets were resuspended in Tris Buffer and transferred to new test tubes. Each pellet was washed one additional time with Tris Buffer. In preparation for SDS-polyacrylamide gel analysis, the pellets were each resuspended in "sample buffer": 0.125 M Tris-chloride, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.002% (w/v) bromphenol blue. The samples were heated at 100° for 5 min. Low molecular weight standards from BioRad Laboratories, Inc. and ^{125}I-labeled microsomes were diluted 1:10 and 4:1 respectively with sample buffer and also boiled for 5 min. All samples were subjected to electrophoreses on 0.75 mm thick discontinuous 10% polyacrylamide gels as described by Laemmli (1970). After electrophoresis, the gels were fixed in 50% methanol and subjected to silver staining (Wray et al., 1981). Stained gels were dried between sheets of cellophane membrane backing on a BioRad model 224 Gel Slab Dryer. For visualization of ^{125}I-labeled proteins, dried gels were exposed to Kodak XAR-5 x-ray film for 12 hr, and the film was developed.

Competitive Antibody Binding Studies

The following experiment was performed to determine whether the antibodies secreted by isn-1 and isn-3 bound the same antigenic determinants on the PGI_2 synthase molecule. First, one ml aliquots of S. aureus previously coupled to rabbit anti-mouse IgG were incubated with 250 µg of mouse IgG_1 secreted by isn-1, isn-3, or cyo-3 for 12 hr; these three antibody-S. aureus complexes were collected by centrifugation, washed with 1 ml of Tris Buffer and resuspended to a

total of 1 ml in Tris Buffer. Next, aliquots of solubilized bovine aortic microsomes of solubilized bovine aortic microsomes containing 20 units of PGI_2 synthase activity were mixed with 250 µg of IgG_1 secreted by isn-1, isn-3, or cyo-1. To 0.10 ml aliquots of the different mouse IgG_1-S. aureus complexes was added to 0.2 ml aliquots (5 units) of the different antibody-PGI_2 synthase mixtures (or enzyme alone); the S. aureus cell pellets were then immediately collected by centrifugation, resuspended in 0.1 ml of the Tris Buffer and assayed for PGI_2 synthase activity.

Immunoradiometric Assay for PGI_2 Synthase

The two hybridoma lines (isn-1 and isn-3) were grown in medium free of bovine IgG, and pure mouse IgG_1 was isolated by chromatography on Protein-A-Sepharose. IgG_1 (isn-3) was iodinated Bolton-Hunter reagent. Procedures for isolation and radioiodination iodination of mouse IgG are detailed in Chapter II.

A standard curve for quantitating PGI_2 synthase protein was generated as follows. Aliquots of solubilized bovine aortic microsomes (containing 0-0.05 units of PGI_2 synthase; microsomes solubilized in 0.1 M Tris-chloride, pH 8.0, containing 0.5% Triton-X-100) were added to 6 x 50 mm glass test tubes each containing 100,000 cpm of ^{125}I-IgG_1 (isn-3) and allowed to stand for 30 min at 24°; next, 10 µl of the IgG_1 (isn-1)-S. aureus complex, prepared as for the competitive binding experiment was added, and the tubes were vortexed and immediately centrifuged at 1500 x g for 10 min at 24°. Any long delay (>5 min) before centrifugation increase the degree of nonspecific precipitation of ^{125}I to unacceptable levels. After centrifugation, the supernatant was removed by aspiration and the

pellets were washed once in 0.5 ml of the solubilization buffer. The washed cell pellets present in the 6 x 50 mm test tubes were placed in vials and ^{125}I determined by counting on a Beckman Biogamma counter.

RESULTS

Preparation and characterization of monoclonal antibodies against PGI_2 synthase.

Outbred white mice were immunized with a crude preparation of bovine aorta microsomes (containing PGI_2 synthase activity) which had been solubilized with 0.5% Triton X-100. Splenic lymphocytes from immunized mice were fused with the SP2/0-Ag14 myeloma cell line. Two hybrid lines (isn-1 and isn-3) that secrete monoclonal antibodies capable of causing immunoprecipitation of PGI_2 synthase activity were selected and cloned from a total of about 1200 hybridomas tested. Immunoglobulins secreted by both isn-1 and isn-3 are of the mouse IgG_1 subclass as determined by Ouchterlony double diffusion analysis against allotype specific antisera. Antibodies secreted by both isn-1 and isn-3 react with PGI_2 synthases from mouse, rat, rabbit, sheep, dog and cow as determined either by immunoprecipitation of enzyme activity from solubilized aortic microsomes or immunocytochemical staining of arterial smooth muscle in sections of renal cortex.

To define the antigen precipitated by antibodies secreted by isn-1 and isn-3, antibody secreted into culture media from isn-1, isn-3, and tsn-4 (a control line secreting a nonspecific IgG_1) were coupled to attenuated S. aureus cells. Each antibody-S. aureus complex was then incubated with a mixture of ^{125}I-labeled proteins prepared from bovine aortic microsomes. Immunoprecipitates were analyzed by SDS

Figure 7. Specificity of monoclonal antibodies to PGI_2 synthase.

Aortic microsomes were solubilized and then iodinated. S. aureus cells complexed with IgGs secreted by isn-1, isn-3 or two non-specific mouse hybrid lines were incubated with ^{125}I-labeled microsomes from bovine aorta (Lane a). The immunoprecipitates obtained were analyzed by SDS-polyacrylamide gel electrophoresis: IgG_1 (isn-1)-S. aureus (Lane b); IgG_1 (isn-3)-S. aureus (Lane c); IgG_1 (cyo-3)-S. aureus (Lane d); IgG_1 (isn-4)-S. aureus (Lane e) and rabbit anti-mouse IgG-S. aureus (Lane f).


92,500
66,200
45,000
31,000
a
b
c
d
e
f

TABLE 4.

Experiment	IgG-S. aureus Conjugate	Microsome Pretreatment	PGI_2 Synthase Activity pmole 6-keto-$PGF_{1\alpha}$/pellet
a	isn-1	isn-1	0
b	"	isn-3	750
c	"	cyo-1	1500
d	"	none	2550
e	isn-3	isn-1	0
f	"	isn-3	0
g	"	cyo-1	1200
h	"	none	3250
i	cyo-3	isn-1	0
j	"	isn-3	0
k	"	cyo-1	0
l	"	none	0

Competitive Binding Between Antibodies Secreted by isn-1 and isn-3

S. aureus coupled to the indicated antibodies were mixed with microsomes pretreated with the indicated antibodies. S. aureus cells were precipitated by centrifugation, resuspended and the PGI_2 synthase activity measured.

polyacrylamide gel electrophoresis and autoradiography (Figure 7). Although a large number of radioiodinated proteins were present in the ^{125}I-labeled solubilized aortic microsomes, only one of these iodinated species was precipitated by mouse immunoglobulins isolated from culture media from both isn-1 and isn-3; moreover, the iodinated proteins precipitated by each of the antibodies had the same electrophoretic mobilities (ca. 52,000 daltons). No ^{125}I-labeled proteins were detected in the control (tsn-4 and 2c3) precipitates. The results indicate that isn-1 and isn-3 each secrete an IgG_1 which when bound to S. aureus cells will precipitate (a) PGI_2 synthase activity and (b) a single, 52,000 dalton protein. This is strong correlative evidence that the 52,000 dalton protein is a subunit of PGI_2 synthase particularly because the antibodies secreted by isn-1 and isn-3 are directed against nonidentical antigenic determinants (i.e. binding of ^{125}I-labeled IgG_1 (isn-3) to bovine aortic microsomes is blocked by preincubation with unlabeled IgG_1 secreted by isn-3 but not by IgG_1 secreted by isn-1 (see below)).

Antibodies Secreted by isn-1 and isn-3 Bind Different Antigenic Sites

Competitive binding experiments were performed to determine whether antibodies secreted by isn-1 and isn-3 bind the same antigenic determinant. Solubilized microsomes were pretreated with IgG secreted by either isn-1 or isn-3 and each sample then incubated with IgG_1 (isn-1 or isn-3)-S. aureus complexes. As expected, incubation of microsomes with IgG_1 (isn-1) or (isn-3) completely inhibited precipitation of PGI_2 synthase activity by S. aureus to which the same antibody was conjugated (Table 4; a,f). Moreover, microsomes pretreated with IgG_2 (isn-3) S. aureus complexes (Table 4b) were

precipitated by IgG_1 (isn-1) S. aureus complexes. However, microsomes pretreated with IgG_1 (isn-1) were no precipitated by IgG_1 (isn-3) S. aureus complexes (Table 4e). These results indicate that IgG_1 secreted by isn-3 cannot bind the microsomal PGI_2 synthase if IgG_1 secreted by isn-1 binds the enzyme first. Similar results were obtained when the antibodies secreted by isn-1 and isn-3 were used to quantitate solubilized PGI_2 synthase activity in the immunoradiometric assay (see below).

There are several possible explanations for this anamolous binding behavior. The simplest is that although IgG_1 secreted by isn-1 and isn-3 bind non-overlapping determinants, and binding of IgG_1 (isn-1) interferes sterically with the access of IgG_1 (isn-3) to its epitope.

Immunoradiometric Assay of PGI_2 Synthase

The elements of the immunoradiometric assay for PGI_2 synthase are diagrammed in Figure 8. A positive linear relationship between precipitated ^{125}I-IgG_1 (isn-3) and added PGI_2 synthase activity exists over the range of 0.005-0.05 units of activity when using IgG_1 (isn-1)-S. aureus cells as the precipitating complex (Figure 9); the slope is equal to 300,000 cpm precipitated per unit of PGI_2 synthase. This assay is 50-100 times more sensitive than the enzyme activity assays. When the IgG_1 (isn-3)-S. aureus complex is substituted as a control for IgG_1 (isn-1)-S. aureus cells, no ^{125}I above background is precipitated. A similar lack of precipitation of ^{125}I is observed when non-specific mouse immunoglobulins are conjugated to S. aureus cells and substituted for IgG_2 (isn-1)-S. aureus.

Figure 8. Illustration of the interaction involved in the immunoradiometric assay for PGI_2 synthase.

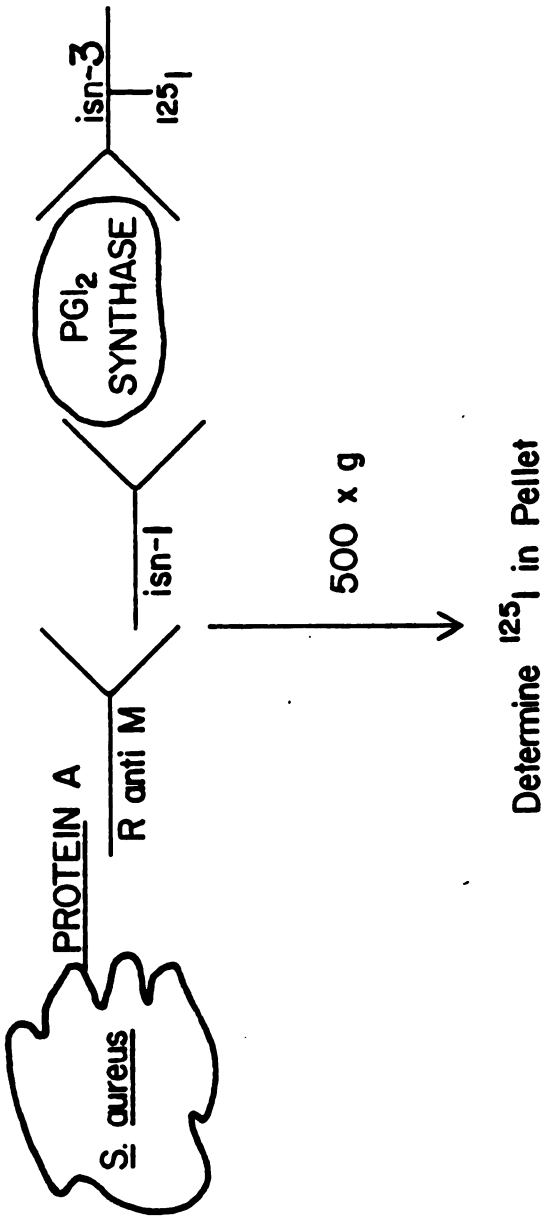
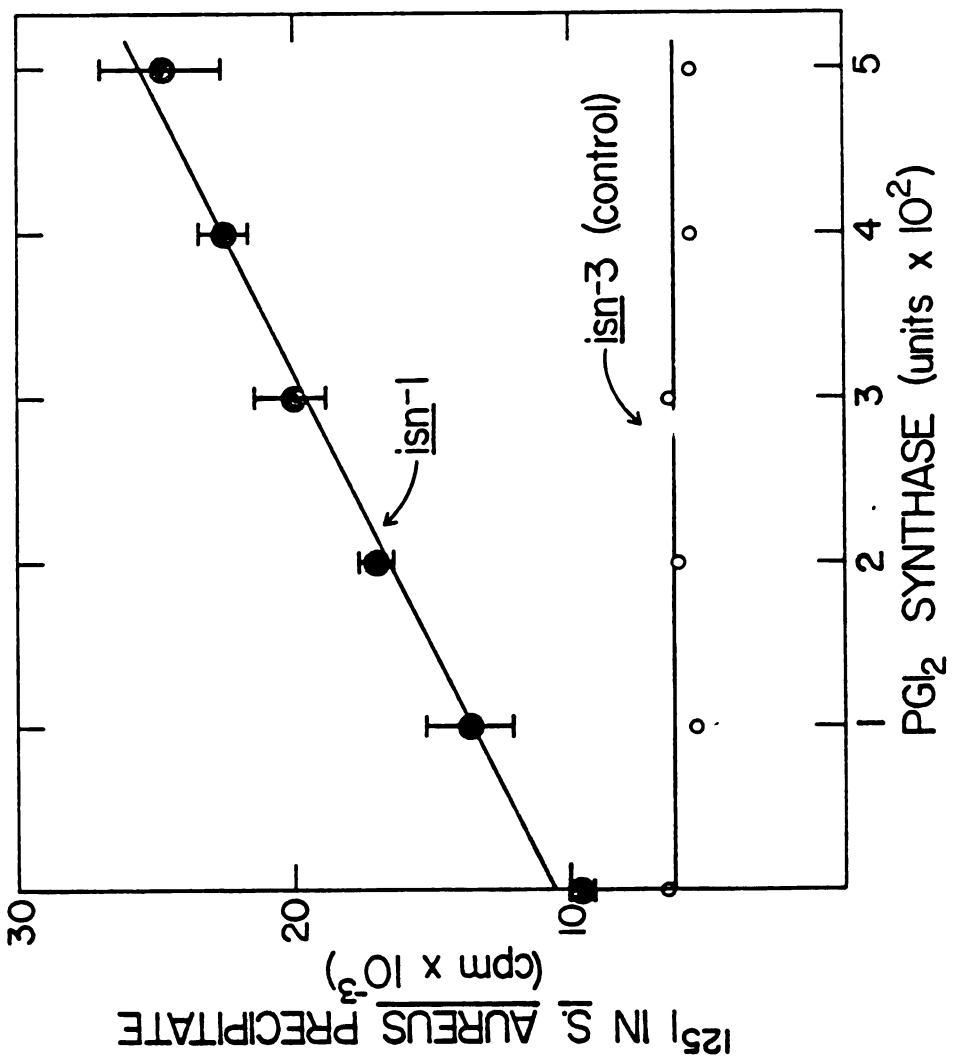

S. aureus
PROTEIN A
R anti M
isn-1
PGI2 SYNTHASE
isn-3
125I
500 x g
Determine 125I in Pellet

Figure 9. Immunoradiometric assay of PGI_2 synthase using solubilized bovine aortic microsomes. PGI_2 synthase was incubated with ^{125}I-IgG_1 (isn-3) for 30 min, then S. aureus cells conjugated to IgG_1 secreted by either isn-1 (●——●) or isn-3 (○——○) were added. The S. aureus cells were pelleted by centrifugation and washed and precipitated ^{125}I quantitated. Results are averages of triplicates (○——○); error bars ± S.D.


^{125}I IN S. AUREUS PRECIPITATE
(cpm x 10^{-3})
30
20
10
isn-1
isn-3 (control)
1
2
3
4
5
PGI_2 SYNTHASE (units x 10^2)

This immunoradiometric assay provides a simple, sensitive and highly specific method for quantitating PGI_2 synthase. The method should be useful for measuring changes in PGI_2 synthase protein concentrations in tissues during physiological stresses such as aging and the development of atherosclerosis.

DISCUSSION

Upon isolation of hybridoma clones producing antibody that precipitated PGI_2 synthase activity, I was faced with the problem of determining the selectivity of the antibodies with no information abou t the physical properties of the PGI_2 synthase enzyme. The results presented in this chapter provide evidence that immunoglobulins secreted by isn-1 and isn-3 both precipitate PGI_2 synthase activity and the same, single 52,000 dalton protein monomer. Moreover, the competitive binding data suggest that the two antibodies interact with different determinants. Isolation of active PGI_2 synthase by immunoaffinity chromatography (Chapter 4) has also indicated that a 52,000 dalton protein monomer can be co-eluted with PGI_2 synthase enzyme activity. These data provide strong evidence that the antibodies secreted by isn-1 and isn-3 are directed against PGI_2 synthase, and that this protein has a subunit molecular weight of 52,000 daltons.

The immunoradiometric assay for PGI_2 synthase may prove to be more versatile than the immunoradiometric assay for PGH synthase because the antibodies secreted by isn-1 and isn-3 cross react with PGI_2 synthase from a broad range of species. However, this assay is still in a developmental stage. For example, I currently standardize the assay using impure microsomal PGI_2 synthase from bovine aorta. Once enough pure protein has been isolated, the assay can be standardized to the mass of PGI_2 synthase measured.

Immunoradiometric assays such as those for the PGH synthase and the PGI_2 synthase are clearly superior to single antibody radioimmunoassays. The reason for this is their increased specificity. The interference by substances that cross react with the antibodies in the entire assay is equal to the product of the percentage cross reaction of one antibody multiplied by the percentage cross reaction of the second antibody. In practice, the specificity of single monoclonal antibodies is so high that a double monoclonal antibody assay should be essentially free from interference.

CHAPTER IV

PURIFICATION AND CHARACTERIZATION OF THE PGI_2 SYNTHASE

In this chapter I describe the purification of PGI_2 synthase by immunoaffinity chromatography and report some spectral and kinetic properties of the purified enzyme.

MATERIALS

Materials

Affigel-10 was obtained from BioRad Laboratories, Inc. Linoleic acid was from NuChek Preps. Type IV lipoxygenase was purchased from Sigma Chemical Co. All other materials were procured from sources described in Chapters II and III.

Preparation of anti-PGI_2 synthase immunoaffinity columns

The IgG_1 (isn-3) AffiGel-10 column used in the purification of PGI_2 synthase was prepared as follows (Staeheln et al. 1981): 3.0 mg of IgG_1 (purified from isn-3 culture media by chromatography on Protein A-Sepharose as described in Chapter III) was dissolved, in 3.0 ml of 0.1 M HEPES, pH 7.5, and incubated at 4° for 12 hr with 6 ml of AffiGel-10. The gel was collected by centrifugation and 6 ml of 0.1 M ethanolamine was added. After 1 hr, the gel was packed in a small chromatography column prepared in a 10 ml plastic syringe and washed exhaustively with Tris Buffer. We have used the columns for several months with no apparent loss of binding capacity. Columns are usually stored in Tris Buffer.

Purification of PGI_2 synthase

Microsomal pellets (0.3 g) were prepared from bovine aorta as described in Chapter III and were resuspended by homogenization in 15 ml of Tris Buffer, (.1 M tris-chloride, pH 8.0, containing, .5%

Triton-X-100 and 1 x 10^{-4} M flurbiprotein) containing 2 mM 2-mercaptoethanol. This suspension was centrifuged at 48,000 x g for 30 min, and the supernatant containing the solubilized enzyme was applied to an IgG_1 (isn-3)-AffiGel-10 column equilibrated with Tris Buffer. Because the flow rate is rapid (5 ml/min) the enzyme solution was rechromatographed 5 times to maximize binding of PGI_2 synthase activity. The column was then washed with 10 volumes of Tris Buffer. PGI_2 synthase activity was eluted with 0.1 M MES-(2-(N-morpholino)ethane-sulfonic acid), pH 6.0, containing 0.5% Triton-X-100, 1 M NaCl and 2 mM 2-mercaptoethanol (pH 6.0 MES-NaCl). Seven 5 ml fractions were collected into 0.75 ml of 1.0 M Tris-chloride, pH 8.0; this adjusted the pH of the eluate to approximately 8.0. A control AffiGel-10 column consisting of 6 ml of resin to which was coupled 3 mg of non-specific mouse IgG_1 was run in parallel with the IGG_1 (isn-3)-AffiGel-10 column.

Solid phase assay of PGI_2 synthase

For certain inactivation and protection studies involving enzyme inhibitors, PGI_2 synthase was bound to S. aureus cells, via IgG (isn-3). The IgG_1(isn-3) S. aureus-PGI_2 synthase complex was incubated with the inhibitors and quickly separated from these agents by centrifugation. By measuring the PGI_2 synthase activity of the washed S. aureus pellets, it was possible to determine whether the inhibitors caused irreversible inactivation of the enzyme. The enzyme was immobilized by attachment to S. aureus using the following protocol. A 10% suspension of rabbit anti-mouse IgG-S. aureus cells (1 ml) prepared as described in Chapter III were incubated with 100 µg of IgG_1 (isn-3) for 12 hrs and the cells collected by centrifugation.

After a single wash with Tris Buffer the resulting 10% suspension of IgG_1 (isn-3)-S. aureus cells (1 ml) was mixed with an equal volume of solubilized bovine aortic microsomes (80 units/ml) and incubated at 4° for 15 min. IgG_1 (isn-3)-S. aureus cojugates will quantitatively bind small amounts of PGI_2 synthase, but excess enzyme was used in these experiments maximize binding of PGI_2 synthase to the S. aureus. The S. aureus cells were then pelleted by centrifugation, the pellet washed once with Tris Buffer, and the cells resuspended in 10 volumes of Tris Buffer. Under these conditions, approximately 12 units of PGI_2 synthase activity were bound per ml of the IgG_1 (isn-3)-S. aureus suspension.

Incubations of immobilized PGI_2 synthase with inhibitors were performed as follows. To 0.1 ml of PGI_2 synthase S. aureus suspension was added 0.1 ml of Tris Buffer and 10 µl of a solution containing the effector. The samples were allowed to incubate at 24° for various times and the S. aureus cells were then collected by centrifugation for 1 min and the supernatant removed. The cell pellets were washed with 0.2 ml of Tris Buffer to remove traces of inhibitors and then resuspended in 0.2 ml of Tris Buffer and assayed with 50 nmole of $[^3H]PGH_2$ (25 µM) to determine the amount of PGI_2 synthase activity remaining. No more than four samples were tested at a single time to insure uniformity in the handling of the samples.

Preparation of 13-hydroperoxy-linoleic acid (13-HP-linoleic acid)

Arachidonic acid (10 mg) was suspended by sonication in 49 ml of 0.1 M sodium borate buffer, pH 9.0; 1 ml of lipoxygenase (Type IV, Sigma, 4.45 10^6 units) was added, and the reaction was allowed to proceed for 5 min at 4° and stopped by acidification to pH 4.0 with 5 N

HCl. The lipid was extracted at 24° with two 50 ml portions of benzene. The benzene extracts were pooled and evaporated to dryness under nitrogen. The residue was redissolved in hexane:ether (95:5; v/v) and applied to a 3-4 g silicic acid column. The column was washed with 150 ml of hexane:ether (95:5) and the 13-HP linoleic acid was eluted with hexane:ether (85:15; v/v). Fractions containing the hydroperoxy acid were pooled and evaporated under reduced pressure at 4°. The residue was redissolved in methanol and stored at -80°. Purity of the 13-HP linoleic acid was determined by thin-layer chromatography on silica gel 60 in diethyl ether/petroleum ether/petroleum ether/acetic acid (50:50:5; v/v/v). 13-HP-linoleic acid has an R_f of .65 in this system. Purity was estimated to be greater than 95%.

Spectrophotometric analysis

All spectra were recorded on a Aminco DW-2 a dual beam/dual wavelength recording spectrophotometer interfaced with a Aminco Midan T analogue computer. Identification and quantitation of the heme content of the preparation was determined by reduced minus oxidized difference spectra of pyridine haemochrome derivatives (Falk, 1964).

Results

Purification of PGI_2 Synthase by Immunoaffinity Chromatography

Solubilized bovine aortic microsomes containing 1080 units of PGI_2 synthase activity were applied to both an IgG_1 (isn-3) AffiGel-10 column and an IgG_1 (control)-AffiGel-10 column (Table 5). About 90% of the PGI_2 synthase activity was bound by the IgG_1 (isn-3)-AffiGel-10 column and no PGI_2 synthase activity was detected in the Tris Buffer washes. Upon elution of the IgG_1 (isn-3)-AffiGel 10 column with pH 6.0-MES-NaCl Buffer, 24% of the starting activity was recovered. In contrast, the IgG_2 (control)-AffiGel-10 column bound less than 10% of the original PGI_2 synthase activity; 100% of the enzyme activity was recovered in the initial elutate and in the Tris Buffer washes; moreover, no PGI_2 synthase activity was eluted with pH 6.0-MES-NaCl Buffer.

Samples of the microsomes before and after application to the different affinity columns, as well as fractions eluted with pH 6.0-MES-NaCl buffer were examined by SDS polyacrylamide gel electrophoresis (Figure 10-11). Silver staining of the gels revealed that fractions eluted from the $IgGa_1$ (isn-3)-AffiGel 10 column with pH 6.0-MES-NaCl Buffer contained a single protein with an apparent molecular weight of 52,000 daltons identical to that of the protein precipitated from radioiodinated bovine aortic microsomes by IgG_1 (isn-1) and IgG_1 (isn-3). A sample of the protein that did not bind

Table 5 Immunoaffinity chromatography of PGI_2 synthase

	Volume (ml)	IgG_1(isn-3)-AffiGel-10 PGI_2 Synthase (units)	IgG_1(isn-3)-AffiGel-10 Activity Recovered (%)	IgG_1(control)-AffiGel-10 PGI_2 Synthase (units)	IgG_1(control)-AffiGel-10 Activity Recovered (%)
Column Fraction					
1. Solubilized microsomes (before column)	15	1080	100	1080	100
2. Solubilized microsomes (after column)	15	87	9	996	92
3. Tris Buffer Wash A	5	0	0	135	13
4. Tris Buffer Wash B	5	0	0	23	2
5. .1 MES, pH 6-1 M NaCl A	5.75	25	2.3	0	0
6. .1 MES, pH 6-1 M NaCl B	5.75	60	5.6	0	0
7. .1 MES, pH 6-1 M NaCl C	5.75	43	4.0	0	0
8. .1 MES, pH 6-1 M NaCl D	5.75	40	3.7	0	0
9. .1 MES, pH 6-1 M NaCl E	5.75	29	2.7	0	0
10. .1 MES, pH 6-1 M NaCl F	5.75	20	1.9	0	0
11. .1 MES, pH 6-1 M NaCl G	5.75	20	1.9	0	0

Figure 10. SDS-Polyacrylamide gel electrophoresis of eluates from IgG_1(isn-3)-AffiGel-10. PGI_2 synthase was purified by immunoaffinity as described in the text and Table 5. Fractions were subjected to SDS-polyacrylamide gel electrophoresis and silver stained. Lane a, solubilized microsomes before the application to immunoaffinity column; lane b, solubilized microsomes after passage through the IgG_1 (isn-3) AffiGel-10 column; Lanes c-i, fractions eluted from the IgG_1 (isn-3)-AffiGel-10 column with pH 6.0-MES-NaCl Buffer (Column fractions 5-11 of Table 5).

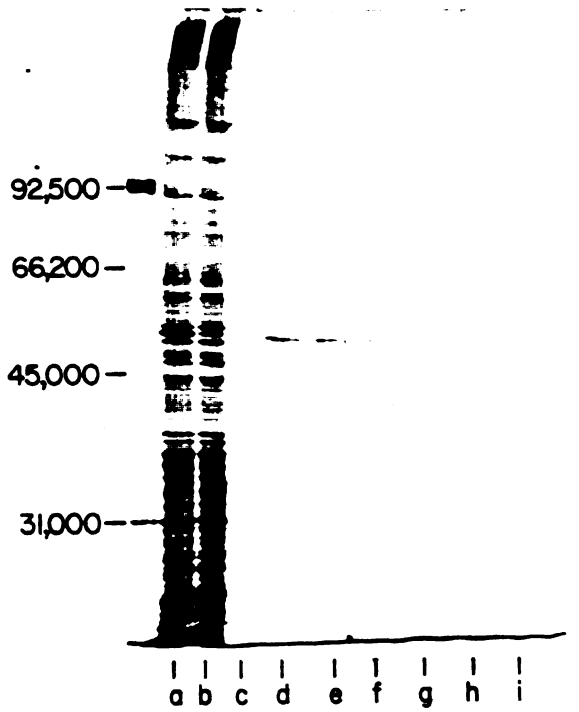

92,500
66,200
45,000
31,000
a b c d e f g h i

Figure 11. SDS-Polyacrylamide gel electrophoresis of eluates from IgG_1 (control)-AffiGel-10 column. PGI_2 synthase was applied to an IgG_1 (control)-AffiGel-10 column, as described in the text and in Table 5. After washing to remove unbound protein, PGI_2 synthase was eluted at pH 6.0. Fractions were subjected to SDS-polyacrylamide gel electrophoresis and silver stained. lane a, solubilized microsomes before application to column; lane b, solubilized microsomes after passage through the column; lanes c-i, fractions eluted from the IgG_1 (control)-AffiGel-10 with pH 6.0-MES-NaCl Buffer (Column Fractions 5-11 of Table 5).

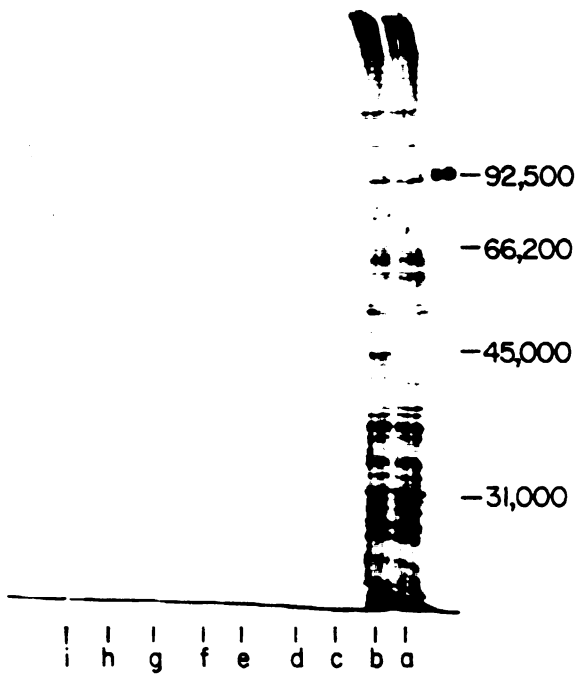
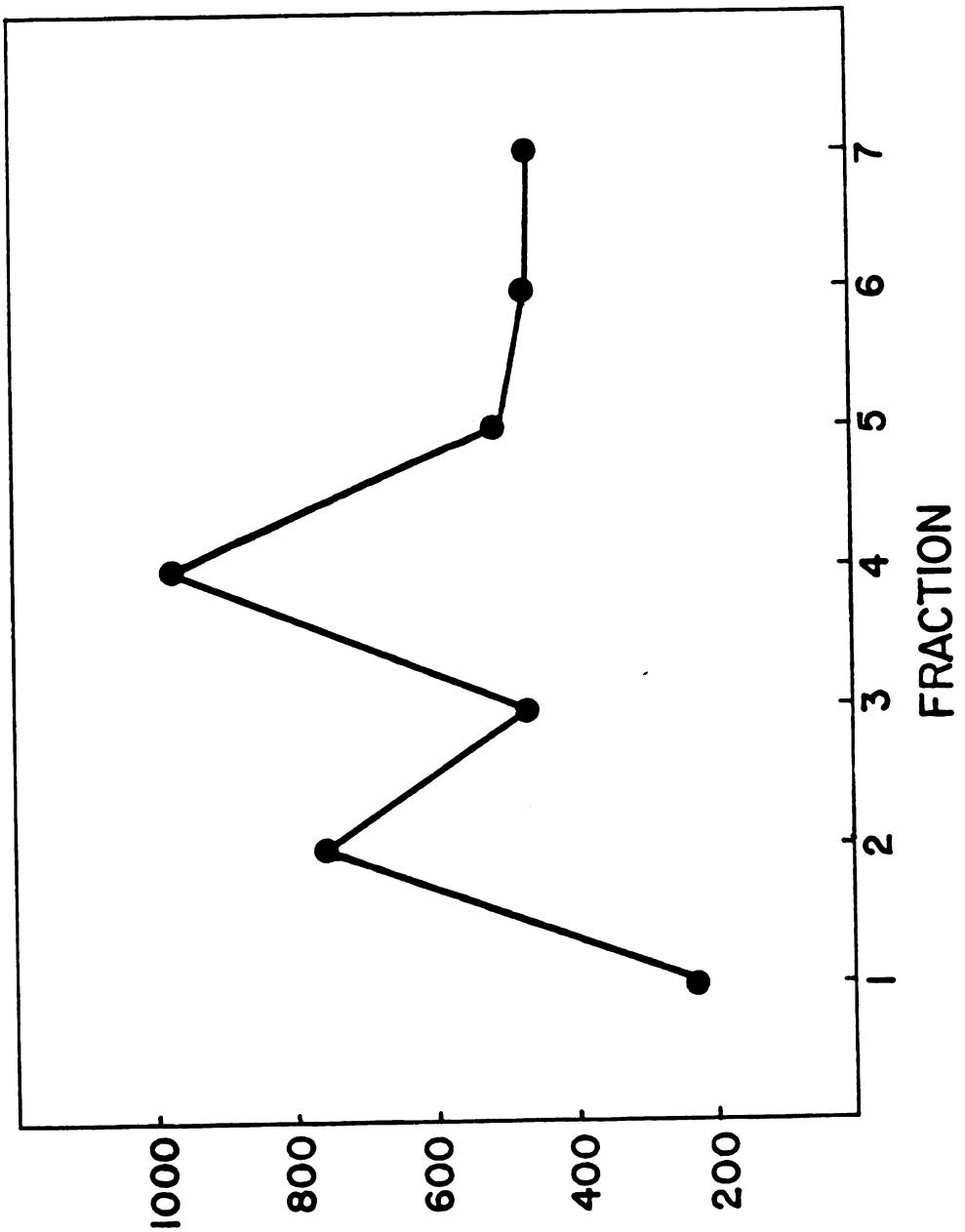

92,500
66,200
45,000
31,000
i h g f e d c b a

Figure 12. Specific activities of PGI_2 synthase eluted from the IgG_1 (isn-3)-AffiGel-10 Column.


1000
800
600
400
200
1
2
3
4
5
6
7
FRACTION

the IgG_1 (isn-3)-AffiGel-10 column showed a reduction in the intensity of staining of the 52,000 dalton band; there was no apparent, staining change in the relative staining intensities of other proteins. No proteins was detected in samples eluted from the IgG_2 (control)-AffiGel-10 column with pH 6.0-MES-NaCl Buffer (Figure 11). These results indicate that a single protein with a molecular weight of 52,000 daltons is selectively bound to the IgG_1 (isn-3)-AffiGel 10 column and that this protein is eluted under conditions in which PGI_2 synthase activity is also eluted.

The specific activity of the isolated PGI_2 synthase varied among fractions; a maximum specific activity of ca 1000 nmole PGI_2/min/mg protein was found in fraction 4 (Figure 12). There is clearly one major protein eluted from the IgG_1 (isn-3) column, and no major protein eluted from the control column. However, a relatively high background level of protein is bound non-specifically to the control column (Figure 13), and this protein is eluted with pH 6.0-MES-NaCl Buffer Apparently, proteins other than PGI_2 synthase are bound uniformly so that while the amount of contaminating protein is significant, no single contaminating protein can be detected on SDS-polyacrylamide gels (Figure 11).

Spectral Characteristics of Purified PGI_2 Synthase

Fractions eluted from the IgG_1 (isn-3) column with pH 6.0-MES-NaCl Buffer exhibited a Soret-like band of absorbance (λ_{max} = 418-420 nm) whose intensity varied in direct proportion to the PGI_2 synthase activity (Figure 14). Only a single band of absorbance was detected between 350 and 650 nm. The absorbance of less intense α and β heme absorption bands in the visible region, are

Figure 13. Protein elution profile for IgG_1 (isn-3)-AffiGel-10 and IgG_1 (control)-AffiGel-10 Columns. Solubilized bovine aortic microsomes were applied to the two columns as described in the text and Table 5. Proteins in each fraction eluted with pH 6.0-MES-NaCl Buffer were assayed by the Lowry procedure . IgG_1 (isn-3)AffiGel-10, (●——●); IgG_1 (control)-AffiGel-10 (○——○).

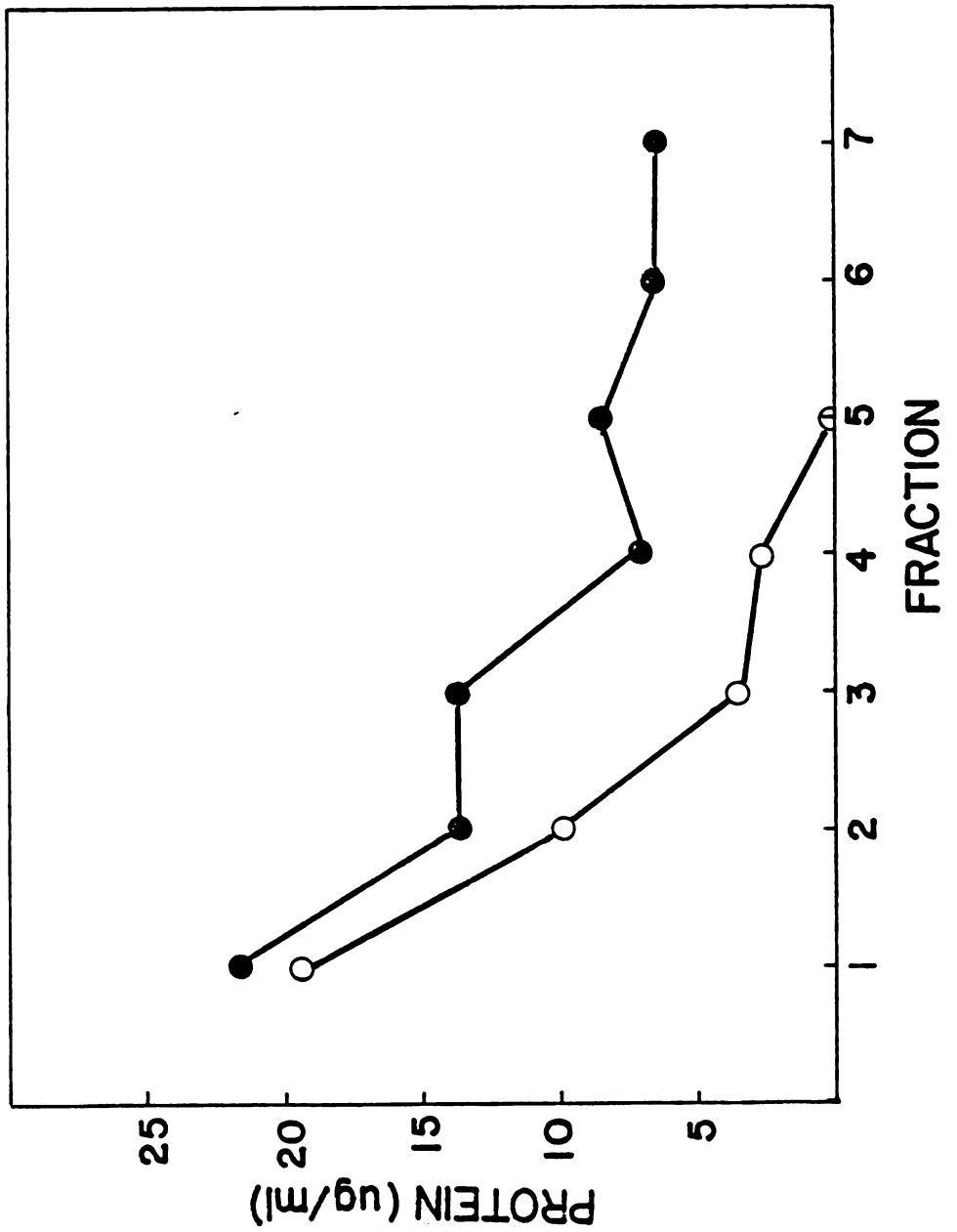
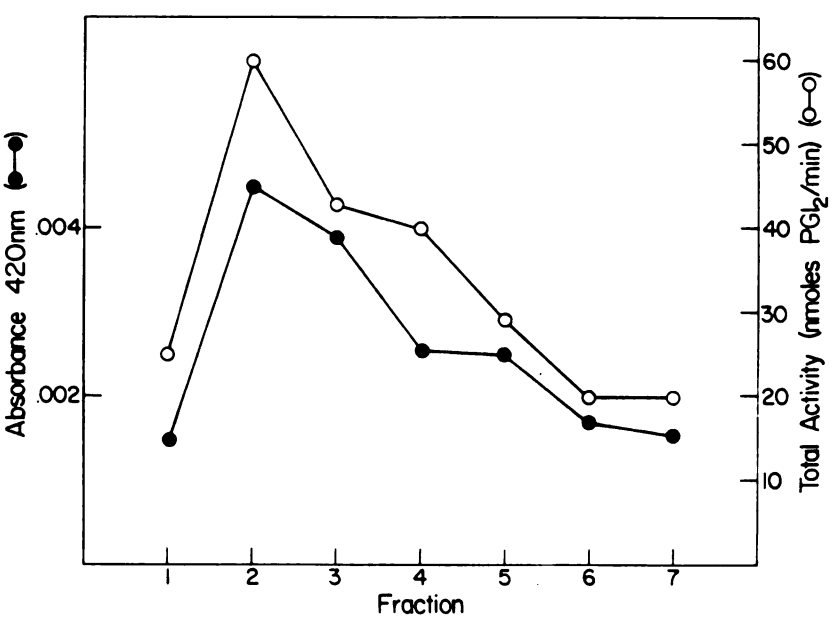

PROTEIN (ug/ml)
25
20
15
10
5
1
2
3
4
5
6
7
FRACTION

Figure 14. Coelution of PGI_2 synthase activity and Heme Absorbance from IgG_1 (isn-3)-AffiGel-10 with 0.1 M MES, pH 6.0, containing 0.5% Triton X-100 and 1 M NaCl.


Absorbance 420nm (●—●)
.004
.002
60
50
40
30
20
10
Total Activity (nmoles PGI2/min) (o—o)
1
2
3
4
5
6
7
Fraction

usually 10% of the Soret absorbance and would be below our detection limits with the protein concentrations obtained (< 5 μg/ml). No absorbance was detected between 350 and 650 nm in the samples eluted from the IgG_1 (control)-AffiGel-10 column with pH 6.-MES NaCl buffer suggesting that the heme-absorbing species is the PGI_2 synthase.

The quantity of heme in various preparations was determined using several approaches. First, a sample of inactive PGI_2 synthase was prepared by eluting the IgG_1 (isn-3)-AffiGel 10 column with 1 M acetic acid, pH 2.5, containing, 0.15 M NaCl and 0.5% Triton X-100. This procedure desorbs most of the bound protein into a single concentrated 5 ml fraction. Pyridine hemochromogen analysis was performed on this inactive enzyme preparation using a difference millimolar extinction coefficient of the absorbance difference between the peak of the α band at 557 nm and the minimum which occurs beween the α band and the β band at 441 (Falk, 1964). Using this value and values for total protein, I computed that the sample contained protoprophyrin IX heme in ratio of one heme per ten 52,000 dalton polypeptides. Estimation of the heme content of active PGI_2 synthase isolated by elution from the (isn-3)-AffiGel 10 column with pH 6.0-MES-NaCl Buffer yielded a ratio of one heme per two-52,000 dalton proteins. For this calculations the concentration of PGI_2 synthase protein was determined as the difference in protein concentrations between the peak activity fraction isolated from the IgG_1 (isn-3)-AffiGel 10 column and the corresponding numbered fraction eluted from the control column, and the absorbance at 420 nm was used to calculate the concentration of heme. The millimolar extinction coefficient for the PGH synthase, $\varepsilon_{mM_{412}} = 120\ cm^{-1}\ mM^{-1}$,

(Roth et al. 1981) was used; however, other protoporphyrin IX containing proteins have similar extinction coefficients (e.g. cytochrome P420: $_{414}$ = 124; Omura and Sato 1964). Results of the heme analysis suggest that the molar ratio of heme to protein subunit (0.1 to 0.5) can account for the recovered activity of the PGI_2 synthase (ca 25%).

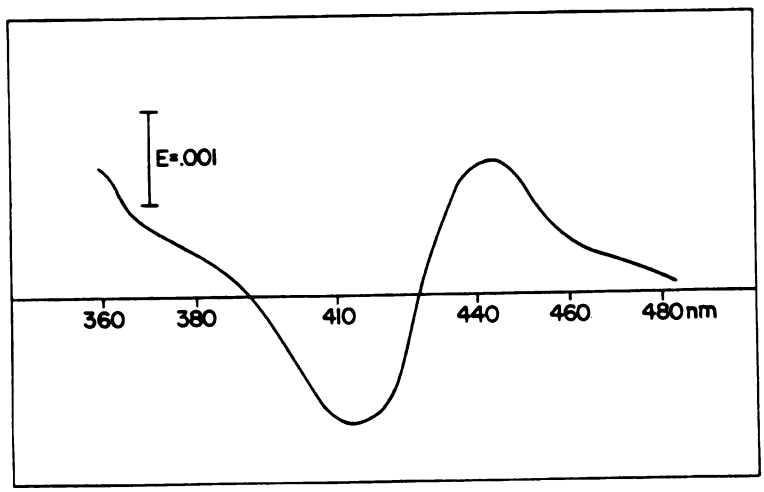
Because the PGI_2 synthase appears to be a heme-containing protein, several heme binding compounds as well as hematin were examined for their effects on enzyme activity. Neither sodium cyanide (1 μM-10 mM), nor sodium azide (1 μM-100 mM) had any effects on the activity of either the microsomal or purified PGI_2 synthase. Both of these nitrogen compounds usually bind ferriheme. Carbon monoxide, a ferrous binding anion, was also ineffective in inhibiting the enzyme. However CO does bind the heme following reduction of the enzyme by dithionite (Figure 15).

It was thought that addition of heme might stabilize the PGI_2 synthase enzyme during purification or restore activity possibly lost by dissociation of the heme during purification. However addition of 10 μM hematin to the buffers used in the purification failed to improve the yield of enzyme isolated from the IgG_1 (isn-3)-AffiGel 10 column; moreover, hematin added after purification failed to increase the activity of PGI_2 synthase isolated in the absence of added heme.

Inactivation of PGI_2 Synthase by 13-hydroperoxy-linoleic acid and PGH_2.

Several specific PGI_2 synthase inhibitors were found to profoundly affect both the activity and the spectral characteristics of the purified PGI_2 synthase. One compound,

Figure 15. Carbon monoxide-dithionite reduced difference spectrum of purified PGI_2 synthase. Purified PGI_2 synthase in the sample cuvette was reduced by adding a few crystals of sodium dithionite. Carbon monoxide was then bubbled through the sample cuvette and the reference cuvette containing unreduced PGI_2 synthase for 30 min.


E=.001
360
380
410
440
460
480nm

9,11-azo-prosta-5,13-dienoic acid (Azo Analog I; Gorman et al., 1978), a PGH_2 analogue differing from PGH_2 in the substitution of a dinitrogen bridge for a dioxygen bridge between carbons 9 and 11 and lacking an hydroxyl group at C-15, caused a dose related decrease (0.3-30 μM) in the activity of PGI_2 synthase (Table 6). As discussed previously, unsaturated hydroperoxy fatty acids, also inhibit the microsomal PGI_2 synthase (Salmon et al., 1978). Addition of 13-HP-linoleic acid (20, 50 or 100 μM) to the purified PGI_2 synthase caused a rapid, time-dependent inactivation of the purified PGI_2 synthase; 100 μM 13-OOH linoleic acid caused 100% inhibition of activity within 5 min (Figure 16).

Accompanying the inhibition caused by both Azo Analogue I and 13-HP linoleic were significant changes in the spectra of purified PGI_2 synthase preparations. Addition of Azo Analogue I at the concentrations studied (0.3 μM-30 μM) caused a shift in the peak of Soret absorbance from 420 nm to 426 nm (Figures 17,18). Presumably, this shift in absorbance in results from coordination of the Azo Analogue I with the heme group of the PGI_2 synthase.

Treatment of PGI_2 synthase with 13-HP-linoleic acid caused a different spectral change. A two minute incubation of purified PGI_2 synthase with 100 μM 13-HP linoleic acid resulted in the loss of 85% of the PGI_2 synthase (Table 6) and concomitant bleaching of the heme spectrum (Figure 17). The reduced absorbance is likely due to chemical modification of the heme. If the heme had simply been dissociated from the protein, one would have expected to see a shift in the absorption maximum to that of free heme.

Figure 16. Inhibition of purified PGI_2 synthase by 13-hydroperoxy-linoleic acid; 20, 50, and 100 μM 13-HP-linoleic acid was added to PGI_2 synthase purified by immunoaffinity chromatography as described in the text. After the indicated incubation times, residual PGI_2 synthase activity was determined as described in the text. For zero time incubations substrate (PGH_2, 50 μM) and inhibitor were added simultaneously.

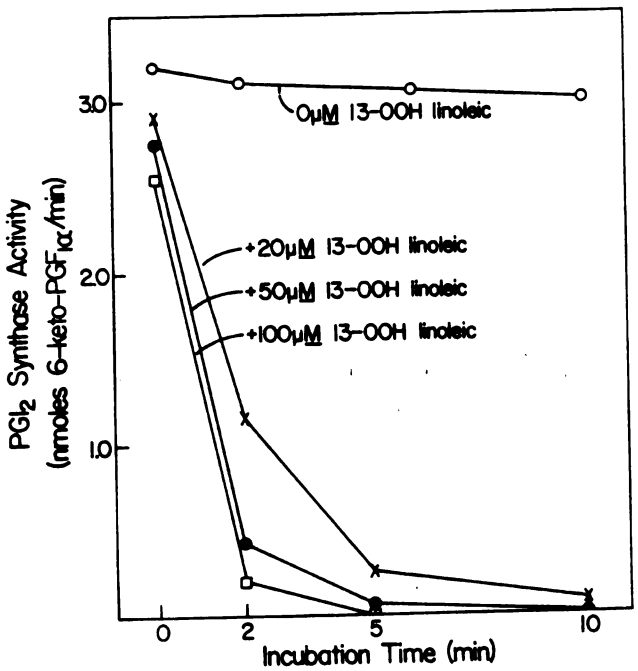

PGI2 Synthase Activity
(nmoles 6-keto-PGF1α/min)
3.0
2.0
1.0
0μM 13-OOH linoleic
+20μM 13-OOH linoleic
+50μM 13-OOH linoleic
+100μM 13-OOH linoleic
0
2
5
10
Incubation Time (min)

Figure 17. Effect of 13-hydroperoxy-linoleic acid on the absorbtion spectrum of purified PGI_2 synthase: protection from bleaching by Azo Analogue I. Spectra were recorded on an Aminco spectrophotometer as discussed in the text. Spectra of PGI_2 synthase purified by immunoaffinity chromatography were taken: (a) following no treatment; (b) after a 2 min incubation at 24° with 100 μM 13-HP-linoleic acid and (c) after adding 30 μM Azo Analog I and then treating for 2 min at 24° with 100 μM 13-HP-linoleic acid. The protein concentration of the PGI_2 synthase preparation used in these experiments was 8 mg/ml.

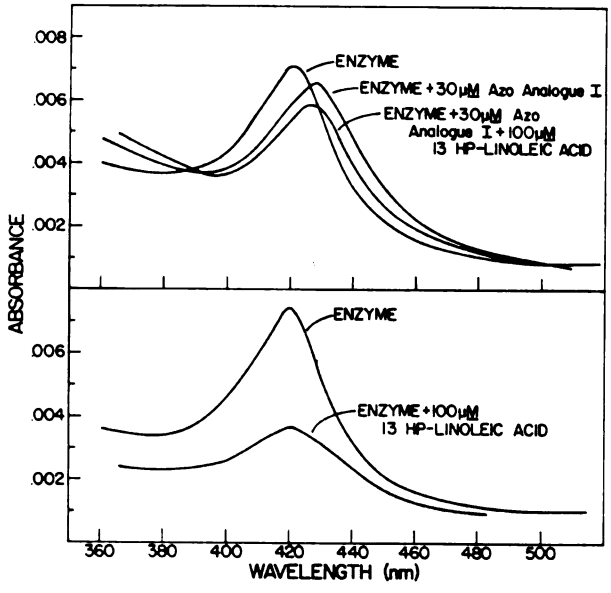
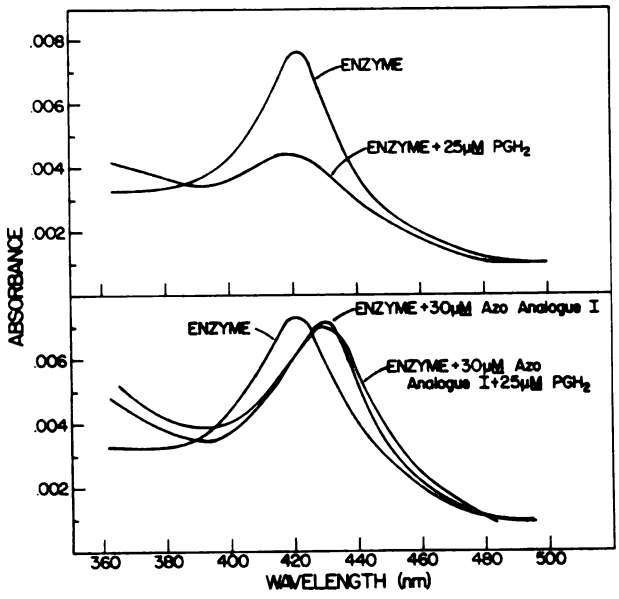

.008
.006
.004
.002
ABSORBANCE
ENZYME
ENZYME + 30μM Azo Analogue I
ENZYME + 30μM Azo Analogue I + 100μM 13 HP-LINOLEIC ACID
ENZYME
ENZYME + 100μM 13 HP-LINOLEIC ACID
360
380
400
420
440
460
480
500
WAVELENGTH (nm)

Figure 18. The effect of PGH_2 on the absorbtion spectrum of PGI_2 synthase: Protection from Bleaching by Azo Analogue I. Spectra of PGI_2 synthase purified by immunoaffinity chromatography were recorded: (a) following no treatment; (b) after a 2 min incubation at 24° with 25 μM PGH_2 and (c) after adding 30 μM Azo Analog I and then treating for 2 min at 24° with 25 μM PGH_2. The protein concentration of the PGI_2 synthase preparation used in these experiments was 8 μg/ml.


.008
.006
.004
.002
ABSORBANCE
ENZYME
ENZYME + 25μM PGH2
ENZYME + 30μM Azo Analogue I
ENZYME
ENZYME + 30μM Azo Analogue I + 25μM PGH2
.006
.004
.002
360
380
400
420
440
460
480
500
WAVELENGTH (nm)

Addition of 25 μM PGH_2 to purified enzyme caused a bleaching of the heme spectrum similar to that observed with 13-HP-linoleic acid (Figure 18). It has been shown that the PGI_2 synthase is inactivated (Watanabe et al. 1979) during the conversion of PGH_2 to PGI_2 and we have confirmed this finding with enzyme immobilized by attachment to S. aureus cells (see below). Under conditions in which the heme spectrum becomes bleached in the presence of PGH_2, the PGI_2 synthase is completely inactivated.

The fact that PGH_2 (an endoperoxide) and 13-HP linoleic acid both cause similar spectral changes and enzyme inactivation suggests that the chemical modifications caused by these agents is similar and perhaps proceeds via a similar mechanism. Moreover, the bleaching of the heme spectrum observed with by both PGH_2 and 13-HP linoleic acid peroxide can be prevented when Azo Analogue I is included in the incubation mixture. As shown in Figure 18, addition of 30 μM Azo Analogue I to the purified PGI_2 synthase preparation before adding 25 μM PGH_2 protected the enzyme from bleaching. The absorbance peak remained unchanged in intensity at 426 nm for at least 3 min after addition of the PGH_2. The same concentration of Azo Analogue I also prevented bleaching of the heme spectrum caused by 100 μM 13-HP linoleic acid (Figure 17).

It was not possible to determine directly whether prevention of the bleaching phenomenon by Azo Analogue I was accompanied by protection of the enzyme activity since Azo Analog I is itself a PGI_2 synthase inhibitor. To circumvent this problem PGI_2 synthase was conjugated to IgG_1 (isn-3) to S. aureus; this provided a way to incubate the enzyme with PGH_2 or 13-HP linoleic in the presence or

absence of Analogue I and then to separate the immobilized enzyme from the effectors.

Employing this immobilized PGI_2 synthase synthase system I observed that Azo Analogue I protected the immobilized PGI_2 synthase from inactivation by both PGH_2 and 13-HP linoleic acid (Tables 6 and 7). Concentrations of Azo Analogue I from 0.3 to 30 μM caused a dose-dependent inhibition of immobilized PGI_2 synthase as assayed with 50 μM PGH_2 (Table 6). In the presence 30 μM Azo Analogue I, 88% of the total S. aureus bound PGI_2 synthase activity remained after a one min incubation with 25 μM PGH_2 (Table 6); in contrast only 22% of the starting activity remained when PGI_2 synthase was incubated for one min Azo Analogue I. When PGI_2 synthase bound to S. aureus cells was incubated with 20-100 μM 13-OOH linoleic for 5 min in the presence of Azo Analog I, 76-86% of the original enzyme activity remained (Table 7). In a parallel incubation without Azo Analogue I, but with 100 μM 13-HP linoleic acid, the PGI_2 synthase activity was completely lost.

Table 6 Azo Analogue I protects PGI_2 synthase from inactivation by PGH_2[a]

Pretreatment	PGI_2 Production During Preincubation (pmoles 6-keto-$PGF_{1\alpha}$)	Final PGI_2 Synthase Activity (pmole 6-keto-$PGF_{1\alpha}$)
none		1180
0.3 μM Azo Analogue I	-	1180
3.0 μM Azo Analogue I	-	1080
30.0 μM Azo Analogue I	-	850
25.0 μM PGH_2	1000	280
25.0 μM PGH_2 + 0.3 μM Azo Analogue I	930	550
25.0 μM PGH_2 + 3.0 μM Azo Analogue I	400	980
25.0 μM PGH_2 + 30.0 μM Azo Analogue I	130	1000

[a]PGI_2 synthase complexed with S. aureus via IgG_1 (isn-3) IgG was incubated with the indicated effectors for 1 min then precipitated by centrifugation. The supernatant from those incubations which included PGH_2 were further analyzed for PGI_2 production. Pelleted cells were washed to remove the effectors, then resuspended and assayed for PGI_2 synthase activity.

Table 7 Azo analogue I protects PGI_2 synthase from inactivation by 13-Hydroperoxy-Linoleic Acid[a]

Pretreatment	PGI_2 Synthase Activity (pmole 6-keto-$PGF_{1\alpha}$)
none	1290
20 μM 13-HP linoleic	275
50 μM 13-HP linoleic	0
100 μM 13-HP linoleic	0
30 μM Azo Analogue I	1125
30 μM Azo Analogue I + 20 μM 13-HP-linoleic	1025
30 μM Azo Analogue I + 50 μM 13-HP-linoleic	950
30 μM Azo Analogue I + 100 μM 13-HP-linoleic	950

[a]PGI_2 synthase complexed with S. aureus via IgG_1 (isn-3) IgG was incubated with the indicated effectors for 5 min then precipitated by centrifugation. The pelleted cells were washed to remove the effectors, then resuspended and assayed for PGI_2 synthase activity.

DISCUSSION

The PGI_2 synthase has been purified to electrophoretic homogeneity and found to be a 52,000 dalton protein that is associated with protoporphyrin IX heme. There is strong circumstantial evidence that the heme is an integral part of the enzyme and necessary for catalytic activity. The most obvious evidence is that the heme and PGI_2 synthase activity both co-elute; no heme is isolated non-specifically by absorbtion to control columns. The molar ratio of protein to heme (0.1-0.5) while not stoichiometric is large enough to account for recovered activity.

The most convincing evidence to show that the heme is a necessary prosthetic group of the PGI_2 synthase are the results of the inhibitor experiments. Three effectors, fairly specific inhibitors of PGI_2 synthase activity, were found to produce changes in the heme absorbance at concentrations at which they also caused enzyme inhibition. Azo Analogue I caused a shift in heme absorbance from 420 nm to 426 nm, and PGH_2 and 13-HP-linoleic acid caused irreversible bleaching of heme absorbance. All of the spectral changes were accompanied by inhibition of PGI_2 synthase activity.

The permanent inactivation of PGI_2 synthase by PGH_2 and 13-OOH-linoleic acid and concurrent permanent bleaching of heme absorbance could be prevented by Azo Analogue I, but this protection required inhibition of PGI_2 synthase by Azo Analogue I. Low

concentrations of Azo Analogue I (0.3 μM) did not inhibit PGI_2 synthase and did not prevent enzyme inactivation or heme bleaching by PGH_2 and 13-HDP-linoleic. Higher concentrations of Azo Analogue I (30 μM) inhibited PGI_2 synthase activity and protected the enzyme from inactivation and heme bleaching. If the heme were not associated with the PGI_2 synthase, it is unlikely that the protection from bleaching would be so tightly coupled with enzyme inhibition.

Other researchers have postulated that the PGI_2 synthase is a heme containing enzyme. Ulrich et al. (1981) obtained cytochrome P-450 like difference spectra from pig aorta microsomes and argued from a mechanistic point of view that the absorbance was due to PGI_2 synthase. Very recently Ulrich and coworkers (Graf et al., May 1982) reported in a poster, at the International Prostaglandin Conference, the purification of PGI_2 synthase to homogeneity from pig aorta. The protein purified was shown to possess a cytochrome P-450 like spectra. While CO-reduced difference spectra of our purified PGI_2 synthase (Figure 15) shows only a broad absorbance near 440 nm, it is likely that the difference between our spectrum and that of Graf et al. is due a difference in conditions used to isolate the enzyme.

This is the first report of the preparation of monoclonal antibodies to an impure enzyme and the subsequent isolation of the enzyme by immunoaffinity chromatography with those antibodies. Others, however, have produced monoclonal antibodies to purified proteins or cell surface antigens and used these antibodies for purification of enzymatically and biologically active proteins. In a particularly elegant study, Hanson and Beavo (1982) isolated anibodies that bind calmodulin associated nucleotide phosphodiesterase only. The

phosphodiesterase could be eluted with EDTA buffer which causes dissociation of calmodulin. Other protein purification reported include: HLA-A and -B antigens (Parham 1979), H-2K antigen (Herrman and Mescher, 1979), nicotinic acetylcholine receptors (Lennon et al., 1980), and coagulation Factor V (Katzman et al., 1981). All of these proteins were isolated, in a biologically active form. They illustrate the potential for use of monoclonal antibodies in purification procedures.

Immunoaffinity purification with columns prepared with conventional antisera are notoriously difficult to elute (Pharmacia, 1979); almost always the conditions that will elute a protein will also denature it. This is probably due to the binding of multiple antigenic determinants on a single protein by the various idiotypic antibodies present in antisera. The difficulty in eluting active proteins, together with the low specificity of most antisera immuno-affinity columns, makes affinity-purification with polyclonal antisera useless for most purposes.

Monoclonal antibodies, however, react with a single antigenic determinant, and the conditions necessary to disrupt a single interaction and effect elution from a column are much less harsh than those for multiple interaction. The PGI_2 synthase was eluted at pH 6.0 with 1.0 M NaCl; acetylcholine receptors were eluted at pH 10.0, with 0.5 M NaCl (Lennon et al., 1980); Factor V was eluted at pH 6.5 with 1.2 M NaCl (Katzman et al.); HLA-A and -B antigen were eluted at pH 11.5, 0.5 M NaCl (Parham, 1979); and H2-K antigen was eluted at pH 8.0 with 0.5% deoxycholate (Herrman and Mescher, 1979). These mild conditions have allowed for recovery of native proteins in each case.

The ability to purify active proteins 1000-5000 fold (Katzman et al., 1981; Secher and Burker, 1980) in a single step makes the use of immunoaffinity chromatography with monoclonal antibodies an attractive purification technique.

The PGI_2 synthase in this purified form has been shown to be quite sensitive to inhibition by 13-HP-linolic acid. The mechanism of inhibition by lipid peroxides has been the subject of controversy for several years. Egan et al. (1976) reported that during the reduction of PGG_2 to PGH_2, PGH synthase produces a nascent oxidizing species that might be responsible for the catalytically-induced inactivation of PGH synthase. They also hypothesized (Ham et al., 1979) that reduction of lipid peroxides to alcohols by PGH synthase in microsomes may release the same oxidizing species, and speculated that this may be the mechanism of PGI_2 synthase inhibition by lipid peroxides. Our work with 13-HP-linoleic shows that the purified enzyme can be inactivated by a direct action of the lipid peroxide on the PGI_2 synthase.

CHAPTER V

QUANTITATION AND LOCALIZATION OF PGH SYNTHASE AND PGI_2 SYNTHASE

In this chapter I describe the quantitation of PGH synthase and PGI_2 synthase in vascular smooth muscle and endothelium using immunoradiometric assays. I also report studies on the immunocytochemical localization of PGI_2 synthase in non-vascular smooth muscle.

MATERIALS AND METHODS

Materials

Monoclonal antibodies secreted by the mouse hybridoma lines isn-1 (IgG_1), isn-3 (IgG_1), cyo-3 (IgG_1) and cyo-5 (IgG_{2b}) were prepared as described previously (Chapters II, III). IgG_1 purified by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography from MOPC-21 culture media was a gift from Dr. Paula Jardieu. Fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG was purchased from Miles Laboratories, Inc. CLS II collagenase was obtained from Worthington Biochemicals. All other materials were obtained from sources documented in Chapters II, III and IV.

Methods

Immunocytofluorescence staining

New Zealand white rabbits (2-3 kg) were sacrificed by intravenous injection of lethal doses of 5% Nembutal via the marginal ear vein. Tissues were removed immediately after sacrifice and placed on ice. Tissue samples were then embedded in 5% gum tragacanth on cork cylinders and quick frozen in either isopentane or hexane cooled to -78° in a dry ice-acetone bath. Tissue sections (10 μm) were cut at -25° using an Ames Lab-Tek cryotome. The sections were transferred to glass cover slips and placed in a desicator under water aspiration for 30 min. Each coverslip was then overlayed with one of five different

"first antibodies"; these included monoclonal antibodies present in complete HT culture media and secreted by lines isn-1, isn-3, cyo-3 or cyo-5; a MOPC-21 IgG_1 was dissolved at a concentration of 5 µg IgG per ml in complete HT media and used as a control first antibody. After a 30 min incubation with first antibody, the coverslips were washed with phosphate-buffered saline (PBS) (composition in mM: 151 NaCl, 45 KH_2PO_4 and 2.5 NaOH), pH 7.2. FITC-labeled rabbit anti-mouse IgG diluted 1:20 in PBS, pH 7.2, was then added to each coverslip. After 30 min, coverslips were again washed with PBS, pH 7.2, and then mounted in glycerol. Epi-fluorescence microscopy was performed using Leitz Orthoplan microscope equipped with a 150 watt Xenon Lamp and appropriate filters for examining FITC fluorescence. Photomicroscopy was performed with an Orthomat camera and Kodak Tri-X Pan film (ASA 400).

Isolation of Bovine Aorta Endothelial Cells and Smooth Muscle

Bovine aortic endothelial cells were isolated by the method of Ingerman-Wojenski et al. (1981). Bovine aorta 20-30 cm long were obtained fresh at slaughter from Michigan State University and immediately placed in 4° Krebs buffer (composition in mM, 118 NaCl, 4.1 KCl, 22 KH_2PO_4, 25 $NaHCO_2$, 2.5 $CaCl_2$, 1.8 mM $MgSO_4$, 14 glucose), pH 7.2. At the laboratory, the aorta were cleaned of adipose tissue and the intercostal vessels were ligated with silk sutures. Aorta were then hung vertically from a ring stand and washed with additional Krebs buffer. The aorta were sealed at the bottom by clamping with hemostats, and the aorta then were filled with Krebs buffer containing 0.5 mg/ml CLS II collagenase. The top of the aorta was covered with foil and the aorta were incubated at 37° for 30 min. The

collagen solution was then drained and discarded. The bottom of the aorta were sealed again and the aorta were carefully filled with Krebs, buffer, which was also drained and discarded. Next, the endothelial cells were harvested. The aorta were sealed at the bottom and the aorta were filled 2/3 full with Krebs buffer. The top of the aorta was clamped shut and the aorta were removed from the ring stand and shaken vigorously. This wash was saved as were two additional washes collected in the same manner. The pooled washes from a single aorta were centrifuged at 1000 x g to collect the endothelial cells into a pellet, and the pellet was washed once with 5 ml of Krebs buffer. After the final centrifugation each cell pellet was resuspended in 1 ml of 0.1 M Tris-chloride, pH 8.0 containing 0.5% Triton-X-100 (v/v). The cell were resuspended by vortexing and then sonicated for 30 sec at setting 50 on a Bisonik sonicator. The homogenate was then centrifuged at 48,000 x g for 20 min to remove the insoluble material. The solubilized protein was used for determination of PGH synthase and PGI_2 synthase protein concentrations.

Aorta denuded of endothelial cells by the above procedure were slit open longitudinally. The luminal surface was scraped gently with a razor and washed with Krebs to assure that all endothelial cells were removed. Several small pieces of smooth muscle were cut from various locations along the length of the aorta. Pieces from an individual aorta were added to 15 ml of 0.1 M Tris-chloride, pH 8.0 and homogenized with a Teckmar homogenizer. Care was taken to maintain the temperature below 15°. Next, Triton-X-100 was added to a final concentration 0.5% (v/v), and the sample was sonicated for 1 min at full power on a Biosonik sonicator. The homogenate was centrifuged at

48,000 x g for 20 min to remove insoluble material and the PGH synthase and PGI_2 synthase concentrations in the solubilized protein was determined.

Immunoradiometric assays for PGH synthase were as in Chapter 2. The assay was standardized using PGH synthase activity from solubilized sheep vesicular gland microsomes. PGH synthase activity was determined polarographically (Hemler et al., 1976). Immunoradiometric assays for PGI_2 were performed as in chapter 3 using PGI_2 synthase activity from bovine aortic microsomes, as a standard.

RESULTS

Quantitation of PGI_2 synthase and PGH synthase in smooth muscle and endothelium

Immunoradiometric assays for quantitating PGI_2 synthase and PGH synthase were developed previously (Chapters II, III). We applied these assays to the measurement of the concentrations of these two proteins in isolated endothelial cells and smooth muscle tissue prepared from bovine aorta (Ingerman-Wojenski et al. 1981). The results are summarized in Table 8. The concentrations of PGI_2 synthase protein were found to be quite similar in both cell layers. The averaged results of the determinations from eleven aorta showed that endothelial cells contained 4.2 ± 1.2 units and smooth muscle contain 5.8 ± 1.0 units of PGI_2 synthase activity per mg of solubilized protein. Surprisingly, the PGH synthase levels differed considerably between the smooth muscle and endothelium with the endothelium having approximately 20 times the PGH synthase concentrations of smooth muscle.

Localization of PGI_2 Synthase in Vascular Smooth Muscle and Endothelium

The distribution of PGH synthase and PGI_2 synthase as determined by immunoradiometric assay was visually corroborated by immunocytochemical localization. The patterns of fluorescent staining obtained with IgG(cyo) and IgG(isn) are analogous to the quantitation

Table 8. Concentrations of PGH Synthase and PGI_2 Synthase in Cell Layers of the Bovine Aorta[a]

Cell Layer	PGH Synthase[b] (units/mg protein)	PGI_2 Synthase[c] (units/mg protein)
Endothelium (7)	50 ± 14	4.2 ± 1.2
Smooth muscle (7)	2.8 ± 1.1	5.8 ± 1.0

[a] Samples from seven different bovine aorta were analyzed using immunoradiometric assays for PGI_2 and PGH synthases as described in the text.

[b]One unit activity is defined as that amount of enzyme which will utilize 1 nmole oxygen per min.

[c]One unit activity is defined as the amount of enzyme which will convert 1 nmole PGH_2 to PGI_2 per min.

results. In a fluorescent micrograph (Figure 19) of an artery of sheep myometrium, staining for PGH synthase with IgG_{2B} (cyo-5) revealed intense fluorescence in the endothelial cell layer; comparatively little staining is seen in the smooth muscle. By contrast, a similar section (Figure 20), stained for PGI_2 synthase with IgG_1(isn-3) shows intense fluorescence throughout the smooth muscle and in the endothelium.

Localization of PGI_2 synthase in nonvascular smooth muscle

The fact that PGI_2 synthase concentrations in vascular smooth muscle were relatively high suggested to us that PGI_2 synthesis might be characteristic of other types of muscle tissue. Accordingly, we examined the smooth muscle from a variety of rabbit tissues and organs for the presence of PGI_2 synthase antigenic reactivity by indirect immunocytofluorescence using procedures similar to those employed earlier in studies on PGH synthase (Smith and Bell, 1978). PGI_2 synthase immunoreactivity was found to be associated with nonvascular smooth muscle in lung, trachea, uterus, urinary bladder, ureter, ductus deferens, and seminal vesicles (Table 9). The longitudinal smooth muscle and muscularis mucosa, but not the circular smooth muscle of the gastrointestinal tract also stained positively for the PGI_2 synthase antigen; in contrast to the results with smooth muscle, we found that neither striated cardiac nor skeletal muscle stained for PGI_2 synthase. Our results indicate that PGI_2 synthesis is characteristic of both vascular and nonvascular smooth muscle cells. Presumably, PGI_2 has a general role as a regulator of the contractile response in smooth muscle.

Figure 19. Immunocytofluorescent localization of PGH synthase in a artery in a cross section of ovine myometrium. Cross sections of ovine myometrium were incubated sequentially with IgG_{2b} (cyo-5) and fluorescein isothiocyanate-labeled rabbit anti-mouse IgG and then examined by fluoresence microscopy. Magnification, 200 X.

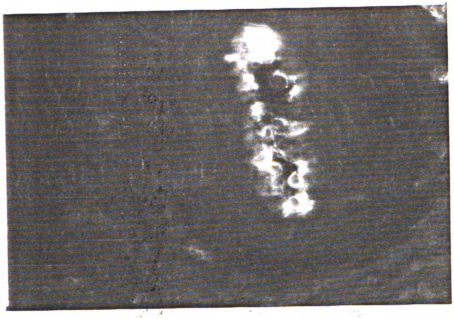
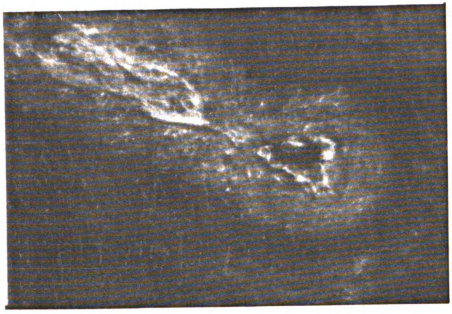


Figure 20. Immunocytofluorescent localization of PGI_2 synthase in an artery in a cross section of ovine myometrium. Cross sections of ovine myometrium were incubated sequentially with IgG_1 (isn-3) and fluorescein isothiocyanate-labeled rabbit anit-mouse IgG and then examined by fluorescence microscopy. Magnification, 200X.



One curious observation from the immunocytochemical studies was that the most intense fluorescent staining in some, but not all, types of smooth muscle was associated with the periphery of the cell. Figure 21 provides an example of this unique pattern of anti-PGI_2 synthase staining as it occurs in the rabbit urinary bladder. Figure 22 shows control staining with control MOPC-21 for comparison. These results suggest that the PGI_2 synthase can be associated with the cell surface in certain cell types. It is not yet clear whether in these instances, the enzyme is on the inside or outside of the plasma membrane. A plasmalemma location for PGI_2 synthase contrasts with the PGH synthase which appears to be associated with the endoplasmic reticulum in most cells (Rollins and Smith, 1980).

Table 9. Distribution of PGH Synthase and PGI_2 Synthase Immunoreactivity in Muscle

Species	Tissue	Muscle Type	PGH Synthase	PGI_2 Synthase
Rabbit	Artery	vascular, smooth	-	+
Rabbit	Stomach (fundus)	longitudinal, smooth	-	+
Rabbit	Stomach (fundus)	circular, smooth	-	-
Rabbit	Stomach (fundus)	muscularis mucosa, smooth	+(nuclear)	+
Rabbit	Large Intestine	longitudinal, smooth	-	+
Rabbit	Large Intestine	circular, smooth	-	-
Rabbit	Large Intestine	muscularis mucosa, smooth		+
Rabbit	Small Intestine	longitudinal, smooth	+(nuclear)	+(cell surface, nuclear)
Rabbit	Small Intestine	circular, smooth	+(nuclear)	±(nuclear)
Rabbit	Small Intestine	muscularis mucosa, smooth	+	+
Rabbit	Urinary Bladder	smooth	-	+(cell surface)
Rabbit	Ureter	longitudinal, smooth	-	+
Rabbit	Ureter	circular, smooth	-	+
Rabbit	Ductus Deferens	smooth	-	+(cell surface)
Rabbit	Prostate	smooth	-	+(cell surface)
Rabbit	Seminal Vesicles	smooth	-	+(cell surface)
Rabbit	Uterus	longitudinal, smooth	-	+
Rabbit	Uterus	circular, smooth	-	+
Sheep	Uterus	longitudinal, smooth	+(nuclear, cyto-plasmic	+(cell surface)
Sheep	Uterus	circular, smooth	+	+
Rabbit	Gall Bladder	smooth	-	+
Rabbit	Lung	Reissen's, smooth	-	+
Rabbit	Trachia	smooth	-	+
Rabbit	Pectoris	skeletal	-	-
Rabbit	Heart	cardiac	-	-

Figure 21. Immunocytofluorescent localization of PGI_2 synthase in rabbit urinary bladder. Cryotome sections of urinary bladder were incubated sequentially with IgG_1 (*isn*-3) and fluorescein isothiocyanate-labeled rabbit anti-mouse IgG and then examined by fluorescence microscopy. Magnification, 200 X.

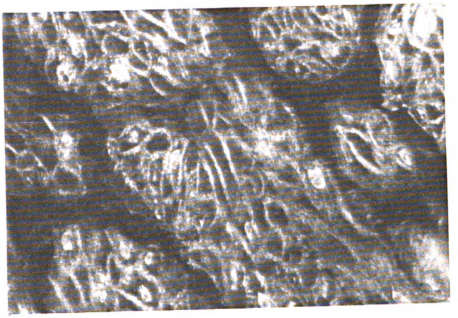
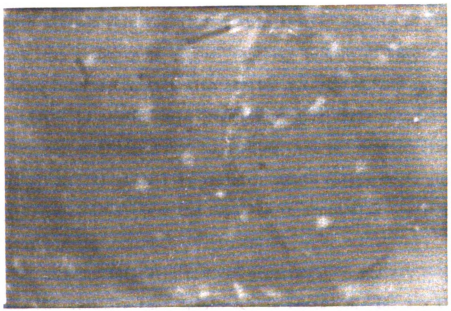


Figure 22. Control immunofluorescent staining of rabbit urinary bladder with MOPC-21 mouse IgG_1. Cryotome sections of urinary bladder were incubated sequentially with IgG_1 (MOPC-21) and fluorescein isothiocyanate-labeled rabbit anti-mouse IgG and then examined by fluorescence microscopy. Magnification, 200 X.



DISCUSSION

We have measured by immunoradiometric analysis the concentration of PGH synthase and PGI_2 synthase in smooth muscle and endothelium. Both tissues were found to contain similar amounts of PGI_2 synthase and thus probably have equal capacity to convert PGH_2 to PGI_2. These results conflict with earlier assays for PGI_2 synthase enzyme activity in rabbit aorta (Moncada et al., 1977) but are consistent with more recent observations suggesting that the denuded vasculature (Ts'ao et al., 1979) as well as isolated smooth muscle cells (Baezinger et al., 1977, 1979) have the capacity to synthesize significant amounts of prostacyclin.

Endothelial cells however were found to contain roughly 20 times the PGH synthase that smooth muscle contained, and are thus able to produce 20 times as much PGH_2 from arachidonic acid. These results are consistent with the relative inability of newly deendothelialized vasculature to synthesize PGI_2 from arachidonic acid (Eldor et al., 1981) and suggest that formation of a PGI_2-forming, neointimal layer following vascular injury (Eldor et al., 1981) is associated with an increase in PGH synthase.

The endothelial also appears to have a greater capacity the synthesize PGH_2 than to convert PGH_2 to PGI_2. The endothelial cell layer has 50 units of PGH synthase activity per mg of protein, which means a mg of endothelial protein contains enough PGH synthase to

utilize 50 nmole/min O_2, and thus can convert 25 nmoles arachidonic acid to PGH_2 per min. The endothelial cells contained 4.2 units of PGI_2 synthase activity per mg protein. Because the PGI_2 synthase assay I have used does not measured initial rates the assay may underestimate PGI_2 synthase activity 2-3 fold. Even so, the endothelial layer has at least twice the capacity to form PGH_2 as it does to convert PGH_2 to PGI_2. It is tempting to speculate that PGH_2 produced by the endothelium may be transported out of these cells, either to the smooth muscle to be converted to PGI_{21} or to the blood to be converted to TxA_2 by platelets. A specific effector such as thrombin, which is known to elicit PGI_2 production in endothelial cells in culture (Weksler et al., 1978), could stimulate PGH_2 production in vivo. Depending on the amount of PGH_2 synthesized, it would either be converted in the endothelial cell to PGI_2 or exported. If transported to smooth muscle the PGH_2 would be converted to PGI_2 and smooth muscle relaxation would occur. If the PGH were transported to the blood, platelets would produce TxA_2 and aggregation would be enhanced. Endothelial cells could thus be control cells for regulation of smooth muscle tone, or platelet aggregation.

Endothelial cells have already been shown to mediate the responses of acetylcholine in smooth muscle (Furchgott et al., 1981). Acetycholine added to vascular strips with the endothelium intact cause relaxation, while acetylcholine added to vascular strips with the endothelium removed causes contraction. It can be shown that acetylcholine induces the endothelium to release an effector, which though not identified causes the relaxation. Thus, endothelial cells may mediate the effects of humoral stimuli such thrombin thereby

participating in the regulation of vascular tone. Further work will be necessary to determine whether release of PGH_2 from the endothelium actually occurs.

A series of monoclonal antibodies specific for either PGI_2 synthase (secreted by isn-1 and isn-3) or PGH synthase (secreted by cyo-3 and cyo-5 have been used to stain cryotome sections from a variety of tissues by indirect immunocytofluorescence. The two monoclonal antibodies used to detect the two antigens were selected on the basis of their known reactivities with different determinants on PGI_2 and PGH synthases. Positive staining using a monoclonal antibody along with negative staining using nonimmune and enzyme-adsorbed immune controls indicates that the antigen is present. It should be noted, however, that a lack of positive staining with a monoclonal antibody should not be taken as evidence that an enzyme is not present. Rather the enzyme may be present at levels below the detection limit of the immunocytofluorescence procedure or may be in a physical state or isozymic form that renders the antigen unreactive with the monoclonal antibody. For example, PGH synthase in the smooth muscle layer of bovine aorta is not readily detectable by immunofluorescence; however, the enzyme in this smooth muscle layer can be measured by more sensitive immunoradiometric assays.

The fact that the PGI_2 synthase antigen is present in most smooth muscle layers but not in the striated muscles examined in this study (Table 8) suggests that PGI_2 synthesis is a characteristic feature of smooth muscle metabolism. PGI_2 normally causes relaxation of vascular smooth muscle. In this regard, the action of PGI_2 may be

to elevate intracellular levels of cAMP which in turn results in relaxation of muscle tone (Miller et al., 1979).

No PGI_2 synthase was detected in circular smooth muscle in the gastrointestinal tract. Furthermore, there are differences in the subcellular distribution of PGI_2 synthase immunofluorescence among smooth muscles from different tissues. These observations indicate that the synthesis of PGI_2 must be regulated in different ways in different smooth muscle layers. One might therefore anticipate that PGI_2 also plays different roles in modulating smooth muscle activity in different organs and tissues. In fact, longitudinal smooth muscle of the large intestine has been shown to contract rather than relax in response to PGI_2 (Nakahata and Suzuki, 1981).

The subcellular location of PGI_2 synthesis is of interest in understanding the regulation of prostacyclin synthesis. Previous studies employing both differential centrifugation techniques (DeWitt et al., 1981) and light and immunoelectron microscopy (Rollins and Smith, 1980) have indicated that the prostaglandin endoperoxide, PGH_2, is formed on the cytoplasmic surface of the endoplasmic reticulum and on the nuclear membrane. However, the subcellular locations of PGI_2 synthase, TxA_2 synthase and PGH-PGE isomerase have not been defined. The results of the present studies suggest that in many cells the PGI_2 synthase can occur in association with the plasma membrane. Thus, the general concept that formation of PGH_2 occurs proximal to the conversion of PGH_2 to PGI_2 and PGE_2 may need revision. For example, PGH_2 synthesized in association with the endoplasmic reticulum may require a specific carrier system for transport to the cell surface before final metabolism to PGI_2.

Synthesis of PGI_2 from PGH_2 may then occur in conjunction with PGI_2 exit from the cell, or alternatively PGH_2 exit may precede enzymic transformation to PGI_2. In order to answer these questions it will be necessary to determine the transverse orientation of the active site of PGI_2 on the plasma membranes of smooth muscle cells such as those derived from the urinary bladder (Figure 21).

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