To determine whether the prostaglandin endoperoxide (PGH) synthase is located on the cytoplasmic or luminal side of the endoplasmic reticulum, the PGH synthase of intact sheep vesicular gland microsomes was examined for its accessibility to nonpenetrating proteases and to several monoclonal antibodies directed against the enzyme. Treatment of microsomes with proteinase K caused the loss of up to 90% of the PGH synthase (cyclooxygenase) activity while less than 10% of the enzyme was inactivated in control samples. Proteinase K digestion of microsomes in which the cyclooxygenase-active site of PGH synthase was selectively labeled by pretreatment with [3H]acetylsalicylic acid resulted in the release of 90% of the tritium in a soluble but nondialyzable form.

Additional evidence that the PGH synthase is located on the outer surface of microsomes was obtained in experiments using monoclonal antibodies directed against three different determinants on the enzyme. Each of the anti-PGH synthase IgGs caused immune precipitation of microsomes containing cyclooxygenase activity whereas control immunoglobulin did not. Unlike the active site, the antigenic sites were neither destroyed nor cleaved from the membrane by proteinase K.

Our results indicate that the PGH synthase is located on the outer surface of sheep vesicular gland microsomes. The sheep vesicular gland microsomes were prepared under conditions which yield right-side-out rat liver microsomes suggesting that the outer surface of the vesicular gland microsomes corresponds to the cytoplasmic surface of the endoplasmic reticulum. Therefore, we conclude that prostaglandin endoperoxides are formed in the cytoplasm.

The prostaglandin endoperoxide synthase is a membrane-bound protein which has been purified from both sheep (1-3) and bovine (4) seminal vesicles. The enzyme possesses two enzymic activities, a cyclooxygenase which catalyzes the oxygenation of polyunsaturated substrates such as arachidonic acid to form PGG2 and a peroxidase which can use a variety of electron donors to reduce PGG2 to PGH2 (2, 4, 5). Cross-linking experiments as well as ultracentrifugation analyses indicate that PGH synthase is a dimer (2, 6). The dimer is apparently composed of identical subunits since the purified enzyme exhibits only one protein band upon SDS-polyacrylamide gel electrophoresis (M, = 70,000 (1-3)) and because only one NH-terminal amino acid residue, alanine, can be detected by Edman degradation of the homogeneous enzyme (6-8).

One feature of the PGH synthase which has not been explored is its orientation in biological membranes. A description of this arrangement is important in defining the route that newly synthesized prostaglandins must take during their exit from cells and thus what elements within the cell may interact with prostaglandins. We previously presented evidence from an immunoelectron microscopy study of Swiss mouse 3T3 cells that the PGH synthase is localized in the endoplasmic reticulum and the contiguous outer membrane of the nuclear envelope but not in the plasma or mitochondrial membranes (9). Unfortunately, it was not possible in this latter study to distinguish the sidedness of the PGH synthase in the endoplasmic reticulum.

In the experiments described in this report we have used nonpenetrating proteases and monoclonal antibodies to determine if the PGH synthase is on the outer or inner surface of intact right-side-out microsomes, and thus, whether the protein chain projects into the cytoplasm or into the lumen of the endoplasmic reticulum.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were obtained from Sigma Chemical Company: trypsin, sodium diethylthiocarbamate, acetylsalicylic acid, bovine hemoglobin, bovine serum albumin, mannnose 6-phosphate, and Tween 20. Flurbiprofen was generously supplied by Dr. Udo Axen, The Upjohn Company, Kalamazoo, MI. Flufenamic acid was from Aldrich Chemical Co. Fungal proteinase K was from EM Biochemicals. Thermolysin was from Boehringer Mannheim. Arachidonic acid was obtained from Nu-Chek Preps, Elysia, MN.

**Preparation of Microsomes**—Sheep vesicular glands were obtained from a local abattoir, frozen immediately in dry ice, and stored at -80 °C. Female Sprague-Dawley rats (150-200 g) were killed by decapitation, and the livers were removed, frozen on dry ice, and stored at -80 °C. All subsequent steps were performed at 4 °C. Sheep vesicular gland and/or rat liver were homogenized with a Poltron homogenizer in 5-10 volumes of 0.1 M Tris-chloride, pH 8.0, containing 20 mm diethylthiocarbamate and 1 mm phenol. The homogenate was centrifuged at 10,000 × g for 10 min, and the resulting supernatant was centrifuged at 200,000 × g for 35 min. The resulting microsomal pellet was suspended by homogenization in starting buffer to a protein concentration of 1-10 mg/ml as determined by the Coomassie blue protein assay procedure (11). 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 (1-3).

**Cyclooxygenase Assays**—Cyclooxygenase activity was measured polarographically at 37 °C using a Yellow Springs Instrument Company model 53 oxygen monitor essentially as described previously.
Microsomes—Sheep vesicular gland microsomes were prepared as described before and resuspended in its original volume. The microsomes were then incubated for various times at 4°C and the sample was incubated for 45 min at room temperature to inactivate the cyclooxygenase activity. The microsomes were then cooled to 4°C, and unlabeled acetylsalicylic acid was added in excess (1 mM) to prevent further incorporation of radioactivity. The mixture was centrifuged at 50,000 x g for 75 min to precipitate the microsomes, and the pellet was resuspended in 1.0 ml of 0.1 M Tris-chloride, pH 7.4, containing 20 mM diethyldithiocarbamate and centrifuged again as before and resuspended in its original volume.

Protease Digestion of PGH Synthase from Sheep Vesicular Gland Microsomes—Sheep vesicular gland microsomes were prepared as described above. Proteinase K, collagenase, thermolysin, trypsin, or bovine serum albumin were dissolved at a concentration of 1 mg/ml in 0.1 M Tris-chloride, pH 8.0, containing 1 mM CaCl2. A sample (50-100 µg) of each of these solutions was added to 1.0-ml aliquots of sheep vesicular gland microsomes (1-10 mg/ml) to 1.0 ml of 0.1 M Tris-chloride, pH 8.0, containing 1 mM CaCl2. The samples were incubated for 6 h at 24°C. Both samples were then assayed for mannose-6-phosphatase activity before and after prior solubilization with sodium taurocholate by measuring the release of inorganic phosphate (Fig. 1). No hydrolysis of mannose-6-phosphatase occurred with either the protease- or albumin-treated microsomes prior to solubilization while hydrolysis did occur when using solubilized microsomes. Similar rates of hydrolysis were observed with both the protease-treated and untreated microsomes. In contrast, when mixed microsomes were solubilized with sodium taurocholate before treating with proteinase K or bovine serum albumin and then assayed for mannose-6-phosphatase activity, the activity was completely destroyed in the sample treated with protease but retained in the sample incubated with albumin. These results indicate that the mannose-6-phosphatase activity of the rat liver microsomes is inaccessible to proteinase K unless the microsomes are solubilized. We interpret this to mean that the rat liver microsomes are intact and have the expected orientation of microsomal proteins. We further interpret these results as indicating that the microsomes formed in the same sample but derived from the endoplasmic reticulum of sheep vesicular glands are also intact and right-side-out. The ratio of microsomal protein to protease was 20 in the experiment depicted in Fig. 1, and this ratio or a higher ratio was used in all subsequent experiments. In most of the experiments described below only sheep vesicular gland microsomes were used. However, similar results were obtained with mixed rat liver/sheep vesicular gland microsomes.

Proteolytic Digestion of PGH Synthase from Intact Microsomes—In an initial experiment to determine the sidedness of PGH synthase, intact microsomes prepared from sheep vesicular gland (10 mg of protein/ml) were incubated for 45 min at each tissue in a parallel manner from the time of procurement from the animal to the homogenization step.

Mixed rat liver/sheep vesicular gland microsomes were incubated with either proteinase K or bovine serum albumin. Each sample was then assayed for mannose-6-phosphatase activity before and after solubilization with sodium taurocholate (Fig. 1). No hydrolysis of mannose-6-phosphatase occurred with either the protease- or albumin-treated microsomes prior to solubilization while hydrolysis did occur when using solubilized microsomes. Similar rates of hydrolysis were observed with both the protease-treated and untreated microsomes. In contrast, when mixed microsomes were solubilized with sodium taurocholate before treating with proteinase K or bovine serum albumin and then assayed for mannose-6-phosphatase activity, the activity was completely destroyed in the sample treated with protease but retained in the sample incubated with albumin. These results indicate that the mannose-6-phosphatase activity of the rat liver microsomes is inaccessible to proteinase K unless the microsomes are solubilized. We interpret this to mean that the rat liver microsomes are intact and have the expected orientation of microsomal proteins. We further interpret these results as indicating that the microsomes formed in the same sample but derived from the endoplasmic reticulum of sheep vesicular glands are also intact and right-side-out. The ratio of microsomal protein to protease was 20 in the experiment depicted in Fig. 1, and this ratio or a higher ratio was used in all subsequent experiments. In most of the experiments described below only sheep vesicular gland microsomes were used. However, similar results were obtained with mixed rat liver/sheep vesicular gland microsomes.

RESULTS

Microsomal Membrane Integrity—The interpretation of our experiments exploring the orientation of PGH synthase depended on the integrity of our microsomal membrane preparations. Thus, it was of prime importance to establish this property. The most common method to ascertain the integrity of microsomal membranes is to determine if treatment of microsomes with proteases destroys the activity of enzymes such as glucose-6-phosphatase which are localized on the inner surface of microsomal spheres (15). Since sheep vesicular gland microsomes had no detectable glucose-6-phosphatase activity, we used an indirect method to determine if these microsomes were intact. Our approach was to prepare mixed microsomes from rat liver and sheep vesicular gland and then to assay for latency of the rat liver mannose-6-phosphatase (13, 16). The endoplasmic reticulum of both the rat liver and sheep vesicular gland was assumed to behave similarly during the preparation of microsomes. Special care was taken to treat each microsome preparation with protease in a parallel manner from the time of procurement from the animal to the homogenization step.

Mixed rat liver/sheep vesicular gland microsomes were incubated with either proteinase K or bovine serum albumin. Each sample was then assayed for mannose-6-phosphatase activity before and after solubilization with sodium taurocholate (Fig. 1). No hydrolysis of mannose-6-phosphatase occurred with either the protease- or albumin-treated microsomes prior to solubilization while hydrolysis did occur when using solubilized microsomes. Similar rates of hydrolysis were observed with both the protease-treated and untreated microsomes. In contrast, when mixed microsomes were solubilized with sodium taurocholate before treating with proteinase K or bovine serum albumin and then assayed for mannose-6-phosphatase activity, the activity was completely destroyed in the sample treated with protease but retained in the sample incubated with albumin. These results indicate that the mannose-6-phosphatase activity of the rat liver microsomes is inaccessible to proteinase K unless the microsomes are solubilized. We interpret this to mean that the rat liver microsomes are intact and have the expected orientation of microsomal proteins. We further interpret these results as indicating that the microsomes formed in the same sample but derived from the endoplasmic reticulum of sheep vesicular glands are also intact and right-side-out. The ratio of microsomal protein to protease was 20 in the experiment depicted in Fig. 1, and this ratio or a higher ratio was used in all subsequent experiments. In most of the experiments described below only sheep vesicular gland microsomes were used. However, similar results were obtained with mixed rat liver/sheep vesicular gland microsomes.

Proteolytic Digestion of PGH Synthase from Intact Microsomes—In an initial experiment to determine the sidedness of PGH synthase, intact microsomes prepared from sheep vesicular gland (10 mg of protein/ml) were incubated for 45 min at

Fig. 1. Latency of mannose-6-phosphatase activity in mixed rat liver/sheep vesicular gland microsomes. Mixed microsomes prepared from rat liver (2 g) and sheep vesicular gland (2 g) were suspended at a protein concentration of 1 mg/ml in 0.1 M Tris-chloride, pH 7.4, containing 1 mM CaCl2, and 1-ml aliquots were incubated with either proteinase K (50 µg) or bovine serum albumin (50 µg) for 6 h at 24°C. Both samples were then assayed for mannose-6-phosphatase before and after prior solubilization with sodium taurocholate by measuring the release of inorganic phosphate (Aini) from mannose-6-phosphatase as described in the text. △—△, with proteinase K, without solubilization; ○—○, with proteinase K, after solubilization; ▲—▲, with albumin, without solubilization; ●—●, with albumin, after solubilization. Microsomes incubated at 24°C for 6 h were found to retain 85% of the starting mannose-6-phosphatase activity.

1 Portions of this paper, including a description of the isolation, characterization, and analysis of monoclonal antibodies against PGH synthase are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20814. Request Document 81M-975, cite author(s), and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
24 °C with bovine serum albumin, collagenase, thermolysin, trypsin, or proteinase K (100 μg/ml). Thermolysin, trypsin, and proteinase K caused losses of cyclooxygenase activity ranging from 25-45%; no appreciable loss of activity occurred with microsomes that were treated with collagenase or bovine serum albumin. In this paper, we describe a system for the inactivation of cyclooxygenase activity of sheep vesicular gland microsomes by proteinase K. In this manner, about 90% of the starting cyclooxygenase activity was destroyed within 90 min while in two parallel control samples (data not shown) in which microsomes were incubated either at 4 °C with proteinase K or at 24 °C with bovine serum albumin, less than 10% of the cyclooxygenase was inactivated. These results suggested that the cyclooxygenase-active site of PGH synthase was present on the outer surface of microsomes.

In a second part of the experiment depicted in Fig. 2, intact microsomes were labeled by preincubation with [3H]acetylsalicylic acid before treatment with proteinase K or bovine serum albumin. No radioactivity was cleaved from the albumin-treated microsomes, but 90% of the tritium label was released from the protease-treated microsomes into a soluble form (Fig. 2). None of the released radioactivity was dialyzable (16 h at 4 °C) although under identical conditions [3H]acetylsalicylic acid did equilibrate across the dialysis membrane. This last result indicates that the release of radiolabel from the microsomes was caused by cleavage of the protein chain and not by an esterase-catalyzed hydrolysis of tritiated acetylated groups from the acetylated serine hydroxyl group (6, 8) of the enzyme.

Aspirin labeling of microsomes was performed under conditions shown earlier by both ourselves (1) and others (2, 3, 17) to produce selective acetylation of the PGH synthase. Furthermore, incorporation of the tritium label into the microsomes during incubation with [3H]acetylsalicylic acid was inhibited by up to 80% in the presence of several different competitive cyclooxygenase inhibitors including Flurbiprofen, flufenamic acid, or salicylic acid. Thus, some of the radiolabeled peptide(s) released by protease treatment of the microsomes must have been derived from the PGH synthase. We cannot rule out the possibility in these experiments that simple acetylation of the PGH synthase active site did selectively solubilize the sheep vesicular gland microsomes. However, we found that treatment of mixed rat liver-sheep vesicular gland microsomes with acetylsalicylic acid did not solubilize the rat liver microsomes as judged by retention of latent mannos-6-phosphatase activity.

In four different experiments we noted that the rate of release of tritium from aspirin-labeled microsomes in the presence of proteinase K was more rapid than the rate of cyclooxygenase inactivation (Fig. 2). This suggested that a portion of the PGH synthase could be cleaved from the microsomal membrane in an active form. To examine this possibility sheep vesicular gland microsomes were treated for short times (5-30 min) with thermolysin, trypsin, or proteinase K, the mixtures were centrifuged to precipitate the microsomes, and both the microsomal and supernatant fractions were assayed for cyclooxygenase activity. We consistently found 15-26% of the total residual activity in the high speed supernatant; a maximum of 30% of the starting cyclooxygenase activity was found in the supernatant when microsomes were treated with trypsin for 10-20 min. In contrast, no cyclooxygenase activity appeared in the supernatant of control microsomes incubated with bovine serum albumin. These results indicate that it is possible to produce a soluble fragment of the PGH synthase molecule retaining cyclooxygenase activity; however, the minimum size for a catalytically active fragment has not been determined.

Precipitation of Microsomal PGH Synthase with Staphylococcus aureus-Antibody Complexes—Three hybridoma lines (cyo-1, cyo-5, and cyo-7) which secrete IgG, molecules against three different antigenic determinants on the PGH synthase were selected and cloned. Monoclonal antibodies secreted by these three lines (cyo-1, cyo-5, cyo-7) or by a nonimmune control, IgG2a-secreting hybridoma (2c3), were affixed to S. aureus cells. These four different antibody-cell complexes were then mixed with either intact or solubilized microsomes prepared from sheep vesicular gland, the samples were centrifuged to pellet the S. aureus cells, and cyclooxygenase activity was measured in both the resuspended cell pellet and the supernatant (Fig. 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 20% of the activity was precipitated when a nonimmune monoclonal mouse IgG2 (2c3) was used. Although mixing the various monoclonal antibodies with the enzyme alone has 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 (Fig. 3) suggesting that the PGH synthase is equally reactive antigenically in both solubilized and membrane-bound forms. Since IgG2 molecules secreted by cyo-1, cyo-5, and cyo-7 interact with different antigenic sites on the PGH synthase, our results indicate that at least three antigenic determinants on the sheep vesicular gland cyclooxygenase are situated on the outer surface of microsomal spheres and thus on the cytoplasmic side of the endoplasmic reticulum.

Effect of Protease Digestion on PGH Synthase Antigenic Reactivity—We performed two experiments to determine if the antigenic sites on the PGH synthase were solubilized and/or destroyed by proteolytic digestion of intact microsomes. In the first experiment, samples of intact sheep vesicular gland microsomes were incubated at 24 °C for 6 h with either bovine serum albumin or proteinase K and then analyzed in a quantitative immunoradiometric assay using cyo-1-, cyo-5-, cyo-7-, or 2c3-IgG2-S. aureus cells as precipitating complexes. Similar results were obtained with both albumin- and proteinase-treated microsomes using each of the different IgG2-S. aureus precipitating complexes. These data indicate that the three antigenic determinants reactive with monoclonal antibodies secreted by cyo-1, cyo-5, and cyo-7 were not degraded by proteinase K although the cyclooxygenase-active site was destroyed under these conditions.

To determine if the antigenic determinants were cleaved from the microsomal membrane by protease digestion, we again treated microsomes with bovine serum albumin or proteinase K as described above, prepared supernatant and membrane fractions by high speed centrifugation, and then assayed these two fractions for immunoreactivity. Essentially no reactivity was observed with the supernatant fractions. Similar levels of antigenic reactivities were observed with cyo-5 and cyo-7-IgGm complexes when the membrane fractions from both albumin- and proteinase K-treated microsomes were tested (Fig. 4). A portion (ca. 20%) of the cyo-1 reactivity appeared to be lost following protease digestion of the microsomes. Since the acetyl-labeled cyclooxygenase active site is almost completely solubilized under these conditions, our results indicate that the catalytic and antigenic sites are found in different regions of the PGH synthase molecule.

**DISCUSSION**

A model depicting the arrangement of PGH synthase in cellular membranes is presented in Fig. 5. There are four major features of the model. The first is that the enzyme is situated in the endoplasmic reticulum. This latter conclusion is based largely on the results of a previous immunoelectron microscopic study on the distribution of PGH synthase antigenic reactivity in mouse 3T3 cells (9). Other observations which are consistent with this conclusion are the findings (a) that the cyclooxygenase antigen as determined by immunofluorescent staining is distributed throughout the cytoplasm in each of about 25 different prostaglandin-forming cell types that have been examined (18-21) and (b) that the PGH synthase of the renal medulla co-sediments with marker enzyme activities associated with the endoplasmic reticulum (22). The NH2-terminal region of the PGH synthase is largely hydrophobic (6, 8) and may be embedded in the membrane. The second major feature of the model is that the PGH synthase is located on the cytoplasmic side of the endoplasmic reticulum. This point is suggested in the present study by the demonstration that the enzyme as it exists in intact sheep vesicular gland in microsomes (a) can be inactivated by treatment with proteinase K and (b) can interact with three

---

**Fig. 4.** Immunoradiometric analyses of PGH synthase in microsomal membranes obtained by high speed centrifugation of albumin- and proteinase K-treated sheep vesicular gland microsomes. Microsomes (1 ml containing 10 mg of protein) were incubated for 6 h at 24°C with (a) bovine serum albumin (100 µg) or (b) proteinase K (100 µg) and then centrifuged at 50,000 × g for 70 min at 4 °C. The resulting pellets were resuspended in 0.1 M Tris-chloride, pH 7.4, containing 1% Tween 20, and immunoradiometric assays were performed on both samples essentially as described in the appended supplement and the legend to Fig. 3S. The units of PGH synthase added to the assays with albumin-treated microsomes (top) were determined by cyclooxygenase assays, and equal volumes of sample from the protease-treated sample were used in the radiometric assay (bottom). S. aureus precipitating complexes were with IgG2 secreted by cyo-1, cyo-5, and cyo-7.

**Fig. 5.** Model depicting the orientation of PGH synthase in the endoplasmic reticulum.
monoclonal antibodies directed against different antigenic determinants. The assumption underlying our conclusion is that the endoplasmic reticulum of sheep vesicular gland behaves in a similar manner to the endoplasmic reticulum of rat liver during preparation of microsomes. Although all the experiments reported here were performed using sheep vesicular gland microsomes, qualitatively similar results have been observed in related studies on the asymmetric arrangement of the PGH synthase in microsomes from both human platelets (23) and Swiss mouse 3T3 cells. The third feature of our model is that the cyclooxygenase catalytic site responsible for conversion of fatty acid to PG is situated on a portion of the protein which can be cleaved from the enzyme by proteases. This conclusion is based on two observations: (a) that proteinase K treatment of microsomes in which the cyclooxygenase was selectively labeled with \([3H]\)acetylsalicylic acid caused the release of tritium in a soluble yet nondialyzable form and (b) that thermolysis, proteinase K, and trypsin all caused the release of some cyclooxygenase activity from the microsomal membrane. There may be only one heme group on the PGH synthase molecule (5, 24). Thus, cleavage of the cyclooxygenase from the microsome may also be accompanied by solubilization of the peroxidase. The final feature of the model (Fig. 5) is that the antigenic determinants are present in a different region of the PGH synthase molecule than the cyclooxygenase site and are, in some way, largely protected from proteolytic digestion. This conclusion is based on quantitative immunoradiometric analyses of the antigenic reactivity of the PGH synthase in protease-treated microsomes. A positive response in this assay requires the combined interaction with PGH synthase of \[^{125}I\]labeled IgG derived from the hybridoma line cyo-3 and an unlabeled IgG secreted by cyo-1, cyo-5, or cyo-7. The antigenic reactivity of microsomal membranes was found to be essentially unaffected under conditions in which the cyclooxygenase activity had been destroyed by protease digestion.

To achieve efficient coupling of the release of arachidonic acid and the conversion of this acid to PGH₂ it is reasonable to suspect that arachidonic acid originates from phospholipid precursors present in the endoplasmic reticulum. This is almost certainly the case in platelets where arachidonate is derived from phosphatidylinositol through the sequential action of a soluble, phosphatidylinositol-specific phospholipase C (25, 26) and a membrane-bound diglyceride lipase (27); in these cells, stimulation by thrombogenic agents causes the hydrolysis of approximately 50% of the total cellular phosphatidylinositol (26-28).

The fact that the active site of the PGH synthase is on the cytoplasmic surface of the endoplasmic reticulum means that prostaglandin endoperoxides are formed in the cytoplasm. The most common immediate fate for newly synthesized PGH₂ is probably the conversion to other prostaglandins. Although a portion of the PGH₂ can, at least in certain cells, exit the cell unchanged. For example, there is evidence suggesting that 80% of the PGH₂ formed from endogenous arachidone in platelets is converted to TXA₂ intracellularly (29, 30) and that a portion of the remainder appears extracellularly (29). Most differentiated cells form one prostaglandin as their major product. Thus, platelets synthesize primarily TXA₂ (31), bovine endothelial cells form PGI₂ (32), and renal collecting tubule epithelial cells form mainly PGE₂ (33). In the case of platelets, collecting tubule cells, and sheep vesicular gland, the specific activities of the enzymes which utilize PGH as a substrate (e.g. thromboxane synthase and PGH-PGE isomerase) are 5-10 times the specific activity of the PGH synthase (34) (33). Therefore, in these cells most newly synthesized PGH₂ is probably metabolized immediately to TXA₂ or PGE₂.

The efficiency with which PGH is converted to other products and the instability of the endoperoxide (34) suggest that those enzymes which utilize PGH as substrate are located in close proximity to the PGH synthase. It will be of importance to examine this concept since intracellular synthesis of prostaglandins may imply that at least some of the functions of these compounds result from interaction with intracellular receptors (35).

Acknowledgments—We thank Drs. Victoria H. Freedman, J. Unkeless, and J. Sung of the Rockefeller University for their instruction and advice in the preparation of hybridoma lines. We appreciate the suggestions and assistance of Drs. Melvin Schindler and John L. Wang of Michigan State University in preparing the manuscript.

REFERENCES

1. Hemler, M. E., Lands, W. E. M., and Smith, W. L. (1976) J. Biol. Chem. 251, 5575-5579
2. Van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H., and van Dorp D. A. (1977) Biochim. Biophys. Acta 487, 315-331
3. Roth, G. J., Stanford, N., Jacobs, J. W., and Majerus, P. W. (1977) Biochemistry 16, 4244-4248
4. Miyamoto, T., Ogino, N., Yamamoto, S., and Hayaishi, O. (1976) J. Biol. Chem. 251, 2629-2636
5. Ohki, S., Ogino, N., Yamamoto, S., and Hayaishi, O. (1979) J. Biol. Chem. 254, 829-836
6. Roth, G. J., Siok, C. J., and Ozols, J. (1980) J. Biol. Chem. 255, 1301-1304
7. Van der Ouderaa, F. J., Buytenhek, M., Sikkerveer, F. J., and van Dorp D. A. (1979) Biochim. Biophys. Acta 572, 29-42
8. Van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H., and van Dorp D. A. (1980) Eur. J. Biochem. 109, 1-8
9. Rollins, T. E., and Smith, W. L. (1980) J. Biol. Chem. 255, 4872-4875
10. Shulman, M., Wilde, C. D., and Kohler, G. (1978) Nature 276, 269-270
11. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
12. Smith, W. L., and Lands, W. E. M. (1972) Biochemistry 11, 3276-3285
13. Arion, W. J., Wallin, B. K., Carlson, P. W., and Lange, A. J. (1972) J. Biol. Chem. 247, 2558-2565
14. Leloir, L. F., and Cardini, C. F. (1957) Methods Enzymol. 3, 840-850
15. DePierre, J. W., and Dallner, G. (1975) Biochim. Biophys. Acta 415, 411-472
16. Arion, W. J., Carlson, P. W., Wallin, B. K., and Lange, A. J. (1972) J. Biol. Chem. 247, 2551-2557
17. Roth, G. J., Stanford, N., and Majerus, P. W. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3073-3076
18. Smith, W. L., and Wilkin, G. P. (1977) Prostaglandins 13, 873-892
19. Smith, W. L., and Bell, T. G. (1978) Am. J. Physiol. 235, F451-F457
20. Hugli, R. L., Fogwell, R. L., and Smith, W. L. (1979) Biol. Reprod. 21, 589-590
21. Bebiak, D. M., Miller, E. R., Huslig, R. L., and Smith, W. L. (1979) Fed. Proc. 38, 884
22. Anggard, E., Bohman, S. O., Griffin, J. E., III, Larsson, C., and Maunbach, Ab. (1972) Acta Physiol. Scand. 84, 21-246
23. Smith, W. L., Rollins, T. E., and DeWitt, D. L. (1981) in Progress in Lipid Research (Holman, R. T., ed) Pergamon Press Ltd., Oxford, in press
24. Hemler, M. E., Crawford, C. G., and Lands, W. E. M. (1978) Biochemistry 17, 1772-1779
25. Macio, G., Chap, H., and Doubt-Blazy, L. (1979) FEBS Lett. 100, 367-370
26. Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580-587
27. Bell, R. L., Kennerly, D. A., Stanford, N., and Majerus, P. W. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3238-3241

2 W. L. Smith, unpublished results.
3 S. Yamamoto, personal communication.
4 G. A. Roth, personal communication.
5 W. L. Smith, unpublished results.
Localization of PGH Synthase

28. Broekman, M. J., Ward, J. W., and Marcus, A. J. (1980) J. Clin. Invest. 66, 275-283
29. Marcus, A. J., Weksler, B. B., Jaffe, E. A., and Broekman, M. J. (1980) J. Clin. Invest. 66, 979-986
30. Baenziger, N. L., Becherer, P. R., and Majerus, P. W. (1979) Cell 16, 967-974
31. Hamberg, M., Svensson, J., and Samuelsson, B. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2994-2998
32. Weksler, B. A., Marcus, A. J., and Jaffe, E. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3922-3926
33. Grenier, F. C., Rollins, T. E., and Smith, W. L. (1981) Am. J. Physiol. 241, F94-F104
34. Nugteren, D. H., and Hazelhof, E. (1973) Biochim. Biophys. Acta 325, 448-461
35. Rao, C. V., and Mitra, S. (1979) Biochim. Biophys. Acta 584, 454-466
36. Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W., and Howard, J. C. (1977) Nature 266, 550-552
37. Kessler, S. W. (1976) J. Immunol. 117, 1482-1490
38. Ey, P. L., Prowse, S. J., and Jenkin, C. R. (1978) Immunchemistry 15, 429-435
39. Freedman, M. H., Grossberg, A. L., and Pressman, D. (1968) Biochemistry 7, 1941-1950
40. Bolton, A. E., and Hunter, W. M. (1973) Biochem. J. 133, 529-539
41. Laemmli, U. K. (1970) Nature 227, 680-685
42. Haugen, D. A., Vander Hoeven, T. A., and Coon, M. J. (1975) J. Biol. Chem. 250, 3567-3570
Localization of PGH Synthase

SUPPLEMENTAL MATERIAL TO ORIENTATION OF THE ACTIVE SITE AND ANTIGENIC DETERMINANTS OF PROSTAGLANDIN ENOPEROXIDE (PGH) SYNTHASE IN THE ENDOPLASMIC RETICULUM

by David L. Hewitt, Thomas E. Hultin, Jeffrey S. Day, John C. Gauger and William L. Smith

This supplement describes the isolation and characterization of monoclonal antisera to PGH synthase and the development of a radiometric assay for quantifying the enzyme.

EXPERIMENTAL PROCEDURES

Materials. Tropicalia sp. brood synaptic cistern digest media was from BBI Microbiological Systems (Cleveland, OH),-week-old female C57BL/6J mice sterilized with 70% ethanol, and stored at -20°C. Cotton rat plasma was isolated from by centrifugation at 4°C. Plasmid pSV-β-gal was from Life Technologies, Inc. Plasmid pCMV-β-gal was from Promega Corporation. E. coli transformations were done with the Competent Cells pack (Promega Corporation). Reference preparations were obtained from Sigma Chemical Co. (St. Louis, MO) and Calbiochem-Novabiochem Corp. (La Jolla, CA). Microtubule-stabilizing agent was from Calbiochem-Novabiochem Corp. (La Jolla, CA). Mitochondria-enriched fraction was isolated from rabbit liver mitochondria as described previously. Bovine erythrocyte membranes (3 mg of protein) were prepared from fresh erythrocytes by hypotonic lysis, followed by sonication for 30 seconds. The resulting supernatant centrifuged at 100,000 g for 30 minutes. The supernatant containing the PGH synthase was collected and protein concentration was determined by the method of Lowry et al. (1951) employing bovine serum albumin as a standard.

Preparation of PGH Synthase from Mitochondria. The PGH synthase was isolated from rabbit liver mitochondria by a modification of the method of Jacobson et al. (1982) as follows: The cell-free supernatant was dialyzed against 10 mM Tris-Cl, pH 7.4, and centrifuged at 100,000 g for 30 minutes. The supernatant containing the PGH synthase was collected and protein concentration was determined by the method of Lowry et al. (1951) employing bovine serum albumin as a standard.
Localization of PGH Synthase

Fig. 15. Immunoprecipitation of 125I-labeled sheep vascular gland microsomal proteins from pooled anti-rabbit PGH synthase (0.05 ml) was mixed with 125I-labeled microsomal protein (0.1 ml) and the resulting mixture was added to 1 ml of 10% (vol/vol) Ficoll-70 suspension (0.01 M Tris-HCl pH 7.4 containing 0.1% Tween 20. After incubating for 1 h at 4°C, immunoprecipitates were collected, washed with starting buffer and radioactivity determined.

To determine the nature of the precipitated 125I-labeled material, immunoprecipitates obtained from the equivalence points were solubilized and then analyzed by SDS-polyacrylamide gel electrophoresis. Electrophoretic profiles for the precipitated material were obtained in the UV absorbance range 0.2-1000 ng/ml. The precipitated material in the equivalence points was precipitated with the rabbit PGH synthase (Fig. 15). When precipitated with purified PGH synthase was observed in the ;57 immunoprecipitates (Fig. 15). The precipitated material was precipitated with purified PGH synthase, and the pattern obtained with a sample of 125I-labeled sheep vascular gland microsomes in the amount used in the immunoprecipitation experiments depicted in Fig. 15 is also shown (Fig. 15).

In our initial screen for antibodies reacting against PGH synthase activity, we used a 3,5-dinitro-2-hydroxy-2-nitrophenyl (DNS) assay to precipitate mouse IgG from the culture media. This assay was used in this study to determine whether the antibody recognized the PGH synthase activity, and whether the precipitated antibodies were specific for PGH synthase. The results of the DNS assay were consistent with the results obtained by immunoprecipitation, i.e., antibodies that reacted with PGH synthase activity in the culture media also precipitated the enzyme in the culture media. However, antibodies that did not react with PGH synthase activity in the culture media did not precipitate the enzyme in the culture media. The specificity of the antibodies was confirmed by using 125I-labeled PGH synthase from purified PGH synthase, and the pattern obtained with a sample of 125I-labeled sheep vascular gland microsomes in the amount used in the immunoprecipitation experiments depicted in Fig. 15 is also shown (Fig. 15).

The results of the DNS assay were consistent with the results obtained by immunoprecipitation, i.e., antibodies that reacted with PGH synthase activity in the culture media also precipitated the enzyme in the culture media. However, antibodies that did not react with PGH synthase activity in the culture media did not precipitate the enzyme in the culture media. The specificity of the antibodies was confirmed by using 125I-labeled PGH synthase from purified PGH synthase, and the pattern obtained with a sample of 125I-labeled sheep vascular gland microsomes in the amount used in the immunoprecipitation experiments depicted in Fig. 15 is also shown (Fig. 15).

As determined by immunoprecipitation of antibody with pooled anti-rabbit IgG, 125I and IgG antisera and 125I and IgG antibodies to PGH synthase. The results of the DNS assay were consistent with the results obtained by immunoprecipitation, i.e., antibodies that reacted with PGH synthase activity in the culture media also precipitated the enzyme in the culture media. However, antibodies that did not react with PGH synthase activity in the culture media did not precipitate the enzyme in the culture media. The specificity of the antibodies was confirmed by using 125I-labeled PGH synthase from purified PGH synthase, and the pattern obtained with a sample of 125I-labeled sheep vascular gland microsomes in the amount used in the immunoprecipitation experiments depicted in Fig. 15 is also shown (Fig. 15).

Table I. Analysis of Monoclonal Antibodies Against PGH Synthase

| Antibodies produced by | Subclass | Reactivity with PGH synthase from |
|------------------------|---------|----------------------------------|
| S24-1                   | IgG     | Positive: sheep, rabbit, mouse, rat |
| S25-2                   | IgG     | Positive: sheep, bovine, human, mouse |
| S26-2                   | IgG     | Positive: mouse, rat, rabbit, mouse |
| S27-3                   | IgG     | Positive: sheep, bovine, human, mouse |
| S28-4                   | IgG     | Negative: all species |

* Determined by immunoprecipitation of antibodies against PGH synthase activity from sheep vascular gland, rat, bovine, and human plasma membranes. Additionally, blood plasma and human plasma microsomes and/or blood antigens staining using rat, mouse, guinea pig and rabbit kidneys (17,18) described in the text.

Also summarized in Table I are data indicating the reactivity of different anti-PGH synthase immunocomplexes with antibodies from different animals. The pattern of cross-reactivity was the same for (g) secreted by S24-1, S25-2, and S26-2, and different from that of IgG produced by S27-3. Thus, S24-1 reacted with a different epitope from the determinants of antibody activity.

An immunoradiometric test for quantifying PGH synthase activity was used. The results of this assay indicate that the antibody reacted with S24-1 and S25-2 and showed weaker reactivity with the antibody from dog. A number of different antisera against PGH synthase have been shown to react with yet another antigenic site, i.e., different on PGH synthase. The immunoradiometric assay was performed as described in the text. An immunoradiometric assay was performed as described in the text. The results of the DNS assay were consistent with the results obtained by immunoprecipitation, i.e., antibodies that reacted with PGH synthase activity in the culture media also precipitated the enzyme in the culture media. However, antibodies that did not react with PGH synthase activity in the culture media did not precipitate the enzyme in the culture media. The specificity of the antibodies was confirmed by using 125I-labeled PGH synthase from purified PGH synthase, and the pattern obtained with a sample of 125I-labeled sheep vascular gland microsomes in the amount used in the immunoprecipitation experiments depicted in Fig. 15 is also shown (Fig. 15).

Curiously, substantial precipitation of S24-1 IgG occurs with the 2-10 IgG-hapten complexes (Fig. 15). This latter observation has been made when S24-1 IgG alone was used for the reactions. The 2-10 IgG-hapten complexes were prepared as described in the text and used with purified PGH synthase (from rabbit) for precipitation of PGH synthase activity (Fig. 15). The results of the DNS assay were consistent with the results obtained by immunoprecipitation, i.e., antibodies that reacted with PGH synthase activity in the culture media also precipitated the enzyme in the culture media. However, antibodies that did not react with PGH synthase activity in the culture media did not precipitate the enzyme in the culture media. The specificity of the antibodies was confirmed by using 125I-labeled PGH synthase from purified PGH synthase, and the pattern obtained with a sample of 125I-labeled sheep vascular gland microsomes in the amount used in the immunoprecipitation experiments depicted in Fig. 15 is also shown (Fig. 15).