Mechanism of Prostaglandin Biosynthesis. I. Characterization and Assay of Bovine Prostaglandin Synthetase*

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ABSTRACT: The Zimmerman reaction was found to be adaptable for the assay of prostaglandin synthetase, which is capable of catalyzing the conversion of arachidonic acid into 11α-15α-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid. The bovine seminal vesicle microsomes possess active prostaglandin synthetase activity when a heat-labile, nondialyzable inhibitor, present in the supernatant fraction was removed. Various parameters affecting the rate of this biosynthetic reaction have been quantitatively defined. To achieve maximal rate, both GSH and a cofactor must be present. The following compounds were found to be suitable cofactors: *p*-aminophenol, hydroquinone, L-norepinephrine, L-epinephrine, serotonin, and 5-hydroxyindolacetic acid. The pH optimum of this reaction depends on the specific coenzyme used.

Van Dorp *et al.* (1964) and Bergstrom *et al.* (1964) simultaneously reported that certain C_{20}-unsaturated fatty acids such as 5,8,11-20:3, 5,8,11,14-20:4, and 5,8,11,14-17-20:5, were readily converted into their respective prostaglandins when exposed to ovine seminal vesicle homogenates. Although a variety of other tissues were also capable of catalyzing this oxidative cyclization (Nugteren *et al.*, 1966), the contents of this enzyme were low as compared to that of ovine seminal vesicles. Wallach (1965) and Kupiecki (1965) examined acetone powder preparations of bovine seminal vesicle homogenates for prostaglandin synthetase activity. Because this enzyme activity was found to be low (2–10%) and difficult to reproduce (Daniels and Pike, 1967), further investigations were abandoned in favor of the ovine system.

As ram seminal vesicles are difficult to obtain in quantity, we were compelled to reexamine the bovine prostaglandin synthetase. The objective of our study is to delineate the mechanisms of this unique oxygenase system. Due to the lack of a simple reliable assay method, the properties of prostaglandin synthetase have not been quantitatively defined until now. The development of such a procedure, which allows the quantitative measurement of prostaglandin synthetase and the characterization of its properties, is the subject of this paper.

Materials and Methods

Chemicals. 5,8,11,14-Eicosatetraenoic acid (HP grade) was purchased from the Hormel Institute. Reduced glutathione (GSH), L-epinephrine, folic acid, L-norepinephrine, 5-hydroxytryptamine (creatine sulfate complex), tetrahydrofolic acid, Tris, coenzyme A, thioacetamide, thioglycol, and 2,3-dimercapto-1-propanol were products of Sigma. 5-Hydroxyindol-3-acetic acid was a product of Aldrich. Analytical precoated layers of silica gel F-254 glass plates for thin-layer chromatography were products of Brinkmann. We thank Dr. Pohland of Eli Lilly Co. for the sample of 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine.

Protein was estimated by the method of Gornall *et al.* (1949). The thin-layer chromatographic system consisted of ethyl acetate–acetic acid–isooctane–water (110:20:50:100, v/v) and the relative mobilities were $PGF_{2\alpha}$[1] = 0.22, PGE_2 = 0.33, PGD_2 = 0.40, and PGA_2 = 0.59.

Preparation of Microsomes. Frozen bovine seminal vesicles were thawed, trimmed of fat and other tissues, and cut into small pieces. The seminal vesicles were homogenized in a Waring Blendor for 2 min using two volumes of 0.1 M phosphate buffer, pH 8.0 (80 g of vesicles/150 ml of buffer). The homogenate was centrifuged for 10 min at 12,000*g* in a Servall RC-2 refrigerated centrifuge. The supernatant was filtered through a double layer of cheesecloth and centrifuged at 105,000*g* for 1 hr in a Beckman Model L ultracentrifuge. The precipitated microsomes were collected, lyophilized, and stored in a freezer. One kilogram of seminal vesicle (wet weight) yielded 10 g of lyophilized powder, containing about 70% protein. On the average, the specific activity (millimicromoles of PGE_2 formed per milligram of protein per minute) ranged from 5 to 9 depending on the age of the glands.

Incubation. Unless otherwise stated, the concentrations of the reaction mixture (5 ml) were as follows: arachidonic acid (0.33 mM); microsomes, 5 mg (3.2 mg of protein); GSH (0.98 mM); and cofactor (1.0 mM) in 0.05 M Tris buffer (pH 8.3). L-Epinephrine was used as cofactor in many experiments instead of hydroquinone because it had a lower blank value. Incubation was carried out in Pyrex test tubes at 37°. After 3 min, the reaction mixture was extracted with two 5-ml portions of ethyl ether to remove interfering colored materials. It was then acidified to pH 2.0 with HCl, and extracted three times with equal volumes of ethyl acetate or ethyl ether. The residue obtained after evaporation of the solvent was then analyzed by the Zimmerman reaction.

Assay. A micromodification (Wilson, 1954) of the Zimmerman reaction was used. All components of the reaction mixture were made up in absolute ethyl alcohol. The alcoholic potassium hydroxide is 2.5 N and is stabilized with ascorbic acid and nitrogen (Wilson and Carter, 1947). To 0.1 ml of absolute ethyl alcohol in the case of the reagent blanks, or 0.1 ml of PGE_1 reference standard or of the material being

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[1] Abbreviations used are: PGE_1, 11α,15α-dihydroxy-9-oxo-13-*trans*-prostenoic acid; PGE_2, 11α,15α-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid; $PGF_{2\alpha}$, 9α,11α,15α-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid; PGD_2, 9α,15α-dihydroxy-11-oxo-13-*trans*-prostenoic acid; PGA_2, 15α-hydroxy-9-oxo-5-*cis*,10,13-*trans*-prostatrienoic acid.

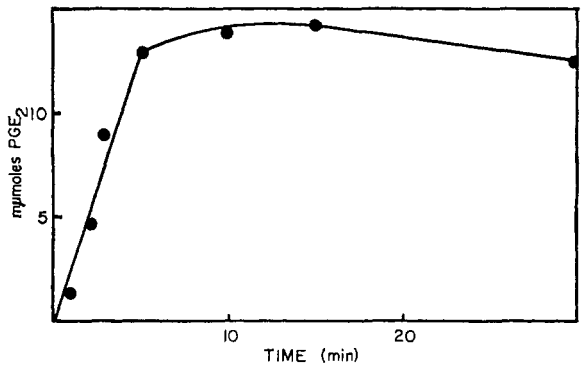


FIGURE 1: The rate of PGE_2 formation. L-Epinephrine was used as cofactor. Incubation and assay conditions are as stated in Methods.

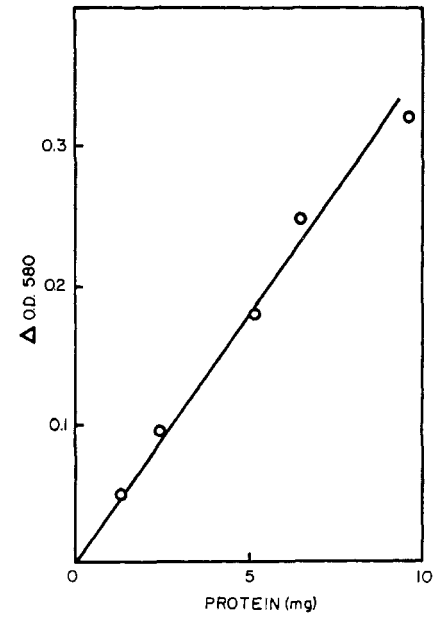


FIGURE 2: Linearity of the PGE_2 assay. Incubation and assay are the same as those described in Figure 1.

assayed, was added 0.3 ml of 1% *m*-dinitrobenzene in absolute ethyl alcohol. After mixing, the tubes were cooled to 0° in an ice–salt bath. Three-tenths milliliter of alcoholic KOH was then added and immediately mixed by shaking each tube briskly. Bright light was avoided during and after addition of alkali. The tubes were allowed to stand in a covered ice–salt bath for 20 min. They were then diluted with 0.8 ml of cooled 80% ethanol and the absorbancy at 580 mμ was read within a 10-min period. Using pure PGE_1 as standards, the linearity of this assay follows the equation $Y = 0.016X$, where Y is the absorbancy at 580 mμ and X is micrograms of PGE_1. The standard deviation of Y for all values of X determined (5, 10, 20, 30, and 40) was less than ±0.01. A standard curve using pure PGE_1 and 4 mg/2 ml of microsomal protein gave a straight line in accordance with the equation: $Y = 0.0094X$. The standard deviation of Y was less than ±0.03 for all values of X (10, 20, 30, 40, and 50). Y is the net absorbance at 580 mμ after subtraction of the value from an identical reaction mixture, containing heat-inactivated enzyme.

Results

Previous studies have shown that when arachidonic acid was incubated with bovine seminal vesicle microsomes in the presence of hydroquinone, a variety of compounds are formed besides PGE_2, PGD_2, and $PGF_{2\alpha}$. Two of which were identified to be 11-hydroxy-12-*trans*-5,8,14-*cis*-eicosatetraenoic acid and 15-hydroxy-13-*trans*-5,8,11-*cis*-eicosatetraenoic acid (Foss *et al.*, 1970). Furthermore, a small amount of PGA_2 was also formed during isolation. When GSH was added to the reaction mixture, the yield of PGE_2 was markedly enhanced and the hydroxy fatty acids and other prostaglandins diminished to a very low level. Under alkaline conditions, it has been well established that PGA_2 and PGE_2 are converted into PGB_2 (Anderson, 1969) and thus they should all give the same chromogen in the Zimmerman reaction. $PGF_{2\alpha}$ however, remains unchanged and will not react. Since the magenta color of the Zimmerman chromogen is specific for molecules bearing the cyclopentanone moiety, the Zimmerman reaction should not be used for the assay of prostaglandin synthetase activity in the absence of GSH. Although the absorption maximum of the prostaglandin chromogen was at 560 mμ, readings were made at 580 mμ to further reduce any absorption caused by interfering materials. Since the chromogen is somewhat unstable, it is imperative that readings be taken within 10-min. Figure 1 clearly illustrates that the formation of prostaglandins is linear for at least 4 min and that a linear relationship exists between microsomal protein and the amount of PGE_2 formed (Figure 2).

Homogenates of bovine seminal vesicles consistently showed low and varied prostaglandins synthetase activity, which confirmed the earlier observations of other workers (Wallach, 1965; Kupiecki, 1965). However, microsomes of bovine seminal vesicles consistently exhibited good activity, which led to the supposition that an inhibitor may be present in the supernatant fraction. This assumption was corroborated by the data shown in Table I. When aliquots of the supernatant fraction was added to the reaction mixture, an inhibition of PGE_2 formation was noted. Since the inhibitory activity was nondialyzable and heat sensitive, it may be proteinaceous in nature.

In the absence of added external cofactors, virtually no conversion of arachidonic acid into prostaglandins took place (Table II). When hydroquinone was included in the reaction mixture, a mixture of prostaglandins (PGF, PGE, and PGD) was formed. In contrast, when GSH was added to the system in the absence of hydroquinone, thin-layer chromatography

TABLE I: Inhibition of Prostaglandin Formation by the Supernatant Fraction.[a]

	PGE_2 (μmole)
Microsomes	0.114
Microsomes + supernatant (1 ml)	0.038
Microsomes + supernatant (1.5 ml)	0.028
Microsomes + boiled supernatant (1 ml)	0.061
Microsomes + boiled supernatant (1.5 ml)	0.068

[a] The reaction mixture contained arachidonic acid (1 mM), GSH (1.3 mM), hydroquinone (0.6 mM), and 4 mg of microsomal protein in 3 ml of 0.05 M phosphate buffer (pH 8.0). After incubation at 37° for 1 hr, the prostaglandins were assayed as described under Methods.

TABLE II: Effect of Hydroquinone and Glutathione on Prostaglandin Formation.[a]

	PGE_2 (μmole)
Microsomes + arachidonic acid	0.042
Microsomes + arachidonic acid + GSH	0.070
Microsomes + arachidonic acid + hydroquinone	0.233
Microsomes + arachidonic acid + GSH + hydroquinone	0.350

[a] The reaction mixture contained arachidonic acid (0.33 mM), GSH (0.98 mM), hydroquinone (0.55 mM), and 5 mg of microsomes (3.2 mg of protein) in 5 ml of 0.05 M Tris buffer (pH 8.3). After incubation for 30 min at 37°, the PG was assayed as described in Methods.

TABLE IV: Cofactor Requirement.[a]

	PGE_2 (μmole)
None	0.073
p-Aminophenol	0.361
L-Ascorbic acid	0.042
Folic acid	0.061
L-Epinephrine	0.258
L-Norepinephrine	0.288
Hydroquinone	0.252
5-Hydroxyindolacetic acid	0.261
Serotonin	0.221
Tetrahydrofolic acid	0.048
2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine	0.000

[a] Conditions are the same as those described under Table II, except 1 mM of the various cofactors were used.

analysis revealed the presence of only PGE_2 (Foss *et al.*, 1970). However, both GSH and hydroquinone or equivalent were required to achieve maximal enzyme activity (Table II).

Effect of SH-Containing Compounds. When GSH was included in the reaction mixture, it enhanced the formation of PGE_2 at the expense of hydroxy fatty acids and other prostaglandins (Foss *et al.*, 1970). Among the SH-containing compounds tested, none were able to duplicate this unique property, exhibited by GSH (Table III). Mercaptoethanol, 2,3-dimercapto-1-propanol, dithiothrietol, and coenzyme A seemed to inhibit prostaglandin synthetic activity. While cysteine afforded some stimulatory effect, hydroxy fatty acids, PGE_2, PGD_2, and $PGF_{2\alpha}$ were all detected in the reaction mixture. An apparent K_m value of 1×10^{-4} M was obtained for GSH at saturating concentrations of arachidonic acid and L-epinephrine.

Effect of Aromatic Compounds. While the requirement for GSH appears to be rather specific, a wide variety of compounds could replace hydroquinone in this enzymatic reaction (Table IV). However, they all possess a common oxidation–reduction system. Among the compounds examined, L-epinephrine, L-norepinephrine, *p*-aminophenol, serotonin, and 5-hydroxyindolacetic acid were all effective in replacing hydroquinone. Thin-layer chromatographic analysis of the reaction mixture revealed that in the presence of GSH, only PGE_2 was formed. Figures 3 and 4 show plots of the rate of prostaglandin formation against hydroquinone and L-epinephrine concentrations, respectively. Double-reciprocal plots of the data afforded apparent K_m values of 4×10^{-5} M for hydroquinone and 1×10^{-4} M for L-epinephrine. Tetrahydrofolic acid, L-ascorbic acid, and tetrahydropeteridine were inactive.

Effect of pH. The pH profiles for hydroquinone, L-epinephrine, and serotonin in the presence of GSH are shown in Figure 5. The rate of PGE_2 formation was maximal at about 8.3–8.5 for hydroquinone and serotonin, and dropped off rapidly on both sides of this pH maximum. At pH 9.0, the rate was approximately 10–15% of that observed at pH 8.3. On the other hand, L-epinephrine exhibited a pH maximum at around 8.8, and at pH 9.0, it still retained over 90% of the maximal activity.

Effects of Substrate Concentrations. The dependence of the rate of the reaction on arachidonic acid concentrations is shown in Figure 6. Double-reciprocal plot of the reaction rate *vs.* the substrate concentration gave an average K_m of 4×10^{-5} M, and apparent maximal velocity was reached with arachidonic acid concentrations of approximately 3.2×10^{-4} M. When the substrate concentration exceeded eight times the value of K_m, substrate inhibition was observed.

Inhibitors. The PG synthetase activity was measured at several concentrations of cyanide (10^{-5}, 10^{-4}, and 10^{-3} M) and CO (1:1 to 3:1, $CO:O_2$) but no inhibition was observed.

TABLE III: Effect of Thiols on PGE_2 Formation.[a]

	PGE_2 (μmole)
None	0.106
GSH	0.164
Cysteine	0.121
Mercaptoethanol	0.070
Thioacetamide	0.100
Dithiothreitol	0.064
2,3-Dimercapto-1-propanol	0.027
Thioglycol	0.109

[a] The conditions were identical with that of Table II, except L-epinephrine (1 mM) was used as the cofactor.

Discussion

Several methods for the quantitation of prostaglandins (Ramwell *et al.*, 1968) have been described in the literature, but none were found to be applicable for the assay of prostaglandin synthetase activity. The measurement of the 278-mμ chromophore of PGB after alkali treatment of PGE was found to be unreliable due to the colored products, generated during cyclization when hydroquinone, adrenaline, or other coenzymes were present. Attempts to follow prostaglandin synthetase activity by the use of oxygen electrodes have been unsatisfactory (Kajita and Nakazawa, 1969), because of the high endogenous activity of the microsomes. On the other hand, the Zimmerman reaction, widely used for the estima-

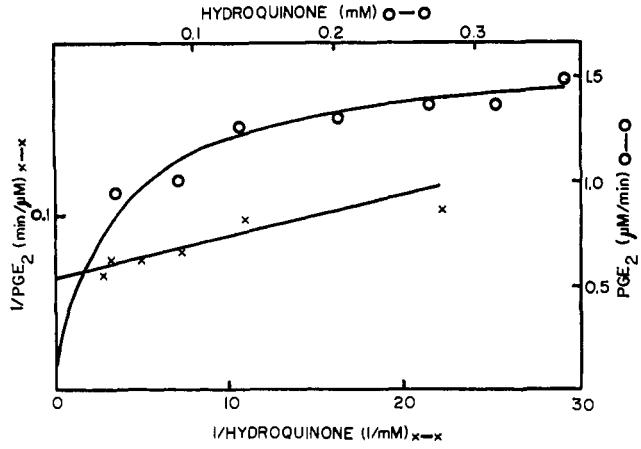


FIGURE 3: The effect of hydroquinone concentrations on the rate of PGE_2 synthesis. Incubation and assay conditions are described in Methods, except 0.65 mM GSH and the indicated concentrations of hydroquinone were used.

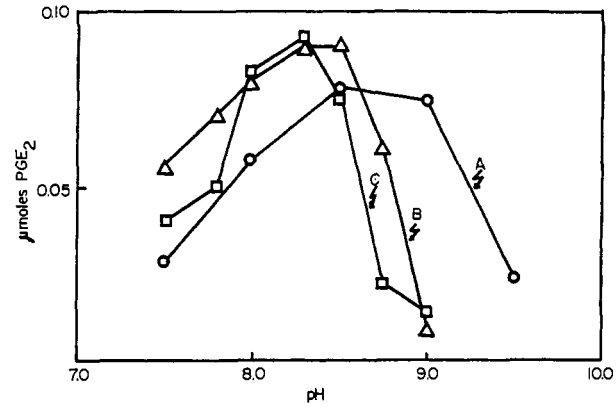


FIGURE 5: The effect of pH on prostaglandin synthetase activity using various cofactors. (A) L-Epinephrine, (B) serotonin, and (C) hydroquinone. Incubation and assay conditions are as stated in Methods, except various pH buffers were used (Tris, pH 7–9, and borate, pH 9.5).

tion of 17-keto steroids was found to be readily adaptable for the quantitation of prostaglandins, for during the oxidative cyclization of arachidonic acid, a cyclopentanone system is formed. Further, in the presence of GSH, arachidonic acid is enzymatically converted only into PGE_2. The reactions between ketones, *m*-dinitrobenzene, and alkali had been investigated extensively (Cänback, 1949). Since the reaction was performed with a large excess of *m*-dinitrobenzene (Corker *et al.*, 1962), and the fact that in alkali, PGE_2 readily dehydrates into PGB_2, one could assign a reasonable structure for the prostaglandin chromogen (Scheme I).

Although the exact roles of GSH in this complex cyclization reaction remain unclear, recent studies (Sih *et al.*, 1970) have shown that the reducing equivalents for this oxygenation are derived from various aromatic compounds such as hydroquinone, L-epinephrine, or serotonin etc. One of the functions of GSH is the reduction of the oxidized forms of the coenzymes back into their respective reduced forms. This explains the observation that PGE_2 was formed from an incubation mixture consisting of arachidonic acid, GSH, and microsomes. GSH probably regenerates the reduced form of the residual endogenous coenzyme in the microsomes. However, this still fails to explain the specificity of GSH. It is possible that GSH may be an allosteric effector.

When the bovine seminal vesicle microsomes are separated from the inhibitor in the supernatant fraction, its properties appear to resemble the ovine enzyme system with respect to coenzyme and GSH requirements. However, bovine seminal vesicle microsomes differ from the ovine enzyme system in that tetrahydrofolate and tetrahydropteridine were inactive in the former whereas it has been reported to be highly active in the latter (Samuelsson, 1967). The parameters affecting prostaglandin formation such as substrate concentration, pH, levels of coenzyme, and GSH have been quantitatively examined. The optimum substrate concentration is around eight times K_m. The pH of the reaction depends on the specific coenzyme. The optimum levels of GSH and coenzyme should

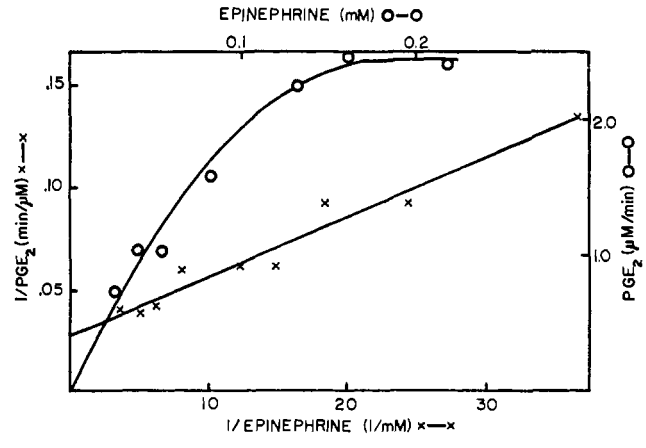


FIGURE 4: The effect of L-epinephrine on prostaglandin synthetase activity. Incubation and assay conditions are the same as those described in Methods, except various epinephrine concentrations were used.

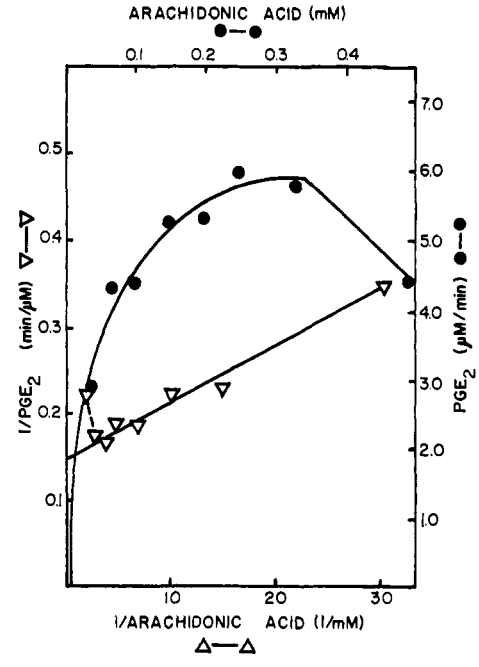
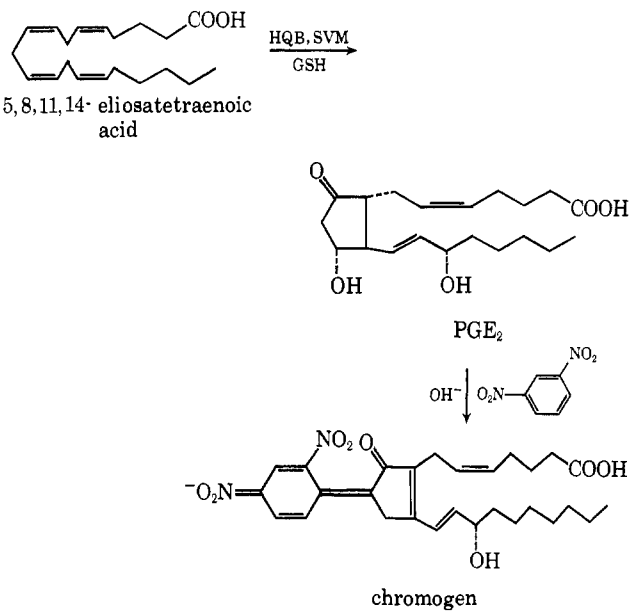


FIGURE 6: The effect of arachidonic acid concentrations on PGE_2 synthesis. Incubation and assay are the same as those of Figure 1, except the concentration of arachidonic acid was varied as indicated.

SCHEME I



be kept at levels around ten times their respective K_m values.

Since prostaglandin synthetase appears to be insensitive to carbon monoxide and cyanide, it does not appear to be a cytochrome P-450 (Sih, 1969) or cytochrome *b* type of oxygenase.

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