Characterization of Prostaglandin Synthetase in Guinea Pig Lung. Isolation of a New Prostaglandin Derivative from Arachidonic Acid†

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ABSTRACT: Prostaglandin biosynthesis was examined using [^{14}C]arachidonic acid as precursor and thin-layer chromatography to separate various biosynthetic products. Prostaglandin synthetase was located in the microsomal fraction of guinea pig lung, and a heat-stable cofactor, necessary for the formation of $PGF_{2\alpha}$ (9α,11α,15-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid), was located in the soluble fraction. In the presence or absence of cofactor, the major "prostaglandin-like" material (MI) was less polar than PGE_2 (11α,15-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid) on thin-layer chromatography and silicic acid column chromatography. Addition of boiled soluble fraction promoted the formation of $PGF_{2\alpha}$ at a rate 1/3 to 1/5 that of MI. MI was not produced from PGE_2 or $PGF_{2\alpha}$ by the lung microsomal fraction plus boiled supernatant system, nor was it produced from arachidonic acid by seminal vesicle microsomal fraction. MI was not converted to PGB_2 (15-hydroxy-9-oxo-5-*cis*-8(12),13-*trans*-prostatrienoic acid) by KOH or to $PGF_{2\alpha}$ by $NaBH_4$. Prostaglandin biosynthesis by the lung microsomal system was stimulated by the addition, *in vitro*, of hydroquinone, norepinephrine, serotonin, and epinephrine, but not by the addition of ascorbic acid, tetrahydrofolic acid, or glutathione. The ratio of $PGF_{2\alpha}$ to MI, produced by lung microsomes, was dependent on the particular compound added; serotonin preferentially enhanced $PGF_{2\alpha}$ biosynthesis, whereas norepinephrine preferentially enhanced MI biosynthesis. In the absence of boiled supernatant, $PGF_{2\alpha}$ production was not appreciably increased by adding serotonin, norepinephrine, or hydroquinone, and stimulation of MI required higher concentrations of the added effectors. Syntheses of $PGF_{2\alpha}$ and MI by lung microsomal fraction were both inhibited equally by added indomethacin. The K_i for this inhibition was found to be 4.6 μM.

Prostaglandins are 20-carbon atom fatty acids which have been implicated in numerous pharmacological, physiological, and pathological processes (Oesterling *et al.*, 1972; Horton, 1969; Von Euler and Eliasson, 1967; Ramwell and Shaw, 1968, 1971). In the lung, prostaglandins have potent effects on bronchial smooth muscle—PGE_2[1] is a bronchial dilator and $PGF_{2\alpha}$ a bronchial constrictor (Oesterling *et al.*, 1972; Horton, 1969). $PGF_{2\alpha}$ is released during anaphylaxis (Piper and Vane, 1969, 1971) and following the administration of vasoactive compounds (Vargaftig and Dao, 1971). In addition, $PGF_{2\alpha}$ has been implicated in the etiology of bronchial asthma (Mathe *et al.*, 1973). Several naturally occurring prostaglandins have been shown to be biosynthesized and degraded by lung subcellular fractions (Oesterling *et al.*, 1972). Metabolism of these compounds takes place in the particle-free fractions of lung homogenates through oxidation of the secondary alcohol group at carbon 15 and reduction of the Δ^{13} double bond (Anggard and Samuelsson, 1967). 15-Hydroxyprostaglandin dehydrogenase has been purified from swine lung and partially characterized (Anggard and Samuelsson, 1967).

In the original work on prostaglandin biosynthesis in the lung, Samuelsson (Anggard and Samuelsson, 1965) demonstrated that arachidonic acid could be converted to PGE_2 and $PGF_{2\alpha}$ by cell-free fractions of guinea pig lung homogenates. Using a similar system, Vane (1971) showed that nonsteroidal anti-inflammatory drugs inhibited prostaglandin biosynthesis in the lung. The conditions used in the studies by Vane and Samuelsson were such that biosynthesis and degradation occurred simultaneously. Our aim was to investigate prostaglandin biosynthesis in the absence of degradation so that environmental effects on the two systems could be measured independently. This paper describes our initial work on the characterization of the prostaglandin synthetase system of guinea pig lung.

Materials and Methods

Arachidonic acid, 99% pure, was purchased from Sigma Chemical Co. Arachidonic acid-*1*-^{14}C (55.5 Ci/mol) was purchased from Applied Science Laboratories and purified by application to a 3-g silicic acid (Mallinckrodt 100 mesh) column and elution with 100 ml of benzene–ethyl acetate 95:5. The purified arachidonic acid was diluted to a concentration of 2.3 μCi/mol with benzene–methanol 1:9 and stored under nitrogen at −20°. [5,6,8,11,12,14,15-^{3}H]Prostaglandin E_2 (5.38 Ci/mol) and [9-^{3}H]prostaglandin $F_{2\alpha}$ (14.3 Ci/mmol) were obtained from New England Nuclear. Prostaglandin standards were the generous gift of Dr. J. E. Pike, the Upjohn Company, Kalamazoo, Mich. Epinephrine, norepinephrine, serotonin, tetrahydrofolic acid, ascorbic acid, glutathione, isoproterenol, and hydroquinone, of the highest purity, were purchased from Sigma Chemical Co. Indomethacin was kindly donated by Merck and Company, Inc. All solvents were freshly distilled prior to use.

Preparation of Subcellular Fractions. All procedures were performed at 4°. Lungs from adult male guinea pigs (350–450

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[1] Abbreviations used are: PGE_2, 11α,15-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid; $PGF_{2\alpha}$, 9α,11α,15-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid; PGB_2, 15-hydroxy-9-oxo-5-*cis*-8(12),13-*trans*-prostatrienoic acid; PGD_1, 9α,15-dihydroxy-11-ketoprost-13-enoic acid; PGD_2, 9α,15-dihydroxy-11-keto-5-*cis*,13-*trans*-prostadienoic acid.

g body weight, obtained from Carworth Breeders) or bovine seminal vesicles (from local slaughter houses) were freed of extraneous tissue, cut into small pieces, and homogenized in 0.25 M sucrose (tissue to sucrose ratio of 1:2) using a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 9000*g* for 20 min using a Sorvall RC-2B refrigerated centrifuge. The resulting supernatant was pipetted off and centrifuged at 100,000*g* for 30 min using a Beckman L3-50 ultracentrifuge. The 100,000*g* pellet was washed once by resuspension in 0.25 M sucrose and centrifugation at 100,000*g* for 20 min. The washed pellet was resuspended in 0.25 M sucrose to a protein concentration of 10 mg/ml. The first 100,000*g* supernatant was boiled on a hot plate for 2 min and centrifuged at 9000*g* to remove denatured protein. For subcellular distribution studies, the homogenate was first centrifuged at 600*g* for 20 min to remove nuclei and cell debris and then centrifuged at 9000*g* as described above. The 9000*g* pellet (mitochondrial fraction) was washed once and resuspended in 0.25 M sucrose to give a protein concentration of 10 mg/ml. Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Incubation Conditions and Assay for Biosynthesis. Each incubation vial contained the following: 20 μg of arachidonic acid, 0.23 μCi of [^{14}C]arachidonic acid, 2.0 ml of boiled 100,000*g* supernatant (or in some cases 2.0 ml of 0.25 M sucrose), 1.0 ml of 0.25 M phosphate buffer (pH 8.0), 3 or 4 mg of microsomal protein, and sufficient distilled water to bring the total volume to 4.0 ml. Compounds to be tested were added in either distilled water or in 95% ethanol. Incubations were carried out at 37° in a Dubnoff metabolic shaker. After 8 min (or in some cases 30 min), reaction was terminated by the addition of sufficient 1 N HCl to bring the pH to 3.5. Samples were extracted with 8 volumes of *n*-hexane to remove most of the unreacted arachidonic acid. The hexane wash was discarded and the aqueous phase extracted with 8 volumes of ethyl acetate. The resulting organic phase was transferred and evaporated to dryness under reduced pressure. Residues from evaporation were dissolved in 100 μl of absolute methanol, and 20-μl aliquots were applied to silica gel G (Analtech 250 μ) thin-layer plates. Following development in either benzene–dioxane–acetic acid 60:35:2 or acetone–methylene chloride–acetic acid 40:60:1.5, the thin-layer plates were scanned on a Packard radiochromatogram scanner. Zones corresponding to the radioactive peaks were scraped into counting vials containing 10 ml of dioxane scintillation cocktail (214 g of fluoralloy [Beckman Instrument Company]/2 l. of scintillation grade dioxane), and assayed for radioactivity in a Beckman LS-250 liquid scintillation counter. Recovery of [^{3}H]$PGF_{2\alpha}$ and [^{3}H]PGE_2, when carried through the entire procedure, was found to be 90–95%.

Column Chromatography. Crude extracts were applied to 5 mm i.d. glass columns which had been packed to a height of 250 mm with silicic acid (Mallinckrodt 100 mesh) in benzene–ethyl acetate 70:30. Following a preliminary wash with 150 ml of benzene–ethyl acetate 70:30, the compounds were eluted with a continuous linear gradient between 150 ml of benzene–ethyl acetate 70:30 and 150 ml of ethyl acetate. Fractions (5 ml) were collected and 0.2-ml aliquots were counted in dioxane scintillation cocktail. In some cases [^{3}H]-PGE_2 and [^{3}H]$PGF_{2\alpha}$ were added prior to chromatography.

Gas–Liquid Chromatography. A Tracor MT-220 gas–liquid chromatograph was used. The gas chromatograph was equipped with dual flame ionization detector and a Packard proportional counter for the simultaneous determination of ^{14}C. The column was a 6-ft U tube, 4 mm i.d., packed with either 3% OV-225 or 3% OV-1 on 80–100 Gas Chrom Q (Applied Science Laboratories). Temperatures of the inlet, column, and detector were maintained at 240, 210, and 240°, respectively. Carrier gas was helium at a flow rate of 60 ml/min.

Preparation of Derivatives. Methods for preparing derivatives were essentially as described earlier with slight modifications (Albro and Fishbein, 1969; Pace-Asciak and Wolfe, 1971).

$NaBH_4$ Reduction. Purified extracts were dissolved in 2 ml of absolute methanol, cooled to 0°, and 10 mg of $NaBH_4$ was added. Reaction was allowed to proceed at 0° for 20 min and at room temperature for 60 min. The reaction mixtures were diluted with 2 ml of H_2O, acidified to pH 3.5 with 1 N HCl, and extracted with 8 volumes of ethyl acetate. The organic phases were transferred and evaporated to dryness under reduced pressure.

Reaction with KOH. Samples were dissolved in 2 ml of 0.5 M KOH. After 1 hr at room temperature, they were acidified to pH 3.5 with HCl and extracted with ethyl acetate as above.

Preparation of Methyl Esters. Diazomethane was freshly prepared from diazald using a diazomethane generating kit (Aldrich Chemical Co.). The purified extracts, or their reduced derivatives, were dissolved in 100 μl of absolute methanol, 0.9 ml of the diazomethane was added, and they were placed in the dark for 60 min. Following this period, the solvent was evaporated to dryness under nitrogen.

Preparation of Trimethylsilyl Ethers. Methyl esters were diluted to a concentration of 1 μg/ml with trimethylsilylimidazole–bis(trimethylsilyl)trifluoroacetamide 2:1 (Pierce Chemical Co.). After 2 hr at room temperature, aliquots were injected directly into the gas chromatograph.

Chemical Ionization Mass Spectrometry. Conditions for mass spectrometry were as described by Oswald *et al.* (1974). Chemical ionization mass spectrometry were obtained with a Finnigan 1015C Quadrupole mass spectrometer, interfaced with a Finnigan Model 9500 gas chromatograph. The manifold temperature was maintained at 175° with an interface temperature of 225°. For methane and isobutane, the ion source pressure was maintained at 600–1000 μ with an overall pressure of 10^{-5} Torr. A 152 × 0.2 cm i.d. glass column, packed with 3% OV-1 on Gas Chrom Q (80–100 mesh), was used with a carrier gas (methane or isobutane) flow rate of 35–40 ml/min. The chromatography column temperature was maintained at 220° for 5 min and then programmed at 10°/min to 240°. The inlet temperature was maintained at 250°. The ionizer was initiated 5 min after injection of the sample.

Results

Prostaglandin synthetase activity in guinea pig lung was located mainly in the microsomal fraction (Table I). Some activity was found in the mitochondrial fraction while the 100,000*g* supernatant fraction was inactive. Incubation of [^{14}C]arachidonic acid in the presence of guinea pig lung microsomes produced a single compound (MI) which was slightly less polar than PGE_2 on thin-layer chromatography (R_F's in benzene–dioxane–acetic acid 20:10:1 [Anderson, 1969a], MI = 0.65, PGE_2 = 0.55). Addition of 100,000*g* supernatant fraction to the lung microsomal preparation inhibited the production of MI and gave rise to another ^{14}C-labeled compound (MII), more polar than MI, with chromatographic mobility identical with $PGF_{2\alpha}$ (Ref benzene–dioxane–acetic acid 20:10:1 = 0.35). Addition of boiled 100,000*g* supernatant fraction to microsomes, in place of untreated super-

TABLE I: Subcellular Distribution of Prostaglandin Synthetase in Guinea Pig Lung.[a]

Subcellular Fraction	ng formed mg of protein^{-1} 15 min^{-1}		Total ng formed (MI + $PGF_{2\alpha}$)	Total ng formed per g of lung
	MI	$PGF_{2\alpha}$		
Microsomal fraction 100,000*g* pellet	210	8	218	655
Mitochondrial fraction 9000*g* pellet	60	<5	60	222
Soluble fraction 100,000*g* supernatant	<5	<5	<10	
Microsomal fraction + 2.5 ml of 100,000*g* supernatant	140	35	175	525
Microsomal fraction + 2.5 ml of boiled 100,000*g* supernatant	235	55	290	870
Microsomal fraction + 2.5 ml of dialyzed, boiled 100,000*g* supernatant	190	10	200	600

[a] Each incubation vial contained 10 μg of [^{14}C]arachidonic acid (400,000 cpm), 4 mg of protein, 2.5 ml of 0.25 M sucrose (or 2.5 ml of appropriate supernatant fraction), 1.0 ml of 0.25 M phosphate buffer (pH = 8.0), and sufficient distilled H_2O to bring the total volume to 4.0 ml. Incubations were carried out at 37° for 15 min. Extraction, chromatography, and quantitation were as described under Methods. Each value represents the mean of duplicate samples.

natant, resulted in increased production of both MI and MII. This effect was likely due to the binding of arachidonic acid to supernatant protein since the difference observed could be eliminated either by increasing substrate concentration in the nonboiled system, or by the addition of bovine serum albumin to the boiled system. When boiled supernatant was dialyzed for 24 hr against 0.25 M sucrose, it could no longer serve as a cofactor in prostaglandin biosynthesis (Table I).

To ensure that MI was not a metabolite or a degradation product of PGE_2 or $PGF_{2\alpha}$, tritiated PGE_2 or $PGF_{2\alpha}$ were incubated with the "microsomal-boiled supernatant" system and carried through the same extraction and chromatography procedures as described for arachidonic acid. No conversion of the tritiated standards to MI or any other products was observed.

Large scale incubations were prepared to produce sufficient labeled material for structural identification. Following extraction with ethyl acetate, [^{3}H]PGE_2 and [^{3}H]$PGF_{2\alpha}$ were added as internal standards. One-tenth of the extract was subjected to thin-layer chromatography and the remainder to continuous gradient elution column chromatography. Tritium and carbon-14 were determined separately in each zone (Figure 1). In both systems, MII cochromatographed with the added [^{3}H]$PGF_{2\alpha}$ while MI appeared less polar than the [^{3}H]PGE_2. Column chromatography fractions containing MI were pooled, and aliquots were treated with either $NaBH_4$ or KOH. Thin-layer chromatography of the reaction products showed that MI had been converted to a more polar derivative by $NaBH_4$ and a less polar derivative by KOH. The ultraviolet spectrum of MI, following reaction with KOH, lacked the 278-nm absorption band which is characteristic of PBG_2 formation (Andersen, 1969b). The methyl ester, trimethylsilyl ether derivatives of MI, reduced MI, $PGF_{2\alpha}$, and PGE_2 were compared by gas–liquid chromatography and the retention times of these derivatives were as follows: PGE_2, 7.5 min; $PGF_{2\alpha}$, 8 min; MI, 16.5 min; and reduced MI, 8.5 min. The methane and isobutane chemical ionization mass spectra of the methyl ester, trimethylsilyl ether derivatives of MI are shown in Figure 2. The simple isobutane spectrum shows prominent fragments at *m/e* 511, 421, 331, and 69 with a base peak at *m/e* 91. The methane spectrum, while more complex, shows a similar pattern with additional fragments at 495, 301, 129, and 119 with a base peak at *m/e* 75. There is an additional fragment at *m/e* 585 which is thought to result from contamination since it did not appear in the isobutane spectra.

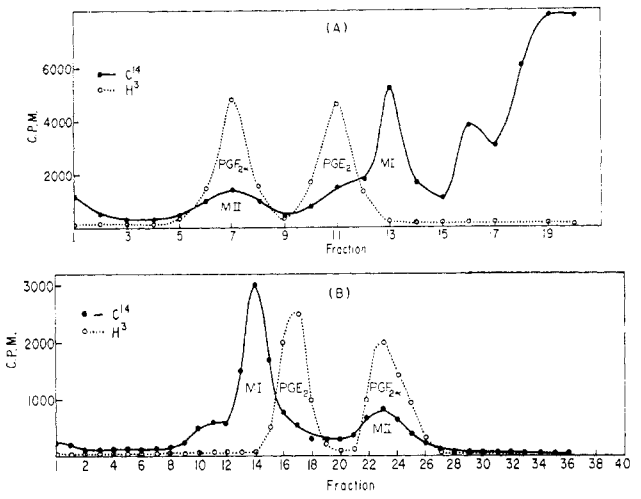

FIGURE 1: Chromatography of crude extract of incubation mixture using guinea pig lung. [^{14}C]Arachidonic acid, guinea pig lung microsomes, and boiled 100,000*g* supernatant were incubated and extracted as described in Methods. [^{3}H]-PGE_2 and [^{3}H]-$PGE_{2\alpha}$ were added at end of the incubation: (—) ^{14}C; (- - -) ^{3}H standards. (A) Thin-layer chromatograph on silica gel G. Benzene–dioxane–acetic acid 20:10:1. (B) Continuous gradient elution column chromatography using 150 ml of benzene–ethyl acetate 70:30 and 150 ml of ethyl acetate following preliminary wash with benzene–ethyl acetate, 70:30.

To ensure that MI was not formed from arachidonic acid as a result of the particular incubation and isolation procedures used, bovine seminal vesicle microsomes were substituted for guinea pig lung microsomes and the dual-label experiments were repeated. Three "prostaglandin-like" compounds were formed by seminal vesicles, one which cochromatographed with $PGF_{2\alpha}$, a second which was less polar than PGE_2 (possibly PGD_2) (Foss *et al.*, 1972), and the major product which cochromatographed with PGE_2 (Figure 3). The 100,000*g* supernatant fractions from seminal vesicles and guinea pig lung, used interchangeably as cofactors with either of the microsomal preparations, were capable of stimulating synthesis. However, the nature of the products formed depended on the organ source of the microsomal

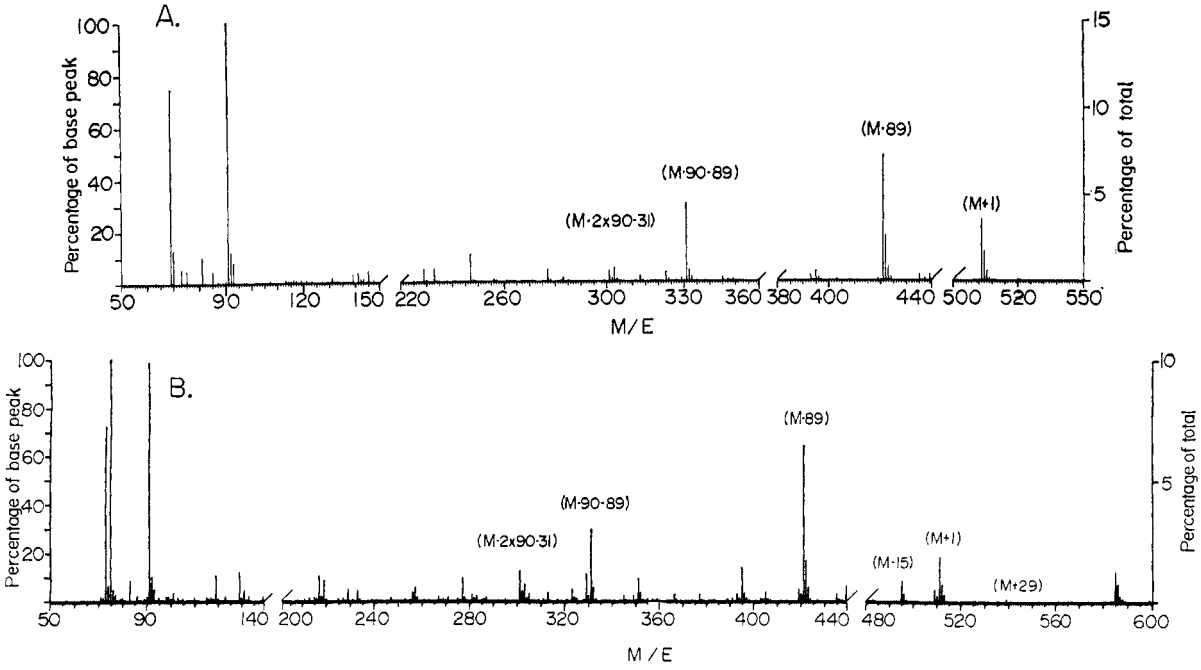

FIGURE 2: Chemical ionization mass spectra of the methyl ester, trimethylsilyl ether derivative of MI: (A) isobutane; (B) methane.

preparation. Regardless of the source of 100,000*g* supernatant fractions, bovine seminal vesicle microsomes formed primarily PGE_2 while guinea pig lung microsomes formed primarily MI.

The prostaglandin synthetase system of guinea pig lung was studied to determine optimal conditions with respect to time, cofactor, protein, and substrate. The reaction rate was linear for 8 min, linear up to 3 mg of protein, and the optimum amount of boiled supernatant was 2.0 ml. The K_m for arachidonic acid was approximately 5 μM. Substrate inhibition was noted at arachidonic acid concentrations greater than 15 μM. Addition of boiled 100,000*g* supernatant fraction to the preparation had no effect on the K_m for arachidonic acid.

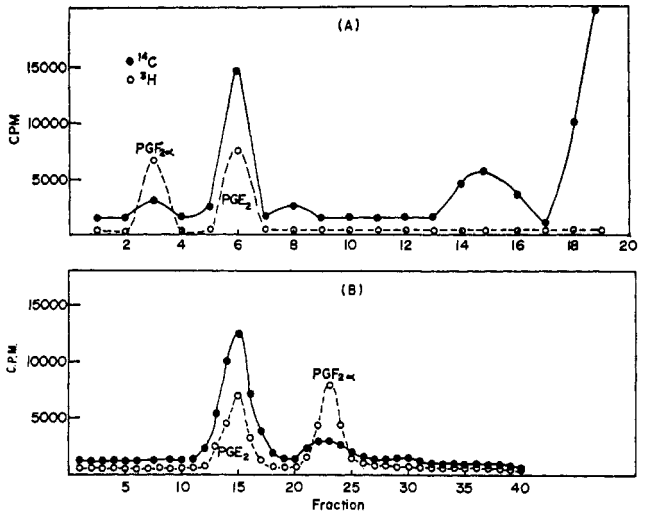

FIGURE 3: Chromatography of crude extract of incubation mixture using bovine seminal vesicles. [^{14}C]Arachidonic acid, seminal vesicle microsomes, and boiled 100,000*g* supernatant were incubated and extracted as described in Methods. [^{3}H]PGE_2 and [^{3}H]$PGF_{2\alpha}$ were added at the end of incubation: (—) ^{14}C; (- - -) ^{3}H standards. (A) Thin-layer chromatograph on silica gel G using benzene–dioxane–acetic acid, 20:10:1. (B) Continuous gradient elution column chromatography using 150 ml of benzene–ethyl acetate 70:30 and 150 ml of ethyl acetate following preliminary wash with benzene–ethyl acetate 70:30.

Several compounds were examined for their possible effects on prostaglandin biosynthesis by guinea pig lung microsomes. When added *in vitro* at a concentration of 5×10^{-4} M, epinephrine, norepinephrine, hydroquinone, *l*-isoproterenol, and 5-hydroxytryptamine stimulated the formation of MI but not $PGF_{2\alpha}$ (Table II), while reduced glutathione, ascorbic acid, and tetrahydrofolic acid had little or no stimulatory effect on the production of either MI or $PGF_{2\alpha}$. Hydroquinone, 5-hydroxytryptamine, and norepinephrine were examined over a range of concentrations, *in vitro*, in the presence and absence of added boiled 100,000*g* supernatant fraction (Figures 4–6). In the absence of boiled supernatant, none of

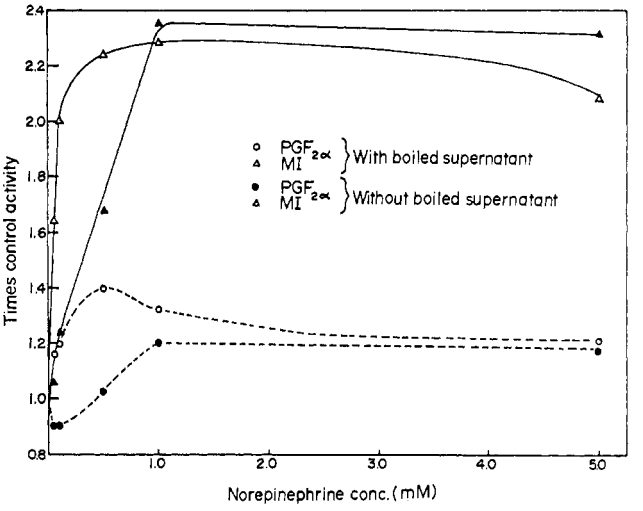

FIGURE 4: Effect of added norepinephrine on prostaglandin biosynthesis in guinea pig lung. Each incubation vial contained 20 μg of [^{14}C]arachidonic acid (400,000 cpm), 3 mg of microsomal protein, and 1.0 ml of 0.25 M phosphate buffer (pH 8.0), either 2 ml of boiled 100,000*g* supernatant or 2 ml of 0.25 M sucrose various amounts of 2×10^{-2} M norepinephrine and sufficient distilled H_2O to give a final volume of 4 ml. Incubations were carried out at 38° for 8 min. Extraction, chromatography, and quantitation were as described under Methods. Each point represents the mean of two determinations. Control values (ng 3 mg of protein^{-1} 8 min^{-1}) without boiled supernatant were 698 for MI and 32 for $PGF_{2\alpha}$; with boiled supernatant, 765 for MI and 183 for $PGF_{2\alpha}$.

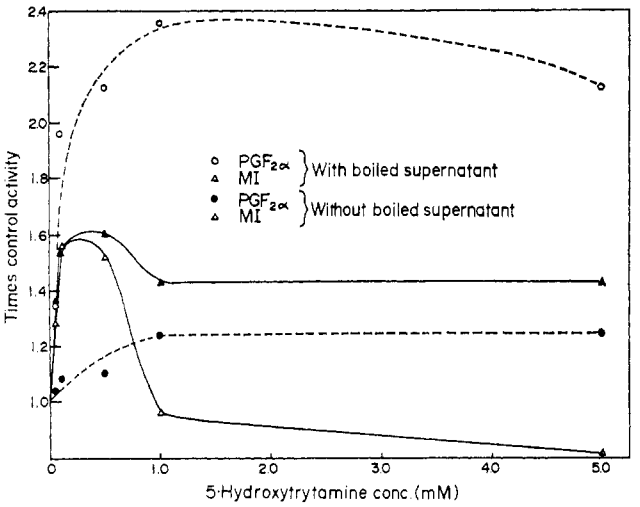

FIGURE 5: Effect of added 5-hydroxytryptamine on prostaglandin biosynthesis in guinea pig lung. Incubation and assay conditions were as described in Figure 4 except that varying concentrations of 5-HT were added. Points represent the means of duplicate determinations. Control values (ng 3 mg of protein^{-1} 8 min^{-1}) without boiled supernatant were 540 for MI and 69 for $PGF_{2\alpha}$; with boiled supernatant, 767 for MI and 172 for $PGF_{2\alpha}$.

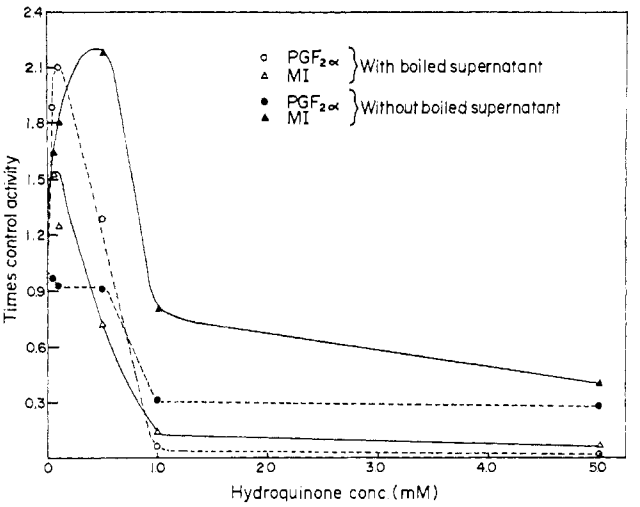

FIGURE 6: Effect of added hydroquinone on prostaglandin biosynthesis in guinea pig lung. Incubation and assay conditions were as described in Figure 4 except that varying concentrations of hydroquinone were added to the incubation vials. Points represent means of duplicate samples. Control values (ng 3 mg^{-1} 8 min^{-1}) without boiled supernatant were 433 for MI and 60 for $PGF_{2\alpha}$; with boiled supernatant, 880 for MI and 128 for $PGF_{2\alpha}$.

the compounds when added *in vitro* increased $PGF_{2\alpha}$ formation, whereas norepinephrine (Figure 4) and hydroquinone (Figure 6) increased MI formations 2.5-fold. In the presence of boiled supernatant, hydroquinone (Figure 6) and 5-hydroxytryptamine (Figure 5) stimulated $PGF_{2\alpha}$ biosynthesis, and the apparent K_m for hydroquinone and norepinephrine, in relation to MI biosynthesis, was decreased. The ratio of MI to $PGF_{2\alpha}$ was also altered by added norepinephrine and serotonin; norepinephrine stimulated mainly MI biosynthesis while serotonin stimulated mainly $PGF_{2\alpha}$ biosynthesis. When added to the microsomal-boiled supernatant system, 1×10^{-3} M hydroquinone inhibited the synthesis of both MI and $PGF_{2\alpha}$, and serotonin inhibited the synthesis of MI. With sheep vesicles gland preparations of prostaglandin synthetase, copper–dithiol complex has been shown to promote $PGF_{2\alpha}$ formation at the expense of PGE_2 and PGD_2 (Lee and Lands, 1972); however, when added to guinea pig lung microsomes, 1 mM dithiotheitol in combination with 0.5 mM copper sulfate did not alter the biosynthesis of either MI or $PGF_{2\alpha}$.

Indomethacin, when added *in vitro*, inhibited the biosynthesis of PGE_2 by seminal vesicle microsomes (Smith and Lands, 1971) and the biosynthesis of $PGF_{2\alpha}$ by cell-free fractions of guinea pig lung (Vane, 1971). In the microsomal-boiled supernatant system, added indomethacin inhibited the synthesis of MI and $PGF_{2\alpha}$ to the same degree. The K_i for indomethacin was found to be 4.3 μM.

Discussion

The microsomal-boiled supernatant system of guinea pig lung appears to form two prostaglandins from arachidonic acid, one which behaves chromatographically like $PGF_{2\alpha}$ and another which has properties similar to PGE_2. The exact identity of these compounds has yet to be established; however, since $PGF_{2\alpha}$ has been found in lung tissue (Anggard,

TABLE II: Effect of Various Compounds on Prostaglandin Biosynthesis by Guinea Pig Lung Microsomes.[a]

Enzyme Preparation	Compd (5×10^{-4} M)	Prostaglandin formed ng 3 mg of protein^{-1} 8 min^{-1} $PGF_{2\alpha}$	MI	Total	MI/$PGF_{2\alpha}$
Washed microsomes		20	651	671	31.0
Washed microsomes	Reduced glutathione	10	673	683	67.3
Washed microsomes	Hydroquinone	5	1453	1458	291.6
Washed microsomes	Ascorbic acid	10	812	822	27.1
Washed microsomes	Epinephrine	30	1203	1233	40.1
Washed microsomes	Norepinephrine	45	1301	1351	30.0
Washed microsomes	Tetrahydrofolic acid	15	722	737	48.1
Washed microsomes	5-Hydroxytryptamine	53	1403	1456	27.5
Washed microsomes	L-Isoproterenol	25	997	1022	39.9
Washed microsomes + boiled supernatant		148	852	1000	5.8

[a] Each incubation vial contained 3 mg of microsomal protein, 1.0 ml of 0.25 M phosphate buffer (pH 8.0), 2.0 ml of 0.25 M sucrose, and 20 μg of [^{14}C]arachidonic acid (400,000 cpm). Sufficient quantities of 2×10^{-3} M stock solutions of the compounds were added to give a final concentration of 5×10^{-4} M.

1965) and was biosynthesized from arachidonic acid by cell-free homogenates of guinea pig lung (Anggard and Samuelsson, 1965), it appears that the more polar derivative (MII) is $PGF_{2\alpha}$. The second compound (MI) is less polar than PGE_2, does not form the dienone chromophore when treated with base, and is reducible to a more polar derivative by $NaBH_4$. $PGF_{2\alpha}$ and the $NaBH_4$ reduction product of MI were compared by thin-layer and gas–liquid chromatography, and were found to have slightly different chromatographic properties. For this reason, reduced MI is not $PGF_{2\alpha}$.

Although the mass spectra, presented above, are not sufficient to identify the structure of MI, they do give information as to the molecular weight and the number of functional groups on the molecule. The highest molecular weight fragment in the isobutane spectrum is at *m/e* 511 and probably compares to the quasimolecular (M + 1) ion which is formed through a hydride transfer reaction. The methane spectra has additional fragments at *m/e* 510 and 509 which are thought to be the molecular ion and the M − 1 ion, respectively. Two other fragments which add credence to the identity of the molecular ion are those at 539 (relative abundance $\simeq 1.4\%$) and at 495. The 539 fragment is indicative of the recombination ion, $M + C_2H_5^+$, and the 495 fragment is indicative of the loss of CH_3 from the molecular ion. The latter represents a common fragmentation pattern associated with trimethylsilyl derivatives. Fragments at *m/e* 421, 331, and 301 conform to the loss of TMSO, TMSOH + TMSO, and 2TMSOH + CH_3O, respectively, thus indicating that the molecule contains two reactive hydroxyl groups and a carboxylic acid group. That MI was reducible with $NaBH_4$ shows that the molecule contains a ketone, and the molecular weight (510) conforms to a prostaglandin methyl ester, di(trimethylsilyl ether), with two double bonds and carbonyl. Although it would be possible to speculate on the other fragments in the methane spectrum, we feel that such speculation would be reckless without additional structural information.

One of the main difficulties in the structural elucidation of MI has been to obtain a realistic amount of relatively pure compound. Owing to the low prostaglandin synthetase activity in guinea pig lung, large amounts of tissue must be used in incubation mixtures, and the ratio of MI to endogeneous compounds is low. The extracts are purified extensively by column chromatography; however, there is still a possibility that column fractions containing MI will be contaminated with interfering substances having similar chromatographic properties to MI. Because of the purity problem, this laboratory has not been able to adequately characterize MI. Further work is being undertaken to both purify MI and to find more suitable derivatives for mass spectrometry.

Bovine seminal vesicle microsomal preparations were shown to produce 9α,15-dihydroxy-11-ketoprost-13-enoic acid (PGD_1), an isomer of PGE_1, from 8,11,14-eicosatrienoic acid (Granstrom *et al.*, 1968) and PGD_2 from arachidonic acid (Foss *et al.*, 1972). Initially, the possibility that MI and PGD_2 were identical was considered. Reduction of PGD_2 by $NaBH_4$ produces $PGF_{2\alpha}$ (Hamberg and Samuelsson, 1973), and since reduced MI did not behave like $PGF_{2\alpha}$ on thin-layer chromatography or by gas–liquid chromatography of its methyl ester, trimethylsilyl ether derivative, we concluded that MI and PGD_2 were not identical. However, the chemical ionization mass spectra of the methyl ester, trimethylsilyl ether of untreated MI could conform to a structure similar to PGD_2, and until the location of two hydroxyl groups and the ketone group are determined, the possibility that MI is PGD_2 cannot be ruled out. The relative stability of MI leads us to believe that MI is not rabbit aortic contracting substance (Piper and Vane, 1969) or the endoperoxide intermediate of prostaglandin biosynthesis. This does not preclude its being a degradation product of one of these compounds, or some other biologically active substance.

The prostaglandin synthetase systems of guinea pig lung and bovine seminal vesicle are not identical. Although soluble fractions from the two tissues are capable of stimulating either microsomal system, each organ's microsomal preparation catalyzes the formation of different prostaglandin material. Hamberg and Samuelsson (1973) proposed the existence of an endoperoxide isomerase which catalyzes the formation of PGE compounds and an endoperoxide reductase which catalyzes the formation of PGF compounds (Hamberg and Samuelsson, 1973). Further structural work may reveal that MI has the ring configuration of a PGF compound. This would imply that guinea pig lung contains primarily the endoperoxide reductase system, whereas seminal vesicles contain primarily the endoperoxide isomerase system. The control of type of prostaglandin synthetased (*e.g.*, E or F) may be determined by the particular enzyme system as well as necessary cofactors.

Several investigators reported that epinephrine, norepinephrine, serotonin, and hydroquinone, when added *in vitro*, stimulated the biosynthesis of PGE and PGD (Lee and Lands, 1972; Pace-Asciak, 1972; Samuelsson, 1970; Takeguchi *et al.*, 1971; Nugterem *et al.*, 1966; Sih *et al.*, 1970; Wallach and Daniels, 1971). We found these chemicals to elicit similar stimulatory effects on the synthesis of MI by guinea pig lung microsomes. In contrast, the effects of added norepinephrine and copper–dithiol complexes on $PGF_{2\alpha}$ formation by guinea pig lung microsomes were different from those seen with other preparations. When added *in vitro* to seminal vesicle microsomal preparations, norepinephrine promoted the formation of $PGF_{1\alpha}$ as well as that of PGE_1 and PGD_1 (Sih *et al.*, 1970), and copper–dithiol complexes promoted $PGF_{2\alpha}$ production at the expense of PGE_2 and PGD_2 (Lee and Lands, 1972). In the absence of boiled supernatant, $PGF_{2\alpha}$ synthesis by guinea pig lung microsomes was not increased by the *in vitro* addition of either epinephrine or copper–dithiol complexes, and none of the compounds tested were able to completely replace boiled supernatant as a cofactor for $PGF_{2\alpha}$ biosynthesis. Thus, there exists a dialyzable, heat-stable factor in supernatant fraction, other than those proposed earlier, which is essential for $PGF_{2\alpha}$ biosynthesis but not for MI biosynthesis by lung microsomes.

The significance of serotonin and norepinephrine, as related to prostaglandin levels and their physiological effects *in vivo*, is difficult to evaluate since the biological activity of MI has not been established. If MI is biologically inactive, epinephrine would shift the prostaglandin synthetase system from the production of an active bronchioconstrictor ($PGF_{2\alpha}$) to an inactive prostaglandin (MI), whereas serotonin would have the opposite effect. Therefore, bronchioconstriction induced by serotonin (Okpako, 1972) could involve the stimulation of $PGF_{2\alpha}$ biosynthesis.

Previous investigators used cell-free homogenates of guinea pig lung as the source of prostaglandin synthetase (Anggard and Samuelsson, 1965; Vane, 1971). The homogenates were prepared in buffer solutions which contained nicotinamide. Nicotinamide inhibits lung NAD glycohydrolase (DiAugustine *et al.*, 1973), thereby making NAD available to 15-hydroxyprostanoate dehydrogenase, and increasing the possibility that prostaglandin metabolism will occur. In the biosynthetic system described above, nicotinamide was elimi-

nated and 100,000*g* supernatant (source of 15-hydroxyprostanoate dehydrogenase) was boiled. These steps are sufficient to enable the study of prostaglandin biosynthesis without interference from metabolism. That MI was not found in the earlier biosynthetic studies could be explained by its metabolism in these systems, or inhibition of its synthesis by soluble fraction proteins. Also, bioassays were frequently used to quantitate prostaglandin activity, and if MI is biologically inactive, it would not have been detected. Both Vane and Samuelsson demonstrated the formation of small quantities of PGE_2, from arachidonic acid, in their preparations. We were not able to detect appreciable quantities of PGE_2; however, since MI and PGE_2 have very similar polarities, the MI peak would "mask" any PGE_2 that was present.

Our paper describes a method for studying prostaglandin biosynthesis in guinea pig lung without interference from the highly active prostaglandin catabolism system. The major product formed, from arachidonic acid in the enzymatic reaction, is a previously undetected prostaglandin which remains to be identified and characterized as to its biological activity. Formation of $PGF_{2\alpha}$ by guinea pig lung microsomes requires heat-stable, dialyzable, cofactors which are present in the soluble fraction. The cofactors cannot be replaced by glutathione, tetrahydrofolic acid, ascorbic acid, hydroquinone, norepinephrine, serotonin, or copper–dithiol complexes; however, in the presence of boiled soluble fraction, $PGF_{2\alpha}$ production is stimulated by these compounds.

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