11,15,19-Trihydroxy-9-ketoprost-13-enoic acid and 11,15,19-trihydroxy-9-ketoprosta-5,13-dienoic acid in human seminal fluid

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Abstract Two novel prostaglandins (PG) have been found in human seminal fluid which had been frozen immediately after collection. They were characterized by combined gas–liquid chromatography–mass spectrometry of various derivatives as 19-hydroxy prostaglandin E_1 (11,15,19-trihydroxy-9-ketoprost-13-enoic acid) and 19-hydroxy prostaglandin E_2 (11,15,19-trihydroxy-9-ketoprosta-5,13-dienoic acid). They were present in three to five times the quantity of prostaglandins E_1 and E_2. Incubation of seminal fluid for 3 hr at 25°C reduced levels of 19OH-PGEs 2.5-fold and PGE_2 2-fold, while increasing levels of PGAs and PGBs 2-fold. No 19OH PGA or 19OH PGB was detected in extracts of unincubated fluid. The PGAs, PGBs and their 19-hydroxy analogs are probably artifacts arising metabolically or as a result of classical isolation techniques.

Supplementary key words gas–liquid chromatography • gas–liquid chromatography–mass spectrometry • *O*-ethyl oxime derivatives • *O*-methyl oxime derivatives • Prostaglandins • trimethylsilyl derivatives

Thirteen different prostaglandins have been identified in human seminal fluid extracts (1–6). PG concentrations in seminal fluid extracts from men undergoing fertility evaluation have been reported (7, 8). However, these measurements were made on seminal fluid samples which were not frozen for storage until several hours after collection. To our knowledge there have been no reports of the PG content of human seminal fluid immediately after ejaculation. We have accordingly examined extracts of seminal fluid samples which have been frozen at −10°C immediately after collection. In the course of this work we have found two novel PGs. Their characterization is now reported.

METHODS

Human seminal fluid from several ejaculates obtained from a healthy fertile donor were pooled on two separate occasions. On a third occasion seminal fluid from three different healthy fertile donors was pooled. Each specimen was cooled to −10°C within 5 min of collection. In one experiment an aliquot of the pooled seminal fluid was analyzed immediately and a second aliquot was analyzed after incubation at 25°C for 3 hr.

Extraction

Separate PG extracts were prepared from each of the three pooled seminal fluid specimens within 3 days of collection. All solvents employed were freshly redistilled and were purged with dry nitrogen prior to use. All apparatus was flushed with dry nitrogen to minimize the oxidation of PGs during their isolation and characterization. Seminal fluid (3–4 ml) was extracted with chloroform–methanol 2:1 (2 × 50 ml). The extract was filtered, the residue was washed with 5 ml of chloroform–methanol 1:2, and the combined extracts were taken to dryness under dry nitrogen. Remaining traces of water (from the seminal fluid) were removed by dissolving the residue in benzene (2 × 5 ml), and evaporating to dryness after each solvent addition.

Abbreviations: PG, prostaglandin; PGA_1, 15α-hydroxy-9-ketoprosta-10,13-dienoic acid; PGA_2, 15α-hydroxy-9-ketoprosta-5,10,13-trienoic acid; PGB_1, 15α-hydroxy-9-ketoprosta- 8(12),13-dienoic acid; PGB_2, 15α-hydroxy-9-ketoprosta-5,8(12),13-trienoic acid; PGE_1, 11α,15α-dihydroxy-9-ketoprost-13-enoic acid; PGE_2, 11α,15α-dihydroxy-9-ketoprosta-5,13-dienoic acid; PGE_3, 11α,-15α-dihydroxy-9-ketoprosta-5,13,17-trienoic acid; $PGF_{1\alpha}$, 9α,-11α,15α-trihydroxyprost-13-enoic acid; $PGF_{2\alpha}$, 9α,11α,15α-trihydroxyprosta-5,13-dienoic acid; GLC-MS, gas-liquid chromatography - mass spectrometry; SIM, selective ion monitoring; TLC, thin-layer chromatography; MO, *O*-methyl oxime; EO, *O*-ethyl oxime; TMS, trimethylsilyl; BSA, *N O*-bis (TMS) acetamide; BSTFA, *N O*-bis (TMS) trifluoroacetamide.

These results were presented at the 22nd Annual Conference on Mass Spectrometry and Allied Topics, Philadelphia, May 1974.

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Isolation of PGs

Isolation of PGs from the unwashed total lipid extract was accomplished by a modification of the procedure of Daniels and Pike (9). Neutral lipids were eluted from the silicic acid column with chloroform, followed by elution of a PG fraction with chloroform–methanol 8:1, and then a more polar fraction with pure methanol. The PG fraction eluted from the column and taken to dryness was derivatized and examined by GLC-MS without further purification.

Recovery studies

Tracer amounts of ^{3}H-labeled PGs (PGB_1, PGE_1, and $PGF_{1\alpha}$) added to seminal fluid prior to extraction were used in separate experiments to verify that these PGs were extracted in reasonably good yields from seminal fluid, and were recovered quantitatively in the crude PG fraction eluted from the silicic acid columns. No tracer or carrier PGs were added to any seminal fluid or to any extracts which were subsequently used for GLC-MS.

Derivatives for GLC-MS

The PG fraction from the first seminal fluid sample was converted to EO-TMS derivatives, the PG fraction from the second sample was converted to MO-TMS and MO-[$^2H_9$]TMS derivatives. After dividing the third sample into two equal parts (and allowing one half to incubate as described above), EO-TMS derivatives were formed.

EO-TMS derivatives were prepared in two steps. The sample was dissolved in dry pyridine (250 μl) and ethoxylamine hydrochloride (1 mg) was added. After 12 hr, the reaction mixture was blown to dryness under nitrogen. The residue was dissolved in BSTFA and heated at 50°C for 3 hr. Aliquots of the reaction mixture were used directly for analysis by GLC-MS.

MO-TMS derivatives were prepared in a similar manner, but using methoxylamine hydrochloride.

MO-[$^2H_9$]TMS derivatives were prepared as for MO-TMS derivatives, but substituting [$^2H_{18}$]BSA for BSTFA.

Chromatography

GLC-MS was performed using an LKB 9000 instrument equipped with a 9 ft × 0.25 in silanized glass column packed with 1% SE-30 on Gas-Chrom Q (100–200 mesh) under the following conditions: flash heater, 270°C; column temperature programmed from 140°C to 290°C at 2°C per min; molecular separator, 250°C; electron energy, 22.5 eV; trap current, 60 μA; accelerating voltage, −3.5 kV. The instrument was modified by the installation of a continuous dynode electron multiplier (10). For mass spectral scans the electron multiplier voltage was 1.5 kV, and for SIM, 2.5 kV. When used in the latter mode, the output signal was displayed on a pen-and-ink recorder and high-frequency noise was filtered using a 12,500 μF capacitor, as previously described (11). Quantitative estimates of the PG content of the extracts were made by comparison of peak areas by cutting out and weighing the GLC peaks. The values for PGE_1 and PGE_2 reported recently by Singh and Zuspan (12), were used for calculating absolute amounts (μg/ml) for the 190H-PGEs from our own GLC traces which contained both PGEs and 190H-PGEs.

Thin-layer chromatography was used to evaluate and monitor extraction and column chromatographic fractionation of total lipids isolated from seminal fluid. Silica gel HR (0.25 mm) on glass plates (20 × 20 cm) was activated for 1 hr at 110°C, and stored in a desiccator prior to sample application. Aliquots of fractions from the silicic acid columns were applied to the plates in a narrow band and developed in benzene–dioxane–acetic acid 20:10:1 in unlined chromatographic tanks. The separated components were visualized by charring (50% aqueous H_2SO_4) or by spraying with 5% phosphomolybdic acid in ethanol (13). None of the PG fraction which was later to be characterized by GLC-MS was subjected to TLC at any time.

RESULTS

A portion of each fraction eluted from the silicic acid column was compared to standards co-chromatographed on TLC. The neutral lipids (fraction I) gave two distinct bands (free fatty acids R_f 0.85, other neutral lipids R_f 0.95) very close together, which migrated near the solvent front, while the phospholipids (fraction III) were found in a single band which remained near the origin (R_f 0.05). The PGs (fraction II) were found in the region between the origin and the solvent front, well separated from both the neutral and polar lipids. The primary families of PGs are well separated from each other in this system. Evaluation by TLC of aliquots of each of the three fractions of seminal lipids eluted from the column clearly indicated that the neutral lipids (fraction I) were free of visible contamination, the PGs (fraction II) contained some neutral and some phospholipid contamination, while the polar lipids (fraction III) appeared to be free of other lipids. The remaining portion of the PG fraction eluted from the column was evaporated to dryness and derivatized for subsequent analysis by GLC-MS without exposure to TLC or other purification steps. It would appear that this approach has two advantages: (*i*) it is reasonably efficient in removing the bulk of the other lipids from the PGs, and (*ii*) the elimination of a TLC step precludes the possible inadvertent loss of PGs which might be separated from the primary PGs, and thus overlooked in subsequent elution and characterization steps.

Single ion monitoring (11) of the EO-TMS derivatives of the PG fraction was performed at *m/e* 611 ($M^{+\cdot}$) to detect selectively the derivatives of PGE_2 (14). Peaks due to the *syn* and *anti* isomers were observed at the expected retention time but components of longer retention time were also found to contain ions of *m/e* 611. Mass spectra of the additional compounds as their EO-TMS, MO-TMS, and MO-[$^2H_9$]TMS derivatives were obtained and retention data were measured on SE-30.

Fig. 1 shows the chromatogram obtained for the MO-TMS derivatives. It can be seen that the additional components were present in much higher concentrations than

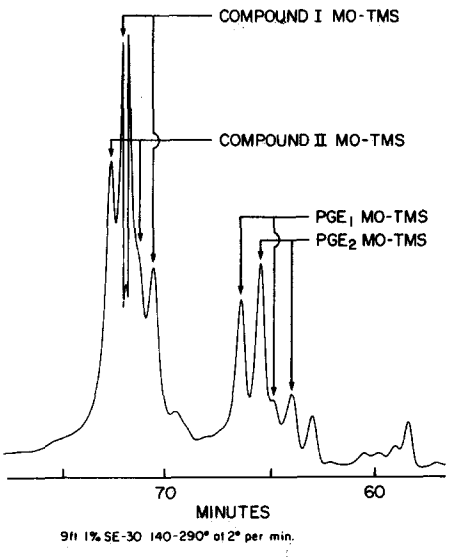


Fig. 1. Gas chromatogram of MO-TMS derivatives of PG fraction of human seminal fluid.

derivatives of PGE_1 and PGE_2. **Table 1** lists GLC data for MO-TMS and EO-TMS derivatives of the additional compounds together, for comparison, with data for the derivatives of PGE_1 and PGE_2 (15). The additional components are denoted as I and II. Since the order of elution of the *syn* and *anti* isomers is unknown, the first eluted isomers of the MO-TMS derivatives of I and II will be denoted as Ia and IIa, and the other isomers as Ib and IIb, respectively (16).

Mass spectra of Ia and Ib are shown in **Figs. 2** and **3.** These spectra contain weak ions presumed to be molecular ions at *m/e* 685 (Ia, 2%; Ib, 3%). There are more abundant

TABLE 1. GLC data for derivatives of novel PGs[a] and of PGE_1 and PGE_2[b]

	MO-TMS	EO-TMS
Compound I	3040	3095
	3090	3130
Compound II	3070	3105
	3120	3145
PGE_2	2830	2860
	2880	2915
PGE_1	2860	2890
	2910	2945

[a] 9 ft 1% SE-30 on Gas-Chrom Q, 100–120 mesh, programmed from 140°C at 2°C per min.

[b] 6 ft 1% SE-30 on Gas-Chrom Q, 100–120 mesh, isothermal at 200°C.

ions at $[M-15]^+$ (*m/e* 670: Ia, 10%; Ib, 3%), $[M-31]^+$ (*m/e* 654: Ia, 11%; Ib, 16%), $[M-90]^{+\cdot}$ (*m/e* 595: Ia, 5%; Ib, 11%), and $[M-105]^+$ (*m/e* 580: Ia, Ib 3%) which appear to be formed, respectively, by loss of a methyl radical, a methoxyl radical, trimethylsilanol, and trimethylsilanol with a methyl radical from the molecular ion.

The mass spectra of the MO-TMS derivatives of PGE_2 (17) contain abundant ions of *m/e* 526, formed by cleavage of the C-15/16 bond, and more intense ions of *m/e* 436, produced by elimination of trimethylsilanol from these ions. The presence of such ions in the spectra of Ia and Ib (*m/e* 523: Ia, 24%; Ib, 8%; *m/e* 436: Ia, 27%; Ib, 10%) indicates that the structure of compound I is similar to that of PGE_2, but with a hydroxyl group on the C-16/20 terminal chain.

Other ions in the spectra of MO-TMS derivatives of PGE_2 which do not contain any part of the C-16/20 chain (17) are present also in the spectra of Ia and Ib. These include ions of *m/e* 133 (i: Ia, 43%; Ib, 80%); *m/e* 204 (ii: Ia, 19%; Ib, 14%); *m/e* 217 (iii: Ia, 45%; Ib, 31%); *m/e* 243 (iv: Ia, 9%;

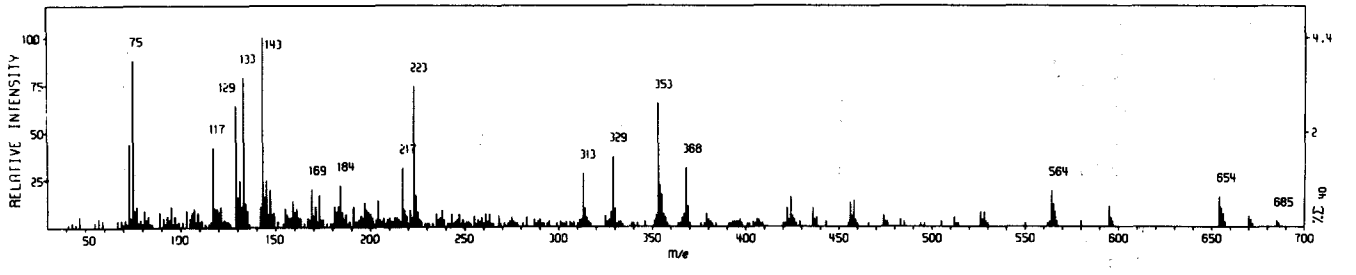


Fig. 2. Mass spectrum (22.5 eV) of compound Ia.

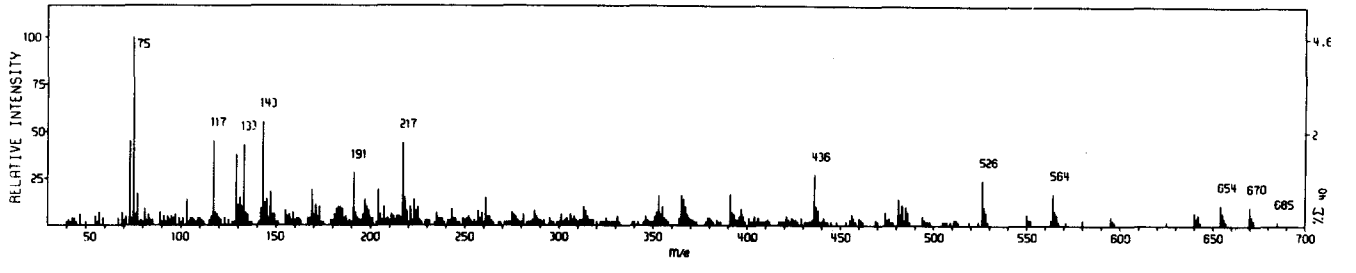


Fig. 3. Mass spectrum (22.5 eV) of compound Ib.

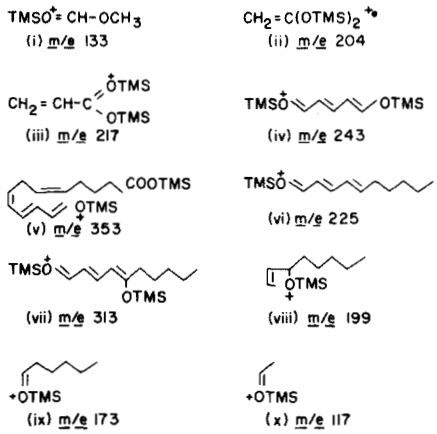


Fig. 4. Formulae of selected ions in the mass spectra of derivatives of selected prostaglandins.

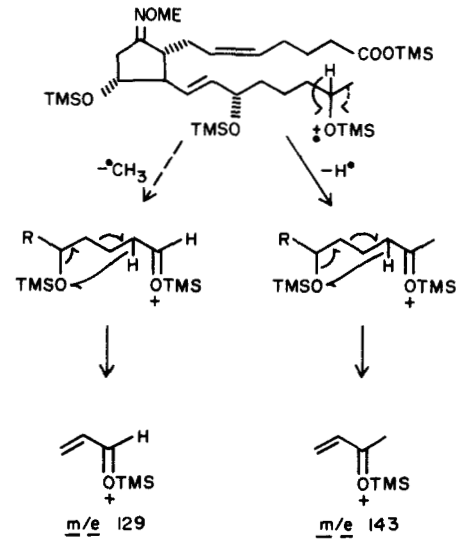


Fig. 5. Postulated mechanisms of formation of ions of *m/e* 129 and 143 in mass spectra of Ia and Ib.

Ib, 7%); and *m/e* 353 (v: Ia, 16%; Ib, 65%) (**Fig. 4**). Ions ii and iii, formed by transfer of an ether TMS group to the ester moiety, are significantly more abundant in the spectra of Ia and Ib than in the spectra of MO-TMS derivatives of PGE_2.

Ions in the spectra of MO-TMS derivatives of PGE_2 which have been shown to contain the C-16/20 chain (17) are absent from the spectra of Ia and Ib. There are, however, several ions 88 amu higher or 2 amu lower in mass than these ions. The former ions apparently contain a trimethylsilyloxy group in place of a hydrogen atom, whereas the latter are probably produced from the former ions by elimination of trimethylsilanol. For example, the MO-TMS derivatives of PGE_2 afford very abundant ions of *m/e* 225 (vi) (Fig. 4), and the spectra of Ia and Ib exhibit corresponding ions of *m/e* 313 (Ia, 10%; Ib, 18%) (Fig. 4) and *m/e* 223 (Ia, 14%; Ib, 74%). There is an ion of *m/e* 313 (vii) (Fig. 4) also in the spectra of MO-TMS derivatives of PGE_2, but the analogous ions appear at *m/e* 401 (Ia, 4%; Ib, 2%) and *m/e* 311 (Ia, 3%; Ib, 4%) in the spectra of Ia and Ib.

TMS derivatives of most of the primary PGs afford ions of *m/e* 199 (viii) and *m/e* 173 (ix) (Fig. 4) formed by cleavage of C-12/13 and C-14/15, respectively (16–22). Ia and Ib afford ions corresponding to vii at *m/e* 287 (Ia, 8%; Ib, 4%) and *m/e* 197 (Ia, 14%; Ib, 13%), whereas ions analogous to ix are seen at *m/e* 261 (Ia, 15%; Ib, 7%) and *m/e* 171 (Ia, Ib: 11%). The ions of *m/e* 199 (Ia, Ib: 8%) presumably comprise C-1/7 (17).

The mass spectra of methyl ester TMS ether derivatives of 19-hydroxy PGB_1 (23) and 15,19-dihydroxy-9-keto-1,2,3,4-tetranorprosta-8(12),13-dienoic acid (24) both contain characteristic ions of *m/e* 117 (x) (Fig. 4). The presence of such ions in relatively high abundance in the spectra of Ia (45%) and Ib (42%) indicates that compound I contains a 19-hydroxy group. The formation of the very abundant ions of *m/e* 129 (Ia, 38%; Ib, 65%) and *m/e* 143 (Ia, 55%; Ib, 100%) can therefore be rationalized as in **Fig. 5.**

Appropriate mass shifts for all of the ions discussed were observed in the spectra of EO-TMS and MO-[$^2H_9$]TMS derivatives of compound I. The mass spectral data are fully compatible with compound I being a 19-hydroxy PGE_2.

Compound II was present in smaller quantities in the PG fraction of the seminal fluid extracts. Its derivatives were not fully separated from those of compound I (see Fig. 1). The mass spectra obtained for derivatives of compound II contained contributions from Ia and Ib. Nevertheless, the mass spectral data strongly indicated that compound II is a 19-hydroxy PGE_1.

Gas–liquid chromatographic data have been reported for derivatives of many primary prostaglandins (15, 18, 19), but there is only a limited amount of data for derivatives of 19-hydroxy analogs (23, 24). However, the data reported for PGB_1, 19-hydroxy PGB_1, and 20-hydroxy PGB_1 indicate that the retention increments[5] for 19- and 20-trimethylsilyloxy groups are +230 and +310, respectively. Inspection of the data in Table 1 shows that the retention increments for the additional trimethylsilyloxy groups in derivatives of compounds I and II are in the range 200–235. This is further evidence for compounds I and II being 19-hydroxy PGE_2 and 19-hydroxy PGE_1.

Comparison of peak areas from our GLC traces indicates that levels of 190H-PGE_1 + 190H-PGE_2 in human seminal fluid are 2.3 times those of PGE_1 + PGE_2. Multiple analyses indicate that the ratio ranges between 2 and 5, but we have used 2.3 in our estimates since this is the approximate ratio found in the chromatogram shown in Fig. 1. By assuming levels of PGE_1 + PGE_2 to be approximately 41 μg/ml of

[5] Israelsson, Hamberg, and Samuelsson (23) and Green (24) reported gas–liquid chromatographic data as "C-values" related to retention times of fatty acid methylesters. They are equivalent to "M-values" (27, 28), but their use has been largely superseded by Kovats' retention indices. However, "C-value" increments are exactly 100 times smaller than increments in Kovats' retention indices, which are related to retention times of *n*-alkanes.

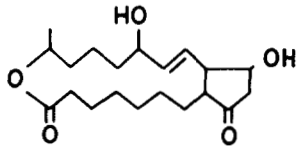
human seminal fluid, from the recent data of Singh and Zuspan (12), we calculate levels of 19OH-PGE$_1$ + 19OH-PHE$_2$ to be 94.3 μg/ml of fresh human seminal fluid.

Single ion monitoring of GLC-MS scans showed that incubation of seminal fluid for 3 hr at 25°C reduced combined levels of 19OH-PGE$_1$ and 19OH-PGE$_2$ 2.5-fold (from 94.3 to 31.4 μg/ml). Incubation also reduced PGE$_2$ levels 2-fold (from 23.7 to 11.8 μg/ml), while increasing levels of PGA and PGB 2-fold. No 19OH-PGAs or 19OH-PGBs were detected in extracts of unincubated fluid but 19OH-PGA$_2$ was indicated after incubation.

DISCUSSION

The gas–liquid chromatographic and mass spectrometric data all provide evidence for the presence of 19-hydroxy PGE$_1$ and 19-hydroxy PGE$_2$ in the PG fraction of total lipid extracts of human seminal fluid. It would be reasonable to assume that freezing the seminal fluid immediately after ejaculation should minimize the possibility of enzymatic interconversion or modification of the PGs originally present. This point is particularly pertinent in view of the many known changes in the chemical or enzymatic composition of human seminal fluid during the first several hours after its collection, when kept at room temperature. Moreover, the fact that the methods of extraction and purification used in this study have avoided the usual extremes of pH, exposure to oxidation, and possible loss or alteration during TLC (the TLC step is eliminated in our method) should reflect more accurately the true picture of the PGs present in freshly collected seminal fluid. It seems very probable therefore, that the 19-hydroxy PGs of the E series were present in the human seminal fluid samples. Furthermore, there was no evidence for the existence of lactones such as III (25).

III



Hamberg and Samuelsson (4, 5, 26) found 19-hydroxy PGs of the A and B series in about four times the concentration of PGs of the E series. We found no 19-hydroxy PGs of the A or B series, but compounds I and II were present in our extracts in up to five times the concentration of PGs of the E series.

The third sample studied here unequivocally demonstrates that 19OH-PGE$_1$ and 19OH-PGE$_2$ exist in fresh seminal fluid, and decrease during incubation at room temperature with a concomitant increase in PGA and PGB as well as the previously found 19OH-PGA. It is possible therefore, that the 19-hydroxy PGs of the E series, which we have found to be present in human seminal fluid immediately after ejaculation, are precursors of the 19-hydroxy PGs of the A and B series previously reported by others. These findings emphasize the need for careful consideration of the metabolic or chemical changes which may occur during sample collection and analysis.

We are indebted to J. E. Pike and U. Axen of The Upjohn Company, Kalamazoo, Michigan, and to K. Sano of Ono Pharmaceutical Co., Osaka, Japan, for gifts of prostaglandins used in this work. The authors are indebted to Drs. Evan C. Horning and Robert E. Anderson, Baylor College of Medicine, Houston, who provided the facilities for the prostaglandin characterization. We also thank Mr. Michael Papantonakis and K. Lyman for their technical assistance during this study.

This work was supported by grants from the National Institutes of Health (GM-13901) and South Carolina Appropriations for Research.

Manuscript received 19 September 1974 and in revised form 28 April 1975; accepted 5 September 1975.

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