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Biosynthesis and Chemistry of 9α,15(*S*)-Dihydroxy-11-oxo-13-*trans*-prostenoic Acid†

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ABSTRACT: Serotonin, when used as a cofactor in the biosynthesis of prostaglandins from *all-cis*-8,11,14-eicosatrienoic acid by bovine seminal vesicle microsomes, was found to enhance the overall synthesis of all the postaglandin and especially 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid. This prostaglandin, which is coproduced with PGE and PGF$_{1\alpha}$, was purified and found to possess the structure 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid. The chemistry of PGD$_1$ and related products as well as the absolute configuration were investigated.

Nugteren *et al.* (1966) first found that a variety of products were formed from *all-cis*-8,11,14-eicosatrienoic acid by an enzyme fraction of sheep vesicle glands when reduced glutathione was omitted from the incubation mixture. One of these products, when treated with methanolic KOH, resulted in rapid decomposition and a small increase in ultraviolet absorption at approximately 245 mμ was observed. Subsequently, Granstrom *et al.* (1968) reported the isolation of a small quantity of a similar compound, which had a weak absorption at 235 mμ after alkali treatment. This product was assumed to be 9α,15-dihydroxy-11-oxo-13-*trans*-prostenoic acid by both groups because a more polar compound with the same chromatographic behavior as PGF$_{1\alpha}$[1] was formed after treatment with $NaBH_4$.

In view of the finding that a small quantity of 8-iso-PGE$_1$ is produced in the incubation mixture and 8-iso-PGF$_{1\alpha}$ methyl ester is virtually indistinguishable from PGF$_{1\alpha}$ methyl ester with respect to chromatographic behavior, infrared, nuclear magnetic resonance (nmr), and mass spectra (Pike *et al.*, 1969) a more rigorous examination of the chemistry of this prostaglandin appears mandatory. Furthermore, the conditions governing the formation of this prostaglandin were unclear (Granstrom *et al.*, 1968). In this paper, we record the

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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; PF$_{1\alpha}$, 9α,11α,15α-trihydroxy-13-*trans*-prostenoic acid; PGF$_{2\alpha}$, 9α,11α,15α-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid; 11-epi-PGF$_{1\beta}$, 9α,11β,15α-trihydroxy-13-*trans*-prostenoic acid; PGA$_1$, 15α-hydroxy-9-oxo-10,13-*trans*-prostadienoic acid; PGB$_1$, 15α-hydroxy-9-oxo-8(12),13-*trans*-prostadienoic acid; iso-PGD$_1$, 9α,15α-dihydroxy-11-oxo-12(13)-*trans*-prostenoic acid; PGD$_1$, 9α,15α-dihydroxy-11-oxo-13-*trans*-prostenoic acid; dihydro-PGD$_1$, 9α,15α-dihydroxy-11-oxoprostanoic acid.

exact conditions favoring the biosynthesis of 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid (PGD_1) by bovine seminal vesicle microsomes and the experiments which are significant in establishing its absolute configuration.

Experimental Procedure

Materials and Methods. *all-cis*-8,11,14-Eicosatrienoic acid (better than 85% chemical purity) was obtained from the Upjohn Co. through the courtesy of Dr. John Pike. Tris and serotonin (5-hydroxytryptamine–creatinine sulfate complex) were products of Sigma. Analytical precoated layers of silica gel F-254 glass plates for thin-layer chromatography (tlc) were products of Brinkmann. All solvents were of reagent grade and were redistilled.

Ultraviolet spectra were recorded in ethanol on a Cary 15 recording spectrophotometer. Melting points, determined on a Thomas-Hoover melting point apparatus, are uncorrected. Infrared spectra were taken on a Perkin-Elmer 257 grating infrared spectrophotometer. Mass spectra were obtained with an AEI MS-9 mass spectrometer, using direct probe introduction with an ion source temperature of 70–120° above ambient; electron potential of 70 eV, and an ionizing current of 100 μA. Temperature was found to be an important factor in determining the fragmentation profile of the prostaglandin molecule. Nuclear magnetic resonance spectra were determined on a Varian A 60A spectrometer at 60 Mcycles in deuterated chloroform or deuterated acetone using Me_4Si as an internal standard. Circular dichroism (CD) spectra were taken in a 5-mm cell at 25° using the 6002 attachment to a Cary 60 spectropolarimeter. The dynode voltage was never allowed to exceed 0.35 and the signal to noise ratio was always in excess of 50 to 1. Tlc plates were developed in a solvent system consisting of ethyl acetate–acetic acid–isooctane–water (110:20:50:100, v/v). The prostaglandins were visualized by spraying with 3% ceric sulfate in 3 N H_2SO_4 and heating to 110° for a few minutes. Silicic acid powder (Mallinckrodt 2847; 100 mesh), mixed with 15% Celite, was used for column chromatography. Bovine seminal vesicle microsomes were prepared according to the method of Takeguchi *et al.* (1971).

Biosynthesis and Isolation of 9α,15(S)-Dihydroxy-11-oxo-13-trans-prostenoic Acid. In a number of experiments, 1.0 g of *all-cis*-8,11,14-eicosatrienoic acid and 3.17 g of serotonin, each dissolved separately in 100 ml of 0.05 M Tris buffer (pH 9.0), were distributed equally to ten 2-l. erlenmeyer flasks containing 400 ml of the same buffer. After equilibration at 37° on an incubating rotary shaker for approximately 1 hr, 4.8 g of microsomes, suspended in 80 ml of the same buffer (precooled to 2°), was added to each of the shaking flasks (150 rpm; 1 in. radius). After 1-hr incubation in air, the flasks were acidified to pH 2.5 with 5 N HCl, and the reaction mixture was extracted three times with ethyl acetate. The ethyl acetate layer (5 l.) was dried over sodium sulfate and evaporated to dryness. The residue containing PGE_1, PGD_1, and $PGF_{1\alpha}$ was chromatographed over a silicic acid column (2 × 25 cm). The column was eluted with a mixture of ethyl acetate in benzene: PGD_1 was eluted off the column with 45% ethyl acetate in benzene while PGE_1 and $PGF_{1\alpha}$ were eluted at 65 and 85%, respectively. Recrystallization of the PGE_1 from acetone–petroleum ether (bp 30–60°) afforded 100 mg of crystals, mp 113.5–114°/batch. $PGF_{1\alpha}$ was recrystallized from diethyl ether–petroleum ether to yield 50 mg of crystals, mp 98–100° (literature values: PGE_1, mp 115–117°; $PGF_{1\alpha}$, 102–103°, Ramwell *et al.*, 1968).

The fractions containing PGD_1 were contaminated by an indole metabolite, which caused difficulties in its purification. However, rechromatographing the combined fractions on a silicic acid–Celite column (0.1 × 17 cm), a white waxy material of mp 62–65° was obtained. The column was eluted with 35–45% ethyl acetate in benzene and the fractions containing PGD_1 were pooled and then treated carefully with activated charcoal. The yield of PGD_1 varied from 50 to 60 mg per batch.

Methylation of PGD. Diazomethane, generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonimide, was passed through a solution of PGD_1 (50 mg) in 5 ml of methanol–ethyl ether (1:9, v/v) *via* a stream of nitrogen until it assumed a permanent yellow color. Chromatography of the resulting methyl-PGD_1 on a silicic acid–Celite column (0.8 × 17 cm), using 35–45% ethyl acetate in benzene as eluent, afforded 48 mg of the pure compound; ($M - 2H_2O$) at *m/e* 332.2331; *Anal.* Calcd for $C_{21}H_{32}O_3$: 332.2351.

Hydrogenation of PGD_1. PGD_1 (120 mg), dissolved in ethyl ether (15 ml), was hydrogenated over 50 mg of 5% palladium on charcoal at 1 atm for 3 hr. After removing the catalyst, the mixture was chromatographed over a silicic acid–Celite column (0.8 × 20 cm). The column was eluted with 30–40% ethyl acetate in benzene to give 42 mg of dihydro-PGD_1.

Dehydration of Dihydro-PGD_1. Dihydro-PGD_1 (16 mg), dissolved in 4 ml of a 1:1 mixture of dioxane and 1 N HCl, was allowed to stand at room temperature for 2 hr. At the end of this period, the reaction mixture was diluted with water and extracted exhaustively with ethyl acetate. After evaporation of the ethyl acetate, the residue was chromatographed over a silicic acid–Celite column (0.45 × 12 cm). The column was eluted with a gradient system consisting of 17–23% ethyl acetate in benzene. The products were detected by tlc. After pooling the appropriate fractions, two dehydration products, dihydro-PGD_1-AI (2.1 mg) and dihydro-PGD_1-AII (3.1 mg; M^+) at *m/e* 338.2459 (*Anal.* Calcd for $C_{20}H_{34}O_4$: 338.2457) were obtained.

Sodium Borohydride Reduction of PGD_1. PGD_1 (50 mg), dissolved in 5 ml of absolute methanol, was cooled to 0°. Sodium borohydride (10 mg) was then added. After standing at room temperature for 1 hr, the reaction mixture was diluted with water, acidified, and extracted exhaustively with ethyl acetate. Chromatography of the crude residue on a silicic acid–Celite column (0.8 × 15 cm) and elution of the column with 75–85% ethyl acetate in benzene afforded two isomers of PGF. The major component has a mobility on thin-layer plate identical with that of $PGF_{1\alpha}$. Recrystallization from ethyl ether–petroleum ether afforded white crystals of $PGF_{1\alpha}$ (22 mg), mp 99–101°. The minor component (2 mg) was slightly less polar than $PGF_{1\alpha}$ on tlc and was shown to be the 11β epimer.

Preparation of Iso-PGD_1. PGD_1 (100 mg) was applied to an activated charcoal column (0.8 × 12 cm) and eluted with 45% ethyl acetate in benzene over a period of 1.5 days. The eluent contained a mixture of unchanged PGD_1 and iso-PGD_1. Separation was effected on a silicic acid (impregnated with 10% w/w of silver nitrate)–Celite (15%) column (0.8 × 20 cm) by eluting with a gradient system consisting of 35–50% ethyl acetate in benzene. Iso-PGD_1 (50 mg) and PGD_1 (35 mg) were obtained.

Results

When *all-cis*-8,11,14-eicosatrienoic acid was incubated with

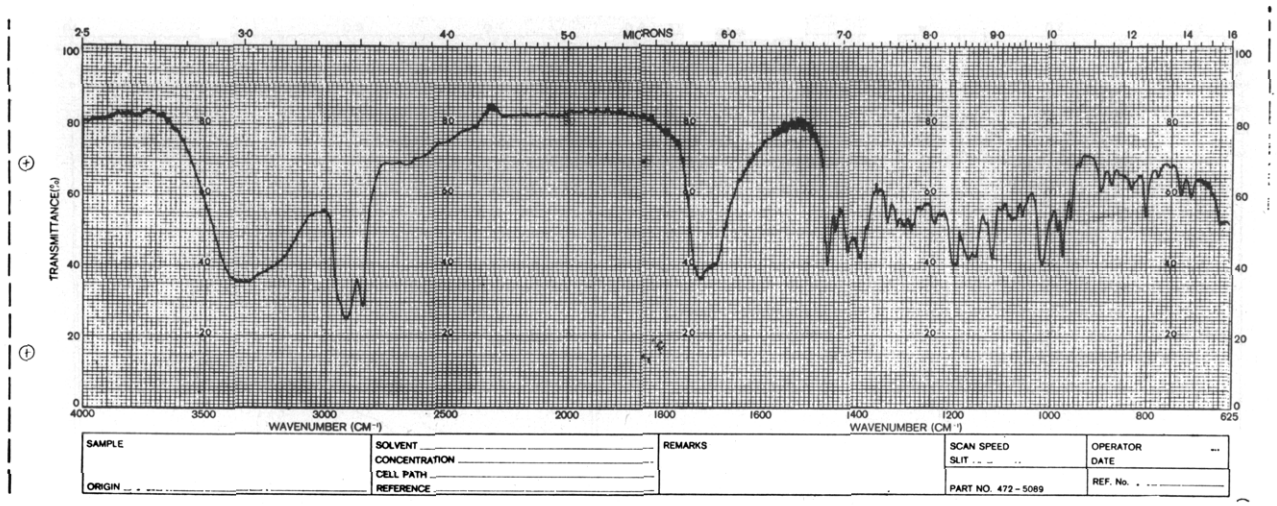


FIGURE 1: The infrared spectrum of 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid.

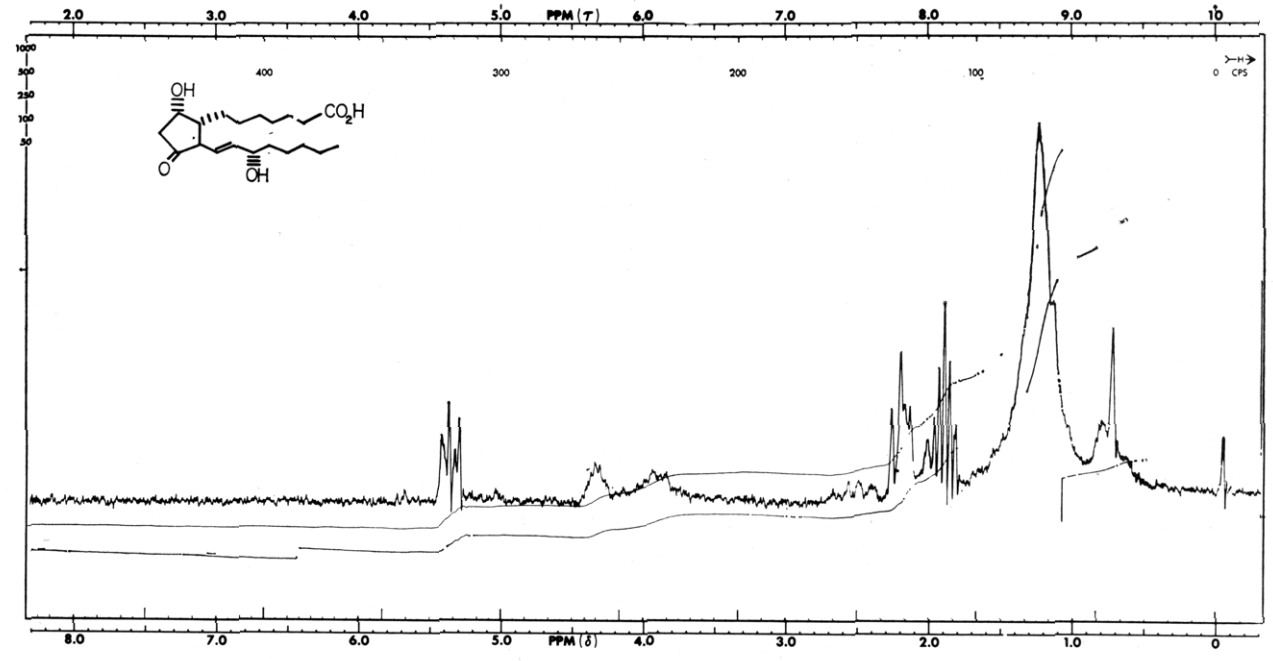


FIGURE 2: The nmr spectrum of 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid.

bovine seminal vesicle microsomes, in the presence of serotonin as described in the methods, another prostaglandin, PGD_1, was formed besides PGE_1 and $PGF_{1\alpha}$. Chromatography on silicic acid afforded a white waxy material, mp 62–65°, whose ultraviolet spectrum showed only end absorption. Its infrared spectrum exhibited strong absorption bands at 5.84 and 5.78 μ and a weak band at 10.3 μ (Figure 1). These data indicated the presence of a carbonyl group in a five-membered ring and a trans double bond. The nmr spectrum of PGD_1 (Figure 2) showed features characteristic of prostaglandin (Ramwell *et al.*, 1968): a pair of doublets centered at δ 5.35 (2 H, vinyl protons at C-13 and C-14), a multiplet at δ 4.35 (1 H, proton geminal to hydroxyl at C-11 or C-15), and a multiplet at δ 3.88 (1 H, proton geminal to hydroxyl at C-11 or C-15). Similarly, the methyl ester of PGD_1 (Figure 3) gave signals at δ 5.58 (2 H) and multiplets at δ 4.55 (1 H), δ 4.17 (1 H), and δ 3.68 (3 H, $COOCH_3$). The main difference in the nmr spectrum of PGE_1 and PGD_1 lies in the appearance of a multiplet in the latter, corresponding to one proton at δ 4.35, tentatively assigned to one of the two protons geminal to the hydroxyl group at C-15 or C-11. In contrast, both the C-11 and C-15 protons in PGE_1 gave a multiplet centered at δ 4.08. The mass spectrum of PGD_1 (Figure 4) gave rise to ions with the following *m/e* values: 336 (M − 18), 318 (M − 2 × 18), 265 (336 − 71), α cleavage with the loss of C_5H_{11}), and 247 (265 − 18). Its methyl ester (Figure 5) in turn gave peaks at 368 (M), 350 (M − 18), 332 (350 − 18), 297 (M − 71), 279 (350 − 71), and 247 (279 − 32; loss of CH_3OH). These data are consistent with a molecular weight of 354 for PGD_1 and the fragmentation pattern further supports the isomeric nature of PGD_1 with PGE_1. The CD curve of PGD_1 (Figure 6) in methanol showed a negative cotton effect ($[\theta]_{max} \times 10^{-3} = -8.4°$ at λ 306 mμ) arising from a n–π^* transition of a carbonyl chromophore. The Cotton effect in the trough

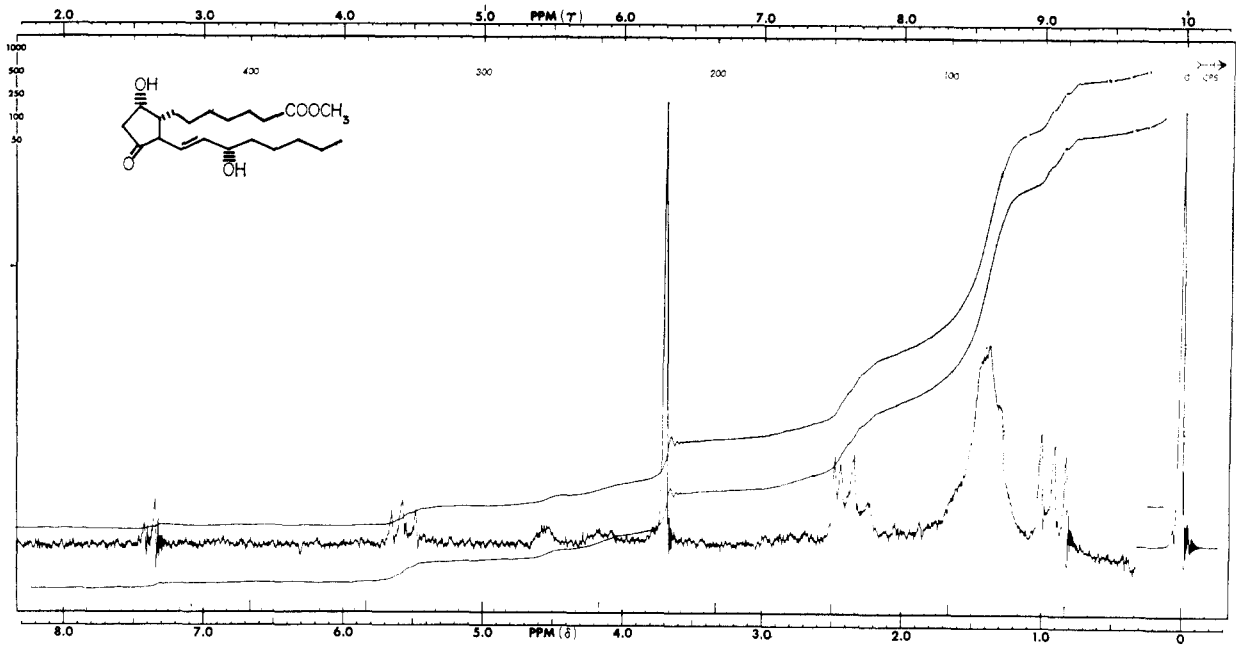


FIGURE 3: The nmr spectrum of 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid methyl ester.

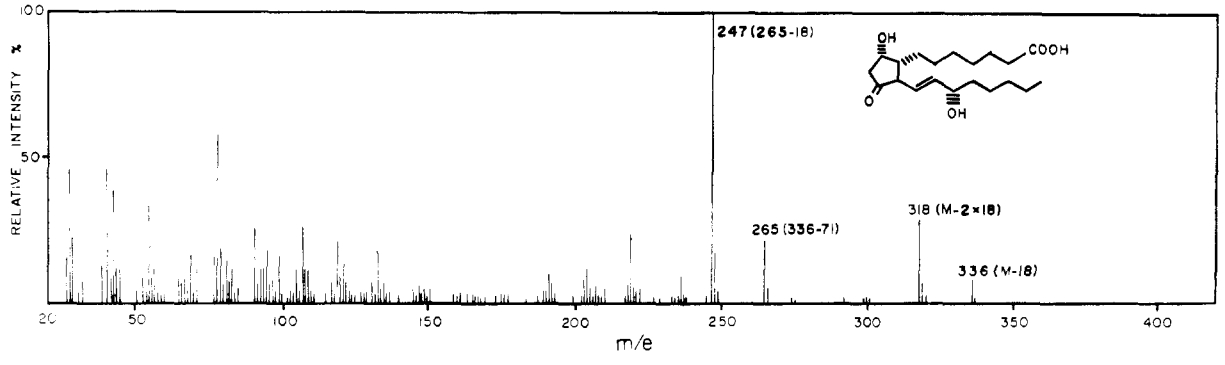


FIGURE 4: The mass spectrum of 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid.

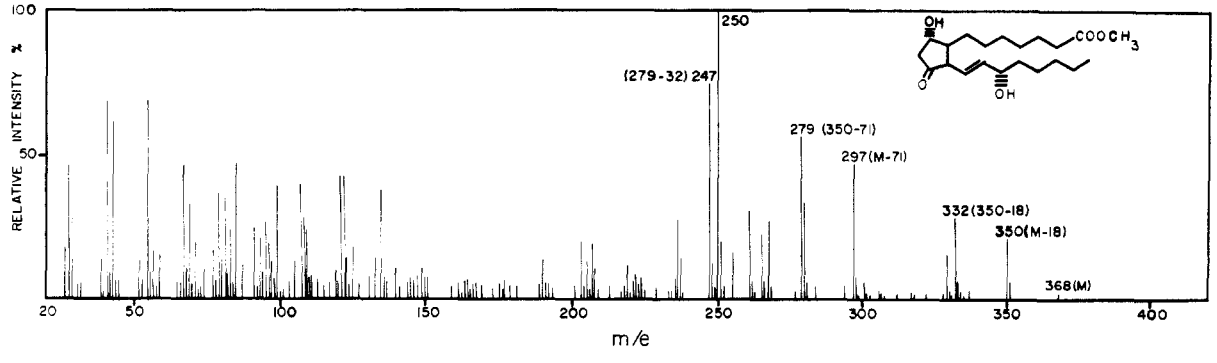


FIGURE 5: The mass spectrum of 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid methyl ester.

may have arisen from the n–π^* transition of a dehydration product of PGD_1.

When PGD_1 was treated with sodium borohydride, two isomers were formed. One of these was found to be identical with $PGF_{1\alpha}$ with respect to infrared spectrum and mixture melting points (99–101°); the other isomer, chromatographically less polar than $PGF_{1\alpha}$, was formed to a lesser degree (approximately one-tenth of $PGF_{1\alpha}$). Because both isomers have identical mass spectra, identical with that of $PGF_{1\alpha}$, the minor component was assigned as 11-epi-$PGF_{1\beta}$.

It was expedient at this point to assume that PGD_1 was isomeric with PGE_1, differing from that molecule in the

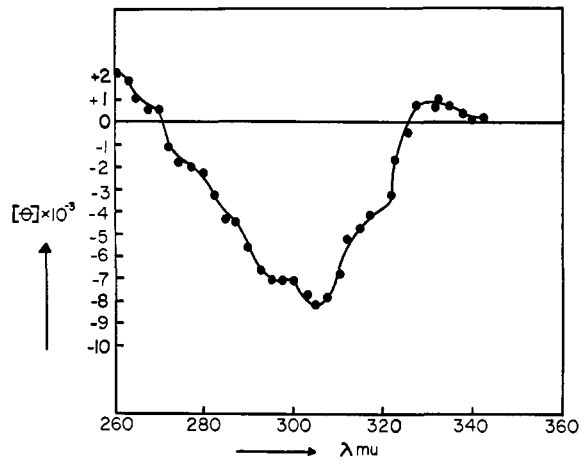
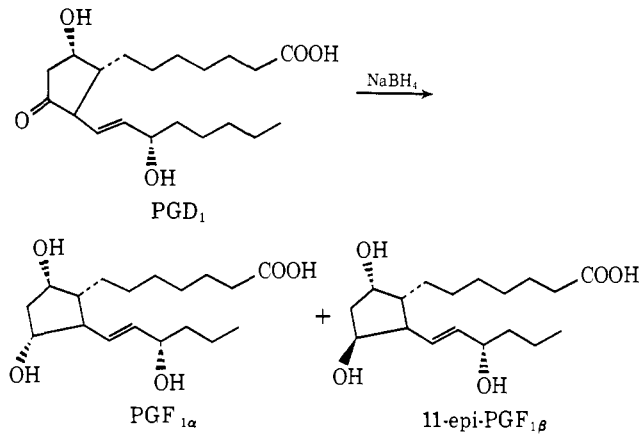


FIGURE 6: The circular dichroism curve of 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid.



positions of the oxygen functions at C-9 and C-11 and/or the stereochemistry of the four asymmetric centers in the molecule.

Hydrogenation of PGD_1 afforded dihydro-PGD_1, whose mass spectrum (Figure 7) showed a parent ion at *m/e* 338 (M − 18) and ions at 320 (M − 2 × 18), 267 (338 − 71), 249 (267 − 18). Nmr analysis revealed the disappearance of the olefinic protons at δ 5.35. Two isomers were obtained when PGD_1 was dehydrated by acid treatment. Dihydro-PGD_1-AII gave an ultraviolet absorption band at λ_{max} 222 mμ (ϵ 6100). Its mass spectrum (Figure 8) gave a parent ion at *m/e* 338 with a fragmentation pattern, illustrating α cleavages around C-15 (*m/e* 267 (M − C_5H_{11}) and 219 (M − $2H_2O$ − 83, C_6H_{11})) and McLafferty rearrangement around

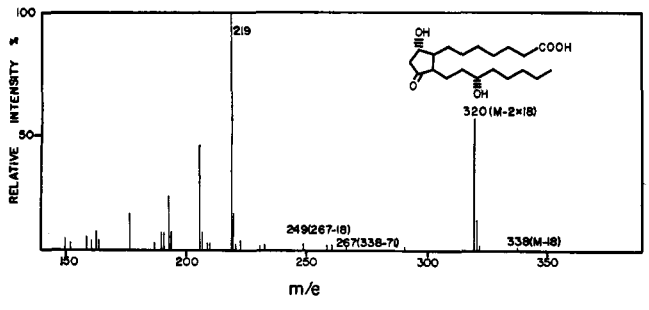


FIGURE 7: The mass spectrum of 9α,15(*S*)-dihydroxy-11-oxo-prostenoic acid (dihydro-PGD_1).

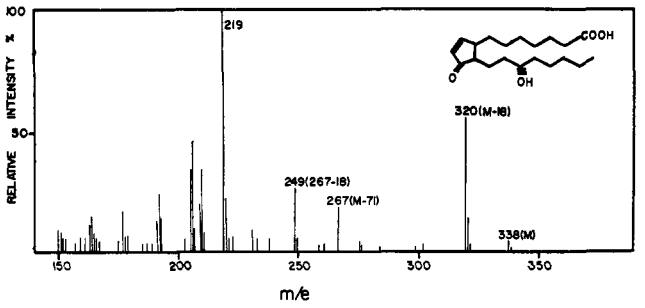


FIGURE 8: The mass spectrum of 15(*S*)-hydroxy-11-oxo-9(10)-prostenoic acid (dihydro-PGD_1-AII).

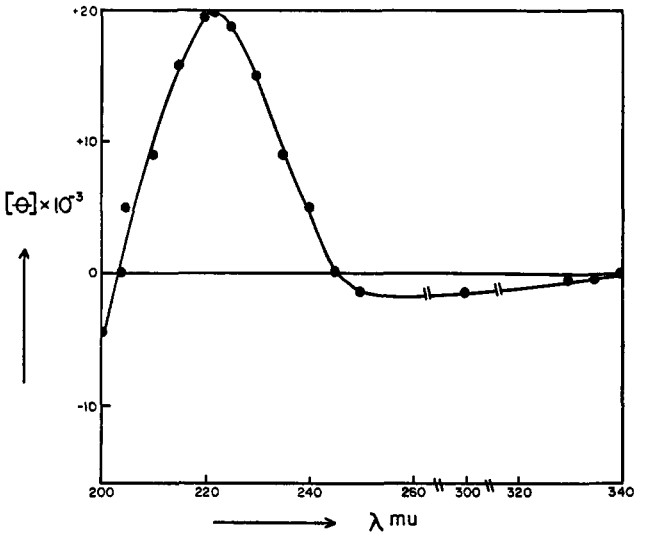


FIGURE 9: The circular dichroism curve of 15(*S*)-hydroxy-11-oxo-9(10)-prostenoic acid (dihydro-PGD_1-AII).

the ketone at C-11 (210 (M − 118)). The CD curve of dihydro-PGD_1-AII gave a positive cotton effect of $[\theta] \times 10^{-3} = +19.8°$ for the π–π^* transition of the enone (Figure 9).

The second dehydration product, dihydro-PGD_1-AI showed only end absorption in its ultraviolet spectrum and its mass spectrum (Figure 10) clearly showed that it was isomeric with dihydro-PGD_1-AII. As the signs of the cotton effects in the CD spectra of PGD_1 and dihydro-PGD_1-AII agree with those of PGE_1 and PGA_1, this confirms the stereochemistry of the prostanoic acid nucleus as that of 1(*R*),2(*R*)-1-(6-carboxyhexyl)-2-octylcyclopentanone. Further, reduction of PGD_1 with sodium borohydride afforded $PGF_{1\alpha}$ as the

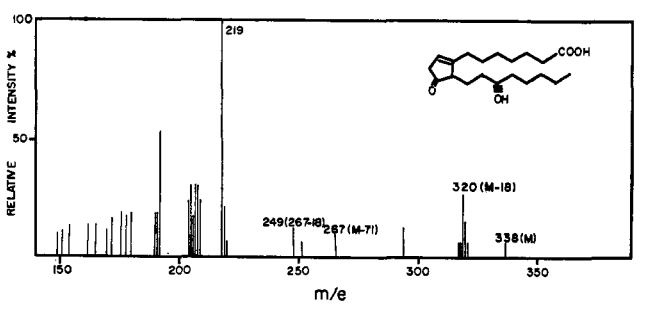


FIGURE 10: The mass spectrum of 15(*S*)-hydroxy-11-oxo-8(9)-prostenoic acid (dihydro-PGD_1-AI).

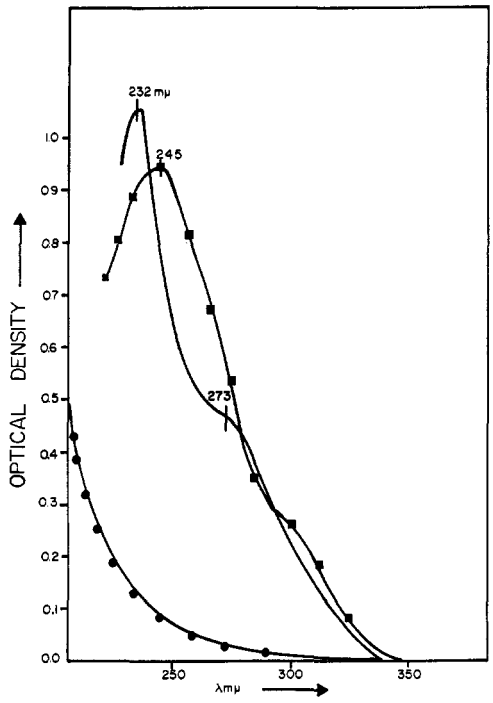
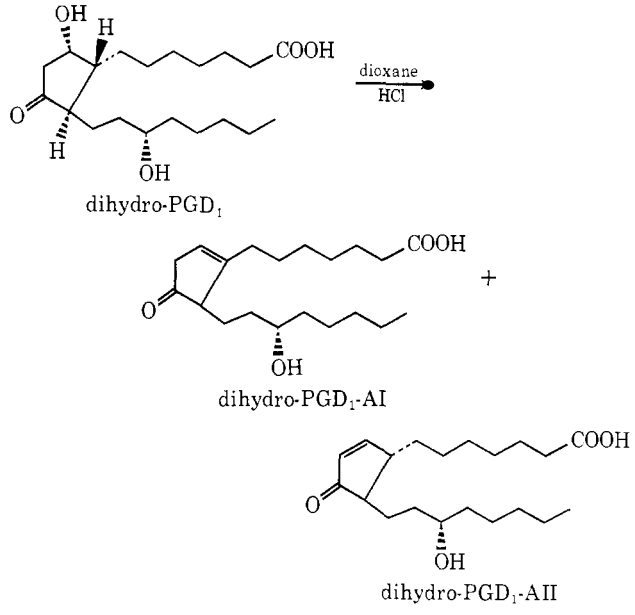


FIGURE 11: Effect of alkali on 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid. The concentration of PGD_1 was 0.0588 mg/ml of 0.05 M NaOH. (●) PGD_1; (■) after 3.5 min; (—) after 145 min.

predominant product, which was found to be identical with an authentic sample with respect to infrared and mass spectra, mixture melting point, and chromatographic behavior. The absolute configuration of PGD_1 was therefore assigned as 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid.



When PGD_1 was treated with sodium hydroxide, a complex mixture of isomers were formed. When this reaction was followed spectrophotometrically, it was seen that the chromophore, first appearing at 245 mμ, gradually gave way to that of 232 mμ with a shoulder at 273 mμ (Figure 11). Mass spectral analysis of the mixture gave a parent ion of *m/e* 336 (M), 318 (M − 18), and 247 (318 − C_5H_{11}). Due to the limited supply of PGD_1 and the difficulty in separating the

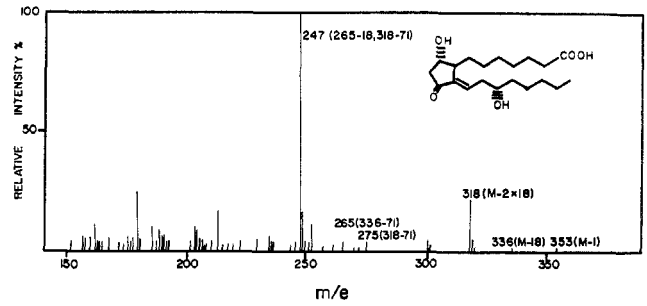
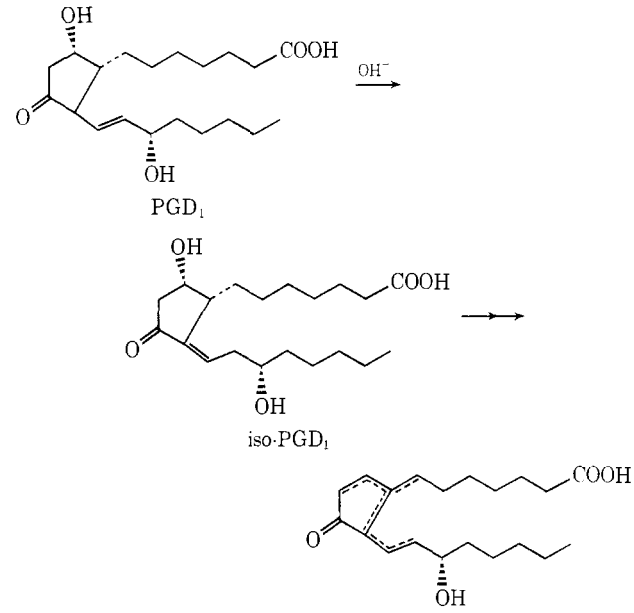


FIGURE 12: The mass spectrum of 9α,15(*S*)-dihydroxy-11-oxo-12(13)-*trans*-prostenoic acid (iso-PGD_1).

alkali reaction products, this avenue was not pursued further. However, the susceptibility of the C-13(14) double bond to isomerization was readilly observed by passing PGD_1 through a charcoal column to yield iso-PGD_1, which possesses an ultraviolet absorption maximum at 243 mμ (ε 8000). Thus, the 245-mμ chromophore formed during alkali treatment of PGD_1 may well be iso-PGD_1, which could then decompose further to yield a complex mixture of isomers. The mass spectrum of iso-PGD_1 exhibited a parent ion of *m/e* 353 (M − 1), 336 (M − H_2O), 318 (M − $2H_2O$), 275 (318 − C_3H_7), 265 (336 − C_5H_{11}), 247 (265 − H_2O; 318 − C_5H_{11}) (Figure 12).



Discussion

We have previously reported (Sih *et al.*, 1970) that the ratio of prostaglandins can be altered during biosynthesis, depending on the coenzyme used in the incubation mixture. The ratio of PGD_2, PGE_2, and $PGF_{2\alpha}$ obtained with hydroquinone was 3:11:1, respectively, whereas this ratio was changed to 3:13:12 with L-epinephrine. Figure 13 illustrates a chromatographic profile of prostaglandins biosynthesized from tritiated arachidonic acid using serotonin as the coenzyme. Serotonin not only stimulated the overall synthesis of all the PG's but, the ratio of PG's was shifted to 6:18:4, indicating that serotonin especially enhanced the formation of PGD_2; the PGA_2 peak was formed nonenzymatically during isolation.

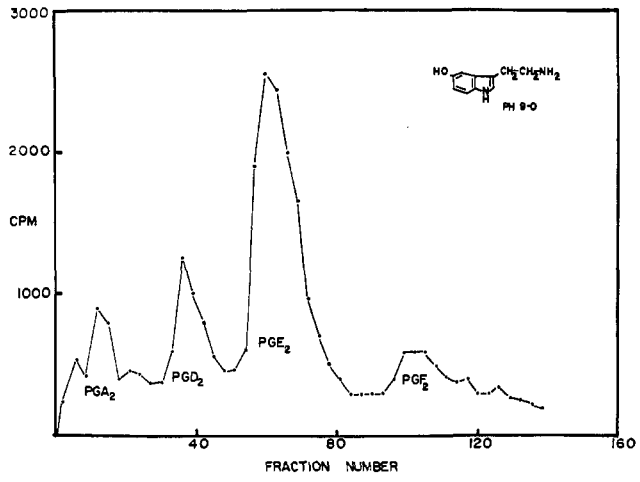


FIGURE 13: Chromatographic separation of PGD_2, PGE_2, and $PGF_{2\alpha}$. The reaction mixture contained 65 μmoles of arachidonic acid (5 μCi of tritium), 1.64 mmoles of serotonin, and 1 g of microsomes in a total volume of 100 ml of 0.05 M Tris buffer (pH 9.0). After incubation for 1 hr at 37°, the contents were acidified to pH 2.0 and extracted with ethyl acetate. The ethyl acetate residue was chromatographed over a silicic acid column (0.8 × 20 cm). The column was eluted with a linear gradient consisting of 300 ml of benzene–ethyl acetate (7:3) in the mixing flask and 300 ml of ethyl acetate in the reservoir flask. Each fraction contained 7.5 ml (Foss *et al.*, 1971).

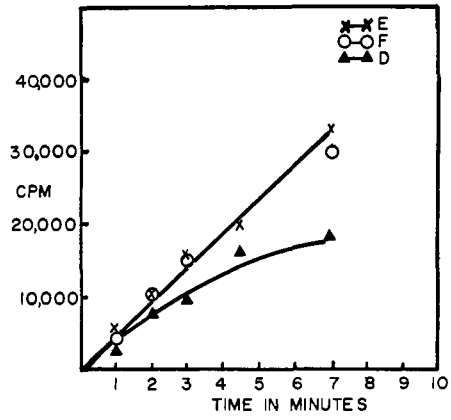


FIGURE 14: The kinetics of PGD_2, PGE_2, and $PGF_{2\alpha}$ biosynthesis from arachidonic acid. The conditions of incubation are same as those described in Figure 12 except L-epinephrine was used as the coenzyme. At the indicated time intervals, samples were taken and analyzed chromatographically. The radioactive peaks were pooled and assayed (Foss *et al.*, 1971).

The compound PGD_1, derived from 8,11,14-eicosatrienoic acid, was identified as 9α,15(*S*)-dihydroxy-11-oxo-13-*trans*-prostenoic acid, which confirms the assignment made earlier by Granstrom *et al.* (1968). The stereochemistry at C-8 and C-12 were defined by circular dichroism studies on PGD_1 and dihydro-PGD_1-AII. The former gave a negative cotton effect while the latter exhibited a strong positive cotton effect. These properties are reminiscent of PGE and PGA, respectively (Korver, 1969). Reduction of PGD_1 with $NaBH_4$ afforded a 10:1 mixture of $PGF_{1\alpha}$ and 11-epi-$PGF_{1\beta}$. Although PGE_1 is readily converted into PGA_1 in acid and into PGB_1 in base, in contrast, PGD_1 yields a complex mixture of isomeric products in both acidic or basic media. Competition of the isomerization of the C-13 (14) double bond with the dehydration of the 9α-hydroxyl group, could produce this complex mixture of isomeric products. The relative simplicity of the product profile of dihydro-PGD_1 in acid and base lends credence to this suggestion.

It was proposed (Granstrom *et al.*, 1968) that an endoperoxide intermediate is involved in the biosynthesis of PGD_1, PGE_1, and $PGF_{1\alpha}$. This would entail the reductive cleavage of the cyclic endoperoxide to $PGF_{1\alpha}$ or the removal of either the hydrogen atom at C-9 or C-11 to yield PGE_1 or PGD_1, respectively. It is interesting to note that $PGF_{1\alpha}$, biosynthesized from [9-^{3}H,3-^{14}C]8,11-14-eicosatrienoic acid retained the ^{3}H label and was enriched 1.36 times with respect to tritium of the precursor. A slight enrichment of tritium in $PGF_{1\alpha}$ (1.12) was also observed with [11-^{3}H,2-^{14}C]8,11-14-eicosatrienoic acid (Granstrom *et al.*, 1968; Hamberg and Samuelsson, 1967). These small isotope effects indicate that the abstraction of hydrogen at C-9 or C-11, which proceeds with isotopic discrimination, is operating at a rate comparable in magnitude to the reduction of the endoperoxide to PGF.

The kinetics of formation of PGD_2, PGE_2, and $PGF_{2\alpha}$ from tritiated arachidonic acid is shown in Figure 14. While both PGE_2 and $PGF_{2\alpha}$ appear to be biosynthesized at a linear rate for at least 7 min, the rate of PGD_2 formation has virtually leveled off after 5 min. If one accepts the endoperoxide intermediate, this kinetic data further substantiate that the abstraction of hydrogens at C-9 and C-11 must be enzymatically catalyzed. If the decomposition of the endoperoxide is nonenzymatic, the rates of formation of these three prostaglandins should then be identical. It was implied (Granstrom *et al.*, 1968) that the enzyme complex may be partially destroyed during isolation and that this results in loss of specificity during isolation and that this results in loss of specificity in the removal of hydrogens at C-11 and C-9. However it is difficult to explain the enhancement of PGD_1 formation by using serotonin as the coenzyme or by the addition of the supernatant fraction to ovine seminal vesicle microsomes (Granstrom *et al.*, 1968; Foss *et al.*, 1971). Further studies are needed to clarify this enigma.

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