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MASS SPECTROMETRIC EVIDENCE FOR THE CONVERSION OF EXOGENOUS ADRENATE TO DIHOMO-PROSTAGLANDINS BY SEMINAL VESICLE CYCLO-OXYGENASE

A COMPARATIVE STUDY OF TWO ANIMAL SPECIES

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SUMMARY

We present conclusive mass spectrometric evidence for the synthesis of 1*a*,1*b*-dihomo-prostaglandin E_2 and 1*a*,1*b*-dihomoprostaglandin $F_{2\alpha}$ by sheep seminal vesicle microsomes and swine whole vesicular homogenates. Swine microsomes prepared according to standard procedure were catalytically inactive, possibly due to autoinactivation of the cyclo-oxygenase caused by metabolism of endogenous substrate released during the isolation procedure. Cyclo-oxygenase activity could be demonstrated, however, when the porcine vesicles were homogenized in the presence of exogenous substrate. Chemical and mass spectrometric evidence presented here allow unequivocal structural assignments.

INTRODUCTION

Prostaglandins (PGs), the products of cyclo-oxygenase (CO) transformation of polyunsaturated fatty acids, are important mediators of intercellular communication and/or modulators of vital biological functions [1, 2]. Therefore, knowledge of the biochemical fate of those fatty acids — whether dietary or lipid store components — should advance our understanding of the complex relationships between nutrition and health. The classical PGs are a group of C_{20} oxygenated fatty acids derived primarily from eicosa-(5*Z*,8*Z*,11*Z*,14*Z*)-tetraenoic acid (arachidonic, 20:4ω6).

Docosapolyenoates, i.e. C_{22} fatty acids, are present in relatively large quantities in some mammalian organs, such as brain, gonads, kidney and

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adrenal glands. The question of their biological role, although a subject of obvious academic and possibly practical interest, is one that thus far has received little attention. Specifically, the fundamental question as to whether the C_{22} fatty acids have a structural function in membranes or are important as PG precursors, or both, has not yet been satisfactorily answered. Sprecher et al. [3] were first to address the question in a systematic way. They reported the conversion of docosa-(7*Z*,10*Z*,13*Z*,16*Z*)-tetraenoic acid (adrenic, 22:4ω6) into dihomo-PGs, -prostacyclin and -thromboxane by renal medullary microsomes of rabbit. Later, the metabolism of adrenate to dihomo-thromboxane was confirmed in human platelets [4].

In this paper we describe the results of a comparative study on the utilization of adrenic acid by ovine and porcine seminal vesicle CO and provide conclusive evidence for the ability of both animal species to synthesize dihomo-PGs of the E and F series. We prepared two different derivatives of each prostaglandin and provided previously unavailable reference mass spectra obtained under standard conditions (electron impact, 70 eV). We also report an apparent age effect on the PGE/PGF production ratio in the ram.

EXPERIMENTAL

Materials and reagents

Seminal vesicles were obtained from sexually mature rams and boars raised on the USDA farm in Beltsville, MD, U.S.A. The organs were excised from anesthetized (pentobarbital) animals, rinsed with cold saline and freed from all visible fat. They were finally blotted dry and used immediately or stored at −70°C until used.

Adrenic acid was purchased from Nu Chek Prep (Elysian, MN, U.S.A.). Glutathione (GSH) was from Sigma (St. Louis, MO, U.S.A.); hydroquinone and O-methylhydroxylamine hydrochloride were from Eastman Kodak (Rochester, NY, U.S.A.). Columns of octadecasilyl-silica (Sep-Pak C_{18} cartridges) were purchased from Waters Assoc. (Milford, MA, U.S.A.); silicic acid was Bio-Sil A (100—200 mesh) from Bio-Rad Labs. (Richmond, CA, U.S.A.). Trimethylsilylimidazole (TSI) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Pierce (Rockford, IL, U.S.A.) and *n*-butylboronic acid from Applied Science Labs. (State College, PA, U.S.A.). All solvents were analytical grade and were glass-distilled before use.

Preparation of microsomes

Portions of the vesicles were partially thawed, diced with a scalpel, then suspended at 7 ml/g of tissue in cold (4°C) 50 m*M* phosphate buffer, pH 7.4, and homogenized on ice with a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 800 *g* for 10 min in a Beckman J-21B centrifuge at 4°C to remove residual fat, nuclei and cell debris. Mitochondria were sedimented by centrifuging the supernatant at 8000 *g* for 10 min. The resulting supernatant was centrifuged for 60 min at 105 000 *g* in a Beckman L8-80 ultracentrifuge using a 50Ti fixed-angle rotor. The resulting pellet of microsomes was gently suspended in pH 7.4 phosphate buffer with a PTFE pestle homogenizer and recentrifuged for another 60 min at 105 000 *g*. The final pellet was

resuspended in a measured volume of 50 m*M* phosphate buffer (pH 7.4) and used as our source of enzyme either fresh or after freezing at −20° C for up to five days.

Biosynthesis and extraction of prostaglandins

From ovine microsomes. In a typical experiment, 2.5 ml of the ram vesicle microsomal suspension, corresponding to 1 g of wet tissue, were incubated at 37° C in a water bath for 20 min with 1 mg of adrenic acid in the presence of GSH (0.14 mg) and hydroquinone (0.28 mg). The reaction was terminated by acidification (formic acid) to pH 3.3. The incubation medium was extracted twice with an equal volume of ethyl acetate—hexane (1:1, v/v). The combined organic phases were evaporated to dryness under nitrogen, the residual moisture was azeotroped with absolute ethanol and the residue was treated with excess ethereal diazomethane. The dry residue after methylation was chromatographed over silicic acid as described below. Control experiments consisted of incubations without microsomes or with boiled microsomes.

From porcine whole vesicles. In a typical experiment, 1 g of swine vesicle was homogenized in 12 ml of 50 m*M* phosphate buffer, pH 7.4, at 4° C in the presence of 0.5 mg of adrenic acid. After centrifugation (500 *g* for 5 min) the supernatant was acidified and passed through a C_{18} Sep-Pak cartridge. The PG fraction was eluted as described previously [5], then it was methylated with diazomethane, chromatographed over silicic acid and further derivatized as described below.

Chromatography

Silicic acid chromatography was performed on activated silicic acid columns (1 g). After the crude residue was deposited on the column, it was washed with 7 ml of ethyl acetate. The primary PG fraction was then eluted with 8 ml of ethyl acetate—methanol (9:1, v/v). The residue after solvent evaporation was derivatized and analyzed as described below.

Derivatizations

The nomenclature for dihomo-PGs used here is that recommended by Nelson [6]. For gas chromatographic—mass spectrometric (GC—MS) analysis, the methyl ester of 1*a*,1*b*-dihomo-$PGF_{2\alpha}$, after elution from the silicic acid column, was trimethylsilylated by treatment with BSTFA—pyridine (1:1, v/v). The 9,11-*n*-butylboronate ester-15-trimethylsilyl (TMS) ether derivative of 1*a*,1*b*-dihomo-$PGF_{2\alpha}$ was prepared by heating (85°C for 30 min) the methyl ester with 250 μg of *n*-butylboronic acid in 50 μl of dry pyridine; the residue from solvent evaporation was then trimethylsilylated with BSTFA in pyridine. The methoxime of 1*a*,1*b*-dihomo-PGE_2 was prepared by heating the methyl ester with 50 μl of a saturated pyridine solution of O-methylhydroxylamine hydrochloride for 1 h at 42° C. The PG derivatives were extracted from the reaction mixture with several portions of diethyl ether after evaporation of pyridine with a stream of nitrogen. The methyl ester of 1*a*,1*b*-dihomo-PGE_2 was converted also to the PGB form by treatment with TSI in piperidine (1:1, v/v) at room temperature [7]. This reagent simultaneously effects the trimethylsilylation of the hydroxyl at the C-15 position [8].

Gas chromatography—mass spectrometry

GC—MS was carried out with Hitachi RMU-6E and Finnigan 3200F instruments interfaced with an INCOS 2300 data system. The glass columns, 1.8 m × 2 mm I.D., were treated with dimethylchlorosilane and packed with 1% OV-101 or 1% OV-17 on 100—120 mesh Gas-Chrom Q. The column temperature was programmed to rise from 180 to 250°C at 4°C/min and remain isothermal thereafter. The injector was heated at 235°C; the separator and the ion source in the Hitachi instrument were kept at 240°C. The Finnigan interface was also kept at 240°C and the analyzer at 95°C. The flow-rate of the carrier gas (helium) was 20 ml/min. We used the Hitachi GC—MS system exclusively to obtain full spectra and the Finnigan system primarily for selected-ion monitoring (SIM) MS. The mass spectra were recorded at 70 eV.

RESULTS AND DISCUSSION

A typical reconstructed ion chromatogram of a dihomo-PG mixture from ram vesicular microsomes incubated with adrenic acid is shown in Fig. 1.

Mass spectra

The mass spectra obtained with the magnetic sector (Hitachi) instrument of the four PG derivatives prepared as described above are shown in Fig. 2—5. All of the spectra had a clearly identifiable molecular ion. The fragmentation patterns, when compared with those of the classical C_{20} PGs (vide infra), appear consistent with the structures indicated in the panels. Specifically, the double

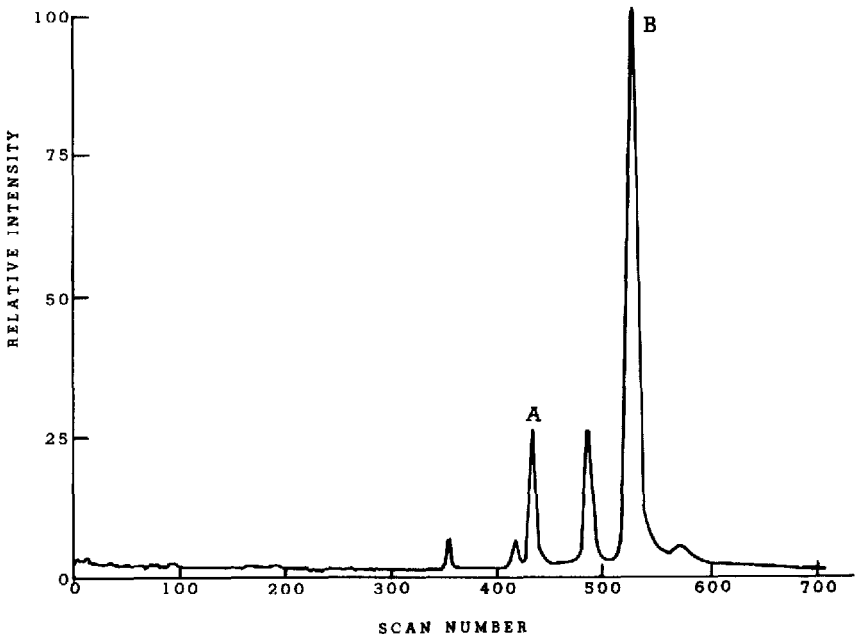

Fig. 1. Typical computer-reconstructed total-ion chromatogram (6-ft. 1% OV-17 column) of a purified dihomo-prostanoid mixture from vesicular microsomes incubated with adrenic acid. Peaks: A = 1*a*,1*b*-dihomo-$PGF_{2\alpha}$-methyl ester-tris-TMS ether derivative; B = 1*a*,1*b*-dihomo-PGE_2-methyl ester-methyloxime-bis-TMS ether derivative (major isomer).

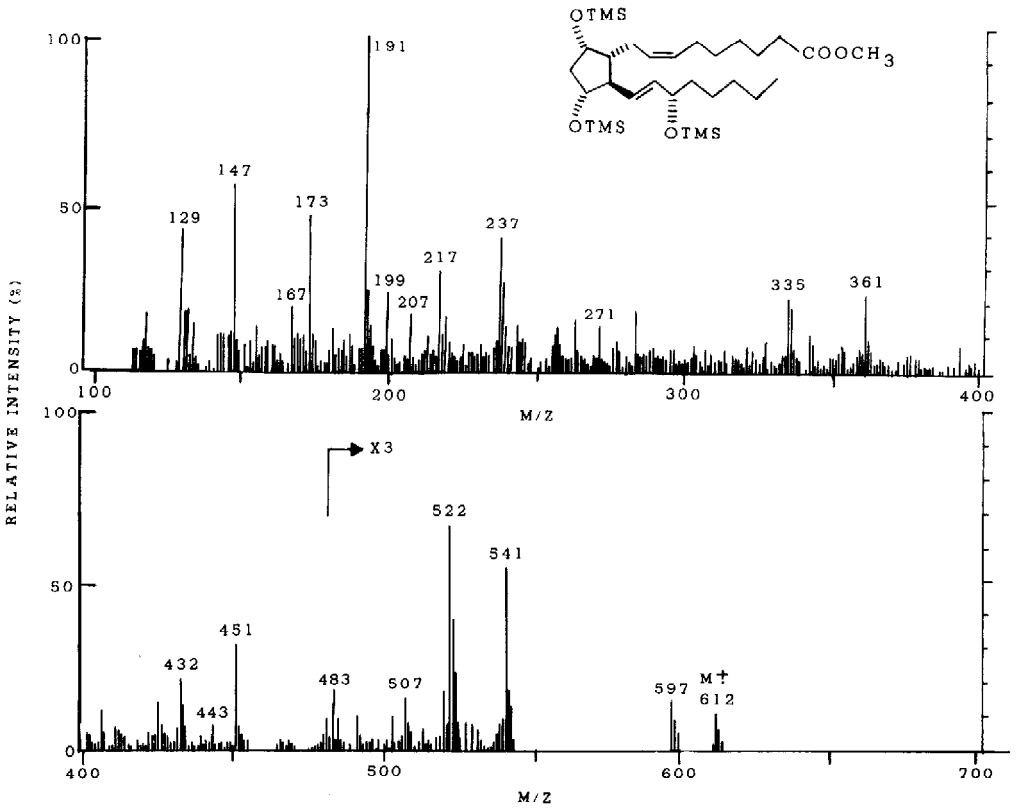

Fig. 2. Electron-impact mass spectrum of 1*a*,1*b*-dihomo-$PGF_{2\alpha}$-methyl ester-tris-TMS ether derivative.

bonds at C-10,11 and C-13,14 in the substrate have been involved in the formation of the cyclopentane ring. Furthermore, the mass spectra of two derivatives of each dihomo-PG provide corroborating chemical evidence for the proposed structures. The steric arrangement of substituents about the asymmetric centers is assumed to be identical to that of the classical PGs.

1a,1b-Dihomo-$PGF_{2\alpha}$-methyl ester-tris-TMS ether derivative. The mass spectrum (Fig. 2) had diagnostically important ions at *m/z* 612 $[M]^+$; *m/z* 541 $[M - 71]^+$, loss of the radical $\cdot CH_2(CH_2)_3CH_3$; *m/z* 522 $[M - 90]^+$, loss of the neutral molecule $(CH_3)_3SiOH$; *m/z* 451 $[M - (71 + 90)]^+$; *m/z* 432 $[M - (2 \times 90)]^+$; *m/z* 361 $[M - (71 + 2 \times 90)]^+$; *m/z* 271 $[M - (71 + 3 \times 90)]^+$. Ions which are homologous to those just described (28 a.m.u. lower), resulting from identical fragmentations, are present in previously published spectra of $PGF_{2\alpha}$ and of its tetradeutero analogue [9]. The equivalent of the ion at *m/z* 483, presumably arising from the loss of the radical $\cdot CH_2(CH_2)_4COOCH_3$ (129 a.m.u.), is more clearly visible in the spectrum of the tetradeutero-$PGF_{2\alpha}$ [9] at *m/z* 483 $[M - 105]^+$, loss of $\cdot C^2H_2C^2H_2CH_2COOCH_3$. Similarly, an *m/z* 443 $[M - 169]^+$ ion resulting from loss of $\cdot CH_2CH{=}CH(CH_2)_5COOCH_3$ (Fig. 2) is also present in the spectrum of $PGF_{2\alpha}$ $[M - 141]^+$ [9]. The fragments at *m/z* 199 and 173 are probably rearrangement ions arising from cleavage of the C-13 to C-20 side-chain as

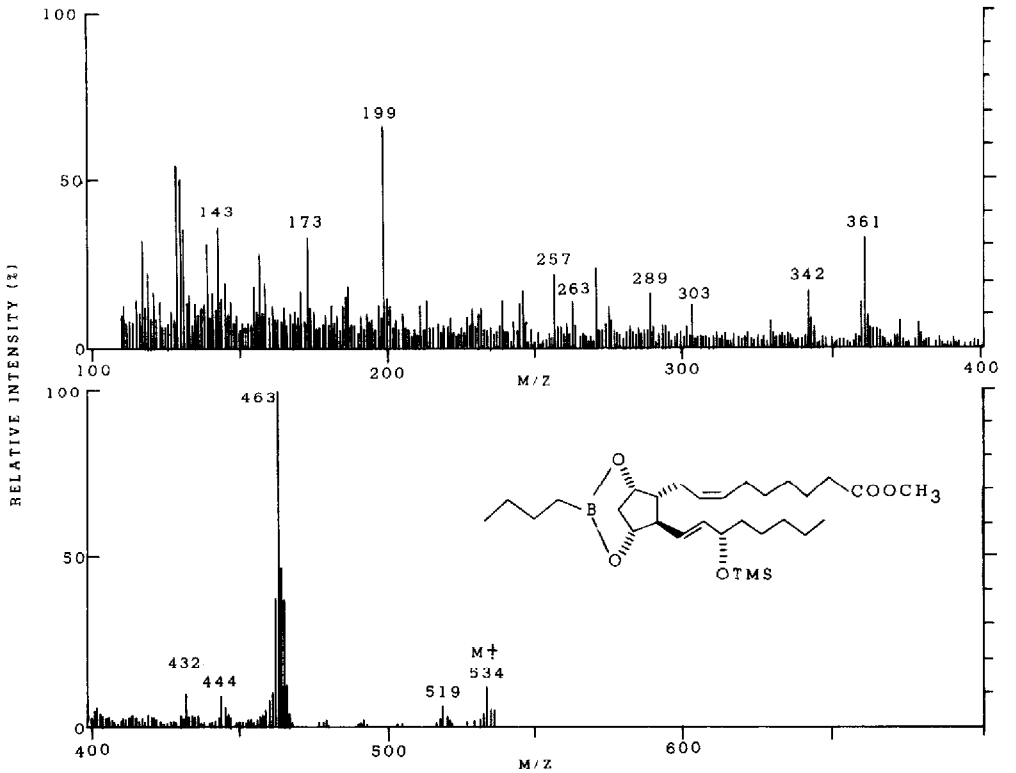

Fig. 3. Electron-impact mass spectrum of 1*a*,1*b*-dihomo-$PGF_{2\alpha}$-methyl ester-9,11-*n*-butylboronate-15-TMS ether derivative.

proposed by Middleditch and Desiderio [10] for the PGE series. Origins and elemental compositions of the ions at *m/z* 237, 217, 207, 191, 167, 147 and 129 in the spectrum of Fig. 2 are less clear. It is noteworthy that the spectrum of the classical $PGF_{2\alpha}$ is strikingly similar in that same mass range [9].

1a,1b-Dihomo-$PGF_{2\alpha}$-methyl ester-9,11-n-butylboronate-15-TMS ether derivative. Prominent ions in the high mass range of the spectrum (Fig. 3) were at *m/z* 534 $[M]^{\cdot+}$; *m/z* 463 $[M - 71]^{+}$, base peak, loss of $\cdot (CH_2)_4CH_3$; *m/z* 444 $[M - 90]^{\cdot+}$, loss of trimethylsilanol; *m/z* 361 $[M - (71 + 102)]^{+}$ and *m/z* 342 $[M - (90 + 102)]^{\cdot+}$. The fragment of mass 102 represents the elements of *n*-butylboronic acid. The elimination of alkylboronic acids from PG derivatives is well documented in the literature [11, 12]. This elimination largely orients the fragmentation on electron impact. Thus, other diagnostically important ions were at *m/z* 432 $[M - 102]^{\cdot+}$; *m/z* 303 $[M - (102 + 129)]^{+}$; and *m/z* 263 $[M - (102 + 169)]^{+}$. As in the spectrum of the tris-TMS derivative (vide supra) the 129 and 169 a.m.u. fragments are presumably the radicals $\cdot (CH_2)_5COOCH_3$ and $\cdot CH_2CH{=}CH(CH_2)_5COOCH_3$, respectively.

1a,1b-Dihomo-PGE_2-methyl ester-methyloxime-bis-TMS ether derivative. The spectrum of this compound (Fig. 4) is characterized by a weak molecular ion (*m/z* 567) and a relatively strong $[M - 31]^{+}$ at *m/z* 536 originating from loss of $\cdot OCH_3$ from the methoxime moiety. Other prominent ions in the high mass range were at *m/z* 496 $[M - 71]^{+}$; *m/z* 477 $[M - 90]^{\cdot+}$; *m/z* 446 [M — (31 +

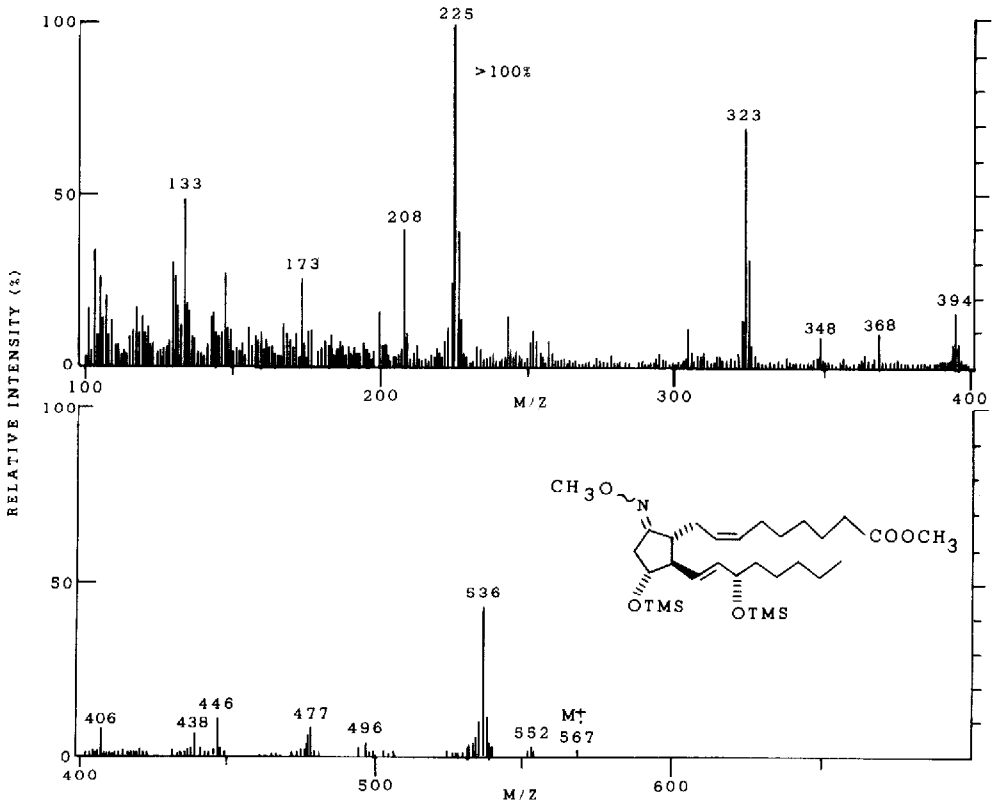

Fig. 4. Electron-impact mass spectrum of 1*a*,1*b*-dihomo-PGE_2-methyl ester-methyloxime-bis-TMS ether derivative (major isomer).

90)] $^+$; and *m/z* 406 [M − (71 + 90)] $^+$. Other relatively weak but diagnostically important ions, the analogues of which were also observed in the spectra of the two dihomo-$PGF_{2\alpha}$ derivatives just discussed, were: *m/z* 368 [M − 199] $^+$, *m/z* 394 [M − 173] $^+$, *m/z* 438 [M − 129] $^+$ and *m/z* 348 [M − (129 + 90)] $^+$. The *m/z* 225 ion (base peak) is very prominent also in spectra of methyloxime derivatives of C_{20} PGE_2. It has been proposed that it arises from ring cleavage at C-10/11 and C-8/12, based on high resolution data and on spectra of isotopically labeled species [10]. The genesis of the second most intense ion at *m/z* 323, possibly homologous to the *m/z* 295 ion in the spectrum of the C_{20} PGE_2 derivative [13], is not clear.

1a,1b-Dihomo-PGB_2-methyl ester-TMS ether derivative. The mass spectrum of this derivative (Fig. 5) is virtually identical to that of the C_{20} PGB_2 homologue [7], except for the fact that all of the relevant fragments have moved 28 a.m.u. higher. This parallelism offers another valuable criterion for structural confirmation of the C_{22} species. Prominent diagnostic ions are: *m/z* 448 [M] $^+$; *m/z* 433 [M − CH_3] $^+$; *m/z* 377 [M − 71] $^+$; *m/z* 349 [M − (71 + 28)] $^+$, base peak; and *m/z* 275 [M − 173] $^+$. The weak ions at *m/z* 419 [M − 29] $^+$ and *m/z* 417 [M − 31] $^+$ are characteristic of methyl esters [14]; their equivalents were observed in the spectrum of PGB_2-methyl ester [7].

In the mass spectra of dihomo-PGE_2 and dihomo-$PGF_{2\alpha}$ previously presented

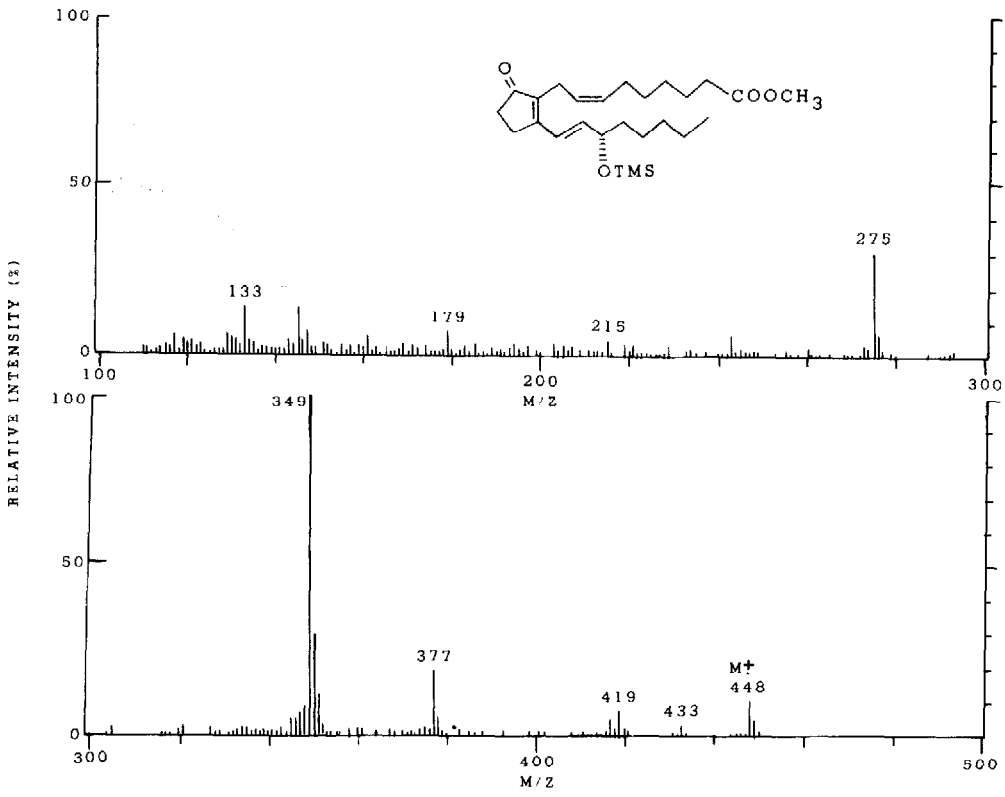

Fig. 5. Electron-impact mass spectrum of 1*a*,1*b*-dihomo-PGB$_2$-methyl ester-TMS ether derivative.

[3] there are several unaccountable major fragment ions 2 a.m.u. higher than those indicated as having diagnostic value. The problem is particularly severe in the critical molecular ion region. This is probably due to either an active GC—MS interface or to the presence of unidentified structurally related impurities. Whereas we do not question the correctness of the authors' conclusions as to the identities and structures of the dihomo-PGs, we believe that the MS evidence they submitted [3] does not adequately support those conclusions. In 1975 Tobias et al. [15] described cyclo-oxygenation of adrenic acid by acetone—pentane powder of sheep vesicular microsomes and identified the metabolites as dihomo-PGE$_2$ and dihomo-PGF$_{2\alpha}$. Those authors based their structural assignments on chemical ionization MS with methane as the reactant gas.

Species differences

Ram seminal vesicle microsomes readily metabolized adrenate to dihomo-PGs. Conversely, swine vesicular microsomes, prepared by identical procedure, appeared totally devoid of CO activity, as judged by lack of PGE and PGF formation from exogenous adrenate as well as arachidonate. That the swine organ does have CO activity in vivo is attested by the fact that boar semen contains PGs, although in much smaller quantities than found in ram semen [16]. We determined that the in vitro biosynthesis of PGs by the

porcine vesicles occurs only if tissue grinding is done in the presence of adrenate or arachidonate (see Experimental section). Even when the substrate was added immediately after homogenization, cyclo-oxygenation did not take place.

We made separate attempts to stimulate CO activity in swine microsomes. Namely: (a) preincubation of microsomes with phenol in phosphate buffer for 30 min [17, 18]; (b) addition of dithiothreitol to the incubation medium; (c) use of norepinephrine [19] in place of hydroquinone as cofactor; (d) the possibility that excess GSH peroxidase activity might be present in the incubation medium [18] was dealt with by adding N-ethylmaleimide to remove the mercaptan. The outcome of these experiments was uniformly and totally negative even when SIM-MS was used to maximize the instrumental sensitivity for detection of PGs. Perhaps autoinactivation of the swine vesicular CO, brought about by metabolism of endogenous substrate released during microsomal preparation, caused loss of activity. The reason why such inactivation occurs with swine tissue and not with the ovine counterpart remains to be established. To our knowledge, the ability of porcine vesicular microsomes to utilize exogenous adrenate has not been studied previously.

Possible age effect in the ram

As expected [20], we invariably obtained a large excess of PGE_2 over $PGF_{2\alpha}$ when we incubated ram microsomes with arachidonate in the presence of GSH and hydroquinone. With adrenate as the substrate, however, 1*a*,1*b*-dihomo-$PGF_{2\alpha}$ was the predominant product with microsomes from an animal that had just reached sexual maturity; conversely, the yield of dihomo-PGE_2 was greater than that of dihomo-$PGF_{2\alpha}$ when the vesicles were from a three-year-old animal. Similar observations were made in repeated experiments conducted under identical preparation and incubation conditions. Quantitative comparisons were based on computer-reconstructed total-ion chromatograms of purified dihomo-PG mixtures from microsomes incubated with adrenate (Fig. 1). If this is indeed an age effect, it might be physiologically significant, e.g. related to variation of the organ function. Seasonal, dietary and other factors, however, might have played a role in producing the observed differences. A clear and definitive test of age requires further experimentation.

CONCLUSIONS

The results of our study confirm that ram seminal vesicle microsomes can utilize exogenous adrenate for the synthesis of C_{22} primary prostaglandins. Furthermore, we demonstrated that: (a) swine vesicular microsomes prepared according to standard procedure are unable to utilize either adrenic or arachidonic acid; (b) CO activity in porcine vesicles can be demonstrated if the tissue is homogenized in the presence of substrate. The GC—MS data and interpretation outlined herein provide more conclusive evidence for the proposed structures of dihomo-PGE_2 and -$PGF_{2\alpha}$. Our observations as well as previous findings from other investigators support the hypothesis that certain C_{22} fatty acids (docosapolyenoates) may have a biological role as PG precursors in mammals. Very recently, the synthesis of dihomo-PGs from adrenic acid by cultured human endothelial cells was reported [21].

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