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PRODUCTION OF PROSTAGLANDINS BY THE PSEUDOPREGNANT RAT UTERUS, *in vitro*, AND THE EFFECT OF TAMOXIFEN WITH THE IDENTIFICATION OF 6-KETO-PROSTAGLANDIN $F_{1\alpha}$ AS A MAJOR PRODUCT

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1 Prostaglandin production by rat uterus homogenates has been studied, *in vitro*, on days 2 to 13 of pseudopregnancy.

2 The highest production of prostaglandins occurred on day 5.

3 The amounts of prostaglandins F and D formed were higher than the amounts of prostaglandin E on every day studied.

4 The ratios of prostaglandins F and D to prostaglandin E produced steadily decreased up to day 6. It then increased with the highest values occurring between days 10 and 13.

5 Progesterone levels in peripheral plasma increased rapidly from days 2 to 5, remained high up to day 9, then steadily decreased between days 10 and 13.

6 The anti-oestrogenic drug, tamoxifen administered on day 2, significantly inhibited the increase of prostaglandin production which occurred on day 5. Prostaglandin E production was inhibited more than the production of prostaglandins F and D.

7 Analysis of the uterine extracts by gas chromatography and mass spectrometry showed prostaglandin $F_{2\alpha}$, $F_{1\alpha}$ (in trace amounts), E_2 and D_2 to be present.

8 The major product detected was 6-keto-prostaglandin $F_{1\alpha}$. Its identification forms an addendum to the paper.

9 Also present as a major product was 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid.

Introduction

Oestradiol treatment of ovariectomized rats increases the prostaglandin $F_{2\alpha}$ synthesizing capacity of the uterus *in vitro* by increasing the amount of prostaglandin synthetase present (Ham, Cirillo, Zanetti & Kuehl, 1975). Oestradiol treatment of ovariectomized guinea-pigs produces similar results (Naylor & Poyser, 1975) and the action of the oestradiol is not affected by the simultaneous administration of progesterone. During the oestrous cycle of the guinea-pig, the prostaglandin $F_{2\alpha}$ synthesizing capacity of the uterus increases towards the end of the cycle (Poyser, 1972) and prostaglandin $F_{2\alpha}$ levels in the uterine venous plasma rise (Blatchley, Donovan, Horton & Poyser, 1972; Earthy, Bishop & Flack, 1975). Oestradiol output from the ovary increases during this period (Joshi, Watson & Labhsetwar, 1973; Blatchley, Maule, Walker & Poyser, 1975), so it is possible that oestradiol is the physiological stimulus for increased synthesis and release of prostaglandin $F_{2\alpha}$ from the uterus.

There is much clinical interest at present in drugs which inhibit prostaglandin synthesis. The non-steroidal, anti-inflammatory drugs inhibit prostaglandin synthetase (Vane, 1971) and thereby prevent the conversion of the precursor fatty acids into prostaglandins. Prostaglandin synthetase is apparently inactivated during the formation of prostaglandins (Lands, LeTellier & Vanderhoek, 1973), so the continual synthesis of new enzyme would appear a critical factor in determining the amounts of prostaglandins formed by a tissue. If the levels of prostaglandin synthetase in certain tissues are under hormonal control, drugs which inhibit the action and/or release of the hormone should maintain low levels of enzyme in the tissue and thereby reduce the prostaglandin synthesizing capacity of that tissue. Such drugs may be of clinical importance in inhibiting prostaglandin synthesis.

In this study, we have tested the effect of the anti-oestrogen, tamoxifen (Harper & Walpole, 1967) on

the prostaglandin synthesizing capacity, *in vitro*, of the pseudopregnant rat uterus. Tamoxifen is an anti-oestrogenic drug in the rat, antagonizing the effects of circulating oestrogen at the tissue level (Harper & Walpole, 1967). It has also been shown to reduce the output of oestradiol from the rat ovary, either by directly inhibiting the ovarian synthesis of oestradiol, or indirectly by blocking follicle stimulating hormone release from the pituitary (Watson, Anderson, Alum, O'Grady & Heald, 1975). Plasma oestradiol levels in the pseudopregnant rat show a small rise up to day 4 (though the peak level may be late on day 3), fall to a lower level which is maintained up to about day 9 or 10, then increase rapidly over the next 3 days (Welschen, Osman, Dullaart, De Greef, Uilenbroek & De Jong, 1975). Initially, we had to study how much prostaglandin was produced normally by the rat uterus on each day of pseudopregnancy. This is of interest also since prostaglandin $F_{2\alpha}$ released from the rat uterus may be the endogenous substance which terminates pseudopregnancy in the rat (Saksena, Watson, Lau & Shaikh, 1974; Weems, Pexton, Butcher & Inskeep, 1975; Castracane & Shaikh, 1976).

Methods

Female Wistar rats were housed in conditions of 12 h day and 12 h night, with the light changes occurring at 8 h 00 min and 20 h 00 min respectively. They received food and water *ad libitum*. Vaginal smears were taken daily and examined microscopically. Day 1 of the oestrous cycle was taken as the day of maximum cornification of the smear, preceding the day of leucocytic infiltration. All rats used had exhibited regular 4 day cycles. Pseudopregnancy was achieved by mating the female rats with a vasectomized male. Mating was assumed to have taken place during the dark period between day 4 and day 1. This latter day now became day 1 of pseudo-pregnancy. All rats, except two, became pseudo-pregnant at the expected time as assessed by the lack of a subsequent oestrus and/or by the determination of peripheral plasma progesterone levels. The pseudo-pregnant rats were used in the following two experiments.

Prostaglandin production by pseudopregnant rat uterus in vitro

Four or five rats per day were killed on the mornings of days 2 to 13 of pseudopregnancy. Each rat was lightly anaesthetized with ether and a 2 ml sample of blood withdrawn from the heart into a heparinized syringe. The blood was centrifuged, the plasma withdrawn and stored at −20°C. Each rat was killed by incising the neck. The uterus was removed, weighed, homogenized and incubated for 90 min in the same manner as described previously for the guinea-pig uterus (Poyser, 1972). The pH of the incubate was then lowered to 4.5 with 1 N HCl and the prostaglandins present extracted with re-distilled ethyl acetate. The ethyl acetate extract was evaporated to dryness on a rotary evaporator at 45°C, and the residue dissolved in 10 ml re-distilled ethyl acetate and stored at −20°C. The amounts of prostaglandins isolated were measured by radioimmunoassay using antisera produced against prostaglandin $F_{2\alpha}$ and E_2. The technique used was identical to that employed for assaying prostaglandins in ethyl acetate extracts of guinea-pig uterus (Mitchell, Poyser & Wilson, 1976). One further cross-reactivity study was performed, using prostaglandin D_2 against the prostaglandin E_2 antibody. Its cross-reactivity was very low (0.003%). Prostaglandin D_2 cross-reacts 17.1% with the $F_{2\alpha}$ antibody.

Progesterone was measured in the peripheral plasma samples of all rats by radioimmunoassay in the manner described previously (Poyser & Horton, 1975), though the plasma usually had to be diluted 10 times with distilled water before assay. The values obtained were in the expected range (Welschen *et al.*, 1975), though marginally lower.

Effect of tamoxifen on prostaglandin production by the pseudopregnant rat uterus in vitro

This study was performed following the results obtained from the first experiment, and in light of the reported finding that tamoxifen administered on day 2 to pregnant rats inhibits the increased output of oestradiol from the ovary which normally takes place on days 3 and 4 (Watson *et al.*, 1975). Five rats were injected subcutaneously at 11 h 00 min on day 2 of pseudopregnancy with 0.05 mg tamoxifen (approx. 0.25 mg/kg). The drug was provided absorbed on to mannitol (1%) and was dissolved in 0.5% aqueous Tween 80 so that 0.5 ml contained 0.05 mg tamoxifen. Five control rats were injected with 0.5 ml aqueous Tween 80 at the same time on day 2. All rats were killed on the morning of day 5. Peripheral plasma levels of progesterone and prostaglandin production by uterine incubates were measured by radioimmuno-assay, as in the first experiment.

Identification of prostaglandins

To facilitate the handling and identification of prostaglandins, samples from several days were pooled together, namely days 2, 4 and 5 (from the first experiment), days 7 and 9, half the samples from each of days 10, 11 and 12, and the samples from the second experiment. This gave 5 pooled samples. (The specific and more detailed analysis of the remaining samples forms an addendum to this paper).

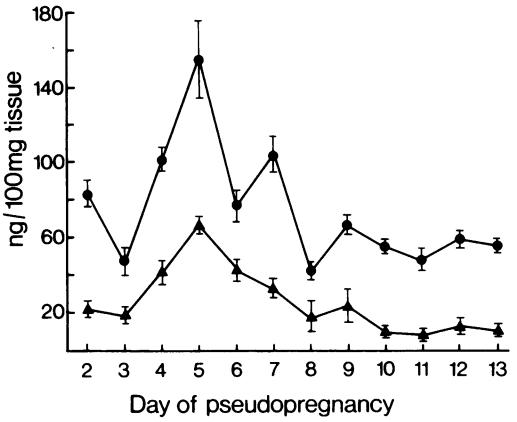

Figure 1 Production of prostaglandins F and D (●) and prostaglandin E (▲) by homogenates of pseudopregnant rat uteri incubated *in vitro*. (See Table 1 for number of animals used per day); s.e. means represent 'between rat' variations.

Each pooled sample was evaporated to dryness and the residue dissolved in 20 ml 67% ethanol. This solution was washed twice with 20 ml petroleum ether (b.p. 60° to 80°C), and then evaporated to dryness. The extract was further purified by silicic acid column chromatography performed as described by Blatchley *et al.* (1972).

Prostaglandins present in the 'F' fraction from the column were converted into the corresponding methyl ester trimethylsilyl ethers (Me-TMS). (Blatchley *et al.*, 1972). The Me-TMS derivatives of authentic prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ were prepared for comparative purposes when the extracts were analysed by combined gas chromatography and mass spectrometry (GC-MS).

Prostaglandins present in the 'E' fraction from the column were converted to the methyl esters by reacting with diazomethane for 15 minutes. The *n*-butyloximes were then prepared by reacting the methyl esters with O-*n*-butylhydroxylamine hydrochloride (5 mg/ml in dry pyridine) for 90 min at 60°C. Finally the trimethylsilyl ethers were prepared by reacting the methyl *n*-butyloximes with 20 µl *N,N*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) at 60°C for 15 minutes. The same derivatives of prostaglandin E_2, E_1, D_2 and 13,14,dihydro-15,keto-prostaglandin $F_{2\alpha}$ were also prepared for comparative purposes when the extracted samples were analysed by GC-MS.

Results

Prostaglandin production by the pseudopregnant rat uterus in vitro

The results are shown in Figure 1 and the amounts of prostaglandins produced are expressed as ng prostaglandin per 100 mg tissue. Levels of prostaglandins F and D were significantly higher on day 2 than on day 3 ($P<0.01$). Levels rose significantly on day 4 ($P<0.01$) and again on day 5 ($P<0.05$), to reach the highest value seen on any day of pseudopregnancy. Levels fell significantly on day 6 ($P<0.01$), rose significantly on day 7 ($P<0.05$), fell significantly on day 8 ($P<0.01$), and rose significantly again on day 9 ($P<0.01$) to remain relatively stable, with small fluctuations, up to the end of pseudopregnancy.

Prostaglandin E levels again showed a peak at day 5, but did not show the same fluctuations from day to day as did the levels of prostaglandins F and D. The levels of prostaglandin E were similar on days 2 and 3, rose significantly on day 4 ($P<0.05$) and again on day

Table 1 Ratio of prostaglandin F and D (PGF and D) to prostaglandin E (PGE) in uterine incubates and peripheral plasma levels of progesterone during pseudopregnancy in the rat

Day of pseudopregnancy	*No. of rats (n)*	*Ratio of PGF and D to PGE*	*Progesterone level ng/ml (mean ± s.e.)*
2	5	3.8	20.1 ± 1.5
3	4	2.6	37.6 ± 3.2
4	5	2.5	50.5 ± 4.0
5	5	2.3	54.0 ± 3.5
6	4	1.8	64.0 ± 6.8
7	5	3.2	56.9 ± 3.2
8	4	2.6	50.8 ± 5.9
9	5	2.9	56.0 ± 3.1
10	4	5.9	42.6 ± 7.4
11	5	5.7	33.4 ± 3.9
12	5	4.7	16.0 ± 3.4
13	4	5.2	8.6 ± 2.4

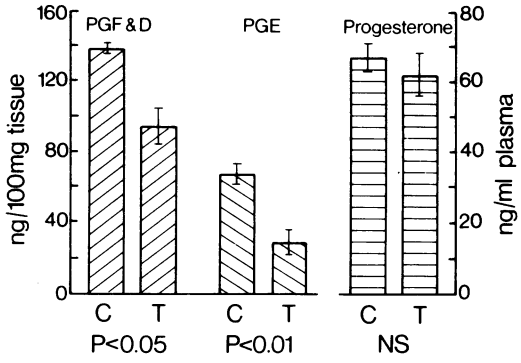

Figure 2 Effect of tamoxifen on production of prostaglandin F and D (PGF & D) and prostaglandin E (PGE) by rat uterus homogenates on day 5 of pseudopregnancy. Peripheral plasma levels of progesterone are also shown. C=control (4 rats); T=tamoxifen-treated (5 rats).

5 ($P<0.01$). From this peak, prostaglandin E levels gradually fell to reach low levels between days 10 and 13.

A comparison of the ratios of the levels of prostaglandins F and D to the level of prostaglandin E is interesting. This ratio steadily decreases from days 2 to 6, possibly indicating that prostaglandin E production is stimulated more than that of F and D. The ratio then shows a sudden rise on day 7, since prostaglandin F and D levels increase on that day while prostaglandin E levels fall. The ratio falls slightly on days 8 and 9 before rising to its highest values between days 10 and 13. This high ratio is due to the very low level of prostaglandin E production rather than to a high level of production of F and D.

Plasma progesterone levels (Table 1) increase rapidly during the first 3 days of pseudopregnancy reaching high levels by day 4 or 5. The levels then show small fluctuations up to day 9, after which they decline to much lower levels by day 13. It may be that the increase in production of prostaglandins F and D from day 8 to 9, and the relatively higher production of prostaglandins F and D in comparison to E from days 10 to 13 may be connected with this decline. It is clear that the much higher production of prostaglandins F and D on day 5 is not connected with any decline in progesterone levels.

Effect of tamoxifen on prostaglandin production by the pseudopregnant rat uterus in vitro

The results are shown in Figure 2. The pretreatment of rats on day 2 with tamoxifen significantly decreased the *in vitro* production of prostaglandins by the day 5 pseudopregnant rat uterus. However, the production

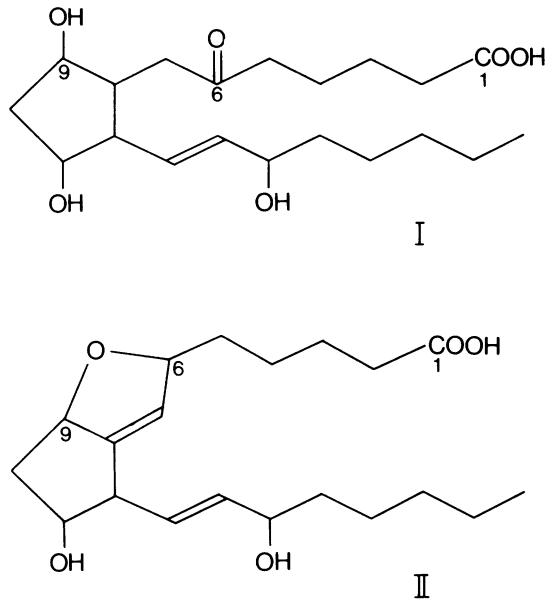

Figure 3 Structures of 6-keto prostaglandin $F_{1\alpha}$ (I) and 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid (II).

of prostaglandin E was apparently affected more since the ratio of prostaglandins F and D to E was 2.0 in the control animals, but increased to 3.5 in the drug-treated animals. Peripheral plasma progesterone levels were high in both groups, as expected, and did not differ.

Identification of prostaglandins

'F' fraction. The predominant prostaglandin present was $F_{2\alpha}$ in all samples studied. Much smaller quantities (1 to 5%) of $F_{1\alpha}$ were also detectable.

'E' fraction. Prostaglandin E_2 was identified as being present in all samples. Smaller quantities of prostaglandin D_2 were also present in each sample. Neither prostaglandin E_1 nor 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ were detected. The major product present in each sample was 6-keto-prostaglandin $F_{1\alpha}$ (Figure 3). The identification of this compound by GC-MS in the remaining uterine extracts forms an addendum to this paper. It was estimated to be present in quantities at least 10 to 20 times more than the quantities of prostaglandin E_2, and, therefore, was also present in greater quantities than $F_{2\alpha}$. It was impossible to measure the quantities accurately since the authentic compound was not available.

Also present was another prostaglandin derivative. Its mass spectrum was identical to that of a compound produced by rat stomach homogenates and which has

been identified as 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid (Pace-Asciak and Wolfe, 1971a) (Figure 3). It was present in quantities less than 6-keto-prostaglandin $F_{1\alpha}$ but probably more than $F_{2\alpha}$. An accurate measurement of the amounts again was not possible.

Discussion

It was unexpected that a major prostaglandin produced by rat uterus homogenates would be 6-keto-prostaglandin $F_{1\alpha}$. The isolation of this prostaglandin from the uterus has not been previously reported. 6-Keto-prostaglandin $F_{1\alpha}$ was produced in amounts at least 5 to 10 times greater than the amounts of $F_{2\alpha}$ the prostaglandin in next abundance. We did not have any authentic 6-keto-prostaglandin $F_{1\alpha}$ to test its cross-reactivity with the $F_{2\alpha}$ antibody. Prostaglandin $F_{1\alpha}$ cross-reacts 100%, but the introduction of a 6-keto group may reduce the cross-reactivity. Perhaps of more significance was the finding that 6-keto-prostaglandin $F_{1\alpha}$ is similar in polarity to prostaglandin E_2. It was eluted in the 'E' fraction from the silicic acid columns and its retention time of gas chromatography was 1 to 2 min longer than that of prostaglandin E_2. This is despite that fact that 6-keto-prostaglandin $F_{1\alpha}$ contains 3 hydroxyl groups while E_2 contains only 2 such groups. This probably indicates that the 6-keto group and 9-hydroxyl group interact in some way, resulting in a reduction in polarity of the molecule. This interaction may also reduce the affinity of 6-keto-prostaglandin $F_{1\alpha}$ for the $F_{2\alpha}$ antibody. It is difficult to predict what the cross-reactivity would be, and, therefore, what contribution 6-keto-prostaglandin $F_{1\alpha}$ makes to the final quantitative answer in the assay of prostaglandin F compounds.

It was also unexpected that 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid would be produced by rat uterus homogenates, though it is known to be produced by the rat stomach (Pace-Asciak & Wolfe, 1971a). This compound probably has low cross-reactivity with the prostaglandin $F_{2\alpha}$ antibody since in comparison to $F_{2\alpha}$ its structure has been modified at both the 6 and 9 positions.

Prostaglandin D_2 was also detected in the uterine incubates. This cross-reacts 17% with the prostaglandin $F_{2\alpha}$ antibody, but since much less of it was produced, it probably contributes only 3 to 5% to the final quantitative value. Prostaglandin $F_{1\alpha}$ cross-reacts 100%, but it was present in the incubates in trace amounts. It will contribute only 1 to 3% to the final answer. Consequently, in the assay using the prostaglandin $F_{2\alpha}$ antibody, prostaglandin $F_{2\alpha}$ is a major component being assayed and possibly 6-keto-prostaglandin $F_{1\alpha}$ is another. However, due to the lack of adequate specificity, one is actually measuring the levels of prostaglandin F and D compounds. This does not include the metabolites of prostaglandin $F_{2\alpha}$ (e.g. 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$) since these do not have significant cross-reactivities.

The antiserum against prostaglandin $E_2$was probably measuring exclusively prostaglandin E_2, unless 6-keto-prostaglandin $F_{1\alpha}$ and 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid unexpectedly have high cross-reactivities. Prostaglandin E_1 is the only prostaglandin which cross-reacts significantly with the E_2 antibody, and its presence was not detected.

From the quantitative results, it is seen that prostaglandin production is highest on day 5 of pseudopregnancy. The level measured is the sum of the amount already present in the uterus plus that synthesized during incubation minus the amount metabolized. 13,14-Dihydro-15-keto-prostaglandin (which is similar in polarity to prostaglandin E_2) was not detected. It would appear that in this *in vitro* system, metabolism of prostaglandins is low and we were actually measuring prostaglandin synthesis by the uterus. However, a detailed study of metabolism is merited. Day 5 is the day of implantation in the rat and it is possible, that a high production of prostaglandins on day 5 is connected with this process.

The increase in production of prostaglandins by the uterus on day 5 of pseudopregnancy was significantly inhibited by treating the rats with tamoxifen on day 2. Tamoxifen, at this dose level, inhibits the output of oestradiol from the ovary (Watson *et al.*, 1975). This would indicate that the increase in prostaglandin production normally occurring from day 3 to day 5 is linked to the increased output of oestradiol from the ovary which occurs late on day 3 and early on day 4. These results also suggest that drugs which inhibit the release and/or action of hormones may be of importance in inhibiting prostaglandin synthesis. In this study, other mechanisms of action of tamoxifen cannot be ruled out.

Ham *et al.* (1975) showed that the treatment of ovariectomized rats with oestradiol stimulated prostaglandin $F_{2\alpha}$ synthesis by the uterus but reduced E_2 synthesis. This resulted in an increase in the ratio of prostaglandins F to E. This did not occur in the day 5 uterus in the present study. Synthesis of all prostaglandins was apparently stimulated with prostaglandin E_2 synthesis actually being stimulated more, as the ratio of prostaglandins F and D to E decreased. It is difficult to explain this difference in findings except that in this study progesterone levels were high, while in the study of Ham *et al.* (1975), the ovariectomized rats were not receiving progesterone supplements.

It was also surprising that prostaglandin production did not increase greatly towards the end of pseudopregnancy when oestradiol levels are high. The ratio of prostaglandin F and D to E did increase, and possibly this is of significance. Uterine levels of pro-

staglandin $F_{2\alpha}$ do not increase greatly towards the end of pseudopregnancy, though they are significantly higher around days 9 and 10 (Weems *et al.*, 1976; Castracane & Shaikh, 1976). Oestrogen does cause the release of prostaglandin F from the uterus of ovariectomized rats though a period of progesterone priming is necessary for the maximum effect to be produced (Castracane & Jordan, 1975). Whether or not prostaglandin $F_{2\alpha}$ produced by the rat uterus is the factor which terminates pseudopregnancy has still to be proven. Our finding that 6-keto-prostaglandin $F_{1\alpha}$ is the major product formed by rat uterus homogenates, raises the possibility that this compound may be the uterine luteolytic hormone in the rat. Consequently, many of the earlier studies may need to be re-appraised in the light of our present findings.

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Addendum

IDENTIFICATION OF 6-KETO-PROSTAGLANDIN $F_{1\alpha}$ BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Introduction

The identification of a compound by gas chromatography and mass spectrometry involves the formation of different chemical derivatives and the interpretation of the mass spectra produced following gas chromatography. It is essential to choose derivatives which identify the nature and position of the functional groups present. Since the extraction and chromatographic procedures described in the preceding paper are directed towards isolating prostaglandins, it was highly probable that the new compound isolated had a prostaglandin-like structure. The determination of the structure of this compound is the subject of this addendum.

Methods

Ethyl acetate extracts of pseudopregnant rat uteri were pooled to produce several larger samples consisting of 4 or 5 individual samples. Each pooled sample was evaporated to dryness, the residue dissolved in 67% ethanol and washed with petroleum ether (b.p. 60° to 80°C). The ethanol solution was evaporated to dryness and the residue further purified by column chromatography, as described in the preceding paper.

The 'E' fraction from each column was subjected to derivatization for analysis by gas chromatography-mass spectrometry. Methyl ester formation, *n*-butyloximation of the keto groups, and trimethylsilyl ether formation were performed as described in the preceding paper. Ethyl esters were prepared using diazoethane, and methyloximes formed with O-methylhydroxylamine hydrochloride (5 mg/ml in dry pyridine). Cyclic *n*-butylboronates were formed by reacting the esterified compound with *n*-butylboronic acid (5 mg/ml in 2,2-dimethoxypropane) for 60 min at 60°C.

The keto group in the unknown compound was reduced also with sodium borohydride in ethanol at room temperature for 30 minutes. After the addition of water and acidification to pH 4.5 with 1 N HCl, the reduced compound was extracted with ethyl acetate. It was then converted into the methyl or ethyl ester, trimethylsilyl ether. Seven derivatives of the unknown compound were prepared.

Analytical procedure

The derivatives were analysed on an LKB 9000 gas chromatograph-mass spectrometer. The glass column, 3 m × 3 mm, was packed with 3% OV1 on Supelcoport. The helium gas flow was 35 ml/minute. The column temperature was 245°C, or 248°C when oximes were analysed. A mixture of straight chain fatty acid methyl esters (consisting of 16 to 24 carbons) was run before each sample in order to calculate its carbon value (C value).

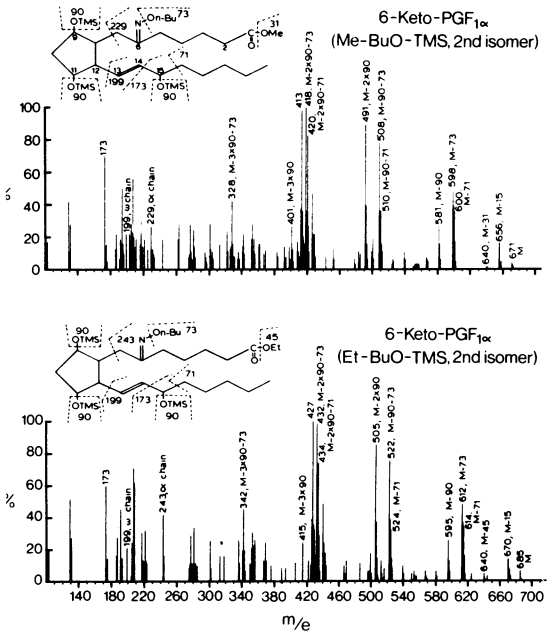

Figure 4 Line diagrams of the mass spectra of the methyl ester, *n*-butyloxime, trimethylsilyl ether (Me-BuO-TMS) and the ethyl ester, *n*-butyloxime, trimethylsilyl ether (Et-BuO-TMS) of the compound isolated and identified as 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$). Ordinate scale: relative abundance of ions (as % of base peak); abscissa scale: ratio of mass (m) to charge (e) (where e = 1).

Results and Discussion

(1) *Methyl ester,* O-n-*butyloxime, trimethylsilyl ether (Me-BuO-TMS)*

Two oxime isomers were obtained which separated on gas chromatography (C values = 26.5 and 26.8). The mass spectrum of the second isomer is shown in Figure 4. The following points are noted:

(a) The molecular ion (mol. ion) occurs at m/e 671 (M) and prominent peaks are found at m/e 598 (M-73), 581 (M-90), 508 (M-90-73), 491 (M-2 × 90), 418 (M-2 × 90 -73), 401 (M-3 × 90) and 328 (M-3 × 90 - 73). These data establish the presence of 3 hydroxyl groups and one keto group and are consistent with a mono-keto prostaglandin $F_{1\alpha}$ structure.
(b) Peaks at m/e 600 (M-71), 510 (M-90-71) and 420 (M-2 × 90 -71) and at 173 and 199 strongly suggest that the ω-chain has the primary prostaglandin structure (i.e. a 13,14 double bond and a 15-hydroxyl group). Also the keto group cannot be situated in the ω-chain.
(c) The peak at m/e 229 indicates that the keto group is probable situated in the α-chain.

(2) *Ethyl ester,* O-n-*butyloxime, trimethylsilyl ether (Et-BuO-TMS)*
Two isomers were detected on gas chromatography with C values of 26.8 and 27.0 respectively. The mass spectrum of the second isomer is shown in Figure 4. This is similar to the Me-BuO-TMS derivative except that the major peaks, with two exceptions, are 14 mass units higher. The most significant finding is a peak at m/e 243 instead of m/e 229. This indicates that the keto group is in the α-chain, and not in the ω-chain which still produces a peak at m/e 199.

(3) *Methyl ester, methyloxime, trimethylsilyl ether (Me-Mo-TMS)*
Two isomers were produced which were resolved by gas chromatography. The first isomer (C value = 24.8) was much smaller than the second isomer (C value = 25.1). A mass spectrum taken of the second isomer showed the mol. wt. of this derivative to be 629. This is due entirely to a difference of 42 mass units between the n-butyloxime and the methyloxime. This clearly indicates that there is only one keto group in the molecule. Furthermore, a peak at m/e 187 (229-42) again showed that the keto group is in the α-chain.

(4) *Methyl ester, cyclic* n-*butyl boronate, trimethylsilyl ether (Me-BuB-TMS)*
The production of butylboronates requires two hydroxyl groups to be present in a 1,3 *cis*-configuration, with the formation of a bridge across these two hydroxyl groups to produce a cyclic derivative. This can only occur in the F_α prostaglandins where hydroxyl groups are situated in the 9α and 11α positions (Pace-Asciak & Wolfe, 1971b; Kelly, 1972). (Both hydroxyl groups could be in the β positions, but such prostaglandins do not occur naturally). Consequently, the very fact that the compound under study formed a cyclic *n*-butylboronate indicates that this compound contains the prostaglandin F_α structure. The mol. ion of the Me-BuB-TMS derivative (C value = 25.2) was 522. This is consistent with the compound having the prostaglandin $F_{1\alpha}$ structure with a keto group in the α-chain. Only two major peaks occurred in its mass spectrum. The smaller peak was at m/e 365, which is equivalent to M–α-chain (522–157). The base peak is at m/e 129 and is probably due to the ion (CH_2=CH–CH=$\overset{+}{O}$-TMS).

(5) *Ethyl ester, cyclic* n-*butyl boronate, trimethylsilyl ether (Et-BuB-TMS)*
The carbon value of this derivative was 25.5 and its mol. wt. was 536. Two major peaks occurred in its mass spectrum, again at m/e 365 (M–α-chain, where the α-chain is now 171) and at m/e 129 indicating

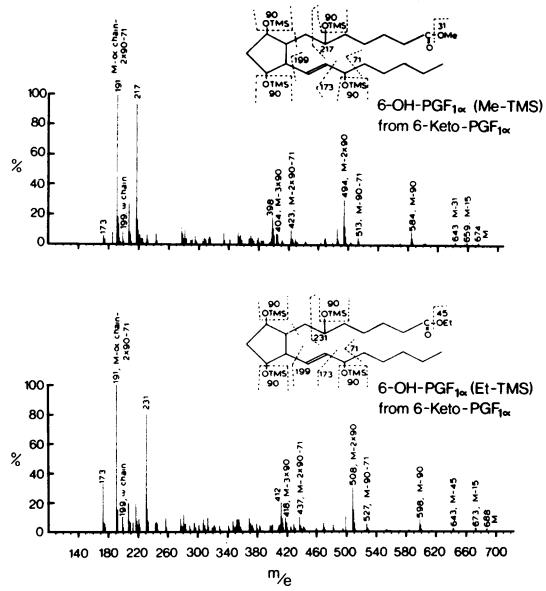

Figure 5 Line diagrams of the mass spectra of the methyl ester, trimethylsilyl ether (Me-TMS) and the ethyl ester, trimethylsilyl ether (Et-TMS) of 6-hydroxyl-prostaglandin $F_{1\alpha}$ (6-OH-$PGF_{1\alpha}$) prepared by the reduction of the isolated compound identified as 6-keto-prostaglandin $F_{1\alpha}$. Ordinate scale: relative abundance of ions (as % of base peak); abscissa scale: ratio of mass (m) to charge (e) (where e=1).

identical fragmentation as in the Me-BuB-TMS derivative.

The evidence accumulated from these 5 derivatives indicates that the compound isolated consists of a prostaglandin $F_{1\alpha}$ structure with a keto group attached to the α-chain. The fragmentation patterns of the derivatives prepared so far give little indication where the keto group is actually located on the α-chain. It was decided, therefore, to reduce the keto group to a hydroxyl group since carbon-carbon bonds split α to a carbon-oxygen bond (as in the loss of 71 and 173 around the carbon-oxygen bond at position 15; Figure 4). The fragmentation pattern of the α-chain should, establish the position of the keto groups.

(6) *Methyl ester, trimethylsilyl ether (Me-TMS) of the reduced compound*

The reduction of the keto group produces two isomers. Our experience of 15(R) and 15(S) prostaglandin $F_{2\alpha}$ has shown that these two isomers do not separate on gas chromatography. It was no surprise, therefore, that the reduced form of the compound under study produced only one gas chromatographic peak (C value=25.0). Its mass spectrum is shown in Figure 5 and the following points are noted: (i) The mol. wt. is 674, which is consistent with the compound being prostaglandin $F_{1\alpha}$ containing one extra hydroxyl group. (ii) A major peak occurs at m/e 217. As shown in the figure, this most probably arises from a split of the bond between carbons 6 and 7, which is α to the carbon-oxygen bond. (iii) The base peak at m/e 191 is due to the loss of the α chain, the two derivatized hydroxyl groups from the cyclopentane ring and the loss of the 5 terminal carbons from the ω-chain. (iv) Since peak m/e 191 is formed preferentially, peaks at m/e 173 and 199 are correspondingly small. (v) There are several significant peaks indicating the loss of derivatized hydroxyl groups (90) and the 5 terminal carbons (71). The peak at m/e 584 (M-90) corresponds to the mol. wt. of prostaglandin $F_{2\alpha}$ (Me-TMS) and the mass spectrum between m/e 300 and 600 is remarkably similar to that of prostaglandin $F_{2\alpha}$ (not $F_{1\alpha}$ since one would have to add 2 protons to compensate for the loss of one derivatized hydroxyl group) (see Poyser, 1972, for a mass spectrum of prostaglandin $F_{2\alpha}$-Me-TMS).

(7) *Ethyl ester, trimethylsilyl ether (Et-TMS) of the reduced compound*

Only one peak was produced on gas chromatography (C value=25.2). The mass spectrum of the Et-TMS of the reduced compound is shown in Figure 5: (i) The mol. wt. is 688, 14 mass units higher than the Me-TMS derivative, as expected. (ii) The base peak is at m/e 191, as expected also, since the production of this fragment involves loss of the α-chain and is, therefore, not affected by the ester derivative. (iii) If the derivatized hydroxyl group is located at position 6, one would now expect the m/e 217 peak present in the Me-TMS derivative to move to m/e 231 in the Et-TMS derivative. A comparison of the mass spectra shows this to be the case, proving conclusively that the hydroxyl group (and, therefore, the original keto group) is located on the carbon at position 6.

Conclusion

The evidence obtained from this study clearly indicates that the new compound isolated from the pseudopregnant rat uterus is 6-keto-prostaglandin $F_{1\alpha}$. This conclusion does make the assumption that both hydroxyl groups on the cyclopentane ring are in the α positions, and that the double bond in the ω-chain is in the 13,14 position. However, naturally-occurring prostaglandins of the F series do have this configuration so hopefully these assumptions are justified. Absolute identification must await the chemical synthesis of the authentic compound and a comparison being made of the mass spectra of the naturally-occurring and synthetic material.

Finally, whilst this study was in progress a short report was published showing that 6-keto-prostaglandin $F_{1\alpha}$ is also formed by homogenates of rat stomach (Pace-Asciak, 1976).

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