THE IDENTIFICATION AND ESTIMATION OF PROSTAGLANDINS AND THEIR METABOLITES

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SUMMARY

Three attempts at developing a radioimmunoassay for prostaglandin E_2 were made. Two of these attempts resulted in the production of anti-sera directed mainly to prostaglandin B_2. The third attempt was designed to produce anti-sera specific to prostaglandin $F_{2\beta}$, a prostaglandin readily formed from prostaglandin E_2 by reduction with sodium borohydride. Antibodies were successfully raised to prostaglandin $F_{2\beta}$ with a low cross-reactivity to the majority of potentially interfering prostaglandins, including prostaglandin $F_{2\alpha}$, the other epimer produced by sodium borohydride reduction of prostaglandin E_2. The anti-sera tested were unable to distinguish between prostaglandins $F_{1\beta}$ and $F_{2\beta}$.

Attempts were made to explain why rabbits immunised with prostaglandin E_2 - bovine serum albumin conjugates produced anti-sera primarily directed to prostaglandin B_2. No evidence for the conversion of prostaglandin E_2 to prostaglandin B_2 during the synthesis of the immunising conjugate was obtained. Although prostaglandins of the A series have been extracted from a variety of sources and these are readily isomerised to the corresponding prostaglandin B, the nature of their biological synthesis remains to be elucidated. The possibility that the prostaglandin E_2 conjugated to the bovine serum albumin was being dehydrated enzymatically to the corresponding prostaglandin A_2 by the immunised rabbits and then isomerised to the corresponding prostaglandin B_2 was investigated. No

evidence for a prostaglandin E dehydrase was obtained in either rabbit blood or lymph nodal tissue.

In experiments designed to evaluate the sheep as a possible alternative animal suitable for raising prostaglandin E_2 anti-sera, an enzyme was discovered in the blood that reduced prostaglandin E_2 specifically to prostaglandin $F_{2\alpha}$. The enzyme has been quantitatively assayed by radiochemical and combined gas chromatography-mass spectrometric methods. The pH and temperature characteristics of the enzyme system were determined by the first assay method. Both assay methods have been used for substrate specificity studies. This enzyme system is active within a narrow pH range with optimum activity at around pH 7. At temperatures above 50 °C the enzyme reaction rate is greatly reduced with optimum activity being around 40-45 °C. The enzyme has been detected in rabbit, horse, guinea-pig and chicken liver and horse heart and chicken brain homogenates. The richest source is rabbit liver. The enzyme was not detectable by the methods employed in blood samples from cat, chicken, dog, guinea-pig, horse, man, rabbit and rat. Preliminary results suggest that a similar enzyme may be present in human liver. The enzyme system was not detectable in liver, kidney or heart samples from the cat, dog, cow and rat.

The influence of age, sex and hormonal state have been studied with reference to the reduction of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ by the enzyme systems present in sheep blood and liver samples from chicken, rabbit and guinea-pig. Ovarian steroid

hormones have been found to influence the enzyme activity and preliminary evidence would suggest a link between these hormones and prostaglandin metabolism.

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CONTENTS

INTRODUCTION

Historical

The presence of smooth muscle stimulating factors in human semen and tissues of the reproductive tract has been reported since the beginning of the century (see von Euler, 1936, for references). Independently Goldblatt (1935) and von Euler (1936) attributed this activity to a new pharmacologically active substance that von Euler termed "prostaglandin". Although the early work of von Euler suggested that "prostaglandin" had a structure compatible with it being a lipid soluble fatty acid, it was not until 1960 that Bergstrom and Sjovall (1960 a; 1960 b.) were able to assign the empirical formulae $C_{20}H_{34}O_5$ and $C_{20}H_{36}O_5$ to prostaglandins E and F respectively that had been extracted from ram seminal vesicles. The first structural elucidation followed in 1963 (Bergstrom, Ryhage, Samuelsson and Sjovall, 1963) when the structures of prostaglandins E_1, $F_{1\alpha}$ and $F_{1\beta}$ were deduced. Since this initial structural elucidation was performed a wide spectrum of prostaglandins and their metabolites have been discovered. Much of this isolation and structural elucidation work was carried out on extracts of human semen (Bergstrom and Samuelsson, 1962; Samuelsson, 1963; Bygdeman, 1964; Hamberg and Samuelsson, 1965; Bygdeman and Samuelsson, 1966 a; 1966 b; Hamberg and Samuelsson, 1966 a; Kelly and Taylor, 1974).

Natural "primary" prostaglandins belong to a series of compounds with certain common features. In their non-metabolised form they are 20 carbon unsaturated fatty acids, containing a 13,14-trans double bond and a 15(S) hydroxyl group that belong to either the A, B, C, D, E or F series. The series differ from each other in the substituents on the cyclopentane ring as shown by structural formulae of some prostaglandins in figure 1. The degree of unsaturation of the side chains is denoted by the subscript 1, 2 or 3 after the letter, although when the 13, 14-trans double bond is saturated the name is prefixed with 13, 14-dihydro. The 1 subscript indicates the presence of only the 13, 14-trans double bond (unless prefixed with 13, 14-dihydro) whilst the 2 and 3 subscripts refer to the presence of a 5, 6 cis plus a 17, 18 cis double bonds respectively. The prefix 19-OH refers to the presence of a hydroxyl group at C-19.

The past decade has witnessed an ever-increasing interest in prostaglandins, particularly by the various national and international drug houses. Much of this interest stems from the high biological activity that various prostaglandins have on a variety of biological systems. Consequently, numerous reports are appearing that describe (or confirm) the routes of biological synthesis and metabolism, the pharmacological, biochemical and physiological effects and the analysis of prostaglandins in samples from a variety of animal species.

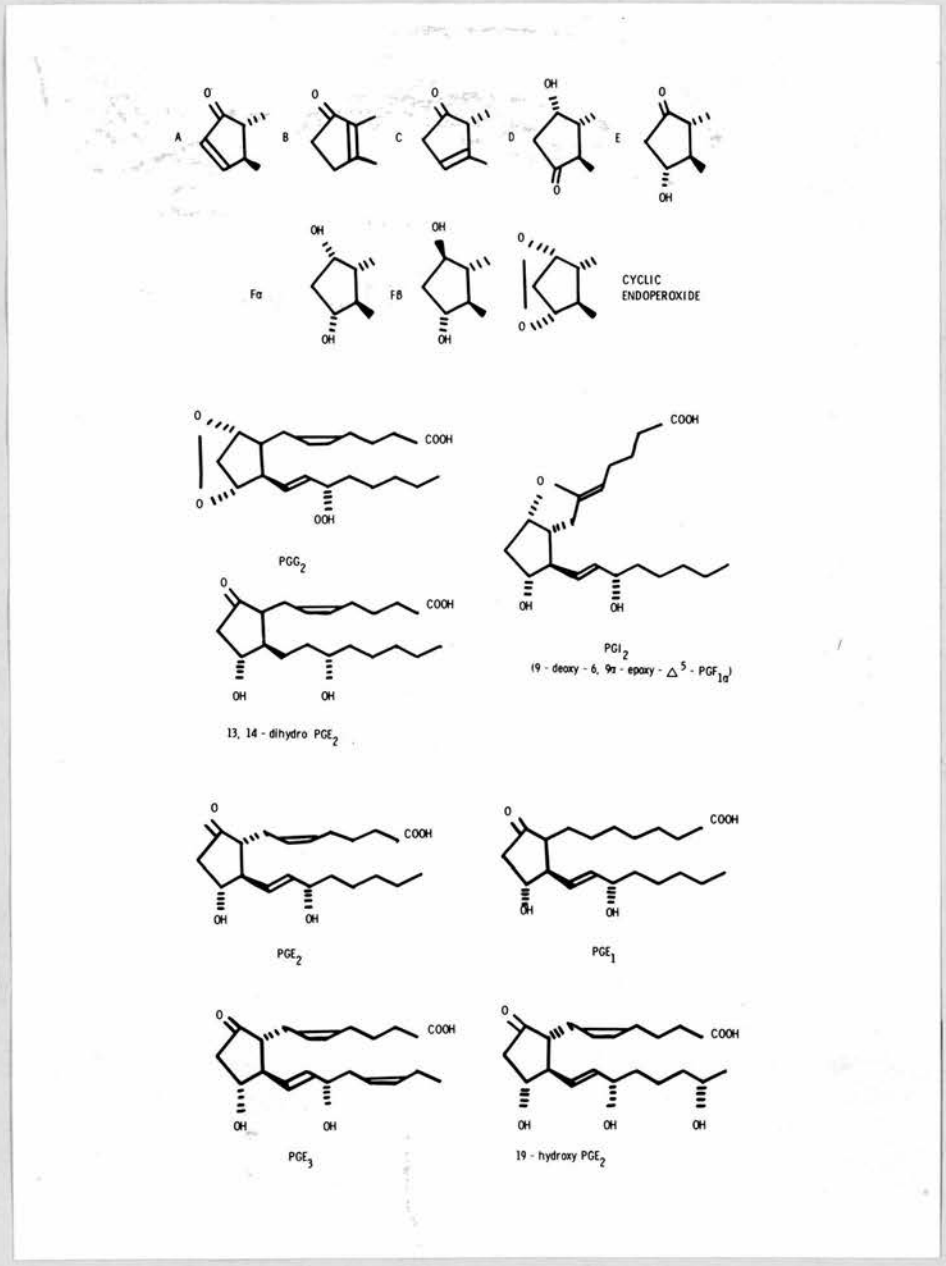

Figure 1. Comparison of the cyclopentane ring substituents in prostaglandins A - F and the side chain substituents in PGG_2, PGI_2, 13,14-dihydro PGE_2, PGE_2, PGE_1, PGE_3, and 19 hydroxy PGE_2.

The discovery of prostaglandin-like substances in a variety of biological situations often leads to the suggestion that a particular prostaglandin may have an important physiological role in that system. An example of this is the suggestion that prostaglandin A_2 (or a metabolite of this prostaglandin) may be the natriuretic hormone produced in response to the increased in the extracellular fluid volume (Lee, 1969; Horton, 1972; Lee, 1975). Evidence has been accumulating for the presence of a circulating hormone produced in response to an increased extracellular volume (de Wardener, Mills, Clapham and Hayter, 1961; Lichardus and Pearce, 1966; Johnston, Herzog and Lauler, 1967). It has been suggested that the origin of this hormone is the kidney (Johnston, Davis, Howards and Wright, 1967) and that it has a short half life in the systemic circulation (de Wardener; Mills, Clapham and Hayter, 1961). This hormone also induces natriuresis in response to saline loading without altering the glomerular filtration rate or the systemic blood pressure (de Wardener et al., 1961) physiological effects also claimed for prostaglandins E_1, E_2 and A_2 (Herzog, Johnston and Lauler, 1968). Further circumstantial evidence supporting the suggestion that a prostaglandin A may be the hormone was derived from the fact that both prostaglandins E_2 and A_2 could be detected in renal venous blood after renal nerve stimulation (Davis and Horton, 1972) or during ischaemia

(McGiff, Crowshaw, Terragno and Lonigro, 1970). Certainly prostaglandins E_2, A_2 and $F_{2\alpha}$ have been conclusively identified in renal medullary tissue (Lee, Crowshaw, Takman, Attrep and Gougoutas, 1967). The origin of the prostaglandin A_2 present in the medullary samples, however, has seriously been questioned by the work of Larsson and Anggard (1975) who have conclusively shown that in their experiments the entire prostaglandin A_2 that was detectable had originated entirely from chemical degradation during extraction and purification procedures. It is a fact that prostaglandin A_2 has a longer half life in the systemic circulation of the cat and dog than prostaglandin E_2 due to it not being inactivated by the lungs (Horton and Jones, 1969; McGiff, Terragno, Strand, Lee, Lonigro and Ng., 1969). Thus A series prostaglandins could be thought of as potential circulating hormones, although it must be remembered that the hepatic circulation can decrease the activity of prostaglandins A_1 and A_2 (Horton and Jones, 1969).

<u>The analysis of prostaglandins and their metabolites</u>

The low concentrations of prostaglandins either present in or produced by the majority of biological systems generally prevents their full rigorous identification by physico-chemical analysis. Consequently the past decade has witnessed the development of a variety of extraction, chromatographic and analytical

procedures that allow an "identification" of prostaglandins to be made. These techniques have been thoroughly reviewed (Shaw and Ramwell, 1969; Horton, 1972; Hensby, in press).

The first step in the successful analysis for prostaglandins of biological origin usually involves a preliminary purification by organic solvent extraction (Horton, 1972). This step produces several problems particularly associated with stability and recovery. The E series prostaglandins in particular are prone to dehydration in the acidic aqueous media associated with extraction procedure (Daniels, Hinman, Johnson, Kupiecki, Nelson and Pike, 1965; Nugteren, Beerthuis and van Dorp, 1966; Larsson and Anggard, 1975). Consequently, the origin of the A series prostaglandins identified in biological systems must remain in doubt until rigorously proven by methods similar to those of Larsson and Anggard (1975). Recently, Taylor and Kelly (1974) have perfected a method for the extraction of E series prostaglandins from semen samples without the problems of dehydration into the corresponding A prostaglandins occurring. This method involves conversion of the E prostaglandins into the corresponding O-alkyl oximes which are more resistant to dehydration during extraction or purification procedures. Using this technique the major prostaglandins present in human semen have been identified as the E series prostaglandins, particularly in the 19-OH prostaglandins E_1 and E_2 (Taylor and Kelly, 1974; Jonsson, Middleditch, Desiderio and

Schexnayder, 1975). Consequently, the previous reports that 19-OH prostaglandins A and B and not the corresponding E analogues (Hamberg and Samuelsson, 1966) were present in human semen may be explained as extraction artifacts.

The problem associated with the recovery in high yields of the small amounts of prostaglandins has been studied in great detail (Horton, 1972). The most efficient and quantitative results are obtained when deuterium isotope dilution techniques are employed. The development of deuterium and tritium labelled prostaglandins and their metabolites has certainly enhanced their qualitative and quantitative analysis. The use of deuterium labelled carrier prostaglandins for the quantitative analysis of prostaglandin E_1 was originally proposed by Samuelsson, Hamberg and Sweeley (1970). However, this method relied on the addition of the deuterium labelled compound after the derivatisation procedures were complete and thus only quantitated the final analysis by gas chromatography-mass spectrometry. The development of high isotope purity tetra deuterated prostaglandins, particularly when used in combination with the corresponding tritium labelled prostaglandins, has proved to be one of the better developments in the quantitative analysis of prostaglandins and their metabolites by combined gas chromatography-mass spectrometry (Axen, Horlin and Samuelsson, 1971; Green, 1973; Green, Granstrom, Samuelsson and Axen 1973; Kelly, 1973; Granstrom, Green, Bygdeman, Toppozada and Wiqvist, 1973; Hensby

and Naylor, 1974). Not all prostaglandin metabolites are available in the corresponding deuterium form, however, methods that are generally applicable for the production of deuterated forms of the derivatised prostaglandins have been described (Samuelsson et al., 1970; Hamberg, 1972; 1974).

Although combined gas chromatography-mass spectrometry has proved to be the most conclusive method for identifying and quantifying prostaglandins and their metabolites, this method entails considerable preparative procedures. These methods must be performed if any useful information is to be obtained from any form of gas chromatography. One of the major problems associated with gas chromatography of prostaglandins is their inherent instability at the high temperatures employed. Consequently a series of derivatives has evolved that stabilise prostaglandins sufficiently to enable them to be subjected to gas chromatography. The most routinely employed derivatives have been reviewed (Green, 1969; Middleditch and Desiderio, 1975). In all cases the carboxylic acid has to be esterified, usually as either the methyl, ethyl or trimethylsilyl derivative, although the pentafluorobenzyl ester has recently been described for the specific use in gas chromatography with electron capture detection (Wickramasinghe, Morozowich, Hamlin and Shaw, 1973; Wickramasinghe and Shaw, 1974). For prostaglandins containing a ketonic function, the corresponding O-alkyl oxime derivative usually has to be prepared. The most commonly employed are the O-methyl and O-benzyl oxime

analogues (Green, 1969; Granstrom, 1972; Baczynskyi, Duchamp, Zieserl and Axen, 1973). Prostaglandins of the A and B series can be subjected to gas chromatography with the ketonic function underivatised. The B prostaglandins when remaining as the free ketone contain a conjugated dienone that has been successfully employed for the electron capture detection of tetranor prostaglandin B_1 (Green and Samuelsson, 1971). The hydroxyl functions of prostaglandins are usually protected for gas chromatography as either the trimethylsilyl ether (Thompson, Los and Horton, 1970), the acetate (Green, 1969), trifluoroacetate (Thompson et al, 1970) or the heptafluorobutyrate (Levitt and Josimivich, 1971). This latter derivative has proved particularly useful for electron capture detection although the choice of derivatising reagent may determine the success with which the derivative is formed (Sugiura and Hirano, 1974). The ring structure of the F prostaglandins enables the choice of derivatives that selectively form when both the ring hydroxly groups are in the cis configuration to be made. Such a derivative is the n-butyl first described for prostaglandin $F_{2\alpha}$ by Pace-Asciak and Wolfe (1971). This derivative has been successfully used for the qualitative and quantitative analysis of prostaglandin $F_{2\alpha}$ (Kelly, 1973; Hensby, 1974).

Another problem associated with the gas chromatography of prostaglandins is that many other lipid-like compounds are also extracted from the majority of biological systems. Thus if meaningful results are

to be obtained by gas chromatography or, indeed, most other analytical procedures, some further purification of the organic extract residue must be performed. The most routinely employed preparative purification procedures include thin layer chromatography (Green and Samuelsson, 1964; Andersen, 1969; Miller, 1974; Horton, 1972), silicic acid and silica gel column chromatography (Daniels and Pike, 1968; Hamberg and Jonsson, 1973; Hamberg, Svensson, Wakabayashi and Samuelsson, 1973), reversed phase partition chromatography (Anggard and Samuelsson, 1964; Granstrom and Samuelsson, 1969; 1971; Hamberg and Wilson, 1973; Nystrom and Sjovall, 1973), and straight phase partition chromatography (Anggard and Bergvist, 1970; Sun and Stafford, 1974; Brash and Jones, 1974; Sun, 1974; Hensby, 1974). The latter two methods in particular have proved to be highly reproduceable and efficient chromatography systems for the small quantities of prostaglandins usually involved.

Although the use of gas chromatography particularly in conjunction with mass spectrometry may provide the most reliable results, the expense and complexity of the operation are limiting factors for this technique. Consequently, many other less conclusive techniques are used for the analysis of prostaglandins. These assays include various bioassay procedures (Horton and Main, 1963; 1965; Vane, 1969; Horton and Jones, 1969), ultraviolet spectroscopy (Bygdeman, Fredricsson, Svanborg and Samuelsson, 1970; Andersen, 1969), enzymatic assay (Anggard, 1968) and radio-immunoassays (Caldwell,

Burstein, Brock and Speroff, 1971; Gershman, Powers, Levine and Van Vunakis, 1972; Jaffe, Smith, Newton and Parker, 1971; Levine, Gutierrez-Cernosek and Van Vunakis, 1971; Kirton, Cornette and Barr, 1972; Levine, Gutierrez-Cernosek and Van Vunakis, 1973).

Biosynthesis, metabolism and interconversion of prostaglandins

The biosynthesis of prostaglandins E and F_{α} has been extensively studied, particularly with acetone dried aggregates of the prostaglandin synthetase from bovine and ovine seminal vesicles. This enzyme complex is widely distributed (see Horton, 1972). The conversion of all cis-5,8,11,14-eicosatetraenoic acid (arachidonic acid) into prostaglandin E_2 was described independently by van Dorp et al. and Bergstrom et al. (van Dorp, Beerthuis, Nugteren and Vonkeman, 1964; Bergstrom, Danielsson and Samuelsson, 1964). Prostaglandin $F_{2\alpha}$ is also enzymatically synthesised from this acid (Anggard and Samuelsson, 1965). The enzymatic conversion of all-cis-8,11,14-eicosatrienoic acid (dihomo-γ-linolenic) into prostaglandins E_1 and $F_{1\alpha}$ (Bergstrom, Danielsson, Klenberg and Samuelsson, 1964; van Dorp, Beerthuis, Nugteren and Vonkeman, 1964; Kupiecki, 1965) and all cis-5,8,11,14,17-eicosapentaenoic acid into prostaglandin E_3 (Bergstrom, Danielsson and Samuelsson, 1964) have also been described. The synthesis of prostaglandin A_2 from arachadonic acid by an enzyme present in the sea whip Plexaura Homomalla is the only report that describes the biosynthesis of prostaglandin A_2 directly from arachidonic acid (Corey, Washburn and Chen, 1973). The enzymatic synthesis of prostaglandins was postulated to proceed via a common cyclic endoperoxide intermediate (Samuelsson, Granstrom and Hamberg, 1967; Samuelsson, 1971) that was subsequently identified (Hamberg and Samuelsson, 1973) and shown to be converted to E and D series

prostaglandins by distinct isomerases and F prostaglandins by a reductase (Hamberg and Samuelsson, 1973; Nugteren and Hazelhof, 1973). The metabolism of arachidonic acid by human blood platelets has been shown to produce compounds other than prostaglandins (Hamberg and Samuelsson, 1974). One compound, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid is produced by a different enzyme system to the prostaglandin synthetase, namely the lipoxygenase enzyme system. The other major products identified were 12L-hydroxy-5,8,10-heptadecatrienoic acid and 8-(1-hydroxy-3-oxopropyl)-9,12L-dihydroxy-5,10-heptadecadienoic acid. This latter compound originally named PHD, has been re-named thromboxane B_2. It has been shown to be a derivative of thromboxane A_2 (Hamberg, Svensson and Samuelsson, 1975), previously known as Rabbit Aorta Contracting Substance and hought to be a prostaglandin intermediate (Piper and Vane, 1969). The major products produced from arachidonic acid that have been identified are shown in figure 2 .

Prostaglandins of the E and E_α series formed from endogenous or radioactively labelled precursors have been demonstrated in a wide range of biological systems (Horton, 1972). These areas include renal medullary tissue of rabbits, cats and dogs (Hamberg, 1969; Muirhead, Germain, Leach, Pitcock, Stephenson, Brooks, Brosius, Daniels and Hinman, 1972; Crowshaw, McGiff, Strand, Lonigro and Terragno 1970; Daniels, Hinman, Leach and Muirhead, 1967; Lee, Crowshaw, Takman, Attrep and Gougoutas, 1967; Crowshaw, 1971) the reproductive organs and fluids of man and animals (Bergstrom and Samuelsson, 1962; Taylor and Kelly, 1974;

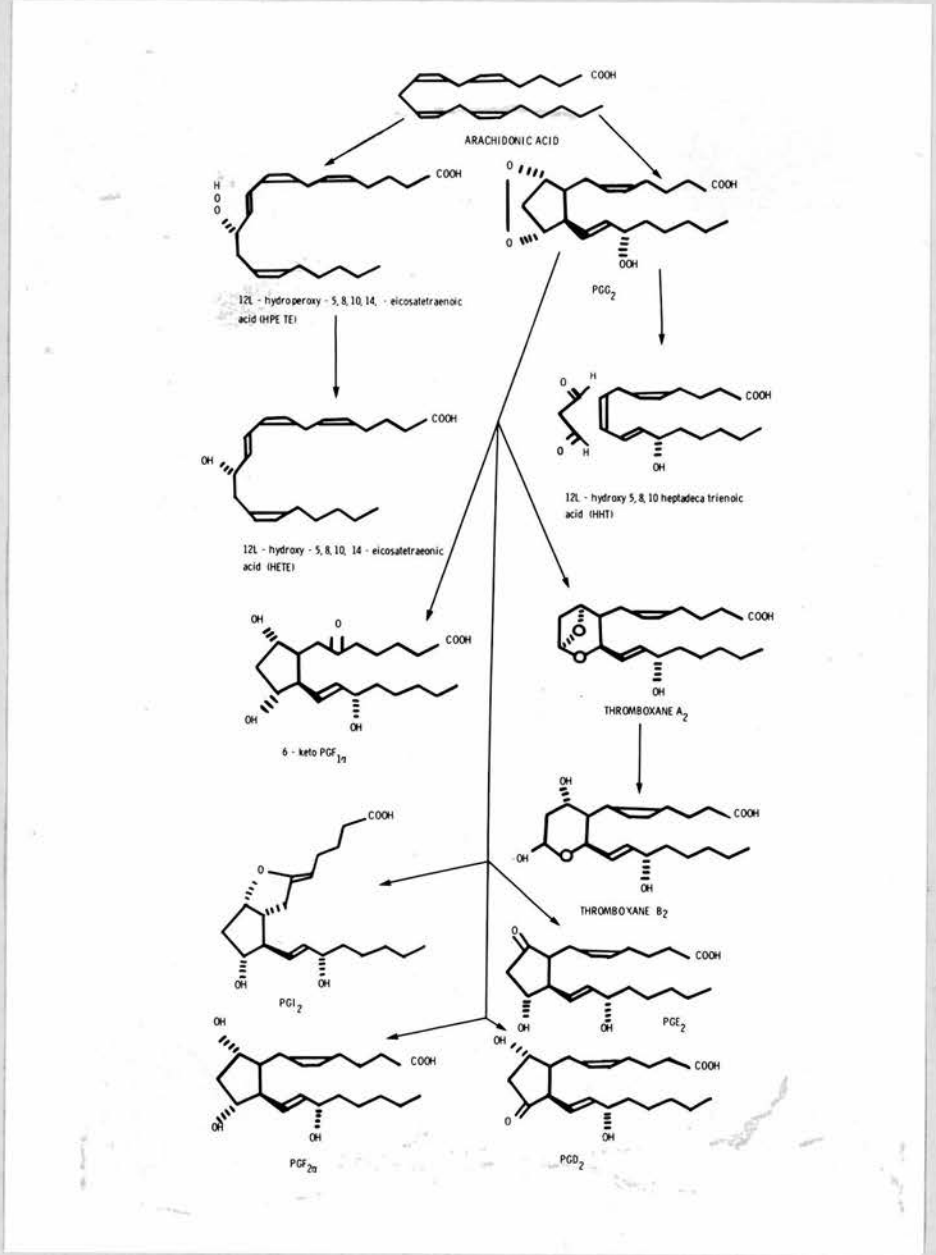

Figure 2. Some of the major transformations of arachidonic acid that have been identified.

Eglinton, Raphael, Smith, Hall and Pickles, 1963; Karim, 1967; Karim and Hillier, 1970; Karim and Devlin, 1967; Horton, Jones, Thompson and Poyser, 1971; Poyser, 1971; Bland, Horton and Poyser, 1971); lung tissue (Anggard, 1965; Bergstrom, Dressler, Krabisch, Ryhage and Sjovall, 1962; Samuelsson, 1964a); the spleen (Davies, Horton and Withrington, 1968; Gilmore, Vane and Wyllie, 1968; Gilmore, Vane and Wyllie, 1969); the central nervous system (Coceani and Wolfe, 1965; Ramwell and Shaw, 1966; Holmes, 1970; Samuelsson, 1964; Horton and Main, 1967; Feldberg, Gupta, Milton and Wendlandt, 1972) and inflamatory exudates and skin (Willis, 1969; Jonsson and Hamberg, 1973; Greaves, Sondergaard and McDonald-Gibson, 1971; Mathur and Gandhi, 1972).

A variety of enzymes that metabolise prostaglandins of the A,C,D,E and F_{α} series has been described, some of them in great detail. The wide range of metabolic pathways that have been described, particularly for the E and F_{α} type prostaglandins explain the often short biological half-lives these prostaglandins have. In addition these enzymes may indirectly influence either site of action or the physiological, biochemical or pathological function of endogenous prostaglandins.

The enzyme that has been most widely reported to metabolise 15 (S) prostaglandin is the 15-hydroxyprostaglandin dehydrogenase (Anggard and Samuelsson, 1966) which converts 15(S) prostaglandins into their 15-oxo analogues. These metabolites were originally thought to be considerably less active (Anggard, 1966; Kloeze, 1969; Pike, Kupiecki and Weeks, 1967), however, recent reports

would indicate that in some biological systems they may have an equal or greater activity than the parent prostaglandin (Dawson, Lewis, McMahon and Sweatman, 1974; Jones, 1975). Detailed studies of the biochemical properties (Nakano, Anggard and Samuelsson, 1969; Shio, Andersen, Corey and Ramwell, 1970) and distribution (Anggard, Larsson and Samuelsson, 1971; Samuelsson, Granstrom, Green and Hamberg, 1971) have been made. Recently reports have appeared that indicate that two distinct types of 15-hydroxy-prostaglandin dehydrogenase may be present in many systems (Lee and Levine, 1974; 1975). The type I dehydrogenase has a requirement of NAD+ as cofactor whilst the type II requires NADP+ as cofactor (Lee and Levine, 1975). The majority of reports, however, have only differentiated one type of 15(S)-hydroxyprostaglandin dehydrogenase This enzyme appears to be specific for 15(S)-hydroxyprostaglandins with a variety of ring structures, although prostaglandins B_2 and D_2 do not appear to be subtrates and α-dinor, α-tetranor and 13,14-dihydro prostaglandins are only poor substrates (Nakano et al., 1969; Sun, Armour and Bockstanz, 1975). This enzyme present in lung tissue is thought to play a principal role in the inactivation of circulating E and F prostaglandins (Vane, 1969). It has been demonstrated that during a single passage through the lungs of a cat, dog and rabbit both prostaglandins E_2 and F_2 lose most of their biological activity (Ferreira and Vane, 1967). Prostaglandins A_1 and A_2, which are good substrates for this enzyme (Nakano et at., 1969) are not, however, inactivated by the pulmonary circulation of either the dog

or the cat (Horton and Jones 1969; McGiff, Terragno, Strand, Lee and Lonigro, 1969) although the guinea-pig lung perfused with Krebs solution will metabolise them (Piper, Vane and Wyllie, 1969). These results may indicate that the A type prostaglandins are either bound to a plasma protein or are not substrates for lung uptake mechanisms. The 15(S) hydroxyprostaglandin dehydrogenase does not accept the corresponding 15(R) hydroxyprostaglandins which, with the 7-oxa-15(R) prostaglandins, are competitive inhibitors of the enzyme (Shio et al., 1970; Marrazziand Matschinsky, 1972). A variety of chemically synthesised analogues of the 15(S) prostaglandins are currently being investigated for biological activity. Many of these contain alkyl functional groups at either C-15 or C-16 which protect them from the action of this enzyme yet retain their biological activity (Bundy, Lincoln, Nelson, Pike and Schneider, 1971; Bundy, Yankee, Weeks and Miller, 1973; Robert and Magerlein, 1973; Karim, Carter, Bhana and Ganer-n, 1973).

Another enzyme which tends to be ubiquitous in the tissues containing the 15(S) hydroxyprostaglandin dehydrogenase is the Δ-13 prostaglandin reductase. This enzyme reduces the C-13,14 double bond of the 15-oxo prostaglandins and was first reported in the guinea-pig lung (Anggard and Samuelsson, 1964). Although the 13,14-dihydro prostaglandin E has been identified in the particle-free fraction of guinea-pig lung homogenates (Anggard and Samuelsson, 1964; 1965) and liver (Hamberg and Samuelsson, 1971) it has been shown that reduction of

the C-13,14 double bond *in vivo* must proceed via oxidation of the 15(S) hydroxyl group (Hamberg and Samuelsson, 1971). The *in vivo* metabolism of the E and F_{α} type prostaglandins is thought to initially proceed, therefore, via the 15(S) hydroxyprostaglandin dehydrogenase and the Δ13,14 reductase. Both prostaglandins E_1 and E_2 are rapidly metabolised by these processes *in vivo* in man (Hamberg and Samuelsson, 1971). When ^{3}H-labelled prostaglandins E_1 or E_2 are administered intravenously in man their half-life is approximately two minutes with the resulting 15-keto-13,14-dihydro metabolites that are produced having a much longer half-life (Hamberg and Samuelsson, 1971).

One of the major problems related to the metabolism of E series prostaglandins concerns the possibility that there may be enzyme systems capable of dehydrating E to A prostaglandins. The E series prostaglandins are known to dehydrate under the extraction conditions usually used (Schneider, Pike and Kupiecki, 1966). Recently, however, several reports of enzymes present in blood or tissue homogenates which will metabolise E series prostaglandins to less polar compounds which behave as the corresponding A prostaglandins have appeared (Levine, Gutierrez-Cernosek and Van Vunakis, 1973; Ramwell and Shaw, 1967; Shaw and Ramwell, 1969; McDonald-Gibson, McDonald-Gibson and Greaves, 1972; Cammock, 1973; Russel, Alam and Clary, 1973; Polet and Levine, 1975). In addition, several reports of A series prostaglandins being identified in seminal fluid samples (Hamberg and Samuelsson, 1966; Hamberg, 1968) and tissue extracts (Lee, Crowshaw, Takman Attrep and Gougoutas,

1967; Presyna, Attallah, Vance, Schodman and Lee, 1973) have appeared. However, as discussed on page 18 unless strict precautions are taken it must always remain debatable as to the origin of A series prostaglandins. There are reports of enzymes that will metabolise A prostaglandins in preference to either E or F_{α} prostaglandins. For example, guinea-pig liver microsomes are reported to contain enzymes that will convert prostaglandin A_1 into the corresponding 19 and 20 hydroxylated derivatives (Israelsson, Hamberg and Samuelsson, 1969). Under identical conditions little (3% or less) of the corresponding compounds were produced when prostaglandin E_1 was used as a substrate. Similarly, the plasma of several mammalian species have been shown to contain an enzyme which induces a single shift of the 10, 11 double bond of PGA_1 to produce the corresponding 9-oxo-11,13-diene isomer, PGC_1 (Jones, 1972; Jones and Cammock, 1973; Jones, Cammock and Horton, 1972; Horton, Jones, Thompson and Poyser, 1971; Polet and Levine, 1971; 1975). In addition, human and rabbit serum is claimed to contain another enzyme that isomerises the PGC to PGB (Polet and Levine, 1975), although, because of the low levels of enzyme activity, these reports require confirmation that it is not a chemically-induced isomerisation. Prostaglandin A_1 and also A_2 are also substrates for the 15(S) hydroxyprostaglandin dehydrogenase (Nakano et at., 1969).

One route of metabolism that appears to apply to A,B,E and F_{α} series prostaglandins is β-oxidation of both the α and ω-side chains. Rat liver mitochondria were found to β-oxidise the α-side chains of these prostaglandins

in vitro (Hamberg, 1968). This route of metabolism may be of particular importance for the in vivo inactivation of prostaglandin analogues that are not substrates for the 15(S) hydroxyprostaglandin dehydrogenase (Granstrom, 1975). Although little published information relating to the biological activity of α-dinor and α-tetranor prostaglandins is available it is known that α-nor prostaglandin E_1 has approximately 10% of the activity of prostaglandin E_1 on several smooth muscle preparations (Horton and Main, 1966).

The E and Fα prostaglandins are not interconverted during their biosynthesis from a common precursor fatty acid (Anggard and Samuelsson, 1965; Hamberg and Samuelsson, 1967). Recently, several reports have indicated that many biological systems including man (Hamberg and Wilson, 1973; Ziboh, Lord and Penneys, 1975); guinea-pig (Hamberg and Israelsson, 1970; Hensby, 1974; 1975), actively fermenting Baker's yeast (Schneider and Murray, 1973) and a variety of mammalian tissues (Lee and Levine, 1974; 1975; Lee, Pong, Katzen, Wu and Levine, 1975; Hensby, 1974; 1975) may possess enzymes capable of interconverting E and Fα prostaglandins. This type of metabolism could be of importance because many of the pharmacological actions of prostaglandins E and Fα differ both qualitatively and quantitatively; an example being that prostaglandin E_2 is a bronchodilator whereas prostaglandin $F_{2}\alpha$ is a bronchoconstrictor (Sweatman and Collier, 1968). Indeed, the possibility that there may be at least two distinct receptors, one being more selective for E type prostaglandins

and the other being more selective for $F\alpha$ prostaglandins has been suggested (Horton and Main, 1965; Eakins and Sanner, 1972). The possibility that three or more prostaglandin receptor types may exist was suggested by Pickles (1967). Recently Jones (1975) has provided pharmacological evidence for the presence of E, D and $F\alpha$ receptors.

The *in vivo* metabolism of prostaglandins E and $F\alpha$ by a variety of mammals has been studied and all of the above metabolic transformations have been detected. One interesting metabolic route not described is the reduction of the 9-keto group of the E series prostaglandins into the corresponding β-hydroxyl configuration. In the guinea-pig *in vivo* prostaglandin E_2 is metabolised to 5β, 7α-dihydroxyl-11-oxo-tetranor-prostanoic acid as determined by the major urinary metabolite (Hamberg and Samuelsson, 1969). This is in contrast to the metabolism of prostaglandin E_2 *in vitro* by guinea-pig liver homogenates where the 9-keto group is reduced to the corresponding 9α-hydroxyl configuration (Hamberg and Israelsson, 1970; Hensby, 1974; 1975). Prostaglandin F_2 is metabolised *in vivo* by the guinea-pig to the major urinary metabolite with the 5α, 7α-dihydroxy-11-oxo-tetranor-prostanoic acid structure (Granstrom and Samuelsson, 1969; Samuelsson, Granstrom, Green and Hamberg, 1971). Consequently, the major urinary metabolite of prostaglandins E_2 and $F_{2\alpha}$ in the guinea-pig differ only in the stereochemical configuration of the hydroxyl group at the C-5 position. The rat has also been shown to metabolise prostaglandin E_2 to 5α,7α-

dihydroxy-11-oxo-tetranor-prostanoic acid as determined by urinary metabolite studies (Samuelsson, Granstrom, Green, and Hamberg, 1971; Green, 1971). Another interesting feature of the *in vivo* metabolism of prostaglandin E_2 by the rat is that four of the excreted urinary metabolites contained a B ring structure. This may indicate that the rat has enzyme systems capable of dehydrating E prostaglandins to the corresponding A prostaglandins which are then isomerised to the corresponding B prostaglandins. It is known that rat plasma contains a prostaglandin A isomerase (Jones and Cammock, 1973). Prostaglandin $F_{1\alpha}$ and $F_{2\alpha}$ are also metabolised *in vivo* by the rat to a variety of metabolites some of which appear to have only undergone one step of β-oxidation prior to excretion (Granstrom, Inger and Samuelsson, 1965; Green and Samuelsson, 1968 Green, 1971). Recently however, 5,7,11-trihydroxy-tetranor-prostanoic acis has been detected as a rat urinary metabolite of prostaglandin $F_{2\alpha}$ (Sun, 1974). This compound obviously was oxidised at C-15 and reduced at C-13,14 to produce the 15-keto-13,14-dihydro derivative prior to being stereo-selectively reduced at the C-15. This may indicate that the α-dinor and the α-tetranor prostaglandins $F_{1\alpha}$ which are excreted may have undergone other routes of metabolism although their structures would not indicate this. The biological inactivation of either prostaglandins E_2 or $F_{2\alpha}$ *in vivo* by rabbit and human subjects has also been studied (Svanborg and Bygdeman, 1972; Hamberg and Samuelsson, 1969; Granstrom and Samuelsson, 1969; Samuelsson, Granstrom, Green and Hamberg, 1971; Hamberg and Wilson, 1973; Granstrom,

1973). The major urinary metabolite of PGE_2 in man was found to be 7α -hydroxy-5,11-diketotetranor-prostane-1, 16-diocic acid (Hamberg and Samuelsson, 1969,1971), whilst the corresponding major human urinary metabolite of prostaglandin $F_{2\alpha}$ was found to be 5α,7α-dihydroxy-11-oxo-tetranorprosta-1,16-diocic acid (Granstrom and Samuelsson, 1969). Thus in both man and guinea-pig the major urinary metabolites of E_2 and $F_{2\alpha}$ differ only in the substituent at C-5. However, it is apparent from these results that there are species differences in the metabolism of prostaglandins. A particularly interesting urinary metabolite for prostaglandin $F_{2\alpha}$ has recently been discovered in man (Granstrom, 1973; Granstrom and Samuelsson, 1972). This metabolite has been identified as 7α, 9α-dihydroxy (α -dinor, ω-tetranor)-prost -3-ene-1,14-dioic acid. The route by which this metabolite is formed remains unknown. However, yields of this metabolite are obtained in urine after the intravenous injection of either 9α,11α dihydroxy-15-ketoprost-5-enoic acid of 9α, 11α, 15α-trihydroxyprost-5-enoic acid but not 7α, 9α -dihydroxy-13-oxo-(α -dinor, ω -dinor)-prost-1,16-diocic acid. The structures of these metabolites are shown in figure 3 .

The Pharmacology and Physiology of Prostaglandins

The entire pharmacology and physiology of prostaglandins that has been reported to date obviously cannot be reviewed; however, areas that are relevant to this thesis, namely cardiovascular and reproductive aspects, will be dealt with in some detail.

The actions of prostaglandins on a variety of smooth

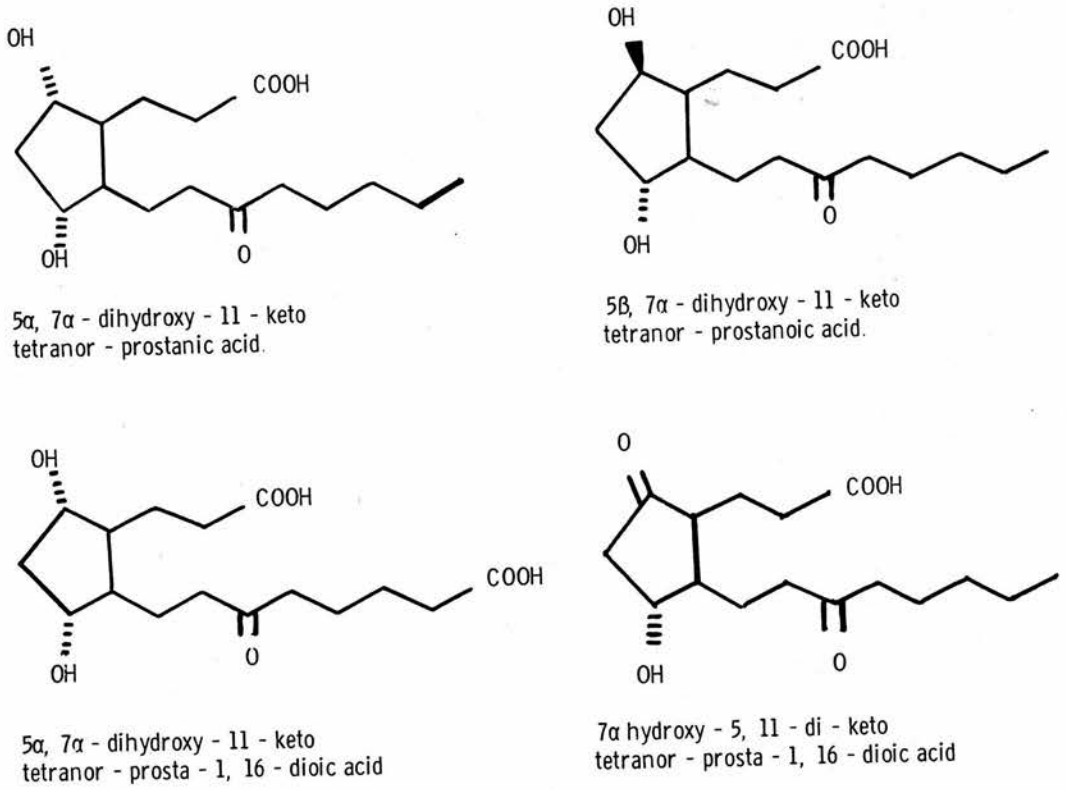

Figure 3. The structures of the major urinary metabolites of PGE_2 and $PGF_{2\alpha}$ in guinea pig (upper) and man (lower) respectively.

muscle preparations have been described (Bergstrom, Carlsson and Weeks, 1968; Horton, 1969; Jones, 1970). In general the majority of reports deal with the E and Fα series prostaglandins, although the actions of A, B and D series prostaglandins have been described. The responses of most smooth muscle are generally thought to be due to a direct action of prostaglandins on the cell. However, a variety of neuronal antagonists including procaine (Harry, 1968), atropine (Horton, 1965; Harry, 1968) and hyoscine (Bennett, Eley and Scholes, 1968; 1969) have been shown to depress the responses of human and guinea-pig ileum to prostaglandins. This, in conjunction with the fact that the responses of aneuronal smooth muscle such as the umbilical blood vessels appears to be unaffected by a variety of pharmacological antagonists (Hillier, 1970), might suggest that on certain smooth muscle preparations prostaglandins may have a direct and indirect mode of action. The fact that many pharmacological antagonists do not appear to block the actions of prostaglandins on a variety of smooth muscle preparations (Bennett, Eley and Scholes, 1968a 1968b; Horton, 1965; Strong and Bohr, 1967; 1967b) indicates that prostaglandins probably have different receptors from those of other agonists. Similarly although E and Fα prostaglandins are known to contract isolated segments of gastro-intestinal longitudinal from all species tested (Bergstrom, Elliasson, von Euler, and Sjovall, 1959; Horton and Main, 1963; 1965; Bennett et al., 1968 a; 1968 b) results from the actions of E and Fα prostaglandins on other types of smooth muscle would suggest

that discrete receptor sites for each of these prostaglandins exist. For example, isolated human bronchiolar smooth muscle is relaxed by E series prostaglandins whilst prostaglandin $F_{2\alpha}$ is spasmogenic on this preparation (Collier and Sweatman, 1968; Sweatman and Collier, 1968; Sheard, 1968; Rosenthale, Dervinis, Begany, Lapidus and Gluckman, 1970; Smith and Cuthbert, 1973). Similarly, evidence from the in vivo actions of various prostaglandins on the sheep cardiovascular system would suggest the presence of at least two distinct receptors (Jones, 1975). Activation of one type results in vasoconstriction and a rise in blood pressure whilst activation of the other results in vasodilation and a fall in blood pressure. Although prostaglandins D_2 and E_2 are the most potent agonists respectively on these two receptor systems, it has been suggested that 15-keto prostaglandin D_2 and 13, 14-dihydro prostaglandin E_1 may respectively be the most selective agonists (R.L. Jones, personal communication). The relative non-selectivity of certain prostaglandin receptors is exemplified by the responses of isolated gastro-intestinal longitudinal smooth muscle preparations which contract to both E and F_{α} prostaglandins. This could be due to the presence of two distinct systems both of which activate the contractile response; however, this would appear not to be the case (R.L. Jones, personal communication). This problem would obviously be resolved by a combination of totally selective E and F_{α} prostaglandins agonists and antagonists.

The central nervous system has been shown to

produce or contain biologically active prostaglandin-like substances (Horton and Main, 1967; Ramwell and Shaw, 1966; Bradley, Samuels and Shaw, 1969). Similarly, a variety of prostaglandins have been found to have marked pharmacological actions on the central nervous system. Prostaglandin E injected into the lateral ventricle of the unaneasthetised cat or intravenously into chicks produced prolonged stupour and sedation (Horton, 1964), whereas prostaglandin $F_{2\alpha}$ has no observable effects in cats but induced an immediate extension of the limbs and dorsiflexion of the chick (Horton and Main, 1965). The sedative actions of A series prostaglandins on the central nervous system of the two animals is qualitatively very similar (Jones, 1970). Detailed reviews of the many pharmacological actions of prostaglandins in the central nervous system have been published recently (Horton, 1972; Karim, 1972).

The analysis of human semen led to the identification and isolation of 13 different prostaglandins (Bergstrom and Samuelsson, 1962; Samuelsson, 1963; Bygdeman, 1964; Hamberg and Samuelsson, 1965; Bygdeman and Samuelsson, 1966 a; 1966 b; Hamberg and Samuelsson, 1966). Recently the 19-hydroxy derivatives of prostaglandin E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ have also been conclusively identified and 8-iso-19-prostaglandin $F_{2\alpha}$ tentatively identified (Taylor and Kelly, 1974; Kelly and Taylor, 1975; Jonsson, Middleditch, Desidero and Schexnayder, 1975). The high concentrations present were postulated as being important for normal fertility (Hawkins and Labrum, 1961; Bygdeman, Fredricsson, Svanborg and Samuelsson, 1970). Seminal prostaglandins

have been implicated in ejaculation, sperm transport, ovum transport or retention and uterine activity for a variety of species (Horton, 1972; Karim, 1972; Poyser, 1973; Labhsetwar, 1973; Chang, Hunt and Polge, 1973; Eskin, Sepic, Azarbol and Slate, 1972; Horton and Main, 1965; Levy and Linder, 1971).

Prostaglandins have been implicated in the ovulatory mechanism since it is known that non-steroidal anti-inflammatory prostaglandin synthetase inhibitors (Vane, 1971) will block ovulation (Orczyk and Behrman, 1972; Armstrong and Grinwich, 1972; Grinwich, Kennedy and Armstrong, 1972; O'Grady, Caldwell, Auletta and Speroff, 1972). The mechanism appears to be related to follicular rupture and not ovum maturation and thus it was concluded that the actions of LH on the ovary are independent of prostaglandins although a modulatory role may be implicated (Linder, Zor, Bauminger, Tsafriri, Lamprecht, Koch, Antebi and Schwartz, 1974).

Other areas of reproduction where prostaglandin production and release have been implicated as having a role are menstruation in women and luteolysis in many animals. The presence of smooth muscle stimulants in menstrual fluid was shown by Pickles (1957) and in the early 1960's these were identified as several substances of lipid origin including prostaglandins E_2 and $F_{2\alpha}$ (Clitheroe and Pickles, 1961; Clitheroe, 1961; Pickles and Hall, 1963; Pickles and Ward, 1965; Eglinton, Raphael, Smith, Hall and Pickles, 1963). From the analysis of human endometrial curettings it would appear that the

levels of prostaglandins E_2 and $F_{2\alpha}$ remain low in the proliferative phase prior to ovulation, although PGE_2 levels may rise slightly in the late proliferative phase. Following ovulation the $PGF_{2\alpha}$ rise but the PGE levels remain low and thus the ratio of PGF to PGE produced during the luteal phase may be important for the menstrual process (Downie, Poyser and Wunderlich, 1974; Pickles, Hall, Best and Smith, 1965).

In many animal species including guinea-pig, sheep and cow, pseudo-pregnant rabbit, rat and hamster but not woman, the presence of the uterus is essential if normal luteal regression is to occur. It has thus been postulated that many animal species release a luteolytic hormone which acts on the ovary to stimulate luteal regression (Poyser, 1973). The release of this hormone by the sheep in luteolytic concentrations occurs only at the end of the oestrous cycle (McCracken, Carlson, Glew, Goding, Baird, Green and Samuelsson, 1972). The evidence to date suggests that in the sheep and guinea-pig prostaglandin $F_{2\alpha}$ is the uterine luteolyic hormone (Bland, Horton and Poyser, 1971; Poyser, 1971; Blatchley, Donovan, Horton and Poyser, 1972; Earthy, Bishop and Flack, 1975; Poyser, 1975; McCracken, Baird and Goding, 1971; Thorburn, Cox, Currie, Restall and Schneider, 1972; Horton, 1972; Poyser, 1974).

Prostaglandins have been implicated in the parturition process of many animals and also women (Poyser, 1974; Karim, 1972). Sharp increases of the prostaglandin $F_{2\alpha}$ present in the uterine venous blood of pregnant sheep

were recorded in the final day of gestation (Thorburn, Nicol, Bassett, Shutt and Cox, 1972; Liggins, Grieves, Kendall and Knox, 1972; Challis, Harrison, Heap, Horton and Poyser, 1972). In women, prostaglandins have been reported to be present in the venous blood during labour (Karim, 1968) or spontaneous abortion (Karim and Hillier, 1970). This evidence, in conjunction with the fact that prostaglandins are only detectable in the amniotic fluid during spontaneous labour or abortion (Karim, 1966; Karim and Devlin, 1967) and prostaglandin $F_{2\alpha}$ released during labour is maximal at the time of peak uterine activity (Brummer and Craft, 1973; Craft, Scrivener and Dewhurst, 1973), would suggest that prostaglandins may have a functional role in parturition. Certainly indomethacin and aspirin, known prostaglandin synthetase inhibitors, will prolong gestation and delay parturition in rats (Aiken, 1972; Chester, Dukes, Slater and Walpole, 1972) and women (Lewis and Schulman, 1973).

A direct consequence of the many physiological pharmacological and biochemical actions of prostaglandins is the requirement for more selective and sensitive qualitative assay procedures. It was with this background that I began my attempt to develop a sensitive radio-immunoassay specific for prostaglandin E_2.

SECTION I

ATTEMPTS TO DEVELOP A RADIOIMMUNOASSAY METHOD FOR PROSTAGLANDIN E_2.

Materials and Methods

Preparation of prostaglandin E_2 solutions for use in prostaglandin E_2 radiommunoassays and metabolism studies

Solid samples of prostaglandin E_2 were generously supplied by Dr. D.R. Maxwell and Dr. K. Crowshaw of May & Baker Ltd. These were transferred to 50ml pear-shaped flasks, dissolved in a small volume (5-10 ml) of Analar methanol and taken to dryness under reduced pressure at 40°C. Analar methanol was used throughout for the stock-prostaglandin solutions. After desiccation under vacuum of the residue until a constant weight was obtained, a calculated volume of methanol was added to give a nominal concentration for prostaglandin E_2 of 10 mg ml^{-1}. From this stock solution 2 ml was transferred to another 50 ml pear shaped flask and the volume adjusted to 20 ml with methanol. After thorough mixing an aliquot (20µl) was removed and added to 1.98 ml of methanol in a silica cell of 3 ml volume and 1 cm pathlength. The spectrum between 350 nm and 200 nm was recorded against a cell containing 2.0 ml of analar methanol using a Pye Unicam SP 800 ultraviolet spectrophotometer equipped with a SP825 programme controller. The concentration of prostaglandin E_2 was then calculated by estimation of the prostaglandin B_2 produced on treatment with freshly prepared methanolic (Molar) potassium hydroxide (Andersen 1969). To each cell in rapid succession 0.2 ml of Molar KOH was added and the contents thoroughly mixed with a battery operated electrical Teflon stirring rod. A spectra was recorded immediately after the mixing and

then a plot of the absorbance at a fixed wavelength of 278 nm (MeOH max) prostaglandin B_2 = 278 nm) against time was obtained. After approximately 25 minutes, the plot plateaued and following a further 5 minute incubation the spectra between 350 nm and 200 nm was recorded. At this time the maximum conversion of prostaglandin E_2 to prostaglandin B_2 ($\doteq$ 99.8%) was complete. The concentration of prostaglandin B_2 was then calculated as follows :

OD_{278} for 10 µg ml^{-1} prostaglandin B_2 = 0.81 (in methanol)

But prostaglandin B_2 present in 2,2 ml MeOH = prostaglandin E_2 in 20 µl MeOH = X µg.

where $X = \frac{OD_{278} \times 10 \times 2.2}{0.81}$

Therefore prostaglandin E_2 present in 1 ml of diluted stock solution = $\frac{X \times 1000\ \mu g}{20}$

Samples of ^{3}H-labelled prostaglandin E_2 (1 to 5 mCi 5,6,8,11,12,14, 15-^{3}H PGE_2 Sp. Ac 145-167 Ci m $mole^{-1}$, Radiochemical Centre, Amersham, England) were transferred to 50 ml pear-shaped flasks, taken to dryness under reduced pressure at 40°C and desiccated at room temperature under vacuum until dry. The residue was then dissolved in 10-20 ml of methanol and an aliquot (5-20 µl) was mixed with 10-20 µg of the diluted (cold) prostaglandin E_2 solution in an Eppendorf tube. This mixture was then subjected to thin layer chromatography on neutral silica gel (50 mm x 200 mm x 0.25 mm; E. Merck A.G.) on glass.

Marker standards containing approximately 10 to 20 µg each of cold prostaglandins E_2 and A_2 (or B_2) were spotted on the plate at the side of the sample being chromatographed. The plates were then developed in either the FVI (Andersen 1969) or the GCM (Miller 1974) solvent systems described on page 56. The plates were developed until the solvent was approximately 2 cm from the top of the plate after which the plates were dried under a stream of air until no scent of solvent residue was detected. The radioactive containing plates were then automatically scanned using a Panax radio thin layer chromatographic plate scanner. From the resulting scans it was possible to determine whether the ^3H-labelled prostaglandin E_2 required purification before use. The remaining prostaglandins were then detected by spraying the plates with a saturated solution of phosphomolybdic acid in ethanol and heating at 115°C for 15-20 minutes. This method of detection produced green/blue spots on a yellow background.

Purification of ^3H-labelled prostaglandin E_2

The high specific activity ^3H labelled prostaglandin E_2 was found to undergo radiochemical breakdown even when stored as a methanol solution at -20°C (as determined by radiochemical purity of thin layer chromatography). Consequently it was desirable to check the radiochemical purity at frequent intervals (≤ 1 month). This decay did not always result in material being produced that co-chromatographed (on t.l.c) with authentic prostaglandin A_2 (or B_2). In certain cases

the specific activity of the ^{3}H-labelled prostaglandin E_2 has been reduced by the addition of cold prostaglandin E_2 and this has been found to prolong the stability of the radio-actively labelled prostaglandin E_2.

When a batch of the ^{3}H-labelled prostaglandin E_2 has been found to be contaminated it has usually been purified by reversed phase partition chromatography (R.P.P.C) on Sephadex LH-20. An example of this isshown in figure 4. In this example a glass column (300 x 10 mm) was packed with graded Sephadex LH-20 (50 $\pm$ 5 μM diameter particle size) previously equilibrated in the solvent mixture (methanol - water - chloroform butanol - acetic acid 50: 50:5:5:0.1 by vol.) and allowed to flow at a constant pressure head overnight prior to use. The column bed volume was 20 ml, the flow rate 7-8 ml hr^{-1} and the room temperature 23$\pm$ 1°C. The sample of ^{3}H PGE_2 (1.5 m Ci$\pm$ 3.5 μg) was applied to the column in 0.5 ml of eluting solvent (+ 2 x 0.2 ml washings) and fractions of 2.0 ml collected on an LKB Ultrorac fraction collecter. From each fraction 5 μl was removed and estimated for radio-activity by liquid scintillation counting. This enabled the elution profile to be plotted and the fractions corresponding with authentic prostaglandin E_2 to be determined. These fractions were then pooled in a 100 ml pear-shaped flask, taken to dryness under reduced pressure at 40°C and redissolved in 10 ml of methanol. This material was then subjected to t.l.c. using the G.C.M. solvent (Miller 1974) and found to be greater than 99% pure.

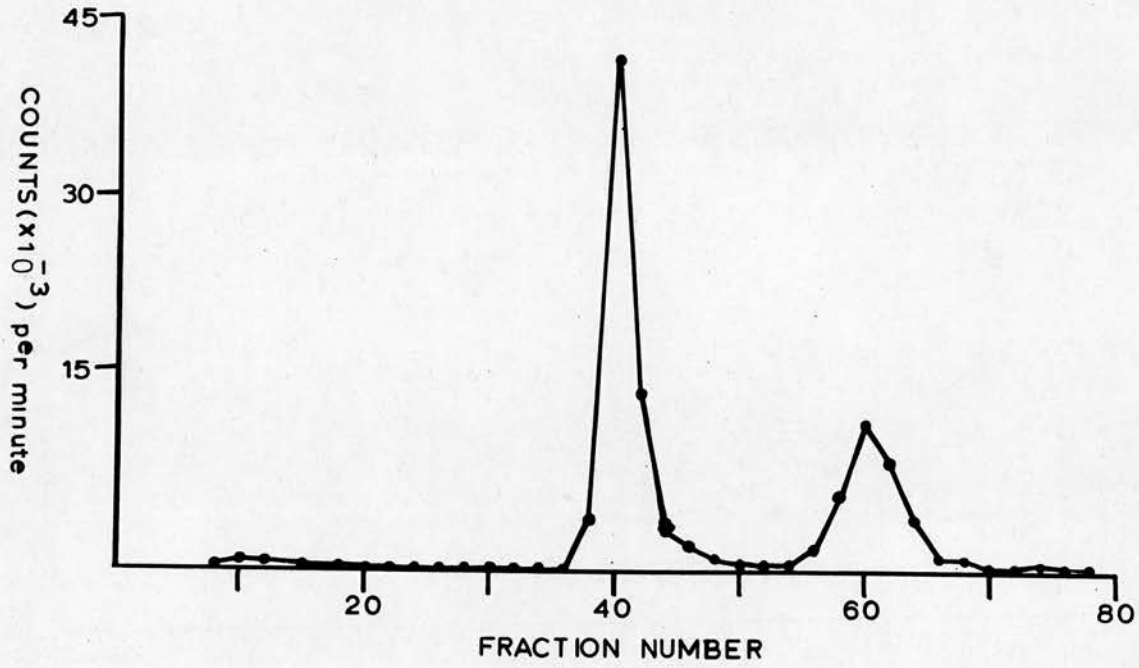

Figure 4. The purification by reversed phase gel partition chromatography on graded Sephadex LH-20 (50 ± 5 uM particle size) of 1.5 mCi ^{3}H-PGE$_2$. The developing solvent was methanol-chloroform-n-butanol-acetic acid (50:50:5:0.1 by volume).

Preparation of prostaglandin E_2 - bovine serum conjugate

All glassware was thoroughly washed with methanol and distilled water before being dried.

Into a 3 dram capped vial (5 ml vol) 6.762 mg of prostaglandin E_2 and 55400 dpm of ^{3}H-labelled prostaglandin E_2 (Sp. Ac. 167 Ci m .mole^{-1}. Amersham) was pipetted (methanol solutions; total vol. 1.0 ml). The methanol was evaporated under a stream of dry air and the dry residue redissolved in 0.5 ml of redistilled 1,4-dioxane. This solution was cooled by partially immersing the vial in a petri dish containing an ice-water mixture and continually stirring with the aid of a magnetic stirrer. To the vial tri-n-butylamine (10 µl) was added and after 5 minutes iso-butylchloroformate (2.5 µl). The reaction was left for 30 minutes and then the reaction mixture was added to another capped 3 dram vial containing a cool solution of British bovine serum albumin (22.41 mg), NaOH (23 µl; 1N) and distilled water (0.6 ml). The original reaction vial was rinsed with 1,4 dioxane (0.6 ml) and the contents added to the second vial. The resulting reaction mixture was kept cool and thoroughly mixed (magnetic stirrer) for a further 4 hours.

The volume of the reaction mixture was then measured (1.6 ml) and 10 µl was transferred to a cellophane dialysis bag containing 5 ml distilled water. This was then sealed with double knots and dialysed for 48 hours against 250 ml of distilled water in a measuring cylinder. The bag was agitated with a fine stream of nitrogen bubbles to ensure maximum efficiency of the dialysis system. The residual

reaction mixture was placed in another dialysis bag and dialysed for 48 hours against running tap water (25 - 50 litres hr^{-1}).

After 48 hours the dialysis bag containing 10 µl of the reaction mixture was washed externally into a beaker and pooled with the contents of the measuring cylinder. The pooled contents were then acidified to pH 5.0 with 10% aqueous acetic acid and extracted twice with an equal volume of redistilled ethyl acetate. The pooled ethyl acetate was washed twice with 20 ml of distilled water then taken to dryness under reduced pressure at 40°C and vacuum desiccated at room temperature. The residue was redissolved in 5.0 ml of methanol and 2.0 ml transferred to a silica cell of 3 ml volume and 1 cm pathlength. The spectrum between 350 nm and 200 nm was recorded against a cell containing 2.0 ml of methanol using a Pye Unicam SP800 ultraviolet spectrophotometer equipped with a SP825 programme controller. The residual 3.0 ml of methanol was reduced to a volume of 0.2 ml at reduced pressure and applied as a thin band (0.2 mm x 20 mm) 3 cm from the base of a neutral silica gel thin layer chromatography plate (50 mm x 200 mm x 0.1 mm on glass). The plate was then developed to a height of 16 cm in the AI solvent system of Green and Samuelsson (1965) (benzene; 1,4-dioxane; glacial acetic acid, 20:20:1 by vol.). Marker prostaglandins E_2 and A_2 (10 µg each) were applied at the side of this methanol residue. After development the plate was scanned for radioactivity using a Panax radio thin layer chromatographic plate scanner. The plate was then sprayed with a solution of 10%

phosphomolybdic acid in ethanol and heated at 110°C for 15 minutes. The spots that developed were outlined in pencil.

The contents of the dialysis bag containing the majority of the reaction mixture after 48 hours was transferred to polypropylene centrifuge tubes and then centrifuged at 5000 x g (at 4°C) for 20 minutes. The supernatant was carefully transferred to a 250 ml round bottomed flask and frozen by external application of dry ice (solid CO_2)-acetone mixture. The frozen contents were then lyophilised overnight under vacuum.

The dry contents were carefully transferred to an 8 dram vial of known weight for the estimation of the recovered PGE_2-BSA conjugate.

Three scintillation vials containing 10 ml of a toluene scintillant mixture (toluene, ethoxyethanol, 2,5-diphenyloxazole (PPO), 1,4-di-(2-(4-methyl-5-phenyloxazolyl))-benzene (dimethyl POPOP) (2000; 400; 8.52; 0.22 v/v/w/w)), 2 ml of Nuclear Chicago Solubiliser and 0.2 ml of distilled water were prepared. To one vial 780 µg and another 529 µg of the PGE_2-BSA conjugate was added and the contents thoroughly solubilised and mixed. The vials were then counted several times on a Nuclear Chicago Mark II scintillation counter and the d.p.m. computed on a PDP 8 I digital computer by the external standard channels ratio method.

The number of prostaglandin E_2 molecules conjugated per molecule of bovine serum albumin was then calculated as follows :

i) Assume molecular weight of British BSA = 66,500

ii) 780 µg and 590 µg of conjugate contained 894 dpm and 582 dpm respectively.

iii) 6.762 mg of PGE_2 contained 55,400 dpm .·. amount of PGE_2 bound in 0.78 mg of conjugate

$$= \frac{894 \times 6.762}{55,400} \text{ mg}$$

$$= \underline{0.10911963} \text{ mg}$$

·.· amount of BSA conjugated with 0.10911963 mg of PGE_2

$$= 0.78 - 0.10911963$$

$$= 0.6708804 \text{ mg}$$

·.· 1 mg of BSA is conjugated with $\frac{0.10911963}{0.6708804}$

$$= 0.1626514 \text{ mg of } PGE_2$$

·.· 66,500 g (1 mole) of BSA is conjugated with 10816.318 g of PGE_2

But molecular weight of PGE_2 = 352

·.· 1 mole of BSA is conjugated with $\frac{10816}{352} = \underline{30.73 \text{ moles of } PGE_2}$

By analogy with the d.p.m. value obtained from 590 µg of the PGE_2-BSA conjugate it can be calculated that 1 mole of BSA is conjugated with $\underline{25.86 \text{ moles of } PGE_2}$.

·.· on average 28.30 moles of PGE_2 are conjugated per mole of BSA

Preparation of PGE_2-BSA conjugate for use in immunisation procedures

Samples of the PGE_2-BSA conjugate (3-4 mg) were weighed, transferred to glass bottles (25 ml) and dissolved in sterile isotonic saline (6-8 ml; 0.9% w/v). An equal volume of Freund's complete adjuvant was added and the resulting suspension thoroughly mixed with a Polytron homogeniser at maximum speed for 30-60 seconds to produce a water-in-oil mixture.

For 'booster samples' of the PGE_2-BSA conjugate the same procedure was employed with the exception that Freund's incomplete was substituded as the emulsifying agent.

Immunisation procedures

a) Multiple intradermal

The method of Vaitukaitis, Robbins, Nieschlag and Ross (1971) was used. Six female New Zealand white rabbits (1.8-2.4 kg) were shaved along the back and given 25-40 injections intradermally (total volume 2 ml) of the Freund's emulsified PGE_2-BSA conjugate. The rabbits were caged and fed water and food ad libidum.

b) Intranodal

The method of Boyd and Peart (1968) was used. Six female New Zealand white rabbits (2.1-2.6 kg) were shaved along the back of each hind limb and subcutaneously injected with lignocaine until pain responses could not be elicited. The popliteal lymph nodes of each leg were exposed under sterile conditions and injected with 100-200 µl of the PGE_2-BSA emulsion. The lymph nodes from two further rabbits were removed for histological examination. After the wounds

had been stitched, each rabbit was injected intramuscularly with benzyl-penicillin (5 mg/kg).

Preparation of anti-sera for evaluation

At four weekly intervals after the primary immunisation procedure the rabbits were bled from the marginal ear vein. The skin covering the ear vein was shaved, coated in a fine film of petroleum jelly and an incision made approximately half-way between the tip and base of the ear. Blood was collected in 50 ml glass tubes and the flow enhanced by covering the remaining area of the ear with xylene. After collection of 25-50 ml of blood during a 15-30 minute period the blood containers were sealed with parafilm and stored at 4°C for a minimum of 16 hours to enable the clotting process to occur.

The resulting serum (anti-serum) was transferred to 35 ml test tubes and centrifuged at 2500 x g (at 4°C) for 20 minutes to remove traces of the blood clot. The resulting supernatant was transferred to clean 35 ml tubes and heated at 55°C for 30 minutes to ensure maximum decomplimentation had occurred. The resulting antisera was then treated with aqueous sodium azide (10 mg/ml) (100 µl:10 ml antisera) and stored in 2-5 ml aliquots at -20°C.

Buffers and solutions

a) Diluent

Tris-HCl buffer (50 mM, pH8) containing gelatin (100 mg/l) was used as the standard buffer for radioimmunoassay incubations unless otherwise specified.

b) Normal rabbit serum (NRS)

Female New Zealand white rabbits were bled from the marginal ear vein and the serum harvested as described for the immunised rabbits. The serum was diluted with the diluent to a concentration of 1% (v/v) unless otherwise stated.

c) Donkey anti-rabbit gamma globulin serum (DARS)

This was supplied by Dr. W. Hunter of the MRC Radioimmunoassay Unit, Edinburgh. The DARS was diluted with the diluent buffer to 20% (v/v) unless otherwise stated.

Liquid Scintillation Counting and Analysis of Results

Liquid scintillation counting was performed with a Mark II Nuclear Chicago liquid scintillation counter.

Individual radioimmunoassay samples (600 µl) were counted in 14 ml of a toluene, ethoxyethanol based scintillant mixture. (toluene-ethoxyethanol 1500; 900 v/v napthalene; 2,5-diphenloxazole (PPO) and 1,4-di-(2-(4-methyl-5-phenyloxazolyl))-benzene (dimethyl POPOP) 112.5; 10.5;0.9 by weight). Quench-calibration curves constructed for the scintillant mixture and the automatic external standard channels ratio method were used to convert c.p.m. into d.p.m. A PDP8I computer was used for these calculations.

Each batch of radioimmunoassay samples were counted in the following sequence :

Vial No. 1 - Background (scintillant and 600 µl diluent)

2 -) Counting standards (scintillant +
3 -) 600 ul of diluent containing

^3H labelled PGE_2 only)

4)
)
5)

6+ - Samples. (scintillant + 600 µl sample)

The values for d.p.m. were then used to calculate the percentage of the ^3H-label bound to the antisera in each sample by the following calculations :

$$\text{Average d.p.m. added} = \frac{2+3+4+5-1}{4} = X$$

d.p.m. in sample = y

$$\% \text{ bound} = 100 - \frac{y}{x} \times 100$$

<u>The effect of diluting the anti-sera on the binding of either ^3H-labelled PGE_2, PGA_2 or PGB_2</u>

An initial dilution of the antiserum ($\frac{1}{100}$) produced by each rabbit was prepared and 1 ml added to a glass tube containing 1 ml of diluent. The sample was vortexed to give 2 ml of a $\frac{1}{200}$ dilution of the antisera. From this sample 1 ml was transferred to another tube containing 1 ml of diluent and vortexed. This dilution procedure was used to produce tubes containing 1 ml of diluted antisera at the following dilutions : 1:200; 400; 800; 1600; 3200; 6400 and 12800. In addition 8 tubes containing 1 ml of diluent only were prepared.

To each tube 50 µl of ^3H PGE_2 (400,000 d.p.m. ml^{-1} Sp.Ac 167 Ci m $mole^{-1}$) was added and the samples vortexed. Four of the tubes containing diluent only were then sealed with parafilm (counting standards). After incubating the samples at room temperature for 1 hour

50 µl of NRS (1:100) was added to the four remaining tubes containing diluent only and the sample tubes containing dilutions of 1:3200 or greater. After a further incubation of 10 minutes at room temperature 50 µl of diluted DARS (1:5) was added to all tubes containing a dilution of 1:1600 or greater and two of the blank tubes. To the remaining tubes 100 µl of the diluted DARS was added. The samples were thoroughly mixed and after an overnight incubation at 4°C, centrifuged at 2000 x g for 30 minutes. From the supernatant 600 µl was transferred to 14 ml of toluene scintillation mixture for liquid scintillation counting.

This experiment was also repeated for ^{3}H PGA_2 and ^{3}H PGB_2.

Factors influencing the binding of ^{3}H PGE_2 to rabbit antisera

Dilution curves for antisera obtained from two rabbits (nos. 25 and 26) were set up as described above. In each dilution curve the incubation time with NRS and DARS varied as described below in table

Incubation Time	
NRS (min)	DARS (hrs)
10	16
20	16
60	16
120	16
10	8
10	16
10	24
10	48

b) Concentration of NRS and DARS

Dilution curves were set up with incubation times for NRS and DARS of 10 minutes and 16 hours respectively. The initial concentration of NRS and DARS incubated with each dilution curve was as follows :

NRS	DARS
1:50	1:5
1:100	1:5
1:200	1:5
1:400	1:5
1:100	1:3
1:100	1:4
1:100	1:5
1:100	1:6

Preparation and purification of ^{3}H-labelled $PGF_{2\beta}$ free acid

Approximately 5m Ci ^{3}H-labelled PGE_2 (167 Ci m mole^{-1}) was taken to dryness under reduced pressure in a 50 ml pear-shaped flask. The vacuum dried residue was redissolved in 1.0 ml of analar methanol and cooled to -10°C with an ice/acetone mixture. A small aliquot of sodium borohydride was added and the reaction vessel stoppered. After 60 minutes at 0 to 10°C the reaction mixture was diluted to approximately 50 ml with distilled water, acidified to pH4 with 10% aqueous acetic acid and extracted twice with an equal volume of diethyl ether. The pooled ether fractions were washed twice with 20 ml of distilled water and then taken to dryness under reduced pressure. The residue was vacuum desiccated and re-dissolved in 0.5 ml of a mixture containing methanol,

water, chloroform, butanol, glacial acetic acid (40; 60; 4: 6: 0.1 by vol.) This was applied to a column (35 ml bed volume) of Lipidex 1000 and eluted with the solvent mixture at a flow rate of 6-8 ml hr^{-1}. Fractions (2.5 ml) were collected in glass test tubes on an LKB Ultrorac fraction collecter and aliquots (10 µl) from alternate fractions assayed for radioactivity by liquid scintillation counting. From the elution profile it was possible to pool several fractions containing prostaglandins $F_{2\alpha}$ and $F_{2\beta}$ respectively. These were found to be greater than 98% pure with respect to each other when subjected to thin layer chromatography in the F VI solvent system (Andersen 1969).

Preparation and purification of ^{3}H-labelled $PGE_{2\beta}$ methyl ester

Approximately 500 µCi of the purified ^{3}H-labelled $PGF_{2\beta}$ free acid was dissolved in 0.5 ml of methanol and treated with excess freshly prepared diazomethane for 10 minutes at room temperature. The sample was then taken to dryness under reduced pressure and vacuum desiccated before being redissolved in 150 µl of redistilled chloroform. This was then made up to 500 µl with n-heptane and applied to a column of Lipidex 5000 (30 ml bed volume) previously equilibrated in this solvent. The column was eluted at 6-8 ml hr^{-1} and fractions of 3 ml were collected on an LKB Ultrorac fraction collecter. The elution profile of the column was determined by assaying alternate fractions (10 µl aliquots) for radioactivity by liquid scintillation counting. The fractions containing the ^{3}H-labelled $PGF_{2\beta}$

were pooled, taken to dryness under reduced pressure and vacuum desiccated. The residue was dissolved in 5.0 ml of methanol and approximately 0.5 µCi was applied to a thin layer chromatography plate. Marker standards of $PGF_{2\alpha}$ and $PGF_{2\beta}$ methyl esters (0.5 µCi each) were applied at the side of the sample and the plate was then developed in the FVI solvent system. After development the plate was scanned for radioactivity on a Panax thin layer chromatography plate scanner. The resulting scans revealed the purified ^{3}H-labelled $PGF_{2\beta}$ methyl esters to be greater than 99% radiochemically pure.

Preparation and purification of $PGF_{2\alpha}$ Me ester

Prostaglandin $F_{2\alpha}$ (1 mg) dissolved in methanol (1.0 ml) was converted to methyl ester by treatment with excess ethereal diazomethane as described on page 61 . The sample was then taken to dryness, vacuum desiccated and purified by straight phase gel partition chromatography on Lipidex 5000. The column (30 ml bed volume) used was equilibrated in an n-heptane-chloroform mixture (70:30 v/v) and fractions of 3.0 ml were collected. Aliquots (30 µl) from alternate fractions were (starting at fraction 20) taken to dryness and redissolved in an equal volume of BSTFA. These were heated at 60°C for 15 minutes and then 5 µl aliquots were subjected to gas chromatography on a Pye Unicam Series 104 gas chromatograph. The instrument was equipped with a glass column (1.5 x 2mm i.d), packed with 3% OV-1 on Supelcoport. The carrier gas (nitrogen) flow rate was 25-30 $ml\,min^{-1}$ and the oven temperature 195°C. The fractions containing the $PGF_{2\alpha}$

methyl ester were pooled, taken to dryness and vacuum desiccated. The residue was redissolved in 10 ml and the concentration of $PGF_{2\alpha}$ me ester present calculated by gas chromatography with $PGF_{2\beta}$ me ester as an internal standard.

Preparation and purification of 15(s)$PGF_{1\alpha}$; 15(S)$PGF_{1\beta}$; 15(R)$PGF_{2\alpha}$ and 15(R)$PGF_{2\beta}$.

Samples (1 mg) of 15(S)PGE_1 and 15(R)PGE_2 were transferred in methanol to 25 ml pear-shaped flasks. These were then cooled to -10°C with ice-acetone mixture after which solid sodium borohydride (Ca 100mg) was added to each sample. The samples were incubated at this temperature for 60 minutes and then distilled water (50 ml) was added to each followed by glacial acetic acid until pH4 was reached. The samples were then extracted twice with an equal volume of ethyl acetate. The pooled ethyl acetate was washed twice with 10 ml of distilled water, taken to dryness and vacuum desiccated. The samples were then dissolved in 0.5 ml methanol and converted to methyl esters by treatment with ethereal diazomethane as described on page 61 . The individual samples were subjected to straight phase gel partition chromatography as described on page 47 to yield the pure epimers.

Preparation of $PGF_{2\beta}$ -BSA conjugate

The method described on page 37 was employed. Pure $PGF_{2\beta}$ (10.5 mg) was conjugated with British Bovine Serum Albumin (BSA) (17 mg) to yield 17 mg of the $PGF_{2\beta}$ BSA conjugate that was calculated to have an average composition of 32 $PGF_{2\beta}$ molecules per molecule of BSA.

In an identical procedure 50 mg of $PGF_{2\beta}$ was conjugated with 90 mg of BSA and the resulting conjugate was found to have a composition of 29 $PGF_{2\beta}$ molecules per molecule of BSA.

Immunisation procedures

a) Multiple intradermal

Twelve female New Zealand White rabbits (2.2-2.9 kg) were immunised by the method of Vaitukaitis, Robbins, Nieschlag and Ross (1971) as described on page 41.

b) Intramuscular

The above twelve rabbits were given booster injections of the $PGF_{2\beta}$ BSA conjugate starting approximately four months after the initial immunisation procedure. The $PGF_{2\beta}$ - BSA conjugate was prepared as a water in oil emulsion as described previously with the exception being that incomplete Freund's adjuvant was substituted as the emulsifying agent. The rabbits were injected (intramuscularly) with 0.5 ml at each shoulder and upper thigh region. The booster procedure was repeated at six-weekly intervals.

Preparation of anti-sera for evaluation

The details of this method are described on page 42. Blood samples were collected at four-weekly intervals after the primary immunisation procedure. Following booster immunisation procedures the rabbits were bled one week after each booster immunisation. In all cases the blood was collected from the marginal ear vein, allowed to clot at 4°C and the anti-serum withdrawn. After centrifugation to remove blood clot fragments the anti-sera were decomplementated by heating at 55°C for 30 minutes and then

treated with sodium azide (10 mg ml^{-1}; 100 ul : 10 ml anti-sera) before being stored at -20°C in 2-5 ml aliquots.

Buffers and solutions

The diluent (tris-HCl buffer 50 nM pH8), normal rabbit serum and donkey anti-rabbit serum are as described on page 42 . Stock prostaglandin solutions were prepared from methanol solutions containing known amounts of the given prostaglandin. Aliquots of the methanol solutions were taken to dryness under a stream of dry air and then redissolved in a calculated volume of diluent to give the required concentration of prostaglandin. From these stock solutions the desired dilutions of the prostaglandins could be made by mixing known volumes of stock solutions with calculated volumes of diluent. All samples were stored at -20°C and thawed immediately before use.

Liquid Scintillation Counting and the Analysis of Results

The liquid scintillation counting was performed with a Mark II Nuclear Chicago liquid scintillation counter using the scintillant described previously on page 43 . Aliquots (600 µl) of individual samples were counted and the automatic external channels ratio method was used to convert c.p.m. to d.p.m. A PDP 8 I computer was used for these calculations and the conversion of d.p.m. into % of ^{3}H-label bound as described on page 43 .

The effect of dilution on the binding of either ^{3}H-labelled $PGF_{2\beta}$ free acid or ^{3}H-labelled $PGF_{2\beta}$ methyl ester to the $PGF_{2\beta}$ - BSA anti-sera

Sequential dilutions of anti-sera (1:200 - 1:12,800) were prepared as described on page 44 . In addition 8 tubes containing 1 ml of diluent only were prepared.

To each tube 50 µl of the required ligand ^{3}H $PGF_{2\beta}$ (400,000 d.p.m. ml^{-1} in diluent. Approximate Sp.Ac. 167 Ci m $mole^{-1}$) was added and the samples vortexed. Four of the tubes containing diluent only were then sealed with parafilm (counting standards). After a 60 minute incubation at room temperature, 50 µl of NRS (1:100) was added to the four remaining tubes containing diluent only and the anti-sera samples of 1:3200 dilution or greater. After a further incubation of 10 minutes at room temperature 50 µl of DARS (1:5) was added to all tubes containing a dilution of 1:1600 or greater and to two of the blank tubes. To the remaining tubes 100 µl or greater of the diluted DARS was added. The samples were thoroughly mixed

and after an overnight incubation at 4°C, centrifuged at 2000 x g for 30 minutes. From the supernatant 600 µl was transferred to 14 ml of toluene scintillation mixture for liquid scintillation counting.

For certain batches of anti-sera, particularly those harvested after booster immunisation procedures, the range of dilutions was extended to include 1:25,600, 1:51,200, 1:102,400. In all but the first batch of anti-sera harvested ^{3}H-labelled $PGF_{2\beta}$ methyl ester was used as the radioactive ligand.

Factors influencing the standard curves for prostaglandin $F_{2\beta}$ methyl ester

From the dilution curves described on page 52 it was possible to ascertain the approximate final dilution of antiserum which gave 60%-70% binding of the ^{3}H-labelled prostaglandin $F_{2\beta}$ methyl ester. A fresh batch of this antiserum was then diluted to 1/24th of this value such that when 50 µl was added to the following reaction vessels, the final dilution was the same as that previously giving 60%-70% binding. Duplicate tubes containing prostaglandin $F_{2\beta}$ methyl ester (in 1.0 ml of diluent buffer) at the following amounts (pico-grams added): 0 (zero standards), 10,30,50,70,150,310,630,1270 and 2550 were prepared. In addition eight blank tubes containing 1.0 ml of diluent buffer only were prepared. To each tube 50 µl of ^{3}H-labelled prostaglandin $F_{2\beta}$ methyl ester was added (Spec. Act. 167 Ci m $Mole^{-1}$; 400pg ml^{-1}), and the tubes vortexed and equilibrated at room temperature for ten minutes.

Four of the blank tubes were then sealed. Then 50 µl of the diluted anti-sera was added to all the tubes (except the four remaining blanks) and the samples vortexed and equilibrated at room temperature for a further 60 minutes. Then 50 µl of diluted normal rabbit serum (NRS) at 1:100 dilution was added to all the tubes (except the four sealed blank tubes) which were vortexed. Following a further incubation of 10 minutes at room temperature, 50 µl of diluted donkey anti-rabbit gamma globulin serum (DARS) at 1:5 was added and the samples were then assayed by the liquid scintillation method described on page 43 .

For each anti-serum tested this was repeated with the NRS and DARS concentration ranges described on pages 45 & 46. but with the range of added prostaglandin $F_{2\beta}$ methyl ester being limited to 0, 10, 30, 50 and 70 pico-grams.

Anti-serum specificity studies

Prostaglandins $F_{2\alpha}$; 11-deoxy-$F_{2\alpha}$; 9,15-dihydroxy-10, 13-trans-prostadienoic acid (9-hydroxy PGA_2) B_1 and B_2 methyl esters were supplied by Dr. R.L.Jones. who had previously prepared and purified them from authentic prostaglandins supplied by the Upjohn Company, Kalamazoo. Prostaglandins $F_{1\alpha}$; $F_{1\beta}$; 15(R)$F_{2\alpha}$ and 15(R)$F_{2\beta}$ methyl esters were prepared and purified as described on page 49 .

Where an anti-serum was capable of binding 60% of the added ^3H-labelled prostaglandin $F_{2\beta}$ methyl ester at a final dilution of 1:3000 or greater, the anti-serum was further characterised for its ability to discriminate between prostaglandin $F_{2\beta}$ methyl ester and the above prostaglandins. In all cases a standard curve for

prostaglandin $F_{2\beta}$ methyl ester over the range of 0, 10, 30, 50, 70, 150, 310, 630, 1270 and 2550 pico-grams was prepared as described on page 53 for each comparison. For prostaglandin $F_{1\beta}$ methyl ester the range of added prostaglandin was identical. For all other prostaglandins the range was : 0.2; 0.8; 3.2; 12.8; 51.2; 204.8; and 820 nanograms of added prostaglandin.

The samples were analysed as described on page 43 .

Factors influencing the reduction of prostaglandin E_2 to prostaglandin $F_{2\beta}$ by sodium borohydride

High specific activity ^{3}H-labelled prostaglandin E_2 (5,6,8,11,12,14,15-^{3}H-PGE_2; 167 Ci m mole^{-1}) was diluted with a methanol solution of prostaglandin E_2 such that 0.2 ml contained approximately 0.5 μ Ci (10 ng). Into twelve clean stoppered test tubes 0.2 ml of this solution was pipetted and the tubes cooled with an ice-methanol solution. Identical tubes containing only 0.5 μ Ci (1.0 ng) of the high specific activity ^{3}H-labelled PGE_2 were also prepared. To each tube 0.2 ml of a methanol suspension of sodium borohydride (Ca. 20 mg ml^{-1}) was added, the contents mixed and incubated for 30, 60 or 120 minutes (quadruplicates). The reaction products were diluted with 2 ml of distilled water and 10-15 drops of 6N HCl were added. The contents were then extracted twice with 4 ml of redistilled ethyl acetate and the pooled organic phases washed with 1.0 ml of distilled water. The ethyl acetate was taken to dryness and the residue applied as a narrow band 2 cm from the base of a thin-layer chromatography plate

(200 mm x 200 mm x 0.25 mm on glass). Marker standards of ^{3}H-labelled prostaglandins $F_{2\beta}$, $F_{2\alpha}$, and A_2 were spotted on the side of each plate. The plate was then developed to a height of 16-18 cm in the FVI solvent system of Andersen (1969). This solvent is composed of ethyl acetate-acetone-glacial acetic acid (90: 10: 1 by volume). The plates were then dried under a stream of cold air and re-developed in the G.C.M. solvent of Millar (1978). This solvent is composed of ethyl acetate-methanol-glacial acetic acid (100:10:1 by volume). After the plates had again been dried under a stream of cold air, they were scanned for radioactivity using a Panax radio thin-layer chromatographic plate scanner. From the resulting scans, zones corresponding with peaks of radioactivity were scraped from the plates into test tubes containing 5 ml of methanol. The contents were vortexed and centrifuged at maximum speed on a bench centrifuge for 5-10 minutes. The supernatants were removed and fresh methanol added to the compacted silica gel. After re-extraction as described above the supernatants for each tube were pooled, taken to dryness under reduced pressure (at 40°C) and vacuum desiccated at room temperature. The residue from each zone was re-dissolved in 1.0 ml of methanol and 4 x 50 µl aliquots were assayed for radioactivity by liquid scintillation counting. The samples were counted in 5 ml of the toluene-ethoxyethanol based scintillant mixture described on page 43 .

In an identical experiment, 10 ng samples (n=4 for each time period) of prostaglandin E_2 were treated

with methanolic sodium borohydride as described above. Prior to extraction 250 ng of 3,3',4,4'-tetra-deutero prostaglandin $F_{2\alpha}$ was added to each sample. The samples were extracted as described above and then converted to the methyl ester, trimethylsilyl ether derivative by the method described on page 61 . These derivatised samples were then subjected to multiple ion detection gas chromatography-mass spectrometry. The instrument used, a Finnigan 3000D mass spectrometer was interfaced with a Finnigan 9500 gas chromatograph equipped with a glass column (1.5 m x 2 mm i.d.) packed with 3% OV-1 on Supelcoport. The oven temperature was 220°C and the carrier gas (helium) flow rate was 25-35 ml min^{-1}. The ions monitored were at M-(90+71) and at M-((2x90) + 71) for both the protium and deuterium forms. For protium prostaglandins $F_{2\alpha}$ and $F_{2\beta}$ these ions were at 333 and 423 whilst they were at 337 and 427 respectively for the deuterium prostaglandin $F_{2\alpha}$. The instrument was calibrated for the protium forms of prostaglandins $F_{2\alpha}$ and $F_{2\beta}$ over the range of 0-60 ng and for deuterium $F_{2\alpha}$ 100 ng (constant) per injection. Samples were dissolved in 50 µl of BSTFA and 5 µl aliquots were injected. Triplicate determinations were made for each sample.

<u>The metabolism of prostaglandin E_2 by rabbit and sheep blood and rabbit popliteal lymph node</u>

A female New Zealand white rabbit was bled from the marginal ear vein as described on page 42 into a tube containing 3.0 ml of citrate anti-coagulant (sodium citrate 2.2 g; citric acid monohydrate, 0.8 g; dextrose, 2.45 g in

100 ml of distilled water) until the total volume collected was 20 ml. Fresh sheep blood (850 ml) was collected into polythene bottles containing citrate anti-coagulant (150 ml) and the contents thoroughly mixed.

Two female New Zealand white rabbits were killed by a blow to the neck followed by exsanguination from the throat. The popliteal lymph nodes were located at the back of each knee joint and removed. These were pooled and homogenised in 20 ml of 100 mM sodium phosphate buffer (NaH_2PO_4, 2.7 g; Na_2HPO_4, 12.54 g; in 1 litre distilled water, pH 7.4) using a Polytron homogeniser at maximum speed. The resulting homogenate and 10 ml aliquots of rabbit and sheep blood were incubated with ^3H-labelled prostaglandin E_2 (1.0 μCi : 10 ng) for 75 minutes at 37°C in a water bath (with agitation). The resulting products were diluted with 100 ml of distilled water and acidified to pH4 with 10% glacial acetic acid. These were then extracted twice with 2 volumes of re-distilled ethyl acetate. The pooled organic phases were twice washed with 25 ml of distilled water before being taken to dryness under reduced pressure at 40°C and vacuum desiccated. The residues were re-dissolved in 25-50 ml of freshly prepared 67% aqueous ethanol and twice partitioned with an equal volume of heavy petroleum (60°- 80°bp). The aqueous phase was taken to dryness and vacuum desiccated. The products were then separated by thin-layer chromatography. The plates used were freshly prepared from a slurry of Kieselgel G (45 g : 80 ml distilled water) spread to an even thickness of 0.25 mm on glass plates (50 mm x 200 mm) previously

cleaned with chloroform. Prior to use the plates were activated by heating at 110°-115°C in an oven for 20-30 minutes. The samples and authentic ^{3}H-labelled prostaglandins E_2 and A_2 were applied as narrow bands approximately 3.0 cm from the base of the plates. The plates were developed with the organic phase of the A VII solvent system (Bygdeman and Samuelsson, 1966). This solvent system was prepared the day prior to use in stoppered separating funnels and is composed of water; ethyl acetate; 2,2,4-trimethyl pentane; glacial acetic acid (100:110:30:20 by volume). The plates were developed to a height of 15-16 cm in closed equilibrated glass tanks lined with Whatman No. 1 filter paper. After development the plates were dried and scanned for radioactivity using a Panax radio thin-layer chromatographic plate scanner.

<u>A comparison of the thin layer development obtained for sheep blood extracts by different solvent systems</u>

Three samples of blood were incubated with ^{3}H-labelled PGE_2 and extracted as described above. The products were subjected to thin-layer chromatography on prepared neutral silica gel thin layer plates (50 mm x 200 mm x 0.25 mm on glass. Merck). Three groups of developing solvents were compared :

Group I - First and Second development G.C.M. solvent (page 84)

Group II - First and Second development FVI solvent (page 84)

Group III - First development FVI; Second development G.C.M. solvent.

The plates were scanned for radioactivity using a Panax radio thin layer plate scanner.

Confirmation that the metabolism of prostaglandin E_2 by sheep whole blood is not artifactual

A sample (50 ml) of freshly collected citrated sheep whole blood (page 58) was heated at 70°C for 5 minutes in a water bath and cooled to room temperature. Aliquots (10 ml) of this sample and a sample of the untreated blood were incubated with ^{3}H-labelled PGE_2 (1.0 μ Ci : 10 ng) as described previously. In addition 10 ml of the untreated blood was incubated in the absence of prostaglandin E_2 in parallel with these samples. At the end of the incubation period 1.0 μ Ci (10 ng) of ^{3}H-PGE_2 was added to the sample and then all three samples were extracted and subjected to multi-development thin layer chromatography as described previously. The plates were then scanned for radioactivity as described previously.

Preparation of derivatives for gas-liquid-chromatography

i) Methyl and Ethyl esters

Diazomethane was freshly prepared by adding approximately 1g of 'DIAZALD' to 10 ml. of diethyl ether and 9.5 ml of 95% aquous ethanol. The reaction vessel was equipped with a 'clear fit' joint to reduce the explosive nature of diazomethane. The glassware was thoroughly cleaned with chromic acid and distilled water at regular intervals to reduce contamination. The diazomethane producing reaction was started by adding 2 ml of KOH (30 g made up to 50 ml with distilled water) to the reaction vessel. The product (diazomethane) was distilled from the reaction vessel by bubbling nitrogen saturated with diethyl ether through the reaction mixture and collecting the vapour in a mixture of diethyl ether / methanol (13.5 : 1.5) chilled with an ice-methanol solution. Diazoethane was prepared in an identical manner by substituting N-ethyl-N'-nitro-N-nitrosoguanidine for Diazald.

Esters (methyl or ethyl) were then prepared by the methods of Green (1969) and Thompson, Los and Horton (1970). The samples to be esterified were transferred in methanol to Eppendorf polypropylene capped tubes and the volume carefully reduced (if necessary) to approximately 0.1 ml under a stream of dry air. The residue was then treated with excess esterifying solution (eg diazomethane) and if excess bubbles (N_2) were evolved the volume was again reduced and fresh esterifying solution added until no further gaseous evolution occurred. The reaction was then

vortexed and left for a maximum of 15 minutes at room temperature. The samples were then taken to dryness under dry air and vacuum desiccated until dry.

II) O-alkyl hydroxylamines

Pyridine solutions of a variety of oximating reagents were prepared by dissolving the respective solid hydroxylamine hydrochloride salt analogue in anhydrous pyridine (stored over KOH pellets) to a final concentration of 5 mg ml^{-1}. The series of oximating reagents used included the hydrochloride salts of the following :

a) hydroxylamine (NH_2OH)

b) O-methyl hydroxylamine (NH_2OCH_3)

c) O-ethylhydroxylamine ($NH_2OC_2H_5$)

d) O-n-propylhydroxylamine ($NH_2OC_3H_7$)

e) O-iso-propylhydroxylamine ($NH_2OCH(CH_3)_2$)

f) O-n-butylhydroxylamine ($NH_2OC_4H_9$)

g) O-n-pentylhydroxylamine ($NH_2OC_5H_{11}$)

The reagents d,e,f and g were generously prepared by Dr. N.H. Wilson of the Department of Pharmacology, Edinburgh University, using the method of Fujii, Wu and Yamada (1967). The others were obtained from commercial sources.

The samples to be oximated were initially converted to the methyl or ethyl ester and thoroughly dried by vacuum desiccation. The oximation process was then performed by adding 100-200µl of the required O-alkyl hydroxylamine hydrochloride pyridine solution vortexing and either :

a) leaving at room temperature for a minimum of 16 hours.

or b) heating at 60°-70°C for 1½ to 3 hours.

The second process has been found to produce higher yields of the derivatised prostaglandin metabolites and the n-butyl and n-pentyl homologues in general.

Following oximation the samples were taken to dryness under dry air and vacuum desiccated.

iii) 9α, 11α cyclic n-butyl boronates

These derivatives were routinely prepared by the method of Pace-Asiak and Wolfe (1970). The esterified (and in certain examples oximated) prostaglandin samples that were suspected of having a 9α, 11α hydroxyl ring structure (eg prostaglandin $F_{2\alpha}$) had to be thoroughly dried by vacuum desiccation prior to forming the 9α, 11α cyclic n-butyl boronate. To these samples 200-300 µl of a solution (5 mg ml^{-1}) of n-butylboronic acid in 2,2-dimethoxypropane was added. The samples were then vortexed and heated at 60°-70°C for 1-1½ hours. The solvent was then removed under a stream of dry air and the samples vacuum desiccated.

An alternative method (R.W. Kelly personal communication) of derivatisation was used for a small number of samples. This method required the addition of 25 µl of n-butyl boronic acid reagent (3 mg ml^{-1} in dry acetone:benzene 4:1 v/v) to the dried esterified samples. After mixing, the samples were placed in a vacuum oven and the pressure was reduced by 25 mm Hg for 15-20 minutes. The residual solvent was eventually removed by increasing the vacuum. This alternative method was not extensively used because of the lower yields of derivative produced.

iv) Trimethylsilyl ethers

The final stage of derivatisation for all prostaglandin samples analysed was the formation of trimethylsilyl ethers. These were prepared by the method of Thompson, Los and Horton (1970). To the prostaglandin samples 50µl of N, N-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) was added and after mixing the samples were either left at room temperature for 3 hours or alternatively heated at 60°C for 15 minutes. In most cases the samples were injected into the gas chromatograph in BSTFA although for certain samples the BSTFA was removed under a stream of dry air and the residue dissolved in an equal volume of n-hexane for injection into the gas chromatograph.

RESULTS

Rabbits immunised by either the multiple intra-dermal or the intra-nodal techniques with a prostaglandin E_2-bovine serum albumin (PGE_2 - BSA) conjugate were found to produce very few antibodies with any affinity for ^3H-labelled prostaglandin E_2. In all the anti-sera tested the maximum binding of the ^3H-labelled PGE_2 obtained at a dilution of 1/200 was never greated than 45% and in most cases and usually less than 35% of the added radiochemical ligand. Consequently the ability of these anti-sera to bind ^3H-labelled prostaglandins A_2 and B_2 was tested. In all cases the ^3H-labelled prostaglandin B_2 was the ligand that was maximally bound by any given anti-serum. In addition the dilution of anti-serum required to bind 60-70% of the added ^3H-PGB_2 was found to increase with time after the primary immunisation procedure. Examples of the binding to anti-sera obtained from rabbits twelve weeks after they have been immunised with PGE_2-BSA by the two methods described is shown in figure 5 on page 66 . The changes in dilution of these anti-sera, giving 60% binding of ^3H-labelled PGB_2 with time after the primary immunisation is shown in figure 6 on page 67 .

It can be clearly seen from these results that the rabbits immunised (by either immunisation technique) with PGE_2-BSA conjugate produced antibodies mainly with a specificity for prostaglandin B_2. In none of the anti-sera tested was there any evidence for large numbers of anti-bodies (as determined by dilution) to prostaglandin E_2.

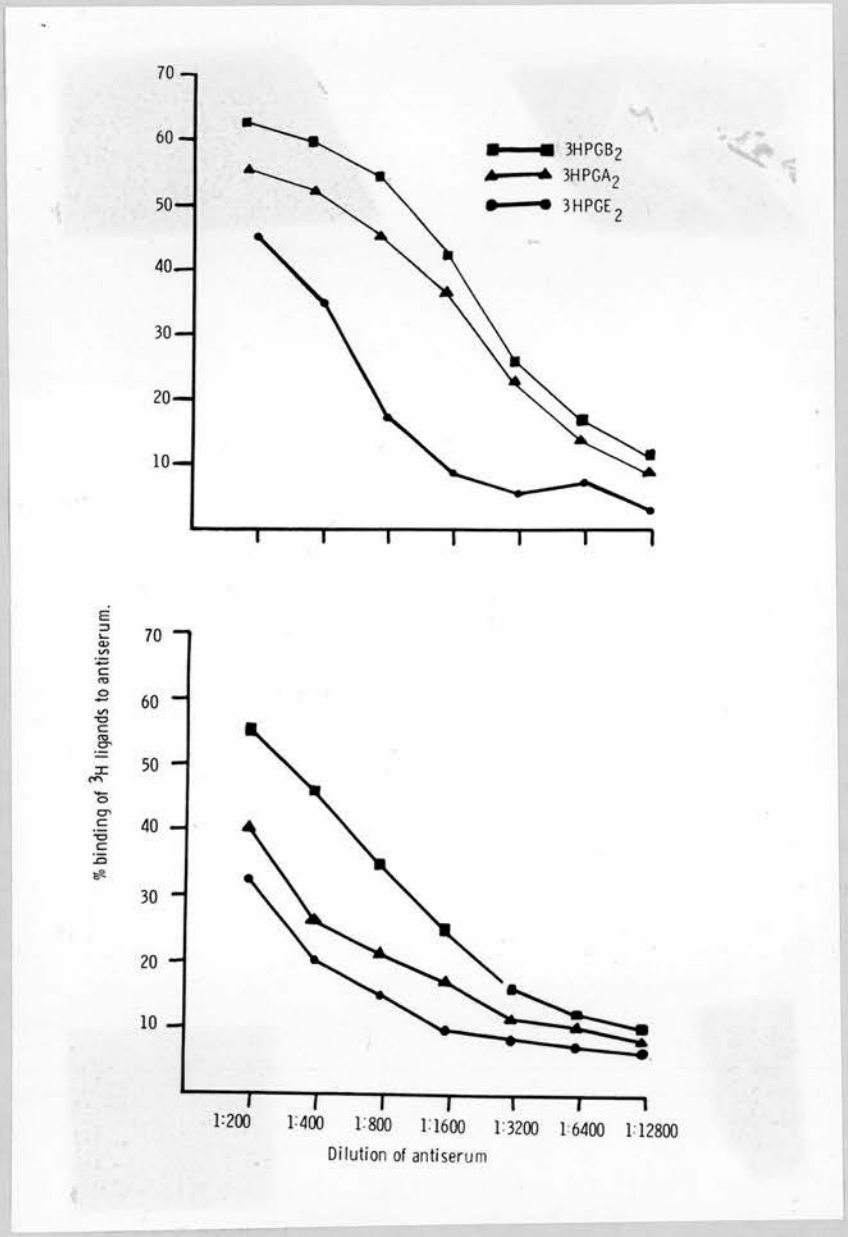

Figure 5. The binding of 3H PGE_2; ^3H P GA_2 and ^3H PGB_2 (400 pg per ligand) to successive dilutions of anti-sera obtained twelve weeks after rabbits were immunised by either the multiple intradermal (upper) or intranodal (lower) technique.

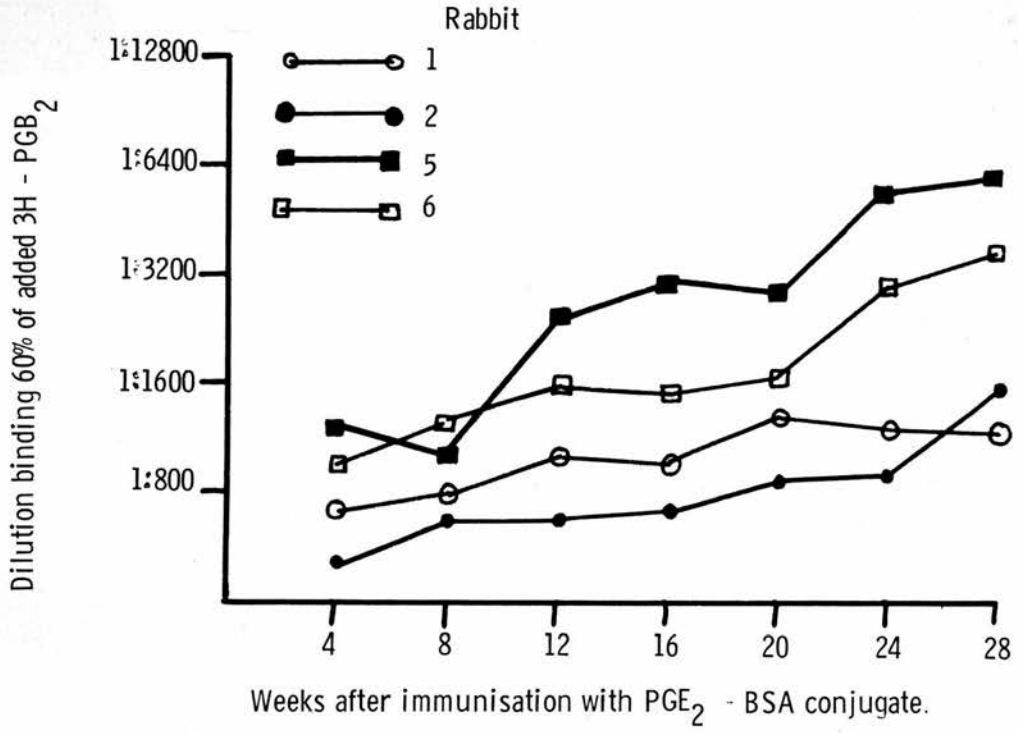

Figure 6. The variation in dilution of antiserum binding 60% of added ^{3}H PGB_2 (400 pg) with time after primary immunisation by the multiple intradermal technique.

However, to confirm that the low levels of ^{3}H-PGE_2 binding antibodies being measured were not artifactual, it was decided to test the effect of increasing the concentration of normal rabbit serum (NRS) and donkey anti-rabbit gamma globulin serum (DARS) being added to the anti-sera under evaluation. In most cases slight increases occurred in the apparent binding of ^{3}H-PGE_2 by a given dilution of anti-serum. However, the levels of non-specific binding also increased and thus any apparent increase was probably due to the added non-specific binding.

The failure to raise antibodies to prostaglandin E_2 in rabbits by either of the two immunisation procedures could not be readily explained. The possibility that the PGE_2-BSA conjugation reaction had induced a chemical dehydration and subsequent isomerisation of the prostaglandin E_2 to prostaglandin B_2, therefore resulting in a PGB_2-BSA conjugate was studied. The unconjugated material recovered after dialysis of the conjugation reaction products was subjected to ultra-violet spectrophotometry and thin-layer chromatography. No evidence for the chemical breakdown of the unconjugated prostaglandin E_2, to either PGA_2 or PGB_2 was obtained by either method. On thin-layer chromatography the recovered material had a mean Rf value of 0.32 whilst authentic prostaglandins A_2 and E_2 had Rf values of 0.45 and 0.31 respectively. Although this did not confirm that the conjugated prostaglandin E_2 had been dehydrated it did provide evidence to suggest the contrary. The probability therefore existed that the prostaglandin E_2 conjugated to the BSA was being dehydrated and isomerised <u>in vivo</u> by the

rabbits after immunisation. Whether this conversion was chemically or enzymatically induced was not known, however, the rabbit was known to contain within its blood plasma a prostaglandin A isomerase (Jones and Cammock, 1973; Jones, Cammock and Horton, 1972; Horton, Jones, Thompson and Poyser, 1971) that isomerises prostaglandin A_2 to B_2. If this enzyme was capable of accepting PGA_2-BSA as a substrate it is possible that the conversion may have proceeded via this enzyme (or a similar undiscovered one in rabbit tissues). The dehydration reaction that converted the conjugated PGE_2 to PGA_2 could have been either enzymatic or due to chemical instability, however, as will be discussed later in this section, attempts to detect a prostaglandin E_2 dehydrase were also made.

The possibilities remaining for the development of a successful radioimmunoassay specific for prostaglandin E_2 were re-evaluated. These possibilities included : the use of an alternative animal for raising antibodies to PGE_2-BSA; the synthesis of a prostaglandin E_2 analogue resistant to dehydration into the corresponding prostaglandin A_2 and its subsequent use as an immunogen; the development of antibodies to a compound that prostaglandin E_2 is readily chemically converted to; the purification of prostaglandin E_2 antibodies from a mixture of E_2, A_2 and B_2 prostaglandin antibodies by affinity chromatography.

The use of sheep for the raising of antibodies was considered, however, the subsequent discovery of an enzyme (see page 77 in this section) in ovine blood capable of reducing PGE_2 to $PGF_{2\alpha}$ and the fact that anti-

sera from sheep immunised with PGE_2-BSA had high cross-reactivities with prostaglandins A_2, B_2 and $F_{2\alpha}$ (M.Withnall, personal communication) suggested that one of the other possibilities be considered. The synthesis of a prostaglandin analogue that was resistant to dehydration was actively considered. Such compounds included 10,10-dimethyl prostaglandin E_2 and the 9-O-methyloxime analogue. The first compound was not available. However, the second could be readily produced in micro-gram quantities by the methods described on page 61 . However, at the time that this work was considered the ability to quantitatively convert sub-nanogram quantities of prostaglandin E_2 into this derivative with any degree of reproduceability was not available. Consequently, it was decided to raise the antibodies in the rabbit to prostaglandin $F_{2\beta}$, a compound readily produced from prostaglandin E_2 by treatment with methanolic sodium borohydride. One problem with this method was that approximately equal amounts of prostaglandins $F_{2\alpha}$ and $F_{2\beta}$ are produced by the reduction reaction. Thus for the radioimmunoassay method to be successful the antisera produced must be able to successfully discriminate prostaglandin $F_{2\beta}$ from prostaglandin $F_{2\alpha}$. Furthermore the conversion of prostaglandin E_2 to prostaglandin $F_{2\beta}$ must be constant and reproducable for the assay method to be successful.

Evaluation of the antisera raised to prostaglandin $F_{2\beta}$ bovine serum albumin conjugate

All the antisera harvested were found to contain antibodies capable of binding ^3H-labelled prostaglandin $F_{2\beta}$

methyl ester. The dilution of each antiserum required for 60% binding of the ^{3}H-PGF$_{2\beta}$ was found to increase with time after the primary immunisation. These increases were found to be enhanced by secondary immunisation in several of the rabbits. Examples of the increases are shown in figure 7 on page 72 . In all examples the incubation had been optimised for NRS and DARS as described previously on pages 45 & 46.

Antisera specificity studies

Several of the antisera were further evaluated for their ability to discriminate between prostaglandin F$_{2\beta}$ methyl ester and various other prostaglandins as their methyl esters. The major potential prostaglandins that were envisaged as being likely to be encountered in any potential use of this assay were tested where possible. These are listed on page 54 . The major prostaglandins not tested that were likely to be encountered were the 13, 14-dihydro derivatives of prostaglandins F$_{2\alpha}$ and F$_{2\beta}$ and their 15(R) epimers. These compounds were not available at the time of testing. Prostaglandin B$_1$ and B$_2$ were tested because under the reaction conditions that convert prostaglandin B$_2$ into F$_{2\beta}$ and F$_{2\alpha}$ sodium borohydride does not reduce the 9-ketonic function also present in the prostaglandins B$_1$ and B$_2$ (R.L. Jones, personal communication). Similarly, prostaglandin A$_2$ when reacted with sodium borohydride produces 3-hydroxy products. Two of these are prostaglandins 11-deoxy F$_{2\alpha}$ and F$_{2\beta}$, in which the 10, 11 double bond has also been reduced. The third compound produced still retains the 10,11 double bond, but in this

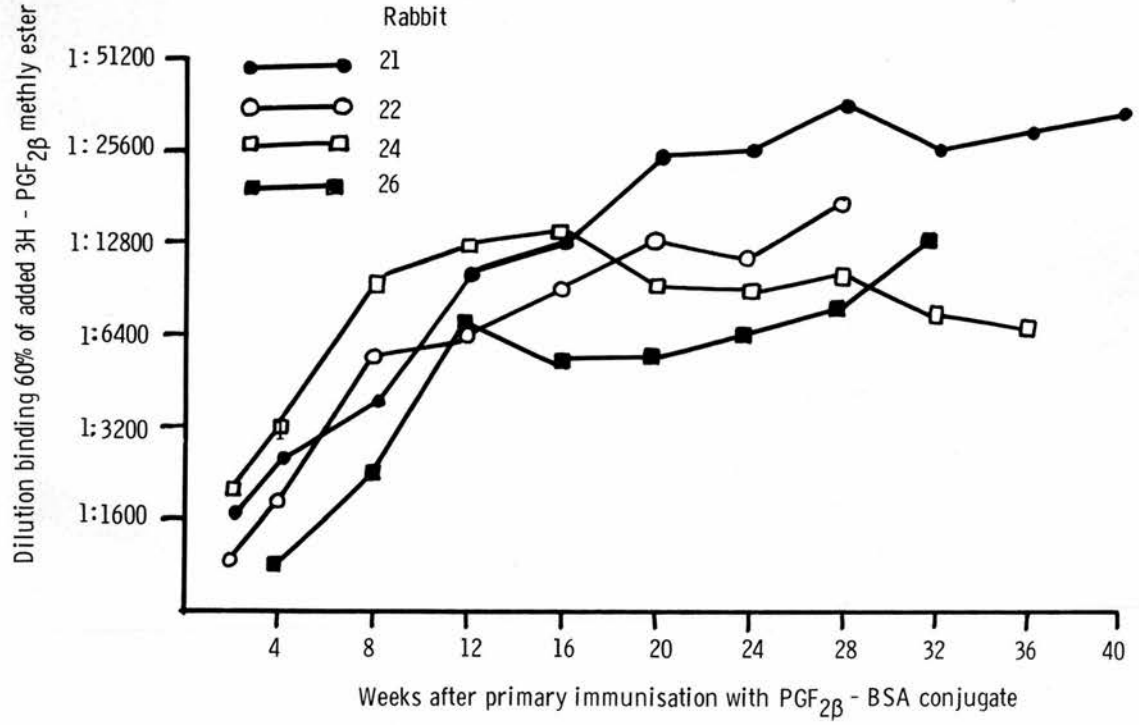

Figure 7. The increase in dilution of antiserum binding 60% of the added ^{3}H $PGF_{2\beta}$ methyl ester (400pg) with time after primary and secondary immunisation procedures.

example only one of the two 9-hydroxy epimers are produced (R.L. Jones, personal communication).

The cross reactivities of the prostaglandins tested were obtained by comparing the quantities of each prostaglandin necessary to cause a fall of 10% in the binding of ^{3}H-$PGF_{2\beta}$ methyl ester from its maximum binding. The amount of prostaglandin $F_{2\beta}$ methyl ester causing the fall was nominated 100% cross reaction, the remaining prostaglandins were expressed relative to this value. The results obtained with several of the anti-sera tested are shown in Table 1 below.

	Rabbit number			
	21	22	24	26
Dilution of anti-serum	1:25600	1:12800	1:12800	1:6400
Weeks after primary immunisation	20	20	16	12
Compound (methyl esters)	percentage cross reaction relative to PGF2 methyl ester			
$PGF_{2\beta}$	100	100	100	100
$PGF_{1\beta}$	104	100	100	108
$PGF_{2\alpha}$	3	1	4	2
$PGF_{1\alpha}$	3	2	2	2
15(R) $PGF_{2\beta}$	7	3	9	6
15(R) $PGF_{2\alpha}$	1	1	1	1
PGB_1	0.1	0.1	0.1	0.1
PGB_2	0.1	0.1	0.1	0.1
9-OH PGA_2	1	1	2	1
11-deoxy $PGF_{2\beta}$	1	1	1	1

Table 1. Relative cross-reactivities of four rabbit $PGF_{2\beta}$ BSA anti-sera to various prostaglandin methyl esters.

Factors influencing the reduction of prostaglandin E_2 to prostaglandin $F_{2\beta}$ by sodium borohydride

The reduction of ^{3}H-labelled PGE_2 by methanolic sodium borohydride results in the production of several products as determined by thin layer chromatography. An example of the products produced is shown in figure 8 on page 74. The two major products were prostaglandins $F_{2\alpha}$ and $F_{2\beta}$. The proportion of prostaglandins $F_{2\alpha}$ and $F_{2\beta}$ produced after 30, 60 and 120 minute incubations of 10 ng prostaglandin E_2 with methanolic sodium borohydride were assayed by two methods. One method was based on the recoveries of radioactivity from the zones of thin-layer chromatography plates. The second method was based on deuterium isotope dilution analysis by multiple ion detection gas chromatography-mass spectrometry. The results obtained by these two methods are shown in tables 2 and 3 on pages 74 & 76 respectively.

Table 2

The percentage conversion to $PGF_{2\alpha}$ and $PGF_{2\beta}$ of 10 ng of ^{3}H-PGE_2 as determined by the radiochemical technique.

	Incubation time (minutes)	Sample 1	2	3	4	mean	S.E.M	Variance
$F_{2\alpha}$	30	43	46	44	41	43.50	1.04	4.33
	60	44	41	42	46	43.25	1.11	4.92
	120	43	49	43	44	44.75	1.44	8.25
$F_{2\beta}$	30	37	41	43	44	41.25	1.55	9.58
	60	38	44	44	40	41.50	1.50	9.00
	120	42	43	43	40	42.00	0.71	2.00

The values quoted are the percentage that each zone comprises of the total radioactivity recovered from thin-layer plates.

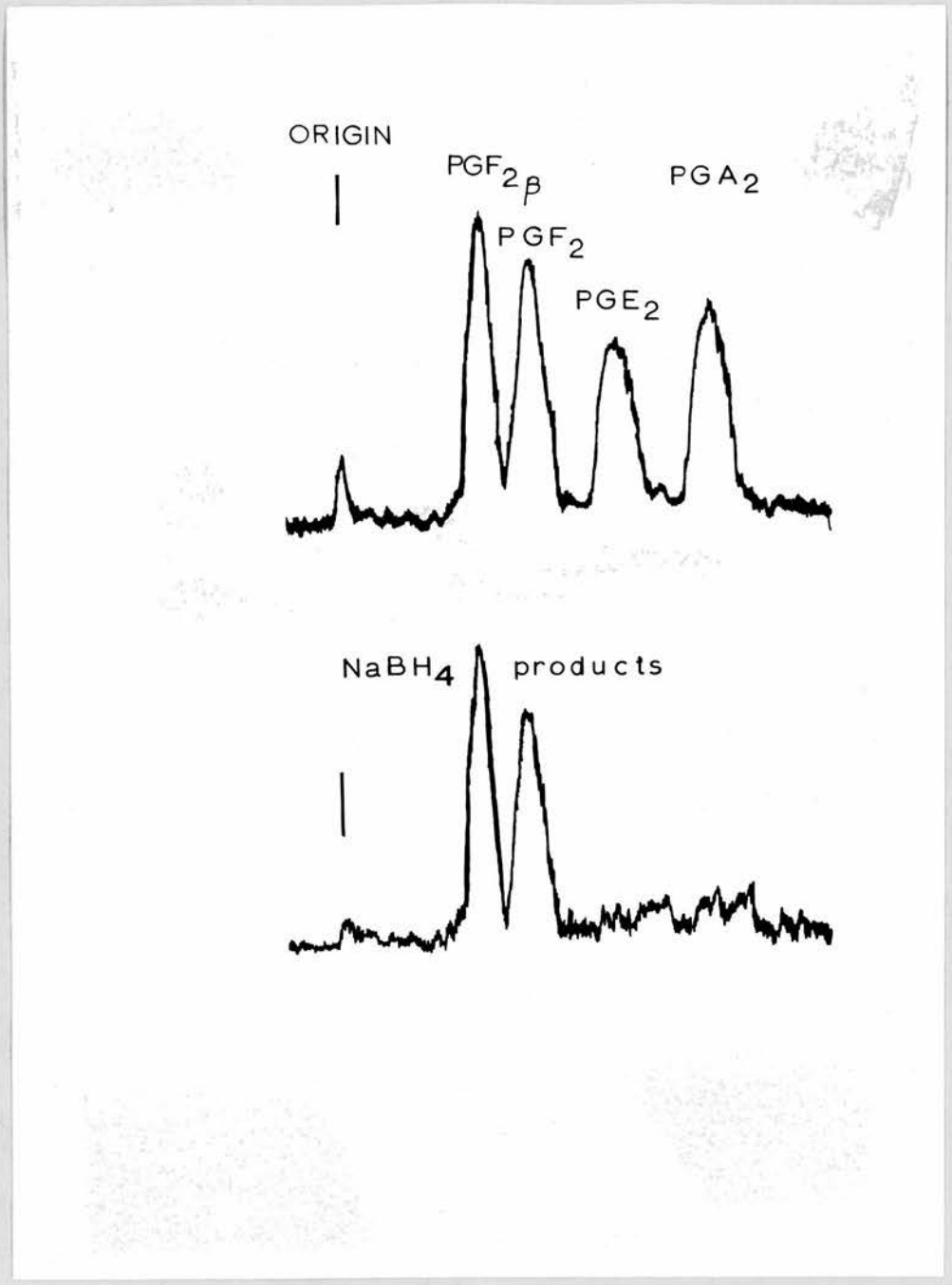

Figure 8. The thin layer radio scans obtained for authentic ^{3}H prostaglandins $F_{2\beta}$; $F_{2\alpha}$, E_2 and A_2 (upper) and the products obtained after treating ^{3}H PGE_2 (10 ng : 0.5 μCi) with methanolic sodium borohydride for 30 minutes as described on page 55.

Table 3

The percentage conversion to $PGF_{2\alpha}$ and $PGF_{2\beta}$ of 10 ng of PGE_2 as determined by the deuterium isotope dilution technique.

	sample						
Incubation time (minutes)	1	2	3	4	mean	S.E.M	Variance
$F_{2\alpha}$ 30	44	48	45	43	45.00	1.08	4.67
60	42	43	40	45	42.50	1.04	4.33
120	43	48	43	46	45.00	1.22	6.00
$F_{2\beta}$ 30	39	40	42	48	42.25	2.02	16.25
60	39	46	42	43	42.50	1.44	8.33
120	41	42	44	42	42.25	0.63	1.58

The values quoted are the percentage that each prostaglandin comprises of the added prostaglandin E_2.

The reduction of 1 ng ^3H-PGE_2 sodium borohydride was also studied by the radiochemical method. The results are shown in Table 4 on page 77 .

Table 4

The percentage conversion to prostaglandin $F_{2\alpha}$ and $F_{2\beta}$ of 1 ng of $^{3}H\text{-}PGE_2$ as determined by the radiochemical technique.

	sample						
Incubation time (minutes)	1	2	3	4	mean	S.E.M	Variance
$F_{2\alpha}$ 30	40	42	49	47	44.50	2.10	17.67
60	39	46	48	43	44.00	1.96	15.33
120	44	43	41	49	44.25	1.70	11.58
$F_{2\beta}$ 30	41	40	40	43	41.00	0.71	2.00
60	34	41	44	48	41.75	2.95	34.92
120	37	46	44	42	42.25	1.93	14.92

The metabolism of prostaglandin E_2 by rabbit and sheep whole blood and rabbit popliteal lymph node

The metabolism of prostaglandin E_2 by the above samples was studied by means of thin-layer chromatography. The resulting scans of the radioactivity present in each sample are shown in figure 9 on page 78 . It can be seen that only the sheep blood samples appeared to metabolise prostaglandin E_2. The metabolite produced was more polar on the thin-layer chromatography system than prostaglandin E_2. Several prostaglandins, including $PGF_{2\alpha}$ and $PGF_{2\beta}$ are more polar than prostaglandin E_2. However, the identity of the metabolite could not be solely determined by thin-layer chromatography Before the identity of this metabolite could be ascertained it was necessary to find a

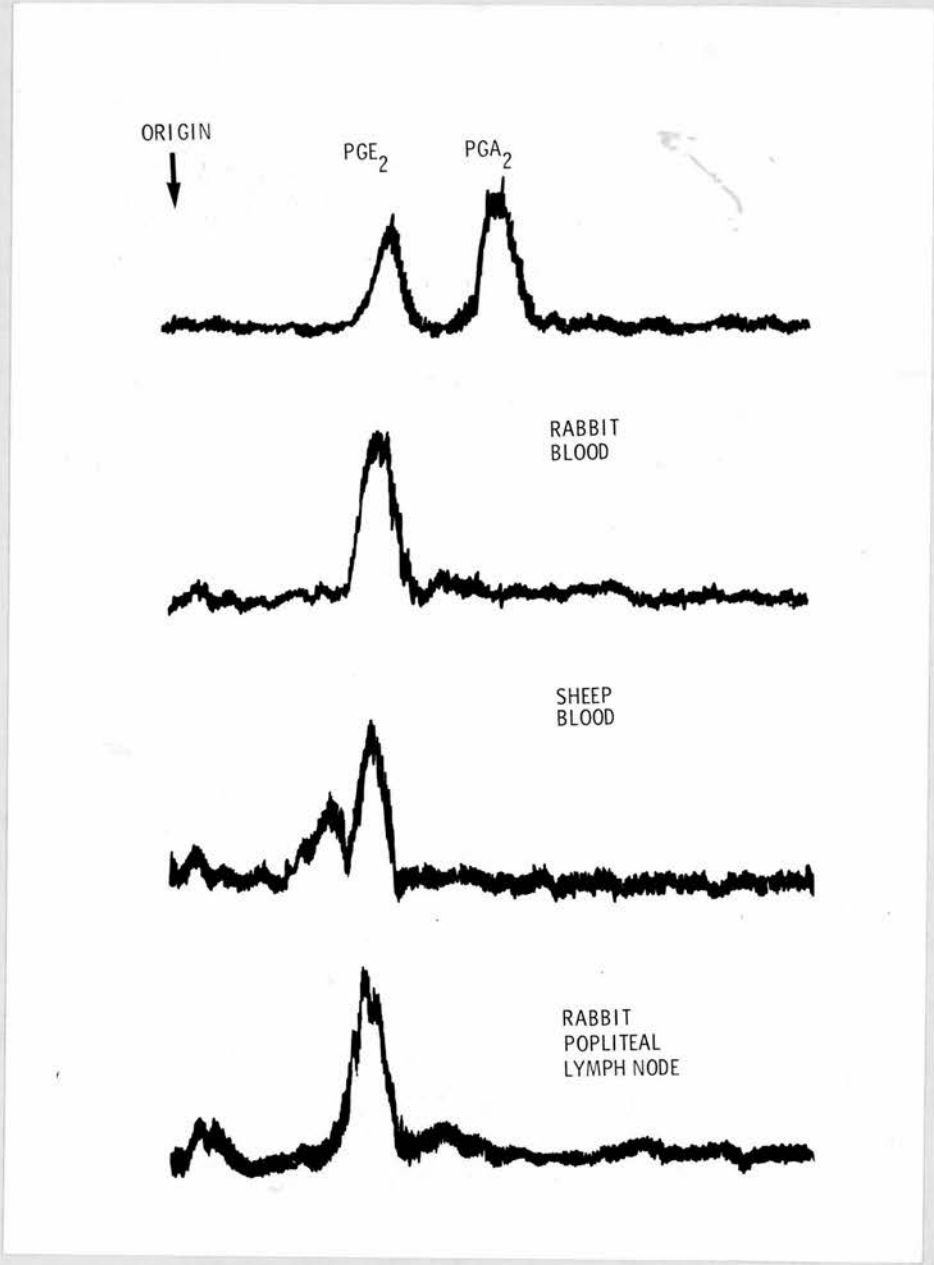

Figure 9. Thin layer radiochemical scans of authentic ^{3}H prostaglandins E_2 and A_2 and the products obtained by incubating rabbit and sheep whole blood and rabbit popliteal lymph node homogenate with ^{3}H PGE_2 as described on page 57-59.

thin-layer chromatography system that not only separated the metabolite from prostaglandin E_2 but also separated this metabolite from the majority of blood pigments that were also extracted from the incubate. In addition, if the metabolite was an F type prostaglandin it would also be necessary to separate the two possible F isomers, namely $PGF_{2\alpha}$ and $PGF_{2\beta}$ that could be produced for prostaglandin E_2. It was known that the G.C.M. solvent system (Millar, 1974) would separate the majority of blood pigments from prostaglandins $F_{2\alpha}$, E_2 and A_2. However, the ability of this solvent to separate prostaglandin $F_{2\alpha}$ and $F_{2\beta}$ was unknown. Consequently the FVI solvent system (Andersen, 1969) which was known to separate these two prostaglandins was compared with and in combination with the G.C.M. solvent. It was found that although the best separation of prostaglandins $F_{2\alpha}$ and $F_{2\beta}$ could be obtained by a multi development in the FVI solvent system, the best separation of these prostaglandins in combination with their separation from the major blood pigments was obtained by an initial development with the FVI solvent and a second in the G.C.M. solvent. In addition, the metabolite had a chromatographic mobility in all the systems conducive to it being prostaglandin $F_{2\alpha}$ and not the alternative epimer prostaglandin $F_{2\beta}$.

<u>Confirmation that the metabolism of prostaglandin E_2 by sheep whole blood was not artifactual</u>

When prostaglandin E_2 was incubated with sheep whole blood at 37°C a metabolite, provisionally identified

as prostaglandin $F_{2\alpha}$, was produced. The possibility that this could have been produced by non-specific reduction of the prostaglandin E_2 during the incubation and extraction procedures had to be discounted. Thus sheep whole blood that had been heated at 70°C for 5 minutes to destroy the enzymes present was used to determine whether the incubation or extraction procedures were responsible for the formation of the metabolite. The results of incubating sheep whole blood at 37°C, before and after incubation at 70°C, are shown in figure 10 on page 81. In addition the effect of the extraction procedure on prostaglandin E_2 is shown. It can be seen that the metabolite is only produced by sheep whole blood that had not been heated to 70°C and thus it was concluded that the production of this metabolite was not artifactual. The identification of this metabolite and further studies on the metabolism of prostaglandin E_2 by other animal blood and tissue samples is described later in this thesis.

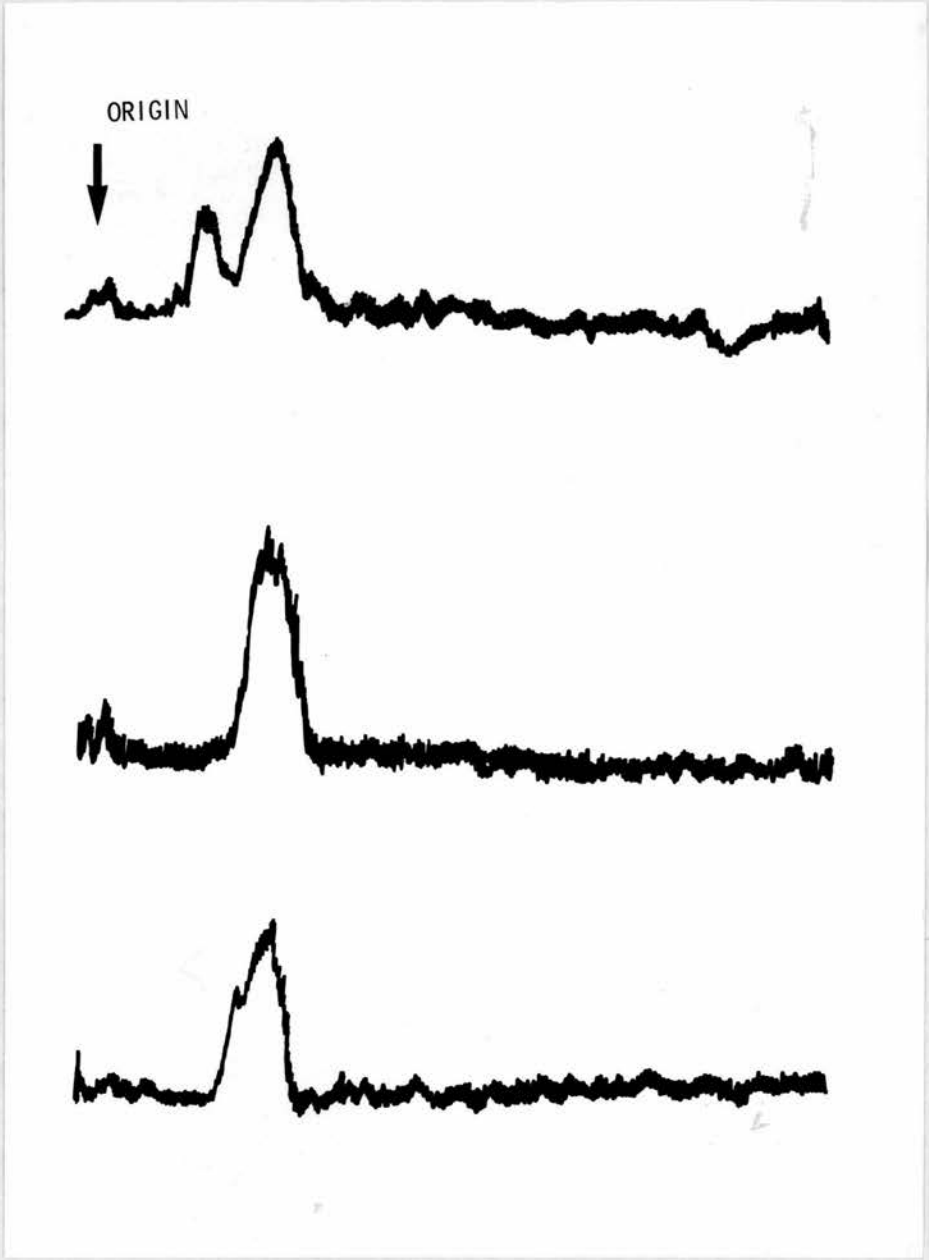

Figure 10. Thin layer radio scans of the organic extraction of products obtained after incubating sheep whole blood at 37°C for 75 minutes before (top) and after (middle) heating at 70°C for 5 minutes and the extraction procedure (bottom) on ^{3}H PGE_2 (1 μCi : 10 ng).

SECTION II

STUDIES ON THE METABOLISM OF PROSTAGLANDIN E_2 BY WHOLE BLOOD FROM THE SHEEP AND OTHER ANIMAL SPECIES

Introduction

During the attempts to raise anti-bodies to prostaglandin E_2 in the rabbit, it was observed that the majority of the antibodies produced had a low affinity for prostaglandin E_2 but a much higher one for prostaglandin B_2. It was therefore concluded that the PGE_2 conjugated to the BSA was being dehydrated and isomerised *in vivo* by the rabbits prior to the antibodies forming. It was already known that rabbit plasma contained a prostaglandin A isomerase (Jones and Cammock, 1973) and thus the possibility existed that after dehydration the PGE_2 was being isomerised through PGC_2 to PGB_2 by this or a similar enzyme. Therefore, an attempt to find the enzyme that was possibly dehydrating the PGE_2 to PGA_2 was made.

No such enzyme was found in the rabbit tissues studied. However, in studies with sheep, which was considered as an alternative animal in which to raise antibodies to PGE_2 because of the absence of a prostaglandin A isomerase within its plasma, it was discovered that a prostaglandin E_2 metabolising enzyme was present in its blood. The metabolite produced was more polar than PGE_2 and had thin layer chromatographic mobilities comparable with it being $PGF_{2\alpha}$. It was therefore decided to study the metabolism of PGE_2 by sheep blood in more detail and to identify the metabolite conclusively. In addition it was decided to screen the blood samples from as many animals as

possible for their ability to metabolise PGE_2 into either more or less polar compounds.

A) The metabolism of Prostaglandin E_2 by blood samples from the sheep; rabbit; cat; dog; guinea-pig; horse; cow; rat; chicken; and man

i) Incubation and extraction procedures

Fresh blood was added to sodium citrate anti-coagulant (8.5 : 1.5 v/v: sodium citrate, 2.2 g; citric acid monohydrate, 0.8 g; dextrose 2.45 g in 100 ml distilled water) and thoroughly mixed. A 10 ml volume of the anti-coagulated blood samples was then incubated with 5, 6, 8, 11, 12, 14, 15-^{3}H-PGE_2 (10 µg : 0.2 to 1.0 µ Ci) at 37°C for 75 min (with agitation) in a water bath. The reaction was stopped by the addition of 5-10 vol. of ice-cold ethanol and thorough mixing. After 30 minutes at room temperature the mixture was centrifuged at 1875 x g for 15 min at 4°C. The supernatant was transferred to a 250 ml flask and the precipitate washed with the same volume of absolute ethanol before being re-centrifuged. The supernatant was then pooled with the first batch and taken to dryness under reduced pressure at 40°C. The resulting oily residue was redissolved in 50-100 ml of distilled water and acidified to pH 4.0-4.5 with 10% acetic acid. This solution was then partitioned twice with an equal volume of re-distilled ethyl acetate and the pooled ethyl acetate fractions were subsequently washed with 2 x 20 ml of distilled water before the ethyl acetate was taken to dryness under reduced pressure at 40°C and desiccated under vacuum at room temperature. The resulting residue was dissolved

in 20 ml of 6 7% aqueous ethanol and partitioned twice with an equal volume of heavy petroleum before the 67% aqueous ethanol phase was taken to dryness and desiccated.

ii) Thin layer chromatography

The residue was then dissolved in 0.2 ml of a methanolbenzene mixture (90 : 10, v/v) and applied with a micro syringe as a thin band (0.3 mm x 25 mm) approximately 2 cm from the bottom end of a thin layer chromatography plate. Neutral silica gel (200 mm x 200 mm x 0.25 mm) on glass plates were used (E. Merck A.G.) and when necessary the plates were pre-washed with methanol to remove fluorescent indicator. Marker standards of ^{3}H-labelled prostaglandins were spotted on the plate at the side of the samples being chromatographed. A multi-run development of the plate was performed using the FVI system of Andersen (Andersen 1969) as the first run (ethyl acetate; acetone; glacial acetic acid 90:10:1 by vol.) and the G.C.M. solvent of Millar (Millar 1974) as the second development (ethyl acetate; methanol; glacial acetic acid 100:10:1 by vol.) In both cases the plates were developed to approximately 2 cm from the top of the plate in closed equilibrated glass tanks containing the developing solvent at a depth of approximately 1 cm. These tanks were lined with sheets of Whatman No. 1 filter paper to assist the equilibration of the solvents. The solvents were changed at regular intervals before use.

The plates were dried thoroughly after each development under a stream of cold air until no smell of solvent was detectable. After the final development the

individual samples on the plates were scanned for radioactivity using a Panax radio thin-layer chromatographic plate scanner. From the resulting scans it was possible to determine whether a particular blood sample metabolised prostaglandin E_2 and also to provisionally identify the metabolites by reference to authentic prostaglandins. (Figure II ; Page 86).

iii) Elution of prostaglandins from thin layer chromatography plates

From the resulting radioactive scans zones corresponding to peaks of radioactivity were scraped from the thin layer plates into test tubes containing 5 ml of methanol. The contents were vortexed for 15-30 seconds and then centrifuged on a bench centrifuge at maximum speed for 5-10 minutes. The supernatant was carefully transferred into a pear shaped flask. The compacted silica gel was washed with a further 5 ml of methanol, centrifuged and the supernatant pooled with the first eluant before being taken to dryness under reduced pressure at 40°C and vacuum desiccated. The residue was then dissolved in 1.0 ml of methanol and 4 x 50 µl aliquots were removed to enable a quantitative determination of radioactivity to be made.

iv) Liquid scintillation counting

The liquid scintillation counting was performed with a Mark II Nuclear Chicago liquid scintillation counter. The sample aliquots were counted in 10.0 ml of a toluene, ethoxyethanol-based scintillant mixture (toluene; ethoxyethanol, 2, 5-diph enyloxa zole (PPO) ; 1,4-di-(2-(4-

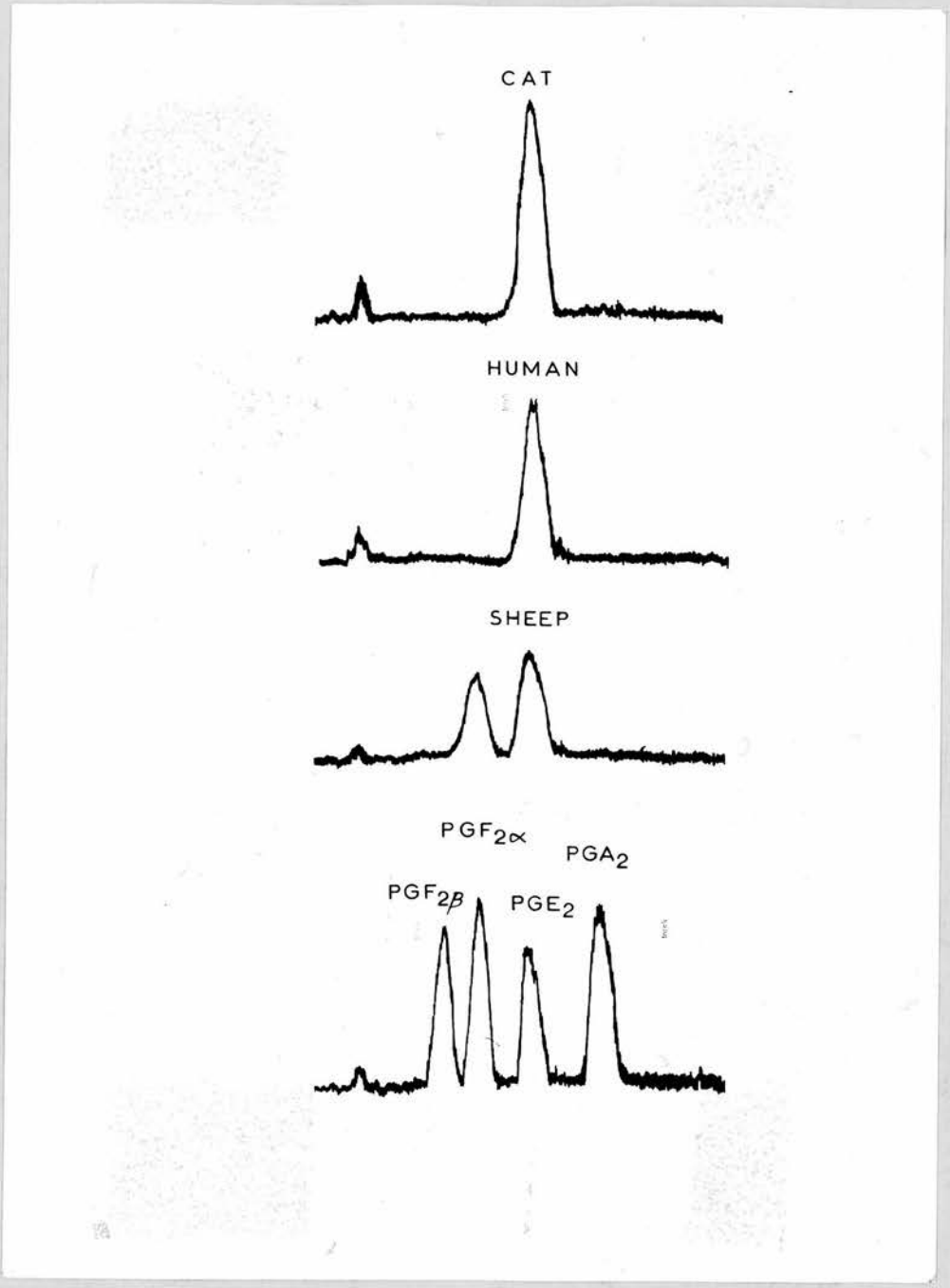

Figure 11. Thin layer radio scans obtained from the products produced by incubating ^{3}H PGE_2 with cat, human and sheep whole blood and authentic ^{3}H prostaglandins $F_{2\beta}$, $F_{2\alpha}$, E_2, and A_2. The experimental details are described on page 84.

methyl-5-phenyloxazolyl))-benzene (dimethyl POPOP) (2000; 400; 8.52; 0.22 v/v/w/w)). Counting efficiency was 35-40% and correction for quenching was made by use of the automatic external standard channels ratio method and a quench-calibration curve constructed for the scintillant mixture.

Preparation and purification of ^{3}H-labelled prostaglandins $F_2\alpha$ and $F_2\beta$

Prostaglandin E_2 (0.4 ml : 1 mg ml^{-1}) was added to 200 µ Ci of ^{3}H-labelled prostaglandin E_2 (Sp. Ac. 167 $Mole^{-1}$. Amersham) in a 25 ml stoppered pear-shaped flask and the mixture cooled to approximately -20°C with an ice-acetone mixture. A small quantity (approximately 100 mg) of sodium borohydride ($NaBH_4$) was added to the reaction vessel and mixed with the contents before incubating at -20°C for 1 hour. The reaction mixture was then brought to room temperature for a further period of 1 hour. During this time 10 µl of the reaction mixture was diluted to 2.0 ml with methanol and 0.2 ml of 1 M KOH added in a spectrophotometer cell. The sample was then assayed for prostaglandin B_2 (Andersen 1969) by scanning at a fixed wavelength of 278 nm with a Pye Unicam SP800 U.V. spectrophotometer and SP825 programme controller. Methanol (2.0 ml + 0.2 ml 1 M KOH) was used as a reference. No prostaglandin B_2 was detectable, thus the reduction of prostaglandin E_2 with sodium borohydride was taken as complete. The reaction mixture was then diluted with 40 ml of distilled water, acidified to pH 4 with 10% acetic acid solution and extracted twice with an equal volume of diethyl ether. The pooled ether fractions were taken to

dryness under reduced pressure, vacuum desiccated and redissolved in 1.0 ml of methanol.

Thin layer chromatography on neutral silica gel (50 mm x 200 mm x 0.25 mm on glass) of an aliquot of the products when developed by the G.C.M. solvent (Millar 1974) revealed a broad major peak of mean Rf 0.30 and minor peak of Rf 0.47 (after detection by spraying with 10% phosphomolybdic acid in ethanol and heating at 110°C for 15 min.). Authentic prostaglandin E_2 used as a marker on the thin layer plate had an Rf of 0.42, thus suggesting that the products of the sodium borohydride reaction were more polar, a chemical feature compatible with the products being prostaglandins $F_{2\alpha}$ and $F_{2\beta}$.

Preliminary results obtained from radio gas chromatography of the methyl ester trimethylsilyl ether derivative indicated that prostaglandin $F_{2\alpha}$ was the major isomer produced. Purification of the reaction mixture by reversed phase partition chromatography on a glass column (350 x 10 mm) containing 6.5 g of LH-20 (graded particle size 40±5 um diameter) was performed. The column was eluted at a flow rate 7-8 ml h^{-1} with a mixture of methanol, water, butanol, chloroform, acetic acid (50;50:5:5:0.1 by volume). Fractions of 1.0 ml were collected in glass test tubes on an LKB Ultrorac fraction collector. From each fraction 10 µl was removed and the radioactive content determined by liquid scintillation counting. This chromatographic step allowed the pooling of several fractions to be performed such that on thin layer chromatography in the FVI solvent system (Andersen 1969)

the prostaglandin $F_{2\alpha}$ and $F_{2\beta}$ were found to be greater than 90% radio-chemically pure with respect to each other.

Preparation of derivatives for gas-liquid chromatography

Samples were treated by the methods described previously (page 61) to produce the following derivatives :

a) Methyl ester trimethylsilyl ether (MeTMS)

b) Methyl ester O-methyloxime trimethylsilyl ether (Me Mo TMS)

c) Methyl ester 9,11-cyclic n-butyl boronate, 15-trimethylsilyl ether (Me NBB TMS).

Radio-gas liquid chromatography

A Pye Unicam Series 104 gas chromatograph was interfaced with a Panax radio gas chromatograph detector for the simultaneous recording of mass (flame ionisation detection) and radioactivity. The gas chromatograph was equipped with a glass column (1.5 m x 2 mm i.d.) packed with 3% OV-1 on Supelcoport (100-120 mesh). The carrier gas (argon) flow rate was 25-40 ml min^{-1} and the oven temperature 190-210°C. Gas chromatography derivatives (Me Mo TMS; Me TMS; Me NBB TMS) were dissolved in 50 µl of either n-hexane of BSTFA and 10 µl was injected. The radio-gas chromatograph was calibrated for carbon values (C values) by plotting the log retention time versus carbon value for normal saturated fatty acid methyl esters (C_{16}-C_{24} in C_2 units).

Combined gas-liquid chromatography-mass spectrometry

This was performed using an LKB 9000 combined gas chromatograph-mass spectrometer. The instrument was fitted with a glass column (1.5 m x 2 mm i.d.) packed with 3%

OV-1 on Supelcoport (100-120 mesh). The gas chromatograph oven temperature was 195-205°C and the carrier gas (helium) flow rate was 20-30 ml hr^{-1}. The instrument was calibrated for 'C' values and the derivatised prostaglandins were prepared as described above for radio gas liquid chromatography. The instrument in addition was calibrated for a variety of authentic prostaglandin derivatives at the beginning of each operation period. Mass spectra was recorded at the crest of the gas chromatography peaks (total ion current detector) corresponding to those calculated from the 'C' value obtained from radio-gas chromatography. The electron energy of the ion source was initially set at 22.5 eV but automatically switched to 27.5 eV when a mass spectral scan was begun.

B) Characterisation of the 9-keto reductase present in sheep whole blood

i) Determination of the blood compartment the enzyme is present in

Freshly collected and citrate anticoagulated sheep whole blood (see page 57) was centrifuged at 4°C for 20 minutes at 1875 x g. The plasma, buffy layer and red cell fractions were carefully separated and stored at 4°C in silanised glass containers. A sample of the red cell fraction was treated with an equal volume of 100 mM sodium phosphate buffer (NaH_2PO_4 12.7 g; Na_2HPO_4, 12.54 g; reduced glutathione, 10 mg in 1 litre of distilled water) and the haemolysed cells recentrifuged at 1875 x g for a further 20 minutes at 4°C. The supernatant was removed and the resulting cell debris resuspended in fresh buffer

solution (2:1 v/v).

A sample of sheep whole blood and each of the following fractions (10 ml) was then incubated with prostaglandin E_2 (10 µg: 1 µCi) at 37°C for 75 minutes in a water bath. The products of the incubation were extracted and purified as described previously (page 58).

a) Plasma

b) Buffy layer

c) Red cells

d) Haemolysed washed red cells

e) Haemolysate

ii) <u>The effect of pH incubation temperature and pre-incubation temperature on the enzyme</u>

In all cases the enzyme preparation used was equivalent to sample 'd' described above (i.e. haemolysed washed red cells). The substrate concentration of prostaglandin E_2 was 3 µM (1 µg ml^{-1}; 1 µ Ci) and the substrate was added in 100 µl of sodium phosphate buffer. The incubation volume was 10 ml and the incubation time 75 minutes.

For the pH study 10% aqueous acetic acid solution was used to acidify from pH 5.0-7.0 and aqueous 0.1M Na OH solution was used for the pH range 7.7-9.0. The substrate was added immediately the pH was attained and then incubated at 37°C.

The pre-incubation temperatures were obtained by heating the enzyme sample (in a glass container) in a water bath (with agitation) at the elevated (or lowered) temperature for 15-20 minutes. The enzyme preparations

were then cooled to 0°C by immersing the container into an incubator containing an ice-methanol mixture. The sample was then incubated with prostaglandin E_2 at 37°C.

The effect of incubation temperature was obtained by heating the sample to the required temperature and then adding the substrate (prostaglandin E_2). The enzyme preparation was then incubated at the required temperature with agitation in a water bath.

iii) Cofactor requirements for the conversion of PGE_2 to $PGF_{2\alpha}$ by sheep red blood cells

a) Haemolysed red blood cells were prepared from freshly collected sheep whole blood as described previously (page 90). The substrate concentration of prostaglandin E_2 was 3 µM (1 $\mu g ml^{-1}$; 1 µCi) cofactor concentration 2 mM and enzyme volume 10 ml. An enzyme preparation without added cofactors was incubated with prostaglandin E_2 for the determination of basal conversion of PGE_2 to $PGE_{2\alpha}$. In two experiments prostaglandin $F_{2\alpha}$ (3 µM; 1 µCi) was incubated with and without NAD or NADP (2 mM) for the determination of the conversion of $PGF_{2\alpha}$ to PGE_2. The cofactors studied for the conversion of PGE_2 to $PGF_{2\alpha}$ were NADH; NADPH ; reduced flavin mononucleotide (FMN - H_2) and reduced flavine adenine dinucleotide ($FADH_2$). Incubations were for 75 minutes at 37°C in a water bath (with agitation). The products were extracted as described previously (page 58), and purified by thin layer chromatography on neutral silica gel (200 mm x 200 mm x 0.1 mm on glass). Marker standards of ^3H-labelled prostaglandins $F_{2\beta}$, $F_{2\alpha}$, E_2 and A_2 were spotted at the side of the samples and the

plates were developed in a multi-run system. The first development was in the FVI solvent (Andersen 1969) and the second in the GCM solvent (Millar 1974). After development the plates were scanned on a Panax radio thin layer chromatographic plate scanner and the zones of radioactivity scraped into test tubes containing methanol (5 ml). After vortexing the tubes were centrifuged at maximum speed on a bench centrifuge for 5 minutes and the resulting supernatant transferred into a pear-shaped flask. The compacted silica gel was re-extracted with methanol (5 ml) as described above. The supernatants for each zone were pooled and taken to dryness and the residue re-dissolved in 1.0 ml of methanol. From each zone 4 x 50 µl was removed and assayed for radioactivity by liquid scintillation counting. The remaining residue from the $PGF_{2\alpha}$ zones transferred to Eppendorf tubes and converted to the methyl ester trimethylsilyl ether as described previously (page 61). These derivatives were then subjected to combined gas chromatography-mass spectrometry on an LKB 9000. The instrument was equipped with a glass column (3 m x 4 mm i.d.) packed with 3% OV-1 on Supelcoport (100-120 mesh). The column temperature was 255°C and the carrier gas (helium) flow rate 35 ml min^{-1}.

b) In an experiment designed to confirm the quantitative results obtained by the radioactive method quantitative gas chromatography-multiple ion detection mass spectrometry was employed.

Groups of fresh enzyme samples (10 ml volume)

as described above were incubated with prostaglandin E_2 (1.4 μM; 1 μCi) in the presence and absence of NADH or NADPH (2 mM). Samples were incubated in all cases for 25, 50 and 75 minutes. At the end of the required time period 200 ul of the reaction mixture was transferred to a test tube containing 2 ml of 10% aqueous acetic acid and 1 μg each of 3,3,4,4 tetra-deutero PGE_2 and 3,3,4,4 tetradeutero $PGF_{2\alpha}$ mixed and left at room temperature for 15 minutes. The remaining reaction mixture was extracted as described above. After 15 minutes aqueous glacial acetic solution was vortexed with 4 ml of redistilled ethyl acetate and then the mixture centrifuged for 5 minutes on a bench centrifuge. The aqueous solutions were then frozen solid by partial immersion in ethylene glycol at -10 to -20°C and the ethyl acetate fraction poured off into a clean 25 ml pear-shaped flask. The frozen aqueous phases were then thawed and re-extracted. The pooled ethyl acetate fractions were taken to dryness at 40°C under a stream of air and then vacuum desiccated.

The extract of the major portion of each incubation sample (9.8 ml) was treated in an identical manner as described on pages 84 and 85

The extract from the minor portion of each incubation sample (0.2 ml) was transferred to capped Eppendorf tubes. The prostaglandin E_2 present in the samples was converted into the methyl ester and either the methyl oxime or the ethyl oxime trimethylsilyl ether. The prostaglandin $F_{2\alpha}$ present in the samples was converted into the methyl ester trimethylsilyl ether by the methods

described previously. The samples were dissolved in 50 µl of BSTFA and 5 µl aliquots subjected to quantitative analysis by the method described briefly elsewhere (Hensby and Naylor 1974). This method was based upon quantitative gas-chromatography-multiple ion detection mass spectrometry. A Finnigan 3000D quadrupole mass spectrometer was interfaced with a Finnigan 9500 gas chromatograph equipped with a 1.5 m x 2 mm i.d. glass column packed with 3% OV-1 on Supelcoport. The carrier gas (helium) flow rate was 25-30 ml min^{-1} and the oven temperature 230-250°C. The instrument was calibrated at the beginning of each run for protium and deuterium by measuring the ratios of the peak heights of given ions over the range 1-100 ng of protium (100 ng deuterium constant). The ions monitered were 295 and 423 for the protium forms of prostaglandin E_2 and $F_{2\alpha}$ respectively and at 299 and 427 for the corresponding deuterium ions. Triplicate determinations for standard and biological samples were made.

iv) Substrate specificity studies

a) PGE_2; 15 (R)PGE_2; PGE_2 methyl ester; PGE_1; 13, 14-dihydro-PGE_2; PGA_2 and PGB_2.

Haemolysed red blood cells were prepared as described previously (page 90). The substrate concentration was 1 µg ml^{-1} (2.8 µM) for all the prostaglandins studied. For prostaglandins E_2; E_2 methyl ester; E_1; A_2 and B_2 1 µ Ci of the corresponding ^3H-labelled prostaglandins was incorporated in the incubation. For prostaglandins E_2 and E_1 the reaction volume was 10 ml and for the rest 50 ml. Blood samples were incubated at

37°C in a water bath (with agitation) for 75 minutes and then extracted into ethyl acetate as described previously (page 58).

Thin layer chromatography

The residue from the extraction procedure was vacuum desiccated and then re-dissolved in 0.2 ml of methanol. For the 8-iso-PGE_1, 15(R)PGE_2 and the 13, 14-dihydro-PGE_1 samples of 0.5 µCi (1.5 ng) of ^3H-labelled PGA_2 was added prior to thin layer chromatography to act as an internal standard for this step. The samples were then applied to neutral silica gel thin layer chromatography plates (200 mm x 200 mm x 0.1 mm, on glass). The extracts from the PGE_2, PGE_1, PGA_2 and PGB_2 incubations were spotted on the same plates as marker standards containing ^3H-labelled prostaglandins $F_{2\beta}$, $F_{2\alpha}$, E_2 and A_2. The 8-iso-PGE_1 13,14-dihydro PGE_1 and 15(R)PGE_2 samples were spotted on the same plate as the PGE_2 methyl ester sample and marker standards of ^3H-labelled prostaglandin $F_{2\beta}$, $F_{2\alpha}$, E_2 and A_2 methyl esters. A standard plate was also prepared containing ^3H PGA_2 and the F_{β}, F_{α} and E standards of the 8-iso, 13,14-dihydro and 15(R) prostaglandins used as substrates.

The thin layer chromatography plates were then subjected to a multi-run development using the FVI solvent of Andersen (Andersen 1969) as the first run and the G.C.M. solvent of Millar [illegible] (1974) as the second development.

not necessary??

The plates were dried thoroughly after each development under a stream of cold air until no scent

of solvent was detectable. After the final development the individual samples were scanned for radioactivity using a Panax thin layer chromatography plate scanner. The cold marker prostaglandins were detected by spraying with a solution of 10% phosphomolybdic acid in ethanol and heating at 110°C for 15 minutes.

Elution of prostaglandins from thin layer chromatography plates

From the scans obtained for the radioactive substrate (eg PGE_2, PGE_2 methyl ester, PGE_1, PGA_2 and PGB_2) residues, zones corresponding to peaks of radioactivity were scraped from the thin layer plates into test tubes containing 5 ml of methanol. For the non-radioactively labelled substrates (eg 8-iso-PGE_1; 13,14-dihydro-PGE_1 and 15(R) PGE_2) the corresponding F_β F_α and E zones were scraped into test tubes containing 5 ml of methanol. The position of the ^{3}H-PGA_2 zones of the biological samples (added as an internal standard) was used to check that the thin layer plates containing the marker standards and the biological samples had chromatographed essentially the same. The prostaglandins were eluted and separated from the silica gel by vortexing with the methanol for 15-30 seconds and then centrifuging on a bench centrifuge at maximum speed for 5-10 minutes. The supernatant from each zone was carefully transferred into pear-shaped flasks and the compacted silica gel re-extracted with a further 5 ml of methanol as described above. The supernatants for each zone were pooled, taken to dryness under reduced pressure, vacuum desiccated and the residue dissolved in 1.0 ml of methanol.

Quantitative and qualitative analysis of the zones obtained from thin layer chromatography

From the radioactively labelled substrate samples, 4 x 50 µl was removed from each zone and quantitatively assayed for radioactivity by liquid scintillation counting. For all the prostaglandin samples 0.5 ml of the methanol solution was transferred to an Eppendorf tube and mixed with either 20 µg of omega-dihomo-PGE_2 (E zones) or 10 µg of omega-dihomo-$PGF_{2\alpha}$ (F zones). The E zones were then converted to the corresponding methyl ester trimethylsilyl ether (Me TMS) by the methods described previously (page 61). The omega-dihomo-prostaglandins E_2 and $F_{2\alpha}$ were a generous gift from Dr. N. Crossley, ICI Ltd.

These derivatives were dissolved in 50 µl of BSTFA and 10 µl was injected into the gas chromatograph of an LKB 9000 combined gas chromatograph-mass spectrometer. This instrument was fitted with a glass column (3 m x 4 mm i.d.) packed with 3% OV-1 on Supelcoport (100-120 mesh). The oven temperature was 240-250°C and the carrier gas (helium) flow rate was 30-35 ml hr^{-1}. Mass spectra for qualitative identification were recorded at the crest of the gas chromatography peaks corresponding to the corresponding authentic prostaglandins. Quantitative analysis was performed by comparing the area of the derivative on gas chromatography with that of the added internal standard. Duplicate quantitative determinations were performed.

b) $\underline{PGD_2}$

Freshly citrated sheep blood was treated as described previously (page 58) to produce six different stock enzyme preparations (A-F). For each stock enzyme preparation 10 ml aliquots were used for the comparison of PGE_2 and PGD_2 as substrates. The incubation was performed at 37°C (with agitation) in a water bath at a substrate concentration of 14 µM. Aliquots (0.2 ml) were removed from each sample at 1, 5, 10, 15, 30, 60 and 90 minutes and incubated with 2 ml of 10% aqueous acetic acid solution containing 1 µg of 3, 3, 4, 4-tetradeutero $PGF_{2\alpha}$. After 15 minutes at room temperature the samples were extracted into ethyl acetate as described previously (page 94). The ethyl acetate for each sample was pooled and taken to dryness at 40°C under a stream of air.

To the enzyme preparation obtained at the end of the incubation period (approximately 100 minutes) from samples D, E and F that had been incubated with prostaglandin D_2 1.0 µ Ci of ^3H-$PGF_{2\alpha}$ (approximately 2.5 ng) was added and the sample was then diluted with 100 ml of distilled water, acidified to pH 4 with glacial acetic acid and extracted twice with an equal volume of re-distilled ethyl acetate. The pooled ethyl acetate was washed twice with 20 ml of distilled water, taken to dryness under reduced pressure and vacuum desiccated. The residue was then re-dissolved in 25 ml of 67% aqueous ethanol and partitioned twice with an equal volume of petroleum spirit before the aqueous phase was taken to

dryness and vacuum desiccated. The residue from each fraction was then re-dissolved in 0.2 - 0.5 ml of methanol and treated with excess diazomethane at room temperature for a maximum of 15 minutes. The samples were then taken to dryness and vacuum desiccated.

Straight phase partition chromatography on a lipophilic substituted LH-20 gel column of prostaglandin methyl esters

A silanised glass column (450 mm x 21 mm) was packed with a lipophilic Sephadex LH-20 gel to a bed volume of 85 ml. This gel was Sephadex LH-20 substituted with 51% (w/w) of C_{11} to C_{14} hydroxyalkyl sidechains and was prepared by Mr. A.R. Brash. Prior to packing the gel had been thoroughly equilibrated with a heptane-chloroform mixture (70:30 v/v) and was further equilibrated *in situ* before being used. The flow rate was 6-8 ml hr^{-1} and fractions of 4.25 ml (5% bed volume) were collected on an LKB Ultrorac fraction collecter. A minimum of 10 bed volumes were collected before a further sample was chromatographed on the column. Samples (standards and biological) were converted to methyl esters prior to chromatography. The vacuum dried residue obtained after methyl esterification was dissolved in 150 µl of chloroform and made up to 500 µl with n-heptane. The total volume applied to the column was never greater than 1.5 ml (including washings).

The column was calibrated for authentic prostaglandin D_2 and three prostaglandin F_2 ring isomers. These were the 9α, 11α ($PGF_{2\alpha}$); 9β, 11α ($PGF_{2\beta}$) and the

9α11β (11β $PGF_{2\alpha}$) isomers. The prostaglandin D_2 and 9α11β $F_{2\alpha}$ were detected by gas chromatography after conversion to the MeMoTMS and MeTMS derivatives respectively. The 9α11α and 9β11α $F_{2\alpha}$ epimers were detected by liquid scintillation counting.

The elution profile of the biological samples D, E and F that had been incubated with PGD_2 was monitored by liquid scintillation counting of an aliquot from alternative samples. This enabled the 9α11α $PGF_{2\alpha}$ zone to be determined (^{3}H-$PGF_{2\alpha}$ added) and from this the position of the 9α11β zone could be calculated. The fractions present in each zone were pooled, taken to dryness under reduced pressure and vacuum desiccated.

Quantitative gas chromatography-mass spectrometry

This was performed on a Finnigan 3000D quadrupole mass spectrometer interfaced with a Finnigan 9500 gas chromatograph equipped with a glass column (1.5 m x 2 mm i.d.) packed with 3% OV-1 on Supelcoport. The carrier gas (helium) flow rate was 25-35 ml min^{-1} and the oven temperature 240-255°C.

Authentic prostaglandin $F_{2\alpha}$ and the biological samples were converted to methyl ester trimethylsilyl ethers, dissolved in 50 µl of BSTFA and 5 µl aliquots injected into the gas chromatograph. Triplicate determinations were made for each sample. The instrument was calibrated for deuterium (100 ng per injection) and protium (0-100 ng per injection) forms of authentic prostaglandin $F_{2\alpha}$. The ions produced in the mass

spectrometer were focussed and repetitively scanned by means of an automatic peak selector. The ions chosen for the analysis were at m/e 423 (m-(90+71)) and 333 (M-(2 x 90 + 71)) for the protium. The corresponding deuterium ions were at m/e 427 and 337.

Qualitative gas chromatography-mass spectrometry

This was performed on an LKB 9000 combined gas chromatograph-mass spectrometer. The instrument was equipped with a glass column (3 m x 4 mm i.d.) packed with 3% OV-1 on Supelcoport. The carrier gas (helium) flow rate was 35 ml min^{-1} and the column temperature 245°C. All prostaglandins (authentic and biological) were prepared as the methyl ester n-butyl boronic acid, trimethylsilyl ether (Me NBBTMS) as described previously (page 61). Under these conditions only F type prostaglandins with both the 9 and 11 hydroxyl groups in the cis configuration (eg 9α11α$PGF_{2\alpha}$) will form the 9, 11 cyclic n-butyl boronate ether. Consequently the 9α11β$PGF_{2\alpha}$ epimer produces the methyl ester trimethylsilyl ether only.

c) 15-keto PGE_1 ; 15 keto PGE_2; 15-keto $F_{1\alpha}$ and 15-keto $F_{2\alpha}$

The 15-keto derivatives of prostaglandins E_1; E_2 and $F_{1\alpha}$ were prepared purified and generously supplied by Dr. R.L. Jones.

Preparation of 15-keto $PGF_{2\alpha}$

A sample of solid PGE_2 (11 mg) was transferred to a 25 ml pear-shaped flask and dissolved in 1.0 ml of absolute ethanol. This was cooled to approximately -10°C

in a beaker containing ice / acetone mixture. To this solution approximately 20 m Ci of tritium labelled sodium borohydride (Amersham, Na B^3H_4; 320 Ci $mole^{-1}$) was added and the contents mixed. After incubating at -10°C for 60 minutes the solution was diluted to 50 ml with distilled water, acidified to pH4 with 1N HCl and extracted twice with two volumes of diethyl ether. The pooled diethyl ether was washed twice with 20 ml of distilled water and then filtered through solid Mg SO_4. The diethyl ether solution was then taken to dryness and vacuum desiccated. The residue was dissolved in methanol (10 ml) and an aliquot spotted on neutral silica gel (50 mm x 200 mm x 0.1 mm; on glass). Marker standards of authentic prostaglandin $F_{2\beta}$ and $F_{2\alpha}$ were spotted at the side of the sample and then the plate was developed in the FVI system (Andersen 1975). After drying the plate under a stream of dry air the plate was scanned on a Panax radio thin layer chromatography plate scanner. Two radioactive peaks were detectable that corresponded with the authentic prostaglandin $F_{2\beta}$ and $F_{2\alpha}$ standards.

An aliquot (3.0 ml) of the methanol solution was treated with diazomethane until it remained pale yellow. After 10 minutes at room temperature the sample was taken to dryness under reduced pressure and vacuum desiccated.

The residue was then re-dissolved in 150 µl of re-distilled chloroform and made up to 500 µl with n-heptane. This was applied (with 2 x 0.2 ml washings) to a 50 ml bed volume column of lipophilic substituted

LH-20 gel. This gel was LH-20 (30 µM particle size) substituted to 62% (w/w) with hydroxyalkyl groups (C_{15}-C_{18}). The column had been thoroughly equilibrated in situ with a n-heptane chloroform mixture (70:30) prior to sample loading. The flow rate was 7-8 ml hr^{-1} and fractions of 2.5 ml were collected on an LKB Ultrorac fraction collecter. From alternate fractions 10 µl aliquots were removed and assayed for radioactivity by liquid scintillation counting. This chromatographic step enabled the separation of the $PGF_{2\alpha}$ and $PGF_{2\beta}$ methyl esters such that on thin layer chromatography in the GCM solvent system they were greater than 99% pure with respect to each other.

The residue of the $PGF_{2\alpha}$ methyl ester fractions was dissolved in methanol (5 ml) and 4 ml was transferred to a 50 ml pear-shaped flask containing approximately 50 mg of $PGF_{2\alpha}$ methyl ester (5 ml methanol) and the pooled sample taken to dryness and desiccated. The residue was re-dissolved in 5 ml of spectroscopic grade 1,4-dioxan and to this 35 ml of a 1,4-dioxan solution containing 2,6 dichloro-3,5-dicyano-para benzoquinone (DDQ; 25 mg ml^{-1}) was added. After mixing the solution was refluxed at 90°C in a waterbath for 3 hours. At one-hourly intervals 50 µl aliquots were removed, mixed with 4 ml of 0.1% $NaHCO_3$ aqueous solution (1g L^{-1}) and then partitioned twice with an equal volume of diethyl ether. The pooled ether fractions were washed, with 2 ml of distilled water and then taken to dryness. The residue was re-dissolved in 5 ml of methanol and 2 ml placed in a silica cell of 3 ml volume and 1 cm pathlength. The

spectrum between 280 nm and 220 nm was recorded against a cell containing 2 ml of methanol using a Carey 116 ultraviolet spectrophotometer. After 3 hours UV absorbence at 235 nm was almost identical with the 2 hour sample. The solution under reflux was then added to 150 ml of 0.1% $NaHCO_3$ solution and solid $NaHCO_3$ added until the pH was 7.5-8.0. This was essential to maximise the amount of DDQ in the ionised form and thus minimise the amount extractable into diethyl ether. The aqueous solution was extracted twice with an equal volume of diethyl ether and the pooled ether washed successively with 100 ml of 0.1% $NaHCO_3$ solution and 100 ml distilled water. The ether phase was then taken to dryness under reduced pressure and the residue vacuum desiccated and then re-dissolved in 5 ml Analar methanol.

The methyl ester was removed by treating the sample with 35 ml of methanolic 0.05N KOH overnight at room temperature. The sample was then diluted with 150 ml distilled water, acidified to pH4 with 10% acetic acid solution and extracted twice with an equal volume diethyl ether as described above.

The vacuum dried residue was purified by reversed phase partition chromatography on Lipidex 1000, a hydroxyalkyl substituted LH-20 gel. The column used was 300 ml bed volume and had been equilibrated with a mixture of methanol, water, chloroform, butanol, glacial acetic acid (50,50,5,5,0.1 by volume). The sample was loaded onto the column in 2.5 ml of this solvent and fractions of 23 ml were collected (flow rate approximately

40 ml hr^{-1}). The elution profile was determined by liquid scintillation counting of 100 µl from each fraction. This step yielded 7.5 mg of 15-keto $PGF_{2\alpha}$ that was essentially pure by thin layer chromatography in the FVI solvent system. Conclusive evidence of identification was obtained by converting an aliquot to the methyl ester, o-n-butyloxime, trimethylsilyl ether (see page 61) and subjecting this to combined gas chromatography-mass spectrometry.

Incubation and extraction procedures

Whole blood (850 ml) from ewes was treated with citrate anticoagulent (150 ml) as described previously (page 58). The enzyme preparation was OBTAINED by treating the red cells with 100 mM sodium phosphate buffer as described previously. The substrate concentration for all the 15-keto prostaglandins was 28 µM (10 µg ml) and the reaction volume 10 ml. The samples were incubated at 37°C in a water bath (with agitation) for 75 minutes.

In all cases the reaction was stopped by the addition of 150-250 ml of distilled water and immediate acidification to pH4 with glacial acetic acid. The aqueous phase was then extracted twice with an equal volume of diethyl ether-ethyl acetate (4:1 V/V) mixture. The pooled organic fractions were then washed twice with 20 ml of distilled water before being taken to dryness under reduced pressure at 40°C and vacuum desiccated.

This residue was then dissolved in 20-30 ml of aqueous 67% ethanol and partitioned twice with an equal volume of heavy petroleum, before again being taken to

dryness and desiccated.

The residue was dissolved in 0.5 to 1.0 ml of methanol and treated with excess ethereal diazomethane at room temperature to convert to methyl esters. Where necessary (eg if material had precipitated out of solution) the resulting solution was filtered through a glass wool plug inserted in a Pasteur pipette. The original flask was washed with methanol (5-10 ml) and this was also filtered. The pooled fitrate was taken to dryness and desiccated.

Straight phase gel partition chromatography on Lipidex 5000

A glass column (350 x 10 mm) was silanized by treatment with a 5% solution (v/v) of dichlorodimethylsilane in toluene for 2 hours at room temperature. The column was then rinsed with successive washings of chloroform and methanol (to remove excess silanising reagent) and plugged at the base with glass wool and sand to a depth of approximately 1.0 cm. The column was then packed to a bed volume of 30 ml with Lipidex 5000 (a lipophilic Sephadex LH-20 derivative) as described previously (Brash and Jones 1974). Prior to packing, the gel had previously been equilibrated with a heptane-chloroform mixture (80:20 V/V) and was further equilibrated *in situ* with this mixture before use. A constant pressure head at the top of the column was maintained and a flow rate of 8-10 ml hr^{-1} obtained. The samples were dissolved in 100-200 µl of chloroform and then n-heptane was added until the solvent composition was the same as the column eluant. The total loading,

including washings, was never greater than 1.5 ml.

Fractions of 3 ml (10% bed volume) were collected on an LKB Ultrorac fraction collecter until the 2000% bed volume point was reached.

The elution profile of the column was obtained by liquid scintillation counting of an aliquot (200 µl) from alternate fractions. Authentic prostaglandins were used to calibrate the Lipidex 5000 column and these were detected by either liquid scintillation counting or gas liquid chromatography.

From the elution profile it has been possible to bulk fractions into zones that have subsequently been taken to dryness and vacuum desiccated. The residue from each zone was re-dissolved in methanol (1.5 ml) and 4 x 50 µl aliquots from each quantitatively assayed for radioactivity by liquid scintillation counting.

Liquid scintillation counting

This was performed with a Mark II Nuclear Chicago liquid scintillation counter.

Aliquots of either the column fractions or the methanol solution containing the pooled fraction residue were counted in 5 ml of a toluene-ethoxyethanol based scintillation mixture (toluene, ethoxyethanol 1500; 900 v/v napthalene; 2,5-diphenyloxazole (PPO) and 1,4-di-(2-(4-methyl-5-phenyloxazolyl))-benzene (dimethyl POPOP) 112.5; 10.5; 0.9 by weight).

Where dpm were required these were obtained by correcting for quenching using the automatic external standard channels ratio method and a quench-calibration

curve constructed for the scintillation mixture. These calculations were performed using a PDP 8 I computer.

Preparation of derivatives for gas-liquid chromatography-mass spectrometry

An aliquot (500 µl) of the methanol residue from the appropriate zone of the Lipidex 5000 columns was taken to dryness under a stream of dry air and vacuum desiccated in capped Eppendorf tubes. The residue was derivatised by the methods described previously (page 61). The derivatives employed were : MeTMS, Me Eo TMS and Me NBBTMS.

Combined gas-liquid chromatography-mass spectrometry

Mass spectra were obtained using an LKB 9000 combined gas-chromatograph-mass spectrometer. The instrument was equipped with a glass column (3 m x 4 mm i.d.) packed with 3% OV-1 on Supelcoport (100-120 mesh). The oven temperature was 225-235°C and the carrier gas (helium) flow rate was 30-35 ml min^{-1}.

Authentic prostaglandins were injected in 5-10 µl of BSTFA.

Solvents and chemicals

All solvents were of analytical grade or re-distilled before use. The chloroform was re-distilled over anhydrous calcium chloride and 1% (by volume) of absolute ethanol was added to the redistillate for stabilisation purposes.

The metabolism of 15-keto PGE_2 by sheep whole blood and cellular fraction

Freshly collected sheep blood was mixed with

citrate anticoagulant (85:15 v/v) and separated into plasma and cellular fractions by centrifugation (at 4°C) for 20 minutes at 1875 x g as described previously (page 90).

Aliquots (10 ml) of whole blood, plasma and cellular fraction were incubated with 15-keto PGE_2 at a substrate concentration of 28 µM (Sp.Ac. 2C:$mole^{-1}$) for 75 minutes in a water bath (with agitation) at 37°C. The products of the reaction were extracted, purified, quantitatively and qualitatively assayed by the methods described on page 107 .

<u>The metabolism of 15-keto PGE_2 by sheep blood cellular fraction with time</u>

Haemolysed sheep blood cells were obtained from freshly collected sheep whole blood after treatment with citrate anticoagulant by the methods described previously (page 90).

Three aliquots (10 ml) were incubated with 15-keto PGE_2 at a substrate concentration of 28 µM (Sp.Ac. 2Ci $mole^{-1}$) for 5, 15 and 75 minutes respectively. The incubation was performed in a water bath (with agitation) at a temperature of 37°C. The reaction products were extracted, purified, qualitatively and quantitatively assayed by the methods described on page 107 .

<u>The effect of incubation pH and temperature on the metabolism of 15-keto PGE_2 and 15-keto PGE_1 respectively by sheep blood cellular fractions</u>

The haemolysed red cell fraction of fresh sheep

blood was prepared as described previously (page 90). In all cases the substrate concentration for both 15-keto PGE_2 (Sp.Ac. 2 Ci $mole^{-1}$) and 15-keto PGE_1 (Sp.Ac. 2 Ci $mole^{-1}$) was 28 µM, the incubation volume 15 ml and the duration of incubation 5 minutes.

The pH study was performed by using 10% aqueous acetic acid solution to acidify the enzyme preparation from pH 5.5 to 7.0 and 0.1M sodium hydroxide aqueous solution for pH 7.5 and pH 8.0. The substrate used for this study was 15-keto PGE_2 and the incubation temperature was 37°C.

The temperature profile was obtained by incubating 15-keto PGE_1 with the enzyme preparation at the given temperature.

The incubation products were extracted, purified, qualitatively and quantitatively assayed by the methods described previously (page 107).

<u>A comparison of the enzyme activity in blood samples from normal female and male with pregnant female and castrated male sheep</u>

Freshly collected sheep whole blood was collected and immediately mixed with citrate anticoagulant (85: 15 v/v) as described previously (page 58).

Prostaglandin E_2 (2.8 µM 1.0 µ Ci) was incubated with 50 ml aliquots of sheep whole blood at 37°C in a water bath (with agitation) for 75 minutes. The incubation was stopped and the products extracted by the addition of 5 volumes of ice-cold ethanol and thorough mixing. After 30 minutes at room temperature

the mixture was centrifuged at 1875 x g for 15-20 minutes at 4°C. The supernatant was transferred to a 1000 ml flask and the precipitate washed with an equal volume of ethanol before being centrifuged. The pooled supernatants were taken to dryness and the residue extracted into ethyl acetate and purified by thin layer chromatography. Quantitative analysis was performed by liquid scintillation counting and conclusive qualitative analysis by combined gas chromatography-mass spectrometry. The details of these methods have been described previously (pages 92 &93).

The metabolism of 15-keto PGE_2 by human blood samples

Fresh human blood (10-30 ml) was collected by venepuncture and mixed with citrate anticoagulant (5:1 v/v). Eight normal healthy males and one female each donated 50 ml of blood for this study. In all cases the 15-keto PGE_2 substrate concentration was 28 µM; the reaction volume 10 ml; the incubation temperature 37°C and the duration of incubation 75 minutes.

The incubation products were extracted by the methods described on page 107 and purified after conversion to methyl esters on a Lipidex 5000 straight phase gel partition chromatography column by the method described previously on page 107 . The quantitative analysis was performed by liquid scintillation counting of aliquots (4 x 50 µl) of the residue (1.5 ml) obtained by pooling the fractions obtained from the Lipidex 5000 column. Conclusive qualitative analysis was performed by combined gas chromatography-mass spectrometry using the derivatives previously described (page 61).

Attempts to prepare prostaglandin E_2 analogues for substrate specificity and metabolism studies

a) **PGE_2-1 alcohol**

The method of Schneider, Pike and Lincoln (1973) was used. A sample of solid prostaglandin E_2 (10 mg) was transferred to a 50 ml pear-shaped flask containing a methanol solution (10 ml) of ^3H-labelled PGE_2 (Amersham; Sp.Ac. 147 Ci m mole^{-1}; 50 µCi). The solid PGE_2 was dissolved in the methanol and the sample converted to the methyl ester by the method described on page 61). The resulting solution was taken to dryness and vacuum desiccated. The residue was re-dissolved in 10 ml of anhydrous pyridine followed by the addition of solid hydroxylamine hydrochloride (Ca. 100 mg). After the solid had been dissolved the resulting solution was heated at 70°C for 2 hours to produce the corresponding oxime of the PGE_2 methyl ester. Whilst the sample was allowed to come to room temperature an aliquot (50 µl) was transferred to a test tube containing 2 ml of distilled water at pH4 (with 6NHCl). This was extracted twice with an equal volume of diethyl ether. The pooled ether fractions were taken to dryness and the residue dissolved in 100-200 µl of a pyridine solution containing O-substituted n-butyl hydroxylamine hydrochloride salt and 50 µl of BSTFA as described on page 62 . When subjected to gas chromatography this sample was found only to contain the methyl ester hydroxylamine oxime analogue of PGE_2 and thus it was concluded that the original

reaction was complete.

The original reaction medium was diluted with 100 ml of distilled water acidified to pH4 with 6 NHCl (to ensure complete ionisation of any residual unreacted oximating reagent) and extracted twice with an equal volume of diethyl ether. The pooled ether phases were washed twice with 20 ml of distilled water and then taken to dryness under reduced pressure. The residue was transferred in methanol to a 50 ml pear-shaped flask, taken to dryness and vacuum desiccated for approximately 60 minutes before being re-dissolved in 2 ml of anhydrous solvent T (Koch Light).

A freshly prepared solution of lithium aluminium hydride in solvent T (c 50 mg ml^{-1}) was added dropwise to the reaction vessel until 3 to 4 ml had been added. The reaction vessel contents were thoroughly mixed and the vessel neck sealed with a cotton wool plug. The contents were allowed to react at room temperature for 60 minutes before ethyl acetate was added dropwise to react with and neutralise the excess lithium aluminium hydride. During this process the reaction vessel was kept cool with an ice/acetone mixture. After the addition of 25 ml of ethyl acetate the reaction mixture was left at room temperature for 15 minutes before being diluted with 100 ml of distilled water. This was then extracted twice with an equal volume of ethyl acetate. The pooled ethyl acetate was reduced to a constant volume (ca. 5 ml; mainly non-volatile solvent T) under reduced pressure. An aliquot (10 µl) of the resulting oil when converted to the trimethylsilyl

ether derivative (by the method described on page 64) had gas chromatographic and mass spectral properties consistent with it being the O-hydroxylamine oxime analogue of prostaglandin E_2-1 alcohol.

The resulting oil was then applied to a 25 g column of silicic acid equilibrated in toluene-ethyl acetate (80 : 20 v/v). The column was eluted (under negative pressure) successively with toluene (250 ml) toluene-ethyl acetate-methanol (250 ml, 40:59:1 by vol.) and methanol (50 ml). Each fraction was taken to dryness under reduced pressure and the residue re-dissolved in 5 ml of methanol. Aliquots of each fraction were assayed for radioactivity by liquid scintillation counting. Fraction 1 was found to contain the majority of solvent T but fraction 2 to contain the majority of radioactivity. The residue from fraction 2 was taken to dryness and vacuum desiccated before being re-dissolved in 5 ml of 90% aqueous acetic acid and cooled to 0-5°C. To this solution 10 ml of 6 M sodium nitrite (at 5°C) was added and the contents allowed to react (at 5°C) for 60 minutes. The products were then extracted twice with an equal volume of diethyl ether and the pooled ether phases washed with 10 ml of distilled water (twice) before being taken to dryness under reduced pressure. The residue from the ether was re-dissolved in 5 ml of methanol and an aliquot (25 µl) transferred to a capped Eppendorf tube. This was taken to dryness and reacted with 100-200 µl of a pyridine solution containing the o-substituted n-butyl hydroxylamine hydrochloride salt and 50 µl BSTFA

as described on page 62 . An aliquot of the resulting solution was subjected to combined gas chromatograph-mass spectrometry using an LKB 9000 instrument.

b) ^{14}C-labelled prostaglandin E_1

Dihomo-γ-linolenic acid (8, 11, 14-eicosatrienoic acid) labelled with ^{14}C in the carboxylic acid position was purchased from New England Nuclear with a specific activity of 58.8 Ci mole^{-1}. The hexane solution purchased contained approximately 750 μCi of ^{14}C radioactivity (Ca 3.9 mg). This was diluted to approximately 10 ml with n-hexane and an aliquot (10 μl) was applied to a neutral silica gel thin layer chromatography plate (50 mm x 200 mm x 0.25 mm on glass). After development in a n-hexane-ether-glacial acetic acid (70:30:1 by volume) mixture the plate was scanned for radioactivity using a Panax radio thin layer plate scanner. The radiochemical purity was found to be greater than 98%. This dihomo-γ-linolenic acid was then incubated with an acetone powder preparation of the ram seminal vesicle prostaglandin synthetase.

The acetone powder was prepared from fresh deep-frozen ram seminal vesicles by the method Wallach and Daniels (1971). Deep-frozen ram seminal vesicles (104.8 g wet weight) were cut into pieces (1-2 cm^3) and stored on ice until homogenised. Homogenisation was performed in 210 ml of 0.154 M (11.6 g L^{-1}) KCl (BDH analar) using a Polytron homogeniser at maximum speed. The homogenate was centrifuged at 2500 x g for 20 minutes

at 4°C. The resulting supernatant was filtered through cheesecloth to remove aggregated fat and cellular debris and then acidified to pH 4.8 by the addition of solid citric acid. This solution was thoroughly stirred and then centrifuged at 4000 x g for 15 minutes at 4°C. The supernatant was carefully decanted off and blended twice with 10-15 volumes of acetone at -35°C. The resulting precipitate was filtered under reduced pressure through a Whatman No. 1 filter paper (at 0°C) and then vacuum desiccated until no solvent scent could be detected.

A sample of this enzyme preparation (102 mg) was incubated with arachidonic acid to check that under the conditions to be employed for dihomo-γ-linolenic acid a sufficiently high yield of prostaglandin E_2 would result. The conversion (measured by U.V. spectroscopy) of arachidonic acid to PGE_2 was 56-58%.

The dihomo-γ-linolenic acid solution (750 µCi 3.9 mg) was taken to dryness in a 50 ml pear-shaped flask and vacuum desiccated. The residue was re-dissolved in 130 µl of absolute ethanol to which 7.8 ml of sodium EDTA buffer (pH8.0; 31.2 mM), 2.6 ml of reduced glutathione (10 mM) and 2.6 ml of hydroquinone (2.5 mM) solutions were added. The sample was incubated at 37°C for 5 minutes and then 435 mg of the acetone powder of the prostaglandin synthetase was added and the mixture incubated at 37°C for 30 minutes in a water bath with constant stirring. The reaction products were diluted with 100 ml of distilled water, acidified to pH4 with glacial acetic acid and extracted twice with two volumes

of diethyl ether. The pooled ether was washed twice with 25 ml of distilled water and then taken to dryness and vacuum desiccated. The residue was re-dissolved in 25 ml of 67% aqueous ethanol and partitioned twice with an equal volume of heavy petroleum. Both phases were taken to dryness for purification by reversed phase partition chromatography.

The 67% aqueous ethanol residue was re-dissolved in 5 ml of methanol and the heavy petroleum residue in 5 ml of n-hexane. An aliquot from each sample was applied to a neutral silica gel thin layer chromatography plate (50 mm x 200 mm x 0.25 mm on glass.) Marker standards of prostaglandins $F_{2\alpha}$, E_2 and D_2 (ca. 5 µg each) were applied at the side of the 67% ethanol aliquot. This plate was developed in the FV1 solvent system of Andersen (Andersen 1969) whilst the other plate was developed in n-hexane-ether-glacial acetic acid (70:30:1 by volume). After development both were scanned for radioactivity using a Panax radio thin layer chromatography plate scanner. The 67% ethanol plate was in addition sprayed with a saturated solution of phosphomolybdic acid in ethanol and then heated for 15 minutes at 110°C to develop the marker prostaglandins. The major peak on thin layer chromatography of the material in the 67% aqueous ethanol residue corresponded to authentic PGE_2 (PGE_2 and PGE_1 are not resolved on this thin layer system) whilst the major component of the heavy petroleum had chromatographic properties compatible with it being unreacted dihomo-γ-linolenic acid. Both fractions,

however, were contaminated with other radioactive compounds and thus required purification.

The methanol solution containing the 67% aqueous ethanol residue was taken to dryness and vacuum desiccated. The residue was re-dissolved in 0.5 ml of a mixture containing methanol, water, chloroform, butanol, glacial acetic acid (50:50:5:5:0.1 by volume) and applied to a 35 ml bed volume of Lipidex 1000 equilibrated in this solvent. Fraction of 3.5 ml were collected on an LKB Ultrorac fraction collecter and assayed for radioactivity by liquid scintillation counting. This step resulted in the purification of the ^{14}C PGE_1 (fractions 24-27) such that it was greater than 98% radiochemically pure on thin layer chromatography in the FV1 solvent system.

The heavy petroleum residue was purified by reversed phase partition chromatography on a column (80 ml bed volume) of Lipidex 5000. The sample was dissolved in 1.0 ml of a mixture containing methanol-water-dichloroethane-glacial acetic acid (800:200:100:1.1 by volume) and applied to the Lipidex 5000 column previously equilibrated in this solvent. Fractions of 8.0 ml bed volume were collected on a LKB Ultrorac fraction collecter and assayed for radioactivity by liquid scintillation counting. This step resulted in the purification of the dihomo-γ-linolenic acid to greater than 98% radiochemical purity as determined by thin layer chromatography.

c) 15-O methyl ether PGE_2 methyl ester

A methanol solution (15 ml) of PGE_2 (1 mg; 10 µCi) was converted to the methyl ester by the treatment with excess ethereal diazomethane as described on page 61. The solution was taken to dryness and vacuum desiccated for 30 minutes before being re-dissolved in approximately 3 ml of freshly prepared ethereal diazomethane. To this solution 2 drops of 1% solution (v/v) of boron triflouride etherate in anhydrous diethyl ether (sodium dried) was added. The diazomethane solution instantly turned from a golden yellow to a pale straw colour after which the solution was diluted with 25 ml of distilled water and partitioned twice with an equal volume of diethyl ether. The pooled ether fractions were twice washed with 10 ml of distilled water before being taken to dryness and desiccated.

The residue was re-dissolved in 0.5 ml of a n-heptane-chloroform mixture (80:20) and purified by straight phase gel partition chromatography on a column of Lipidex 5000 equilibrated in the solvent mixture. The column had a bed volume of 25 ml and fractions of 2.5 ml were collected on an LKB Ultrorac fraction collecter. These were assayed for radioactivity by liquid scintillation counting using the methods described on pages 85 & 88. Two peaks of radioactivity were detected. The first (fractions 6-8) contained material identified by combined gas chromatography-mass spectrometry as the 11,15-di-o-methyl ether methyl ester of PGE_2. The second peak (fractions 11-14) contained material that had mass spectra compatible

with it being a mixture of the 11 and the 15 mono-o-methyl ether methyl esters of PGE_2. The material in this peak was re-chromatographed on a column (55 ml bed volume) containing lipophilic LH-20 (30 μM particle diameter) substituted to 62% (w/w) with C15-C18 hydroxalkyl side chains. The eluting solvent was a mixture of n-heptane-chloroform (90:10 v/v). The sample was loaded on to the column in 0.5 ml of this solvent mixture and fractions of 2.5 ml were collected on an LKB Ultrorac fraction collecter. Alternate fractions were assayed for radioactivity by liquid scintillation counting. Two peaks of radioactivity were detected by this method. The material in the first (fractions 30-35) had a mass spectrum compatible with it being the 11 mono-o-methyl ether methyl ester of PGE_2 whilst material in the second (fractions 70-77) had a mass spectrum compatible with it being the 15 mono-o-methyl ether of PGE_2.

RESULTS

The metabolism of prostaglandin E_2 by blood samples from the sheep; rabbit; cat; dog; guinea-pig; horse; cow; rat; chicken and man

After incubation of ^{3}H-labelled prostaglandin E_2 with the above citrated blood samples, only sheep whole blood had apparently metabolised the prostaglandin as determined from the thin layer radio-scans. In the case of sheep whole blood two peaks of radioactivity were observed as described previously (page 78 figure 9). An example of the thin layer chromatography radio-scans obtained from the sheep, cat, guinea-pig and human blood is shown in figure 11 page 122 . The two peaks obtained from the sheep blood sample had Rf values corresponding with their being the original prostaglandins E_2 (least polar) and prostaglandin $F_{2\alpha}$. The Rf values of several prostaglandins on this thin layer chromatography system are given in Table 5 on page 123 . The metabolite, tentatively identified as prostaglandin $F_{2\alpha}$ comprised 43% of the radioactivity recovered from the thin layer plate. When the metabolite was subjected to radio gas chromatography as either the methyl ester, O-methyl oxime, trimethylsilyl ether or the methyl ester, n-butyl boronate, trimethylsilyl ether derivative, only a peak of radioactivity was observed corresponding to the relative volume of ^{3}H-labelled prostaglandin $F_{2\alpha}$. An example of the radio gas chromatography of authentic ^{3}H-labelled prostaglandins $F_{2\beta}$ and $F_{2\alpha}$ and the sheep blood metabolite is shown in figure 12 on page 124 . When subjected to combined gas chromatography-mass spectrometry

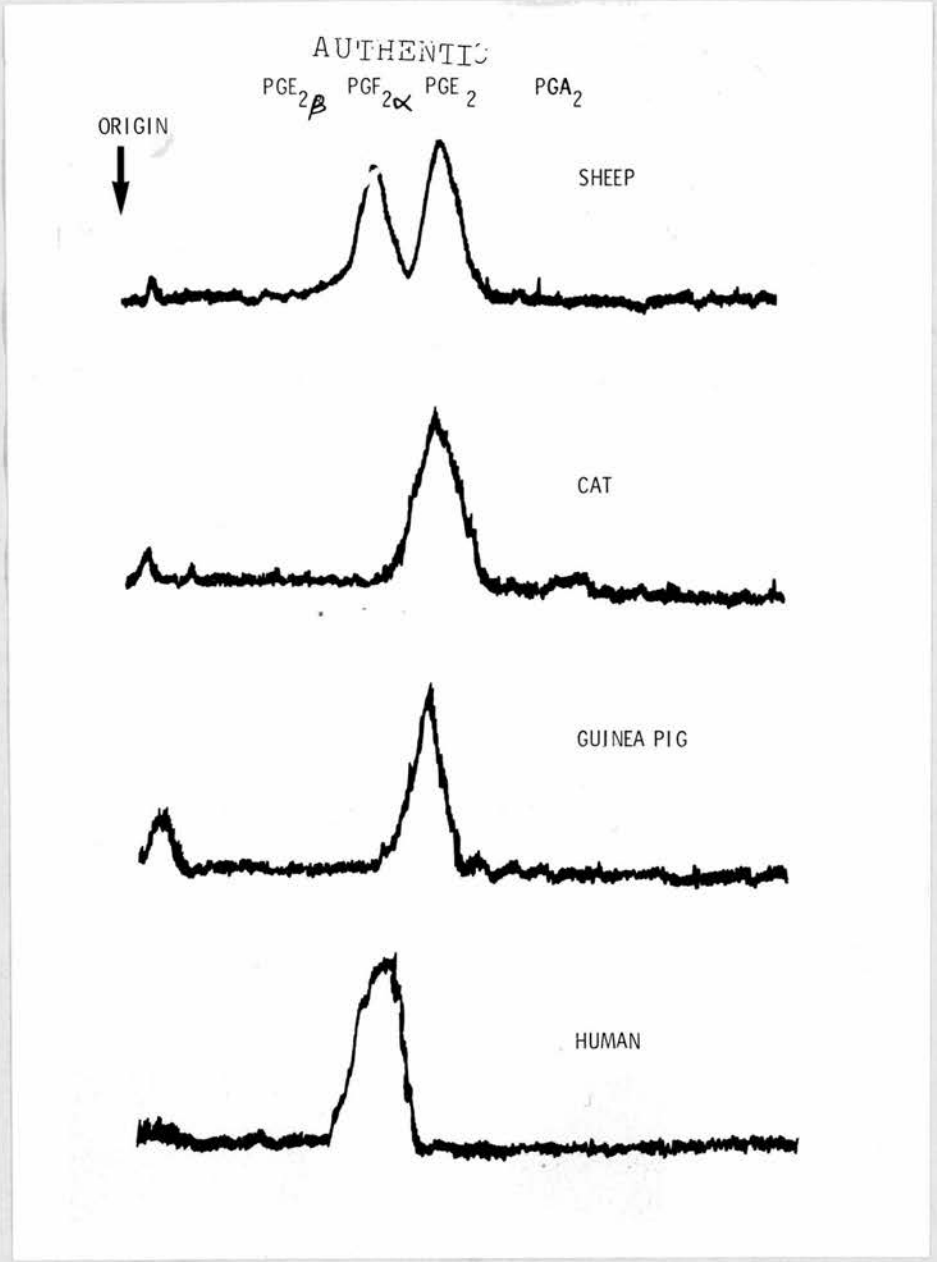

Figure 11a. Thin layer radio scans obtained from the products produced by incubating ^{3}H PGE_2 with sheep, cat, guinea-pig and human whole blood. The experimental details are described on page 84.

Compounds (free acids)	F VI double development	GCM double development	FV1 x 1 plus GCM x 1
$PGF_{2\beta}$	0.10	0.15	0.13
$PGF_{2\alpha}$	0.16	0.24	0.21
PGE_2	0.20	0.35	0.29
15-keto $PGF_{2\alpha}$	0.22	0.42	0.33
15-keto-13,14 dihydro $PGF_{2\alpha}$	0.30	0.50	0.37
15-keto PGE_2	0.52	0.67	0.55
15-keto-13,14 dihydro PGE_2	0.59	0.67	0.57
PGA_2	0.61	0.67	0.57

Table 5. The Rf values of some prostaglandins and their metabolites on thin layer chromatography using two solvent systems(PAGE 56).

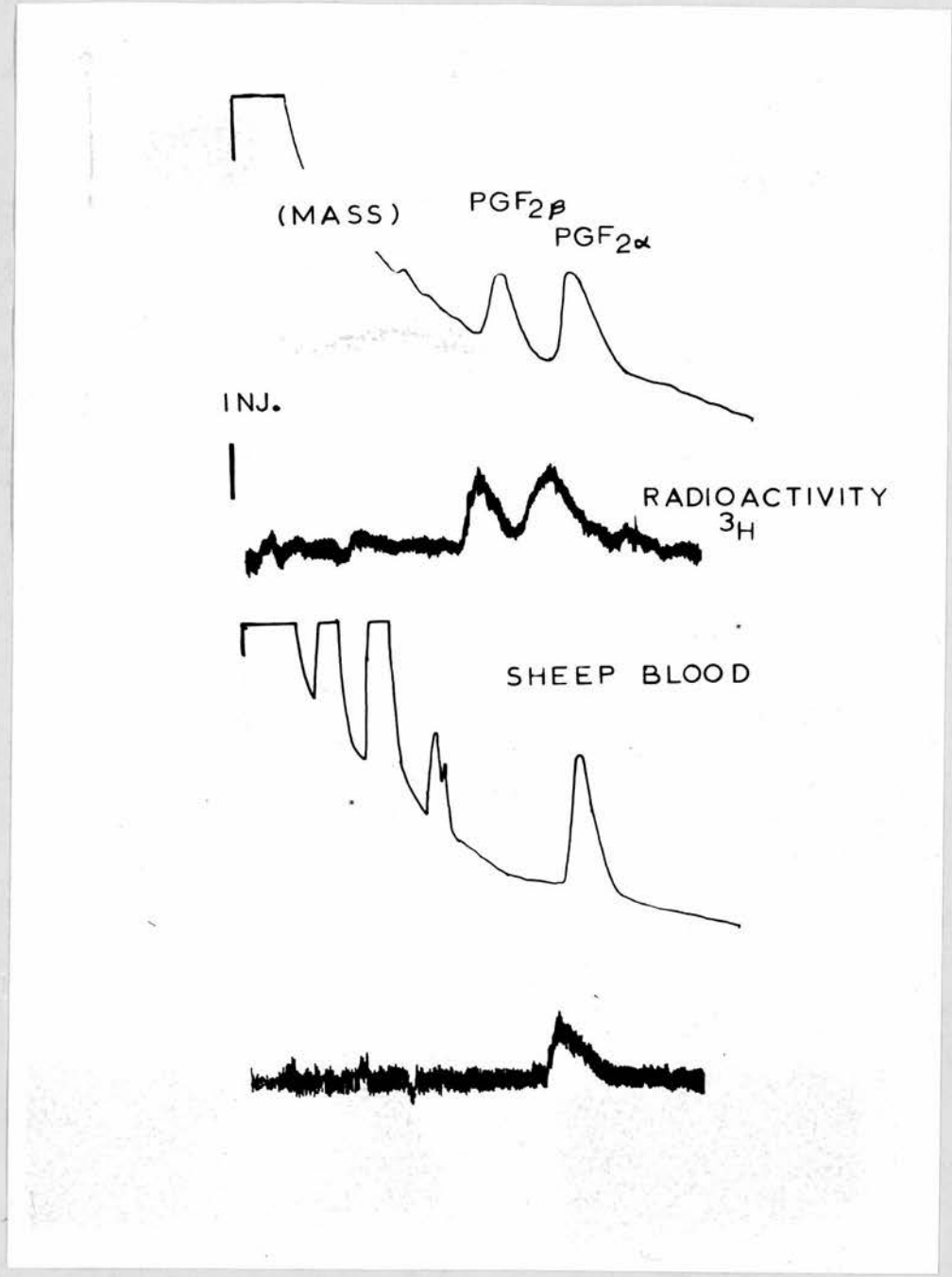

Figure 12. Radio gas liquid chromatography of authentic ^{3}H prostaglandins $F_{2\beta}$ and $F_{2\alpha}$ methyl ester trimethyl silyl ethers (upper) and the corresponding derivative obtained from the $PGF_{2\alpha}$ zone of t.l.c. plates after sheep blood had been incubated with ^{3}H PGE_2 (lower).

as both of the above derivatives, the sheep blood metabolite was conclusively identified as prostaglandin $F_{2\alpha}$. Examples of mass spectra obtained are shown in figure 13 on page 126 .

Characterisation of the 9-keto reductase present in sheep whole blood

When incubated with the plasma; buffy layer; red cells; haemolysed washed red cells and the resulting haemolysate, prostaglandin E_2 was only metabolised to prostaglandin $F_{2\alpha}$ to a similar extent as whole blood by the red cells and haemolysed red cells. The buffy layer did metabolise prostaglandin E_2 to prostaglandin $F_{2\alpha}$ but the conversion was only 6.3% of the corresponding metabolism by whole blood. This was possibly due to contamination of the buffy layer by red cells, however, this requires further clarification. It was thus apparent that the major fraction of the enzyme responsible for the metabolism of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ was in the erythrocyte fraction of sheep blood.

The effect of temperature and pH on the 9-keto reductase

The enzyme present in haemolysed red blood cells and capable of reducing the 9-keto group of prostaglandin E_2 was found to be heat labile. The values quoted in tables 6+7 and on page 129 and represented graphically in figures 14+15 on pages 127&8 show that incubation at temperatures above 50°C, as did prolonged incubation at 50°C, destroyed the ability of the haemolysed red cells to metabolise prostaglandin E_2. It can also be seen fro m these figures 14+15 on pages 127&8 that the enzyme system is

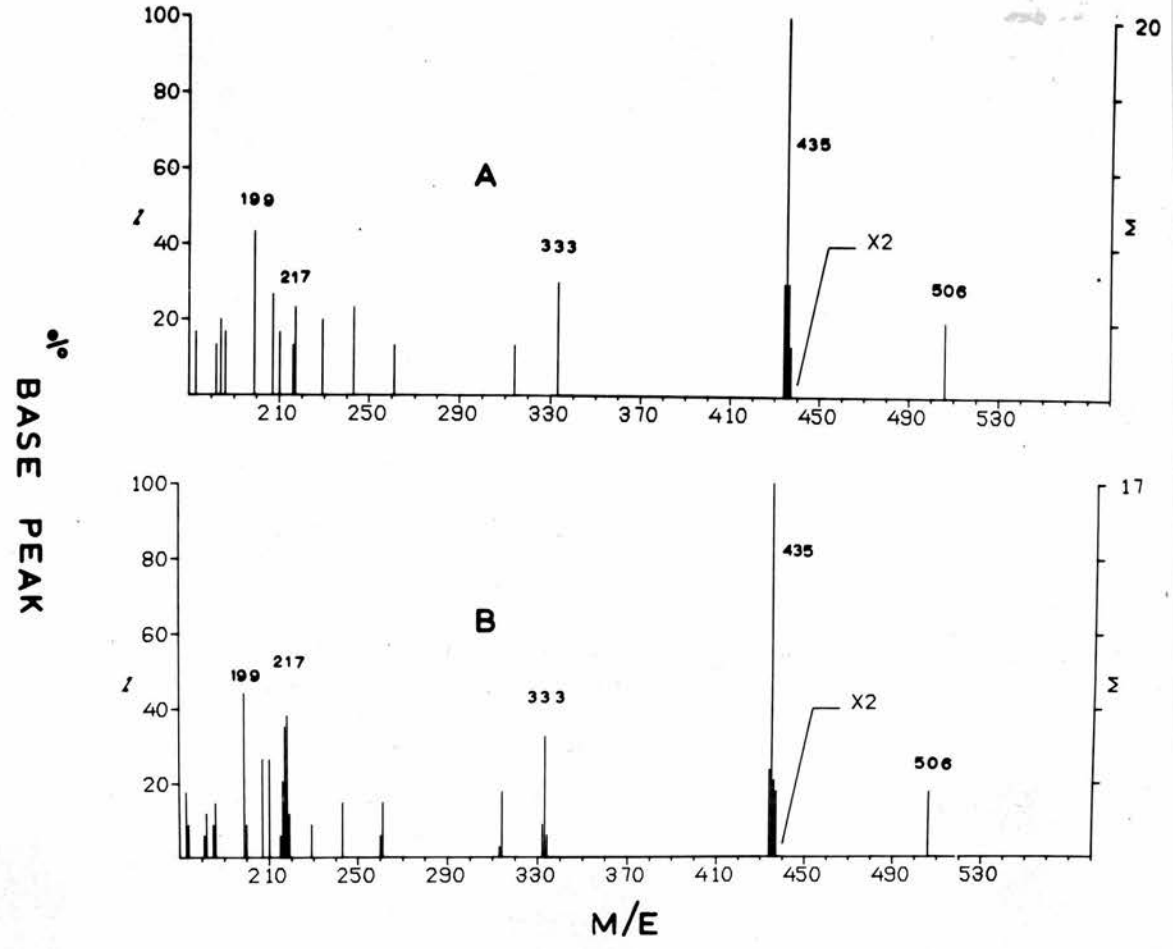

Figure 13. Mass spectra of authentic prostaglandin $F_{2\alpha}$ methyl ester, 9, 11 cyclic n-butyl boronate, 15 trimethyl silyl ether (upper) and the same derivative of the material obtained from sheep whole blood incubated with ^{3}H PGE_2 and previously identified by t.l.c. and radio gas liquid chromatography as $PGF_{2\alpha}$.

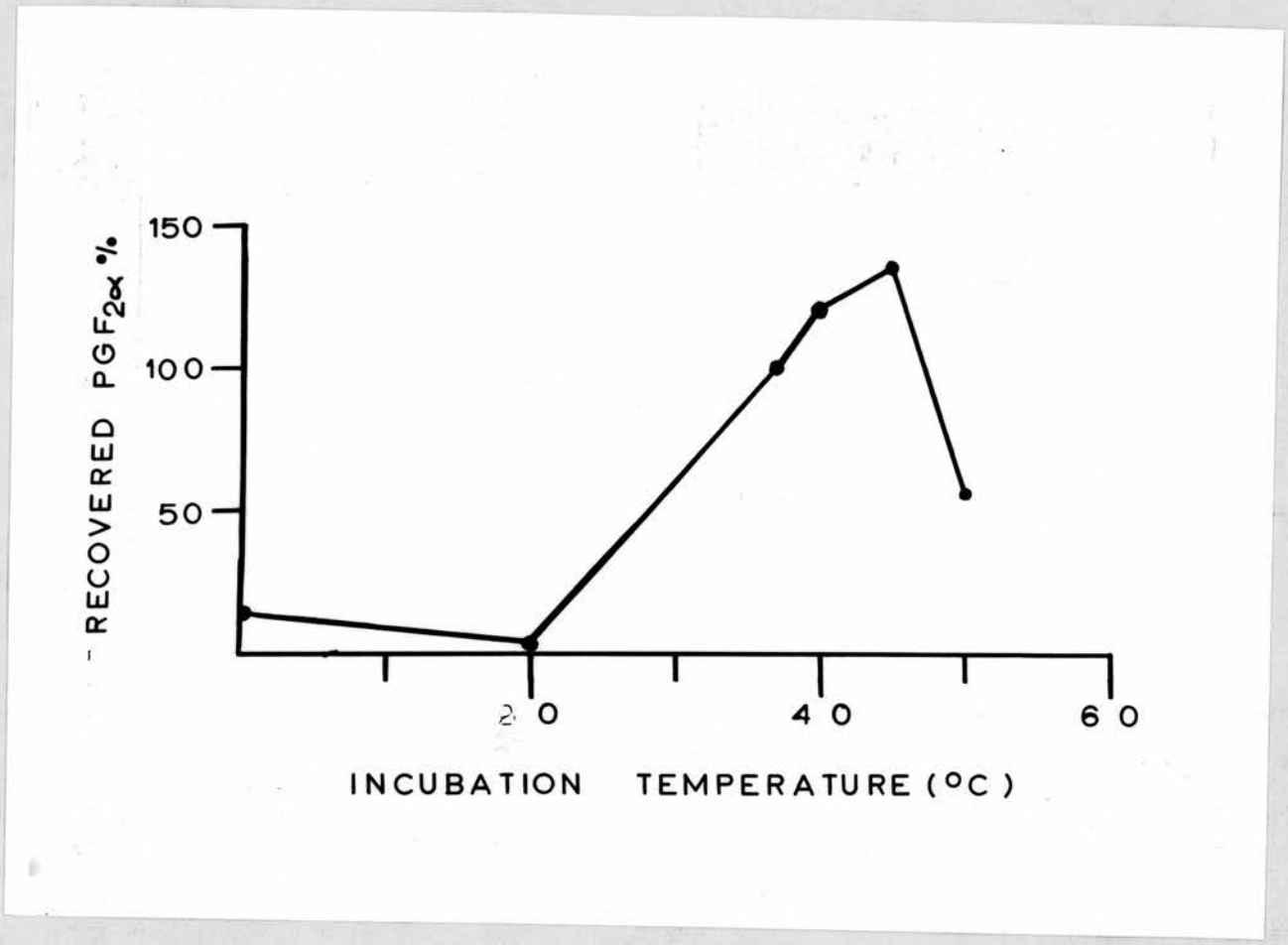

Figure 14. The effect of incubation temperature on the conversion of ^{3}H PGE_2 to ^{3}H $PGF_{2\alpha}$ by sheep blood.

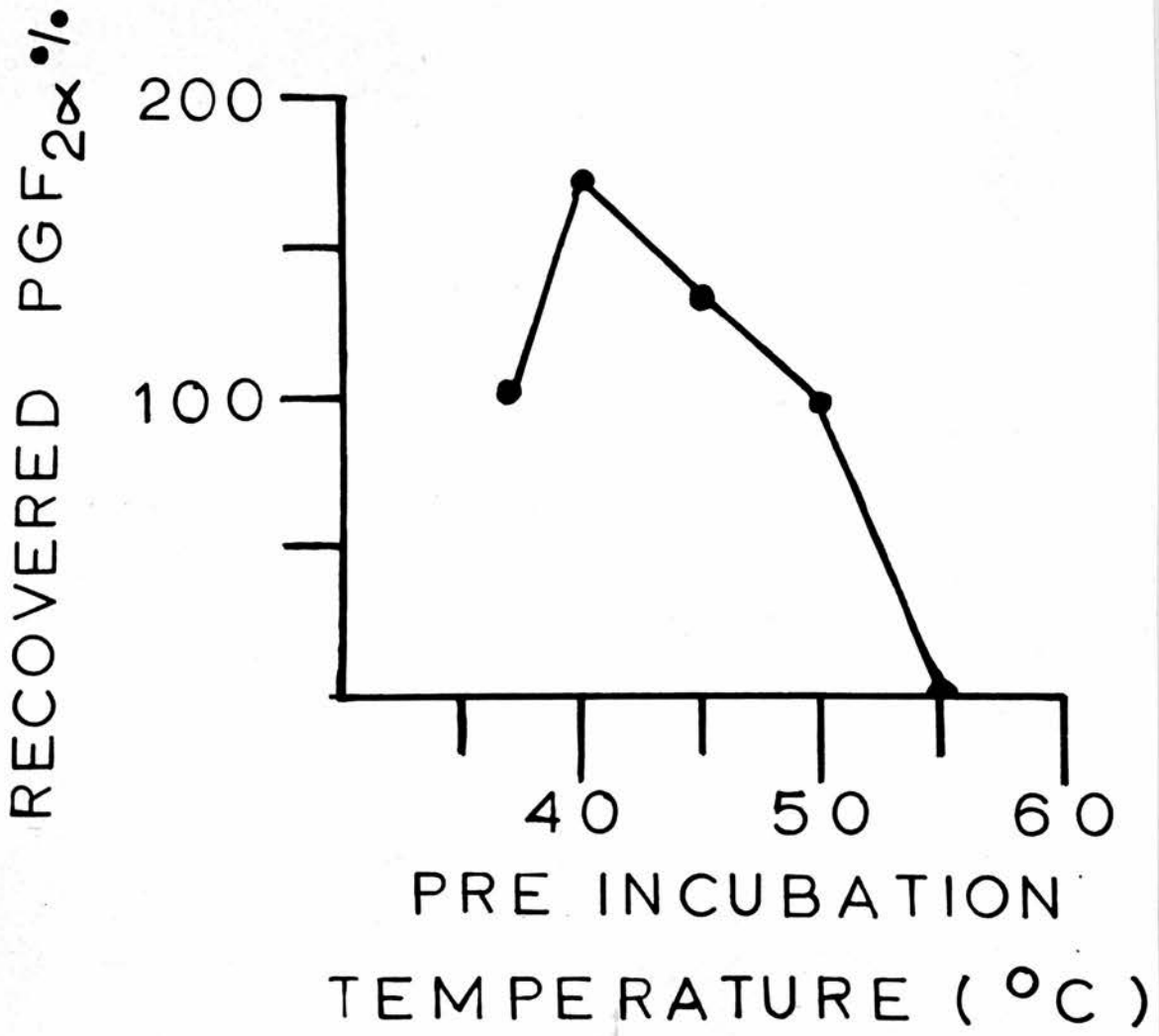

Figure 15. The effect of pre-incubation on the conversion of ^{3}H PGE_2 to ^{3}H $PGF_{2\alpha}$ by sheep blood.

apparently activated by either pre-treatment or incubation at temperatures up to 45°C. The reason for this activation is unknown, but it is possible that it may be due to a heat labile inhibitor being inactivated at these temperatures more quickly than the 9-keto enzyme.

Table 6

The effect of pre-incubation temperature on the 9-keto reductase activity in sheep haemolysed red cells.

pre-incubation temperature (0°C)	sample 1	2	mean value
37	100	100	100
40	174.31	143.15	158.73
45	132.08	126.86	129.47
50	99.76	89.72	94.74
55	0	11.27	5.64

The values quoted represent the recovered radioactive material in the $PGF_{2\alpha}$ zone of thin-layer plates relative to the value recovered from the 37°C incubation control.

Table 7

The effect of incubation temperature on the 9-keto reductase activity in sheep haemolysed red cells.

Incubation temperature (0°C)	Percentage radioactive material in $PGF_{2\alpha}$ zone of thin-layer plates relative to the values recovered from the 37°C incubation control
0	13 . 13
20	3 . 22
37	100
40	120 . 82
45	137 . 08
50	56 . 64

The effect of pH on the 9-keto reductase activity is indicated in table 8 below and graphically shown in figure 16 on page 131 . There, is an apparently sharp pH profile, with the optimum activity around pH 7.0. The substrate (^{3}H-PGE_2) is relatively unstable above pH 9.0 and therefore attempts to obtain values for enzyme activity at pH values greater than 9.0 were not made. Nevertheless, it can be seen that the activity is rapidly lost outside a narrow pH range.

Table 8

The effect of pH on the 9-keto reductase activity in sheep haemolysed red cells.

Incubation pH	Sample 1	2	mean value
5.0	0	13.2	6.6
5.5	16.1	-	16.1
6.4	-	44.5	44.5
7.0	100	100	100
7.7	-	19.8	19.8
8.0	41.4	-	41.4
8.4	-	16.5	16.5
9.0	32.2	23.1	27.6

The values quoted represent the recovered radioactive material in the $PGF_{2\alpha}$ zone of the thin-layer plates relative to the value from the 37°C incubation control.

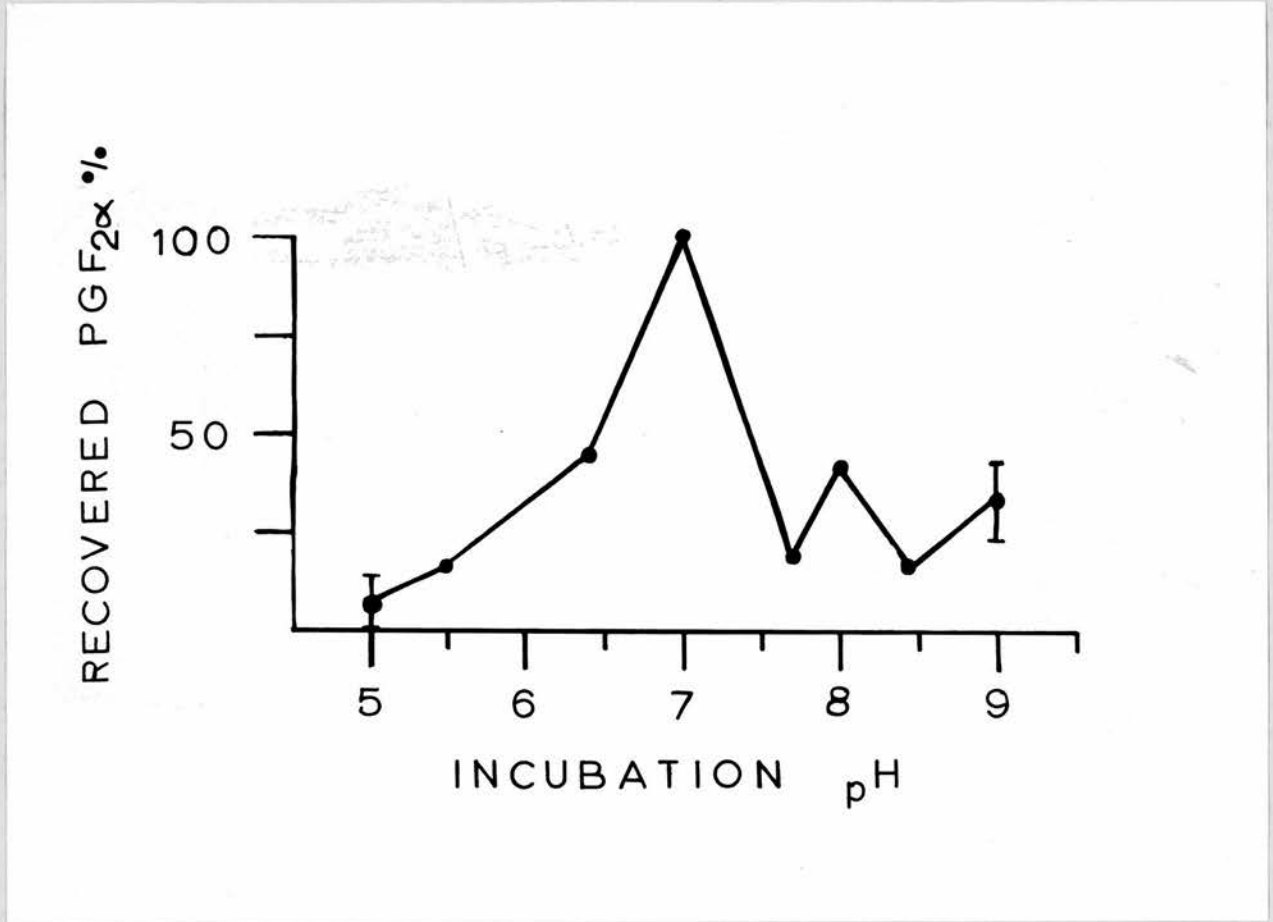

Figure 16. The effect of incubation pH on the conversion of ^{3}H PGE_2 to ^{3}H $PGF_{2\alpha}$ by sheep blood.

Co-factor requirements for the conversion of PGE_2 to $PGF_{2\alpha}$ by haemolysed sheep red blood cells

The values quoted in Table 9 below represent the amounts of prostaglandin $F_{2\alpha}$ (as a percentage of the total recovered radioactivity) produced by haemolysed sheep red blood cells when incubated with prostaglandin E_2 in the presence and absence of various cofactors. None of the cofactors tested produced marked changes in the total conversion of prostaglandin E_2 to prostaglandin $F_{2\alpha}$. The reverse reaction, namely the conversion of prostaglandin $F_{2\alpha}$ to prostaglandin E_2 was studied.

Table 9.

The effect of various reduced cofactors on the conversion of PGE_2 to $PGF_{2\alpha}$ by haemolysed sheep red blood cells.

Sample / cofactor (2mM)	1	2	mean value
None	100	100	100
$NADH_2$	97	107	104
$NADPH_2$	96	99	97.5
Flavin mononucleotide	102	100	101
Flavin adenine dinucleotide	100	106	103

The values quoted represent the recovered radioactive material in the $PGF_{2\alpha}$ zone of thin-layer plates relative to the value recovered from the 37°C incubation control

In none of the examples tested was the recovered radioactivity present in the prostaglandin E_2 zone of the thin-layer plates greater than 2%. It was thus concluded that the reverse reaction was not occuring at a significant rate under the conditions tested. The prostaglandin $F_{2\alpha}$ produced from the prostaglandin E_2 was conclusively identified (after conversion to the methyl ester trimethylsilyl ether) by combined gas chromatography-mass spectrometry. Mass spectra recorded at the crest of the gas chromatography peak corresponding to authentic prostaglandin $F_{2\alpha}$ were essentially the same as that shown in figure 38 on page 211 . Prominent ions were at m/e 584 (M+); 569 (M-15); 513 (M-71); 494 (M-90); 423 (M-90+71); 404 (M-2x90); 333 M-((2x90) + 71); 217; 199; 191 and 173.

The possibility that changes in rate of prostaglandin E_2 to $F_{2\alpha}$ conversion would not be apparent from measurements of the conversion after 75 minute incubations existed. Similarly, it was desirable to confirm the quantitative results obtained by the radiochemical method as being valid. Both these problems were tested, by comparing the values obtained for recovered prostaglandin $F_{2\alpha}$ using the radiochemical method and a deuterium isotope dilution technique for different incubation times, in the presence and absence of added NADH and NADPH. The values obtained for the two methods are shown in Table 10 on page 134 .

Table 10

The effect of NADH and NADPH on the conversion of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ by haemolysed sheep red blood cells as determined by two different isotope dilution methods.

Sample Incubation time (minutes)	no added cofactor		NADH added (2mM)		NADPH added (2mm)	
	^3H	^2H	^3H	^2H	^3H	^2H
25	31	29	32	29	28	31
50	46	45	43	46	44	45
75	58	59	60	61	62	60

^3H = radiochemical method

^2H = deuterium method

The values quoted in the ^3H columns represent the recovered radioactive material in the $PGF_{2\alpha}$ zone as a percentage of the total recovered radioactivity. The values quoted in the ^2H columns represent the prostaglandin $F_{2\alpha}$ as a percentage of the total $PGF_{2\alpha}$ + PGE_2 recovered when assayed by multiple ion detection combined gas chromatography-mass spectrometry.

It can be seen from the results quoted in Table 10 above that both methods give essentially the same result and that neither NADH nor NADPH would appear to influence the conversion of PGE_2 to $PGF_{2\alpha}$ by haemolysed sheep red blood cells.

Substrate specifity studies

a) PGE_2; 15(R)PGE_2; PGE_2 methyl ester; PGE_1; 13,14-dihydro-PGE_1; PGA_2; PGB_2 and 8-iso-PGE_1

Two quantitative assay methods were employed for

the analysis of the zones obtained from the thin-layer chromatography plates. One method employed for prostaglandins E_2; E_2 methyl ester; E_1; A_2 and B_2 was based on the recovery of the ^3H-labelled material. The other method used for prostaglandins 15(R)E_2; 13,14-dihydro-PGE_1 and 8-iso-PGE_1 was a quantitative form of gas chromatography utilising omega-dihomo-PGE_2 and omega-dihomo-PGF_2 as the internal standards respectively for the E and Fα zones obtained from thin-layer chromatography. The results obtained are presented in Table II on page 136 .

Table 11

Enzyme activity with different substrates.

substrate	sample 1	2	mean value
*PGE_2 (free acid)	100	100	100
*PGE_2 methyl ester	100	101	100.5
^{2}15(R)PGE_2	22	19	20.5
* PGE_1	106	104	105
^{2} 8-iso PGE_1	4	10	7
^{2}13,14-dihydro PGE_1	9	11	10
*PGA_2	0	0	0
*PGB_2	0	0	0

* assayed by radiochemical method

2 assayed by the quantitative gas chromatographic method

The values presented represent the recovered material in the PGF_α zones of the thin-layer plates relative to the value obtained from the $PGF_{2\alpha}$ zone when PGE_2 free acid was used as a substrate.

The material from each E and F_α zone was conclusively identified by combined gas chromatography-mass spectrometry. Prostaglandins A_2 and B_2 were apparently not substrates and therefore these samples were not subjected to gas chromatography-mass spectrometry. The mass spectrum of authentic 8-iso-prostaglandin $F_{1\alpha}$ and the material obtained from the 8-iso $F_{1\alpha}$ zone from thin layer chromatography are shown in figure 17 on page 137 .

It was apparent from these substrate specificity studies that the enzyme present in sheep blood and capable

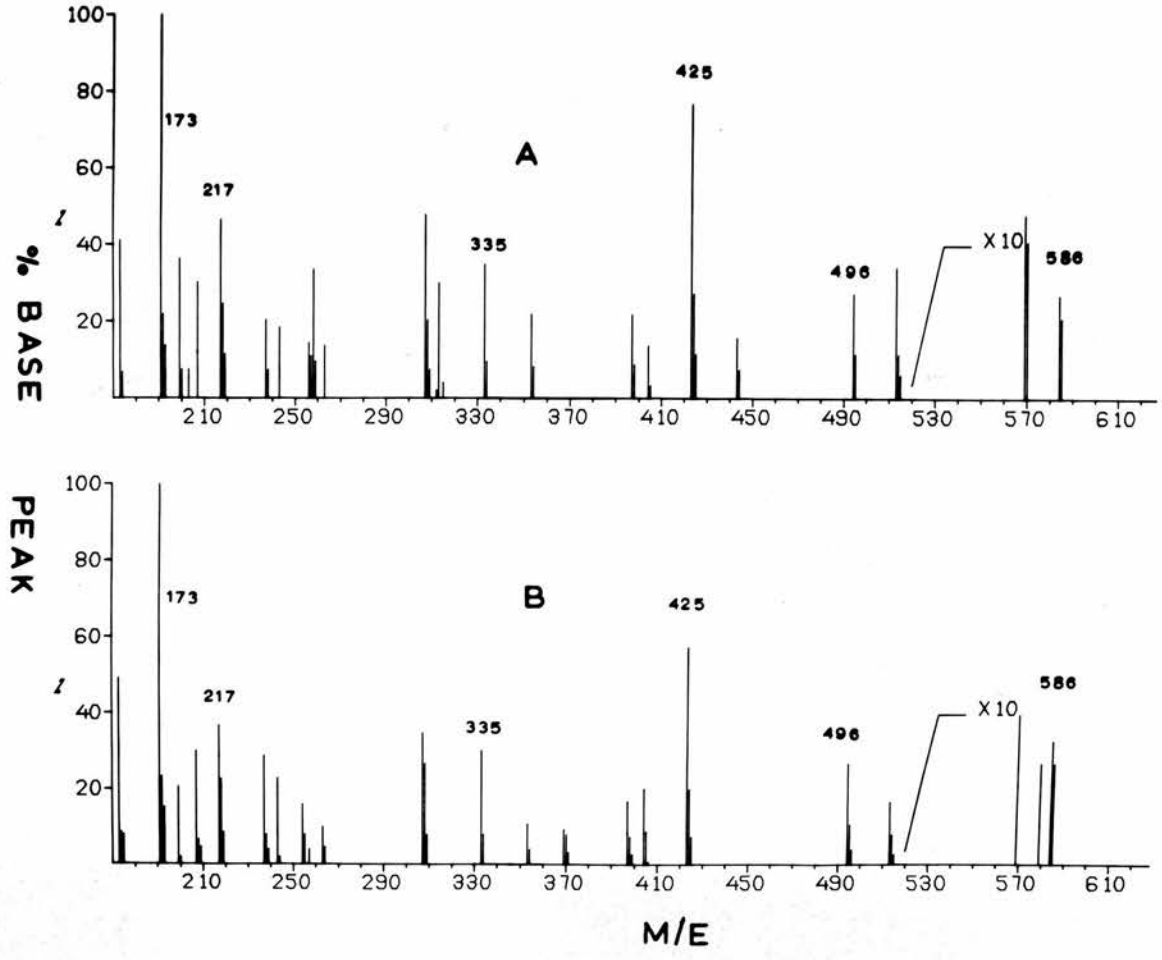

Figure 17. Mass spectra of authentic 8-iso-$PGF_{1\alpha}$ methyl ester trimethyl silyl ether (upper) and the corresponding derivative of the material obtained from the 8-iso-$PGF_{1\alpha}$ zone on t.l.c. of the extract of sheep blood incubated with 8-iso-PGE_1 (lower).

of reducing prostaglandin E_2 to $F_{2\alpha}$ had certain substrate structural requirements for maximal enzyme activity. It was apparent from the results obtained with prostaglandins 8-iso-E_1; 13,14-dihydro E_1; 15(R)PGE$_2$ and PGB$_2$ that the configuration of the atoms around carbons C-7; C-8; C-12; C-13; C-14 and C-15 was essential for maximal activity. Similarly the presence of a hydroxyl group at C-11 is indicated as being essential for enzyme activity from the result with prostaglandin A_2 as substrate. Since prostaglandins E_1 and E_2 methyl ester were equally good substrates as prostaglandin E_2 the 5,6-cis double bond and the C-1 carboxylic acid are apparently less essential for maximal enzyme activity. Consequently it was decided to investigate what effects on enzyme activity would be caused by substituting ketonic functions for the hydroxyl groups at C-11 and C-15. In addition it was hoped to ascertain whether these ketonic functions would also be reduced to hydroxyl groups and therefore further characterise the substrate specificity of the enzyme system.

b) Prostaglandin D_2

Prostaglandin D_2 is a structural isomer of prostaglandin E_2 as shown by figure 1 on page 3. When reduced with sodium borohydride, prostaglandin D_2, unlike prostaglandin E_2 produces only one of the two hydroxyl isomers possible (R.L. Jones, personal communication). The configuration of the resulting hydroxyl group is α and thus the product is prostaglandin $F_{2\alpha}$. The possibility that the enzyme system of haemolysed sheep red blood cells that stereospecifically reduced the 9-keto function of E

prostaglandins to the 9α-hydroxyl group may also reduce the 11-keto group of D prostaglandins to either or both the 11α or 11β hydroxyl epimers had to be considered. Preliminary results indicated that the thin-layer chromatography systems tested (G.C.M., FVI and AI) would not offer a sufficient separation of the 11α and 11β hydroxy epimers of prostaglandin $F_{2\alpha}$ (unpublished results). Consequently the separation of these two epimers had to be performed using a lipophilic substituted LH-20 gel chromatography system. An example of the separation of authentic prostaglandins D_2; 9α, 11α, PGF_2; 9α 11β, PGF_2 and 9β, 11α, PGF_2 on this chromatographic system as their corresponding methyl esters is shown in figure 18 on page 140 . Using this system the separation of the 9α, 11α and 9α, 11β epimers was greater than 98% with respect to each other. The products obtained after a 100 minute incubation of prostaglandin D_2 with three batches of this enzyme preparation were subjected to chromatography on this system, after extraction and conversion to methyl esters. The zones corresponding to 9α11αPGF_2 were detected by liquid scintillation counting and from these the zones corresponding to 9α11β epimer were predicted. When the material from each zone was subjected to combined gas chromatography-mass spectrometry as the methyl ester, n-butyl boronate, trimethylsilyl ether only 9α11α PGF_2 ($PGF_{2\alpha}$) was detectable. An example of the gas chromatographic trace obtained is shown in figure 19 on page 141 . It was thus concluded that prostaglandin

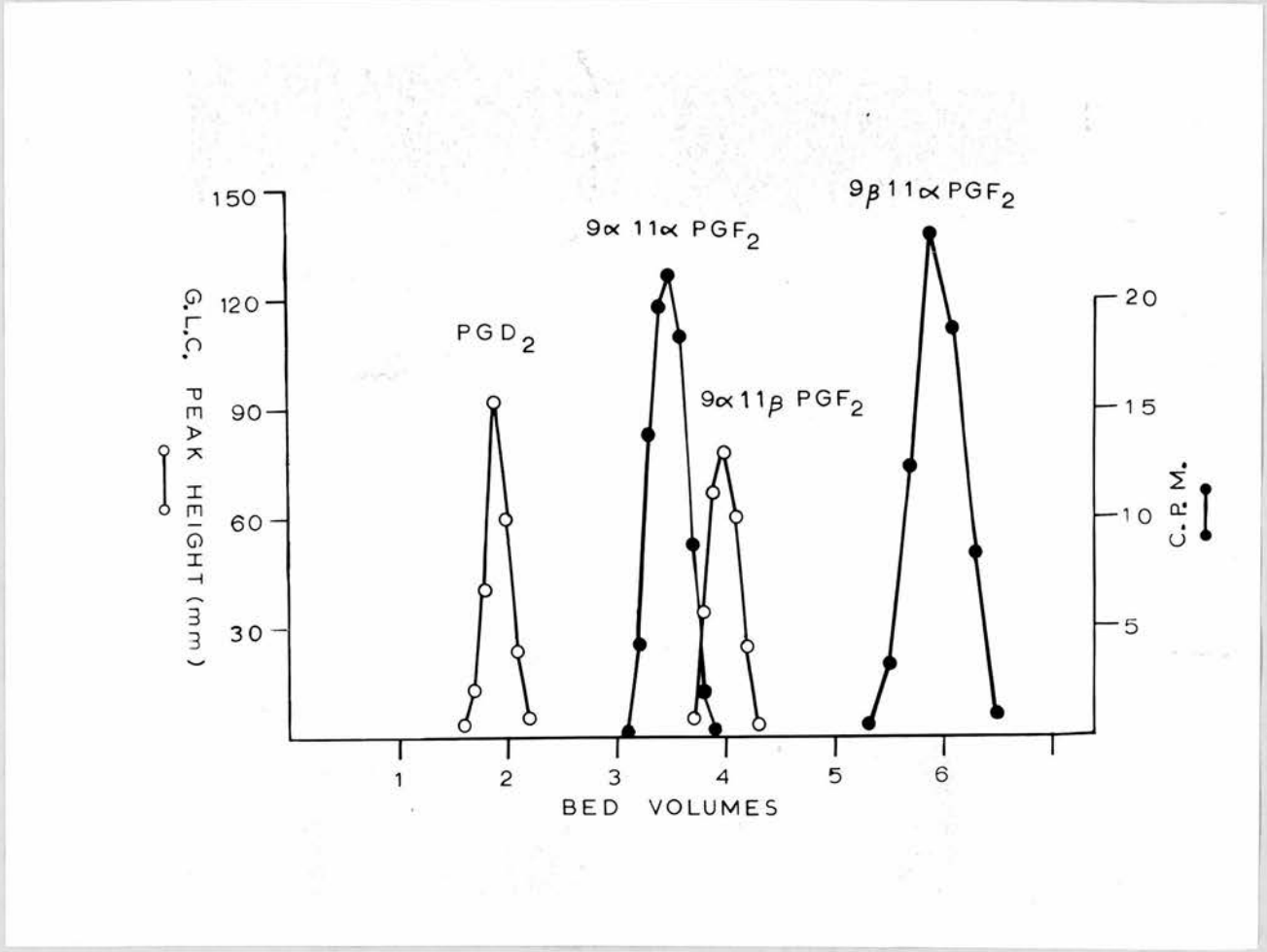

Figure 18. The separation of the methyl esters of prostaglandins D_2, 9α, 11β PGF_2; 9α, 11α PGF_2 and 9β 11α PGF_2 on straight phase gel partition chromatography. The column used was a lipophilic Sephadex LH-20 derivative eluted with heptane, chloroform (70:30 v/v). The details are described on page 100.

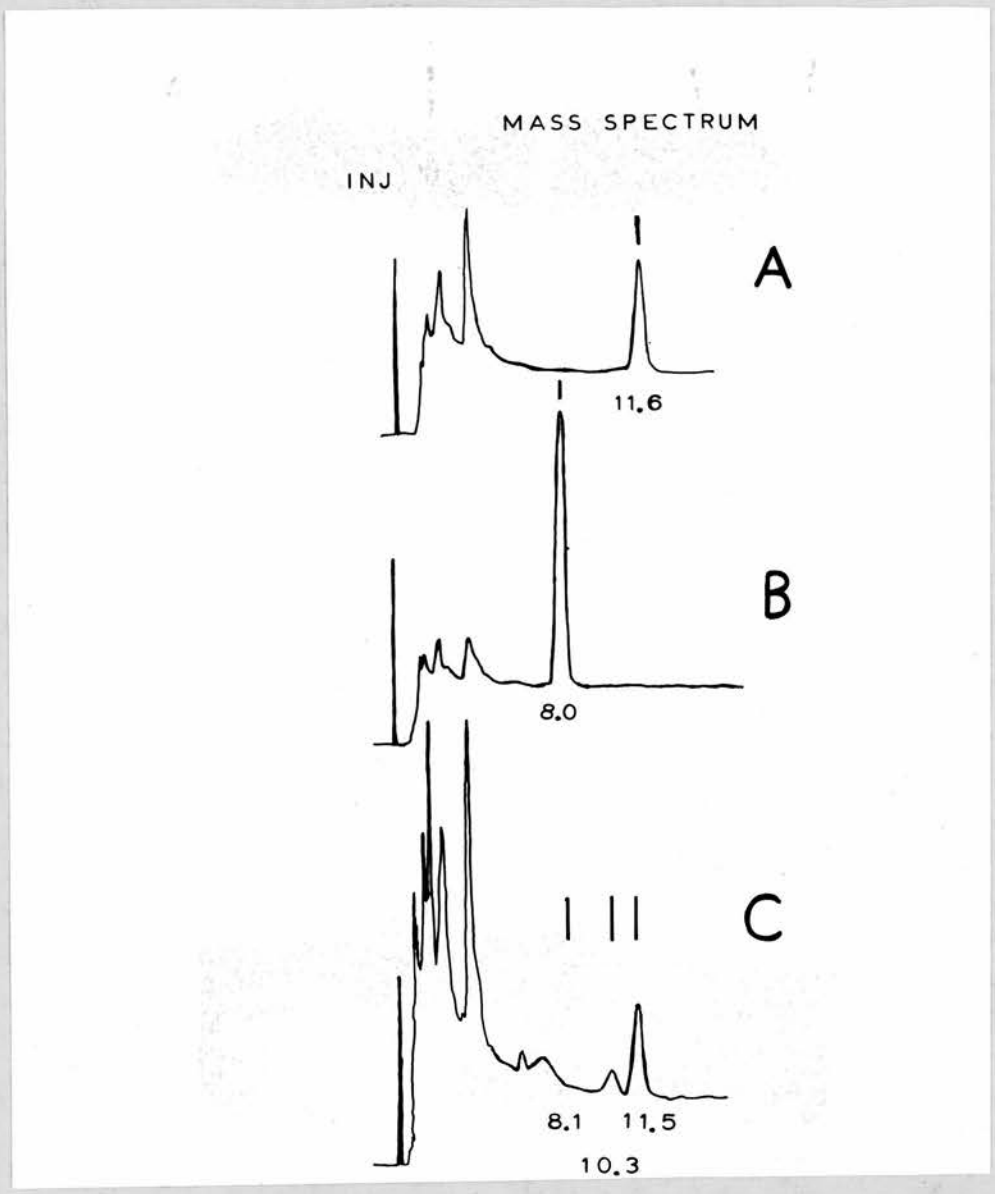

Figure 19. Gas chromatography traces (total ion current) obtained for authentic prostaglandins 9α 11α $PGF_{2\alpha}$ (A), 9α 11β $PGF_{2\alpha}$ (B) and the product obtained by incubating PGD_2 with sheep blood (C) and previously identified as 9α 11α $PGF_{2\alpha}$ -like on straight phase gel partition chromatography when derivatised as the methyl ester, cyclic n-butyl boronate trimethylsilyl ether.

D_2, like prostaglandin E_2, is reduced to prostaglandin $F_{2\alpha}$ by sheep red blood cells.

The rate at which these two substrates are converted to prostaglandin $F_{2\alpha}$ by this enzyme was quantitatively determined for six samples by multiple ion detection combined gas chromatography-mass spectrometry. The samples were assayed as the methyl ester trimethylsilyl ether. The values for the recovered prostaglandin $F_{2\alpha}$ for each substrate obtained from 0.2 ml aliquots of the enzyme preparation are shown in Table 12 on page 143.

Table 12

The quantities of prostaglandin $F_{2\alpha}$ produced from prostaglandins D_2 and E_2 with time

Substrate	Incubation time (minutes)	sample A	B	C	D	E	F	mean value (ng) $PGF_{2\alpha}$	SD	SE	variance
		$PGF_{2\alpha}$ ng									
D_2	1	20	15	20	18	24	21	19.7	0.3	0.12	0.09
	5	75	60	70	55	83	73	69.3	1.02	0.14	1.05
	10	130	120	140	150	190	170	150	2.6	1.06	6.8
	15	190	180	210	230	280	240	222	3.66	1.5	13.4
	30	410	420	350	390	500	390	410	5.0	2.0	25.0
	60	580	550	480	520	590	530	542	4.0	1.66	16.5
	90	660	600	550	610	690	640	625	4.9	2.0	24.3
E_2	1	16	10	18	13	17	12	14.3	0.31	0.13	0.10
	5	45	32	49	36	51	41	42.3	0.74	0.30	0.55
	10	82	79	83	73	94	79	81.6	0.7	0.28	0.49
	15	116	115	118	111	131	118	119	0.75	0.31	0.57
	30	365	370	380	290	340	380	355	3.45	1.41	11.93
	60	562	520	480	450	570	570	526	5.1	2.09	26.37
	90	650	610	520	580	640	630	605	4.8	1.9	23.5

The values quoted are nanograms of $PGF_{2\alpha}$ per 0.2 ml of enzyme preparation. The values for each sample were obtained from triplicate determinations and are the mean value obtained from the 333/337 and 423/427 m/e ratios.

Table 13

Ratio of the d^0 and d4 $PGF_{2\alpha}$ ion peak heights at constant d4 and variable d^0 concentrations

ng d^0 $PGF_{2\alpha}$ per injection	$d^0/d4$ peak heights (m/e 333/337) 1	2	3	mean	SEM	(m/e 423/427) 1	2	3	mean	SEM
0	0.04	0.05	0.04	0.043	0.033	0.09	0.09	0.08	0.0867	0.033
1	0.07	0.06	0.07	0.0667	0.033	0.12	0.11	0.12	0.1167	0.033
2	0.10	0.09	0.09	0.0933	0.033	0.16	0.15	0.15	0.1533	0.033
5	0.18	0.19	0.19	0.1867	0.033	0.25	0.26	0.26	0.2567	0.033
10	0.34	0.37	0.37	0.3600	0.010	0.38	0.37	0.37	0.3733	0.033
20	0.63	0.65	0.65	0.6433	0.0067	0.62	0.64	0.64	0.6333	0.0067
40	1.23	1.23	1.26	1.24	0.0100	1.12	1.15	1.13	1.1333	0.0088
60	1.86	1.88	1.89	1.8767	0.088	1.83	1.85	1.84	1.8400	0.0058
80	2.48	2.51	2.52	2.5033	0.012	2.13	2.11	2.11	2.1167	0.0067
100	3.10	3.15	3.14	3.1300	0.0153	2.68	2.68	2.69	2.6833	0.0033

c) 15-keto PGE_1; 15-keto PGE_2; 15-keto $PGF_{1\alpha}$ and 15-keto $PGF_{2\alpha}$

When incubated with sheep whole blood, 15-keto PGE_2 was found to be metabolised to a variety of compounds that were purified and provisionally identified (as their methyl esters) by chromatography on Lipidex 5000. An example of the elution profile obtained is shown in figure 20 on page 146. The two major metabolites (peaks C and D) were conclusively identified by gas chromatography-mass spectrometry as prostaglandins E_2 and $F_{2\alpha}$. The mass spectrum of the prostaglandin E_2 produced when derivatised as the methyl ester, isopropyloxime trimethylsilyl ether is shown in figure 21 on page 147 and was identical with authentic prostaglandins E_2. Confirmation that the two ring hydroxyl group were both cis (ie. 9α, 11α) in the material identified as prostaglandin $F_{2\alpha}$ was obtained by means of the 9,11 cyclic n-butyl boronate derivative. The major evidence for the assignment of the hydroxyl configuration at C-15 being 15(S) and not 15(R) was that on Lipidex 5000 the 15(S) and 15(R) epimers of PGE_2 are separated (Brash and Jones, 1974). However, although the methyl ester iso-propyloxime, trimethylsilyl ether derivatives of 15(S) and 15 (R) PGE_2 did not separate on the gas chromatography system employed, there were certain noticeable differences in their mass spectra, particularly of the first isomer eluted from the gas chromatograph. For example in the 15(R) PGE_2 derivative major ions are

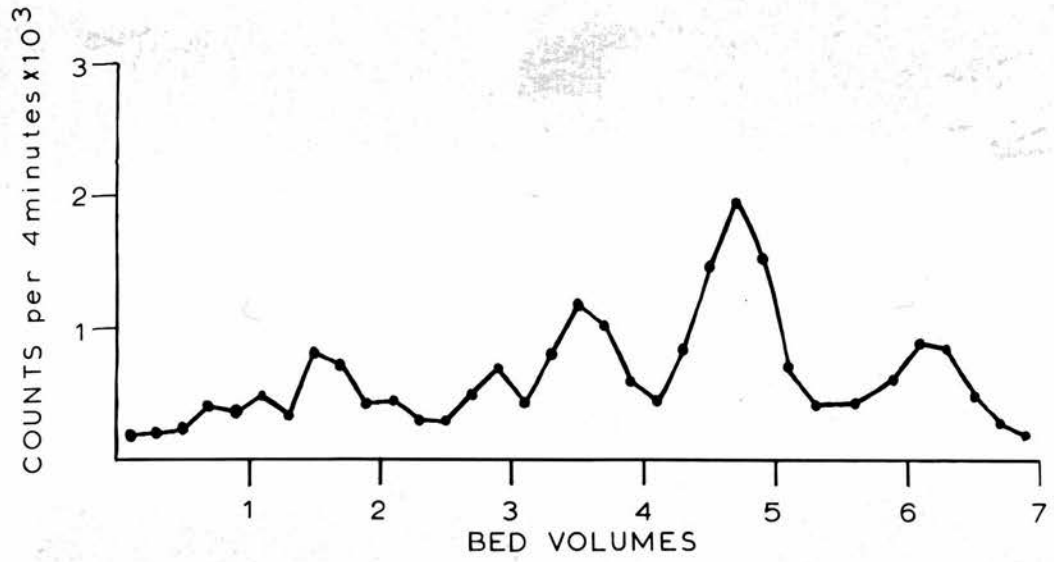

Figure 20. The elution profile from a Lipidex 5000 gel partition column of the products (after conversion to methyl esters) obtained by incubating 15-keto PGE_2 with sheep whole blood at 37°C. The experimental details were given on page 106.

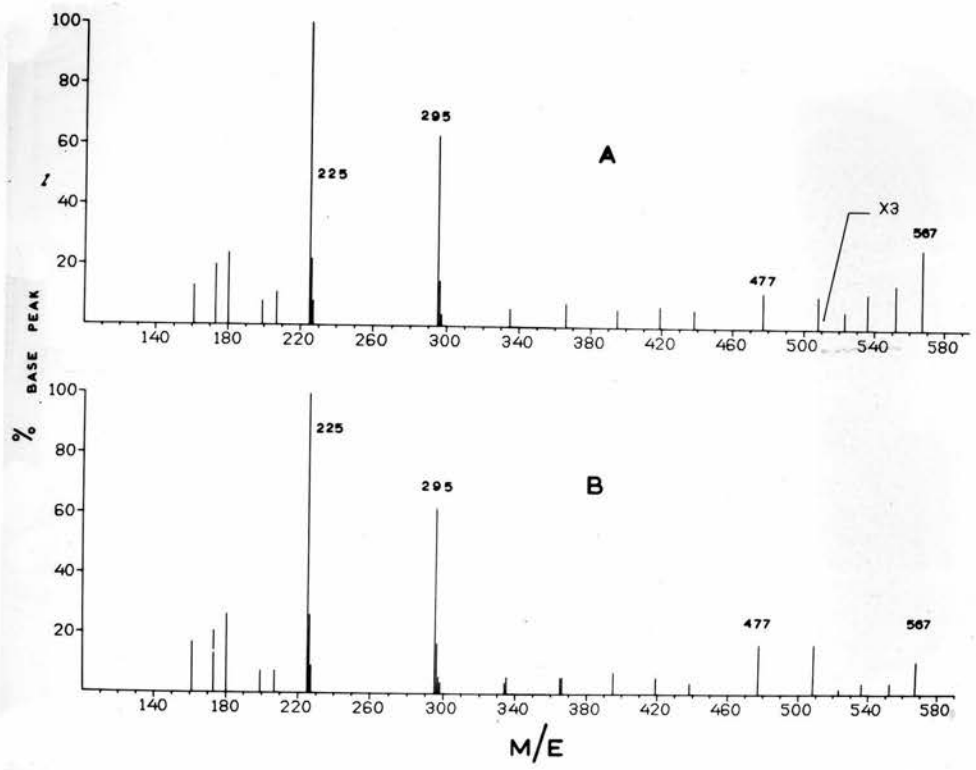

Figure 21. The mass spectra obtained for authentic PGE_2 (upper) and the material in the PGE_2 zone (lower) obtained from Lipidex 5000 (peak C, figure 20, page 146) after 15-keto PGE_2 was incubated with sheep blood. The derivative analysed in each instance was the methyl ester isopropyloxime, trimethylsilyl ether, produced as described on page 61.

present at m/e 437 and 451 whereas in 15(S) PGE_2 these are virtually absent. In the material identified above as a PGE_2 these ions at m/e 437 and 451 were virtually absent and therefore it was concluded that the material was 15(S) PGE_2.

The material eluted in peak B was divided into two portions, B_1 and B_2. The minor portion, B_1 (fractions 23-31) was conclusively identified by combined GCMS as being mainly 15-keto prostaglandin $F_{2\alpha}$. The major portion, B_2 (fractions 32-39) of the recovered material was identified as being a mixture of 15-keto prostaglandin E_2 and 13,14-dihydro-prostaglandin E_2. Although this latter prostaglandin was identified the corresponding 13,14-dihydro $PGF_{2\alpha}$ was not detected in any of the peaks. Had this material been present in the prostaglandin $F_{2\alpha}$ peak (D) then two prostaglandins would have been detected as the cyclic n-butyl boronate derivatives of these two prostaglandins as they readily separate on the gas chromatography system employed. Only prostaglandin $F_{2\alpha}$ was detected.

When 15-keto prostaglandin E_1 was used as substrate an identical pattern of metabolites were produced. The major metabolites were conclusively identified by the combined gas chromatography-mass spectrometry methods described above as prostaglandins E_1 and $F_{1\alpha}$. In addition 15-keto $PGF_{1\alpha}$ and 13,14-dihydro PGE_1 were also identified as metabolites.

When incubated with sheep whole blood both 15-keto prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ were each converted to

one major metabolite. An example of this is shown in figure 22 on page 150 . These metabolites were conclusively identified as the 13,14-dihydro prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ respectively. Preliminary evidence of identification was obtained when these metabolites were found to have gas chromatography retention times and mass spectra identical with authentic 13,14-dihydro prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ methyl ester trimethylsilyl ethers. However, the 13,14-dihydro derivatives did not separate from their respective parent prostaglandins when chromatographed as this derivative on the gas liquid chromatography system employed. Conclusive identification was obtained by use of the methyl ester, 9α, 11α-cyclic n-butyl boronate, trimethylsilyl ethers. This derivative did enable the required separation of these compounds. None of the metabolites produced were identified as the parent prostaglandin; instead the majority were conclusively identified as the 13,14-dihydro prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$.

From the substrate specificity studies with the 15-keto E and F_{α} prostaglandins it was apparent that at least one other enzyme, namely a 13,14 reductase, was present in sheep whole blood. In addition the possibility existed that the enzyme reducing the 9-keto functions of the E series may have been a different one to the enzyme reducing the 15-ketonic functions. It was therefore decided to further characterise the metabolism of 15-keto prostaglandins by sheep blood, in an attempt to define whether the reduction of the 9 and 15-keto groups was by

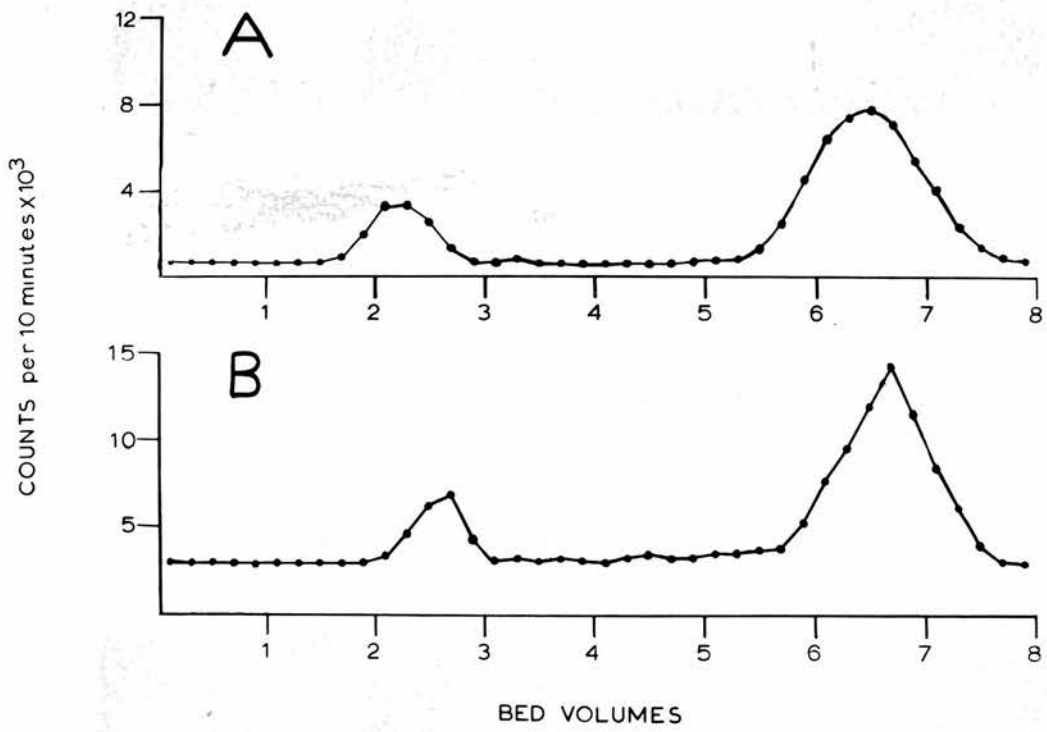

Figure 22. The elution profiles obtained from Lipidex 5000 eluted with heptane, chloroform (80:20 v/v) of the products, after conversion to methyl esters, obtained by incubating ^{3}H 15-keto $PGF_{2\alpha}$ (upper) and ^{3}H 15-keto $PGF_{1\alpha}$ (lower) with sheep blood at 37°C.

the same or different enzyme.

Metabolism of 15-keto prostaglandin E_2 by sheep whole blood, plasma and cellular fraction

After incubation of ^3H-labelled 15-keto prostaglandin E_2 with citrated sheep whole blood for 75 minutes at 37°C, the products were converted to methyl esters and chromatographed on the Lipidex 5000 column described previously. An identical qualitative pattern of metabolites were produced as described in figure 23 on page 152.

After centrifugation at 1875 x g to separate citrated sheep whole blood into plasma and cellular fractions, each fraction was incubated with ^3H-labelled 15-keto prostaglandin E_2 and the extracted products, after conversion to methyl esters, subjected to chromatography on Lipidex 5000. The plasma fraction did not apparently metabolise the 15-keto prostaglandin E_2. However, the cellular fraction did metabolise the 15-keto prostaglandin E_2 in an identical manner to sheep whole blood. Again, prostaglandin E_2, $F_{2\alpha}$ and 13,14-dihydro E_2 were conclusively identified by combined gas chromatography as the major metabolites produced.

The metabolism of 15-keto prostaglandin E_2 by haemolysed sheep red blood cells with time

The pattern of metabolites produced at different incubation times is shown in figure 24 on page 153. These figures represent the elution profiles from Lipidex 5000 of the metabolites produced after conversion to methyl esters.

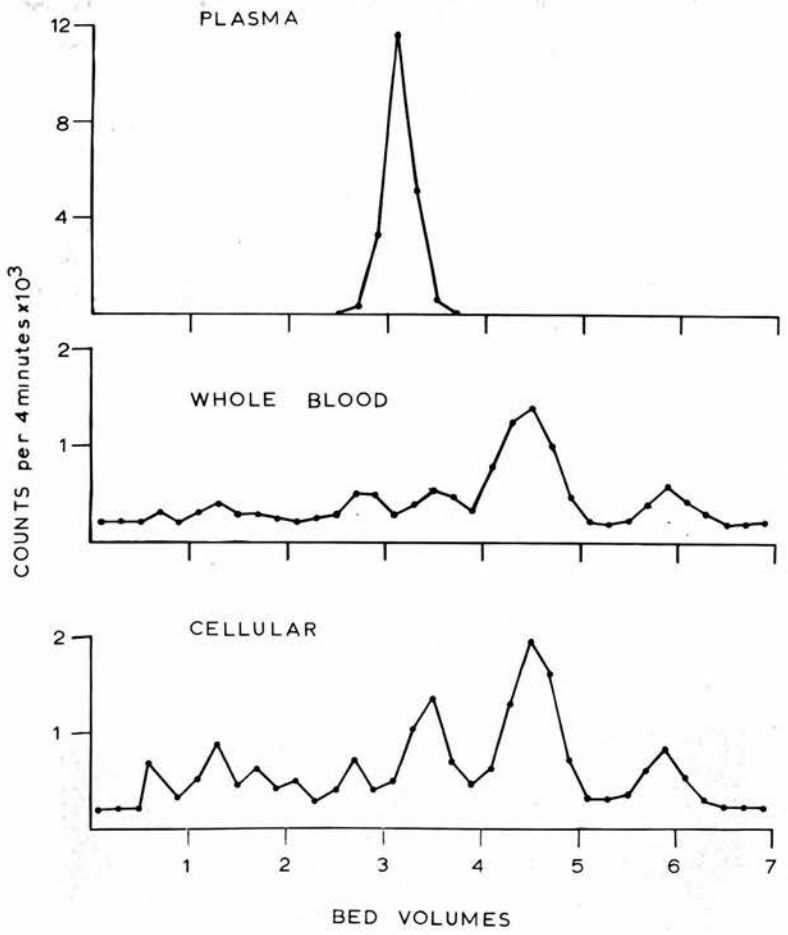

Figure 23. The products obtained (after conversion to methyl esters) when sheep plasma (upper), whole blood (middle) and cellular fraction (lower) were incubated with ^{3}H 15-keto PGE_2. The column used was a Lipidex 5000 eluted with heptane, chloroform (80:20 v/v). The details are described on pages 107 & 109.

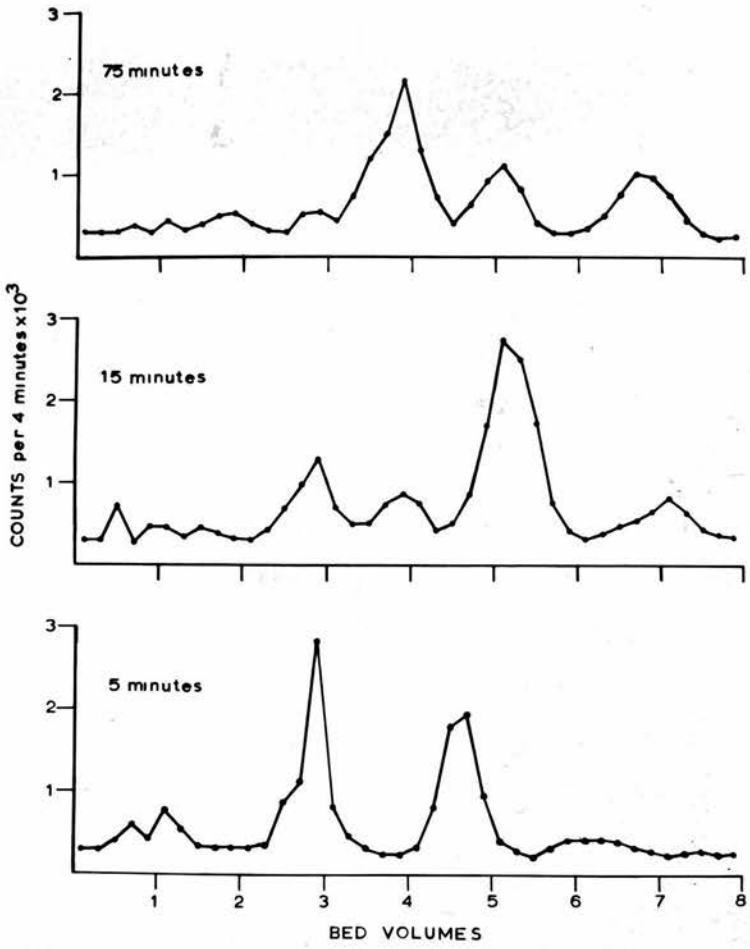

Figure 24. The products obtained (after conversion to methyl esters) when sheep red blood cells were incubated for 5, 15, and 75 minutes with ^{3}H 15-keto PGE_2. The column used was Lipidex 5000 eluted with heptane, chloroform (80:20 V/V). The details are described on pages 107 and 110.

After a 5 minute incubation there are two major and two minor peaks of radioactivity. The material in peak A was not identified. Again peak B was divided into two sections as described on page 148. The first portion was found by GCMS to be mainly unmetabolised 15-keto PGE_2, whilst the second contained a mixture of 15-keto $PGF_{2\alpha}$ and 13,14-dihydro PGE_2. The third and fourth peaks (C and D) were again identified as prostaglandins E_2 and $F_{2\alpha}$ respectively.

After 15 minutes it can be seen that the levels of 15-keto PGE_2 have decreased whilst those of 13,14-dihydro PGE_2, 15-keto $PGF_{2\alpha}$, PGE_2 and $PGF_{2\alpha}$ have all increased. These changes are even further amplified after a 75 minute incubation.

It can be seen from these results that significant reduction of the 15-keto group has occurred after a 5 minute incubation. This is in contrast to the reduction of the 9-keto group (see table 12 on page 143) which is much slower. Thus the possibility existed that the reduction of the 9 and 15-keto functions was performed by different enzyme systems. Consequently it was decided to determine the effect of pH and temperature on the reduction of the 15-keto group in a further attempt to compare its reduction with the reduction of the 9-keto group.

The effect of incubation pH and temperature on the metabolism of 15-keto prostaglandins E_2 and E_1 respectively by haemolysed sheep red blood cells

The values quoted in table 14 on page 157 and graphically represented in figure 25 on page 156 show that reduction of the 15-keto group to the 15(S) hydroxyl group is heat labile. The incubation time was limited to 5 minutes because, as shown previously, (figure 24 page 153) the principal metabolite produced from 15-keto prostaglandin E_2 in this time period is prostaglandin E_2. In addition, the substrate, namely 15-keto prostaglandin E_1, used in the temperature profile study would probably have been non-enzymatically degraded by prolonged incubation at elevated temperatures.

It can be seen that incubation at temperatures above 40°C destroyed the ability of this enzyme system to reduce the 15-keto group. Similarly the reaction is activated by incubation temperatures up to 37-40°C.

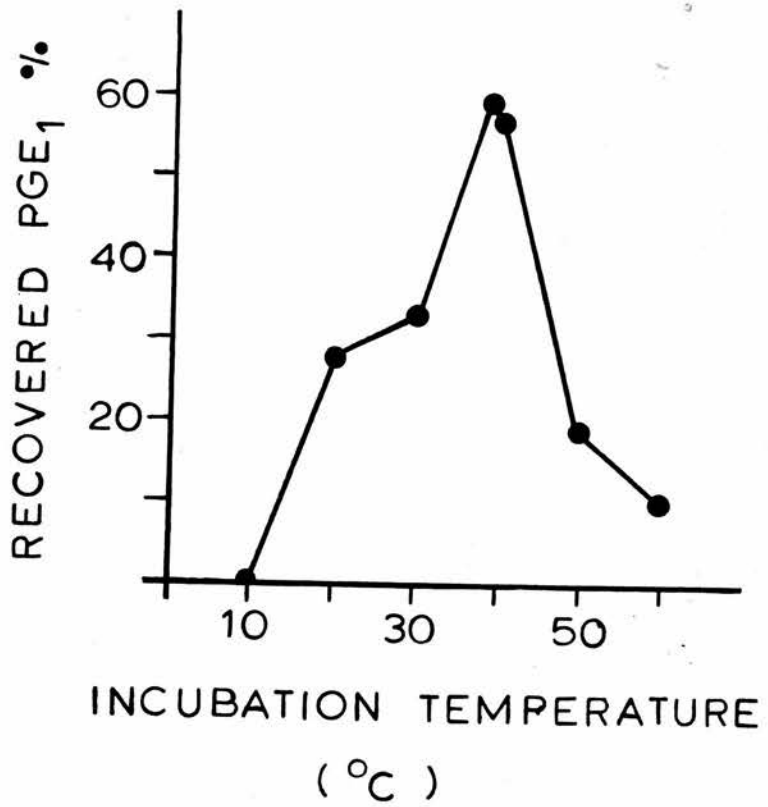

Figure 25. The effect of incubation temperature on the reduction of the 15-keto group in 15-keto PGE_1 to the corresponding 15 (S) hydroxyl group by sheep blood cells at 37°C.

Table 14

The effect of incubation temperature on the metabolism of 15-keto prostaglandin E_1 by haemolysed sheep red blood cells.

Incubation Temperature °C	Percentage of recovered radioactive material in the PGE_1 zone relative to the same material recovered from the 37°C control
10	0
20	46
30	56
37	100
40	98
45	58
50	32
60	19

These results are in contrast with those for the metabolism of prostaglandin E_2 by this system (pages 128 & 129) where the enzyme actively increases with incubation temperatures up to 45°C and it is only at incubation temperatures above this that enzyme activity is lost.

The effect of incubation pH on the metabolism of 15-keto prostaglandin by this system is described in Table 15 below and in figure 26 on page 159 .

Table 15

The effect of incubation pH on the metabolism of 15-keto prostaglandin E_2 by haemolysed sheep red blood cells.

Incubation pH	Percentage of recovered radioactive material in PGE_2 zone relative to the same material recovered at pH 7.0
5.5	35
6.0	68
6.5	85
7.0	100
7.5	131
8.0	34
9.0 *	0 *

* at this pH most of the 15-keto prostaglandin E_2 was degraded to an unidentified compound.

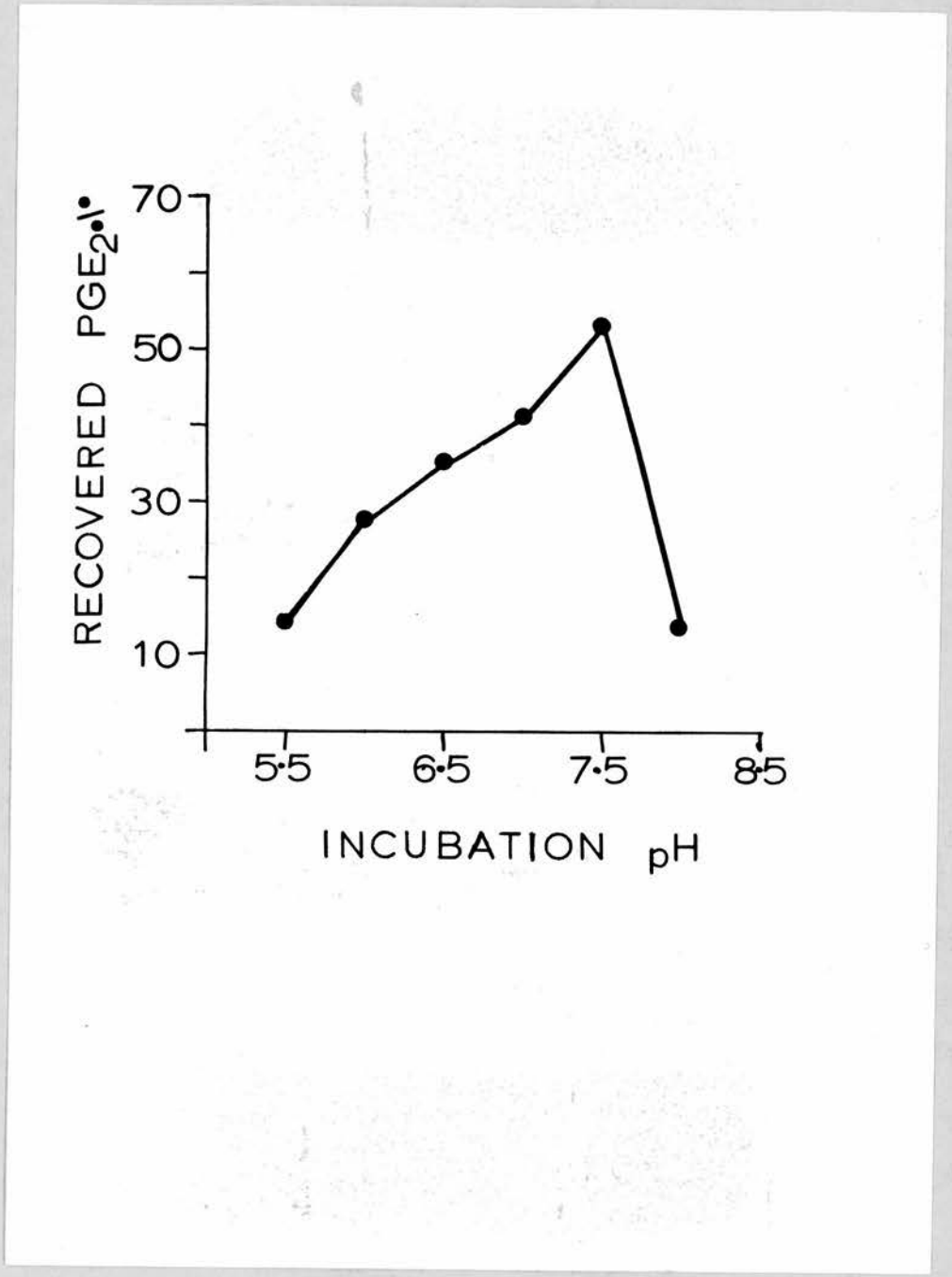

Figure 26. The effect of incubation pH on the reduction of the 15-keto group in 15-keto PGE_2 to the corresponding 15 (S) hydroxyl group by sheep red blood cells.

The optimum enzyme activity is apparently around pH 7.5. This again is in contrast with the reduction of the 9-keto function where optimum activity was found to be around pH 7.0. In both cases the ability to reduce ketonic functions is rapidly lost at pH value above 7.5 and below pH 6.0. From comparisons of the reaction rates, pH and temperature profiles for the reduction of the 9 and 15-keto functions it would appear that there are possibly two different enzymes operating. Alternatively it may be that the enzyme reducing the 15-ketonic functions is a low substrate specific enzyme. This is supported by the fact that the 11-ketonic function of prostaglandin D_2 is also reduced by the cellular fraction of sheep blood. The presence of a further enzyme, namely the 13,14-prostaglandin reductase, was also found in sheep blood cellular fraction. Consequently it was decided to determine whether the blood samples from other species also contained enzyme systems capable of metabolising 15-keto prostaglandins. It was, however, known from the experiments previously recorded in this thesis (pages 124 - 124) that many animal blood samples did not contain enzymes capable of metabolising prostaglandin E_2.

<u>The metabolism of 15-keto prostaglandin E_2 by human blood samples</u>

The pattern of metabolites produced when freshly collected and citrated human venous blood is incubated with 15-keto prostaglandin E_2 are shown in figure 27 on page 161 .

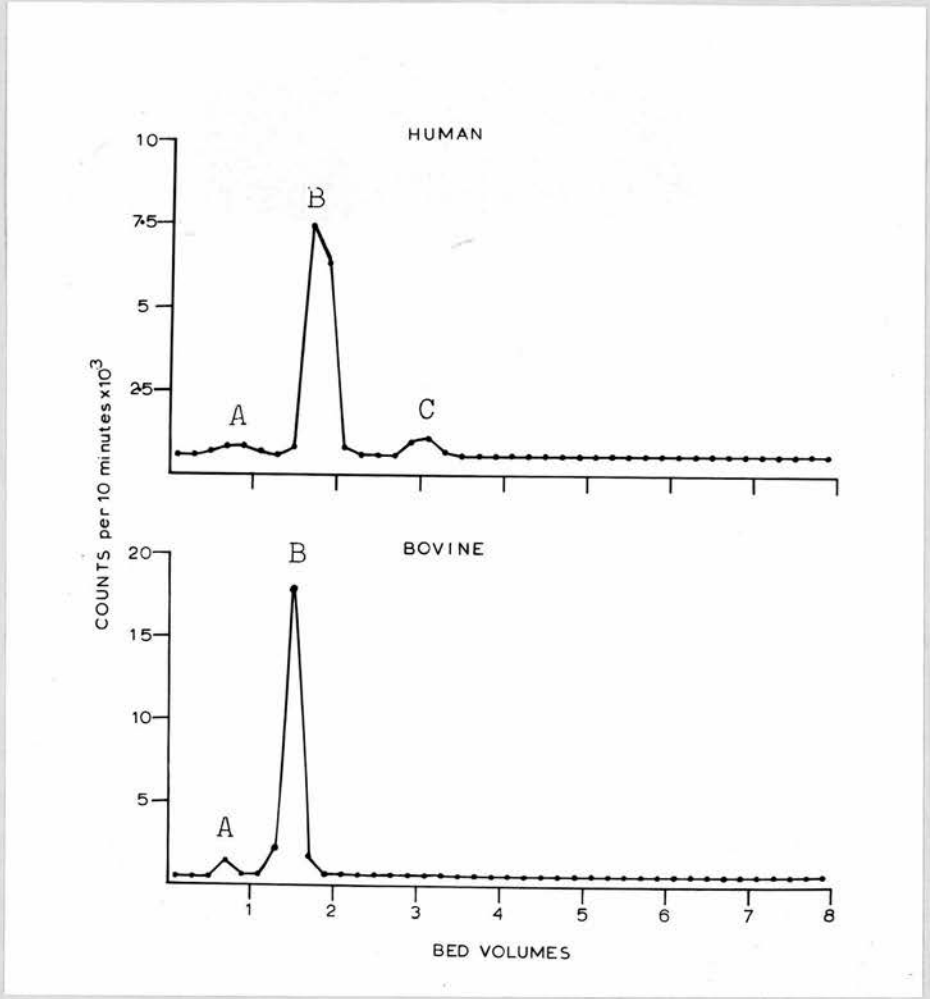

Figure 27. The metabolism of ^{3}H 15-keto PGE_2 by human (upper) and bovine (lower) blood. The incubation products after extraction were converted to methyl esters and separated on Lipidex 5000 columns eluted with heptane chloroform (80:20 v/v). The experimental details are given on page 112.

It can be seen that from the mobility on Lipidex 5000 of the metabolites (after conversion to methyl esters) that they are less polar than the original substrate (15-keto PGE_2). The major metabolite (peak B) was conclusively identified as the methyl ester, isopropyloxime trimethylsilyl ether derivative by combined gas chromatography-mass spectrometry as 15-keto, 13,14-dihydro prostaglandin E_2.

The material in peak C was conclusively identified as un-metabolised 15-keto prostaglandin E_2, whilst the material in peak A was not identified.

In a comparison of eight male (seven Caucasean and one Asian) and one female human venous blood samples for their ability to metabolise 15-keto prostaglandin E_2, a range of prostaglandin 13,14 reductase activity was found. The results are printed in Table 16 on page 163 .

Table 16

blood sample	Lipidex 5000 peak A	B	C
1	21	73	6
2	9	39	52
3	17	61	22
4	7	63	30
5	3	89	8
6	4	77	19
7	16	48	36
8	18	53	39
9	12	35	53

Samples 1 to 7 were male Caucasean blood samples. Sample 8 was a male Asian and sample 9 a female Caucasean.

The values presented are the percentage of the recovered radioactivity (from the Lipidex 5000 zones) that each peak represents.

In an identical experiment 2 samples of female bovine blood were also studied. These were both found to metabolise 15-keto prostaglandin E_2 to 15-keto-13,14-dihydro prostaglandin E_2. The proportion of the total recovered radioactivity that this metabolite contributed was 89% and 93% respectively in the two samples tested. It is therefore apparent that blood samples from at least three species namely: ovine, bovine and human, contain an enzyme system capable of reducing the 13,14 trans double bond of 15-keto prostaglandins. Whether these or other

enzymes are present in blood samples from other animal species remains to be determined. But the absence of 15-keto reductase activity does tend to suggest that the 9 and 15 keto reductase activities have a similar distribution and may be one and the same enzyme.

The metabolism of prostaglandin E_2 by blood samples from normal female and male, pregnant female and castrated male sheep

During an experiment to compare the range of 9-keto reductase activity present in sheep whole blood, it was observed that two samples had much lower activity than the rest. These two samples were identified as originating from castrated male sheep. It was therefore decided to determine the apparent relative 9-keto reductase enzyme activity present in the above four groups of sheep. The results obtained are presented in table 17 below.

State of animal	Blood sample 1	2	3	4	5	6	mean	S.E.M
normal female	48	59	52	43	62	51	52.5	2.86
normal male	58	64	61	-	-	-	61.0	1.73
pregnant female	27	47	35	41	-	-	37.50	4.27
castrated male	39	46	33	-	-	-	39.33	3.76

The values quoted represent the recovered radioactive material in the prostaglandin $F_{2\alpha}$ zone of thin layer plates as a percentage of the total recovered

radioactivity. The length of gestation for the pregnant female sheep and the duration of castration was not known.

Preparation of prostaglandin E analogues for substrate specificity studies

a) Prostaglandin E_2-1 alcohol

The reduction of the 9-hydroxylamine oxime of prostaglandin E_2 methyl ester by lithium aluminium hydride was successful and resulted in the corresponding 1-alcohol. However, attempts to remove the protecting group (hydroxylamine oxime) at C-9 with nitrous acid at 5°C and thereby generate the corresponding prostaglandin E_2-1-alcohol were unsuccessful.

b) ^{14}C-labelled prostaglandin E_1

The elution profile obtained from the Lipidex 1000 column used to purify the 67% aqueous ethanol residue is shown in figure 28 on page 166 . The material in the major peak (fractions 24-27) was found by thin layer chromatography to be radiochemically pure and to have a chromatographic behaviour identical with authentic prostaglandin E_1.

The residue obtained from the heavy petroleum fraction was found by thin-layer chromatography to be radiochemically impure. The residue was purified by reversed phase partition chromatography on an 80 ml bed volume of Lipidex 5000. The elution profile of this column is shown in figure 29 on page 167 . Two main peaks of radioactivity were detected. The first (fractions

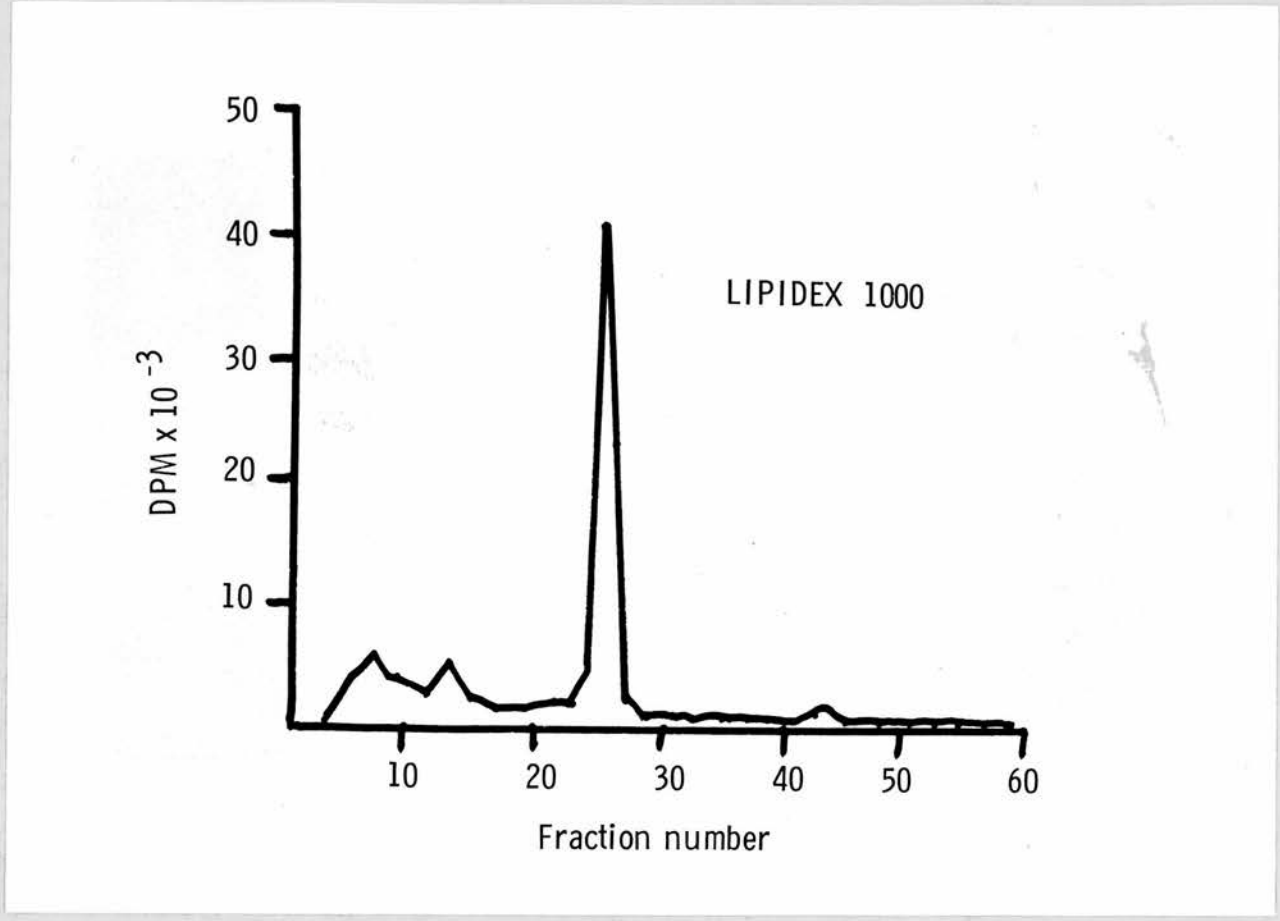

Figure 28. The purification on Lipidex 1000 of the 67% aqueous ethanol extract residue obtained after incubating ^{14}C labelled dihomo -γ- linolenic acid with a ram seminal vesicle prostaglandin synthetase preparation. The experimental details are given on page 116.

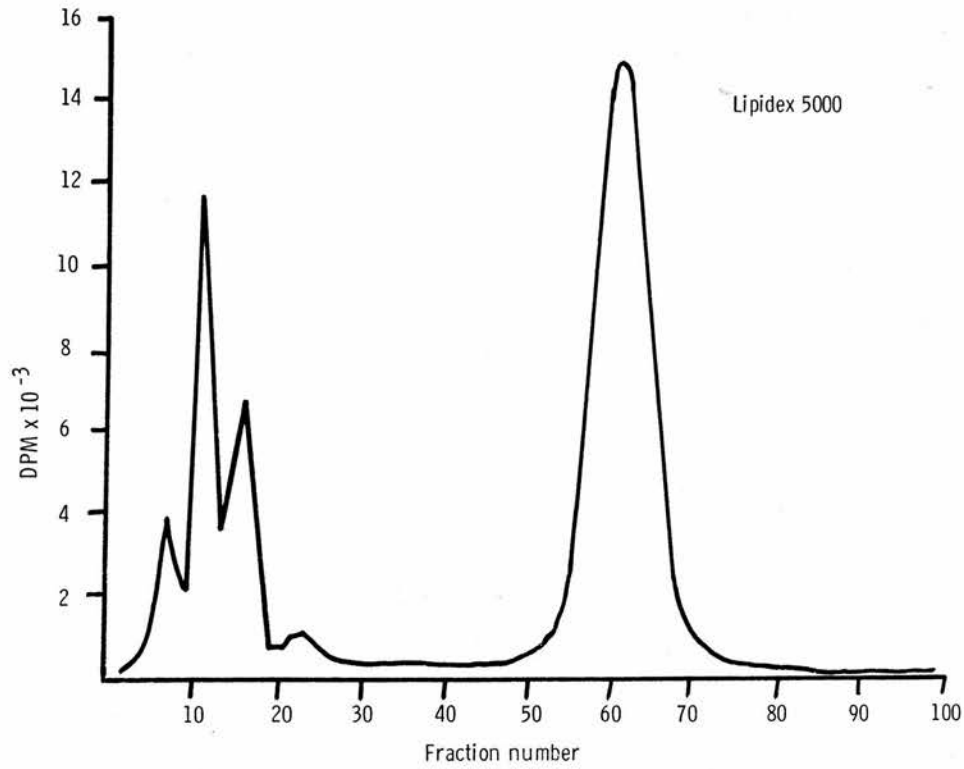

Figure 29. The reversed phase purification on Lipidex 5000 of the impure unmetabolised dihomo-γ-linolenic acid present in the heavy petroleum extract residue obtained after extracting the ram seminal vesicle incubation described on page 116.

5-20) was not further identified. The second (fractions 54-69) was found to be radiochemically pure and to have a mobility on thin-layer chromatography compatible with it being un-metabolised dihomo-γ-linolenic acid (C20: 3ω6).

c) 15-O methyl ether PGE_2 methyl ester

The elution profile from a column (25 ml bed volume) of Lipidex 5000 of the residue obtained after PGE_2-methyl ester was treated with BF_3/diazomethane in ether is shown in figure 30 on page 169 . Two major peaks of radioactivity were obtained. Material in the first peak (fractions 6-8) when converted to the n-butyloxime trimethylsilyl ether as described on page and subjected to combined gas chromatography-mass spectrometry was identified as the 11 , 15 di-omethyl ether methyl ester of prostaglandin E_2. Material in the second peak (fractions 11-14) when converted to the n-butyloxime, trimethylsilyl ether and subjected to combined gas chromatography-mass spectrometry was found to be a mixture of the 11 and 15 mono-O-methyl ethers of prostaglandin E_2 methyl ester. This material was therefore re-chromatographed on a column (55 ml bed volume) containing a lipophilic substituted (62% w/w C-15 to C-18 hydroxyalkyl side chains) LH-20 gel. The elution profile of this material is shown in figure 31 on page 170 . Two major peaks of radioactivity were obtained. The first (fractions 30-35) contained material identified as the 11 -mono-O-methyl ester of prostaglandin E_2. The second peak (fractions 70-77)

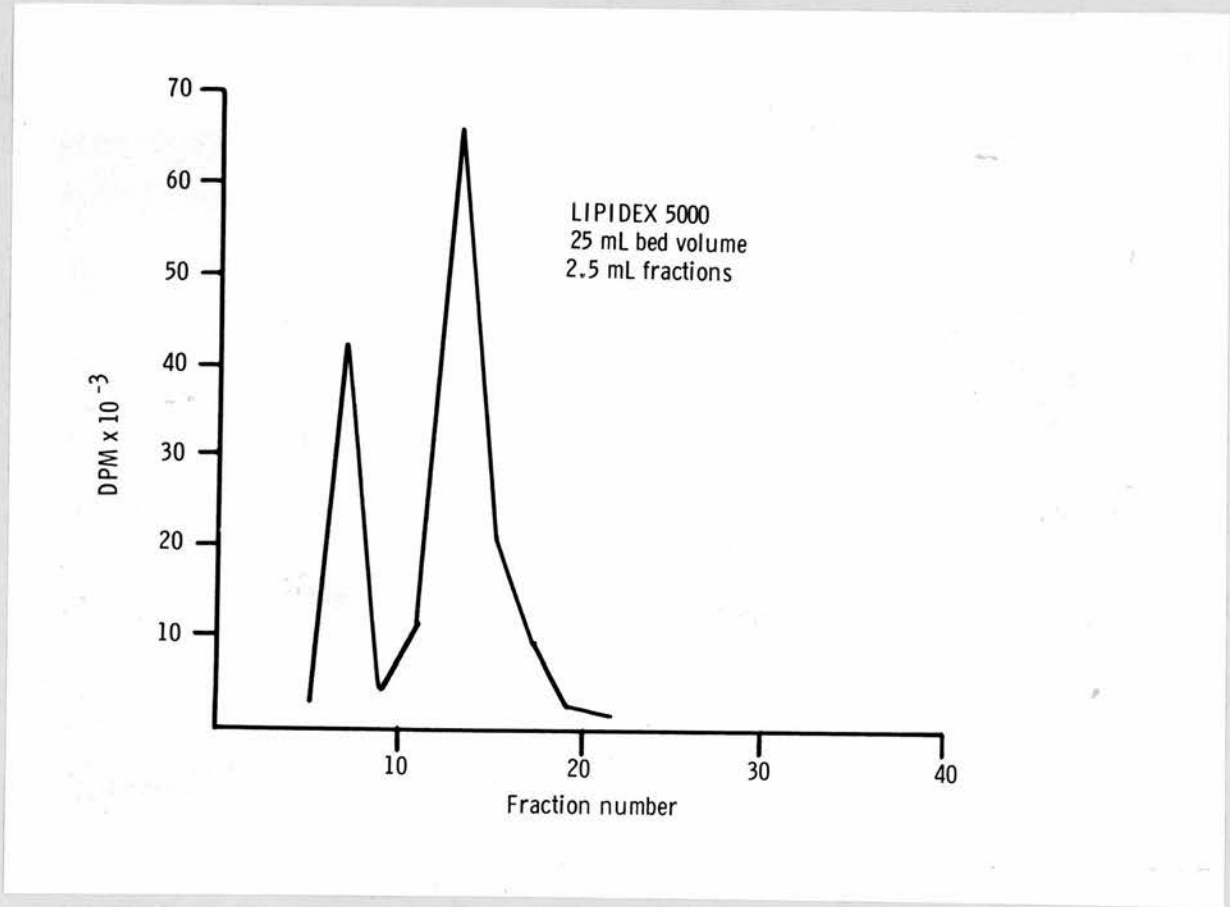

Figure 30. The elution profile from a Lipidex 5000 column (eluted with heptane, chloroform 80:20 v/v) of the reaction products obtained after treating PGE_2 methyl ester with BF_3/diazomethane.

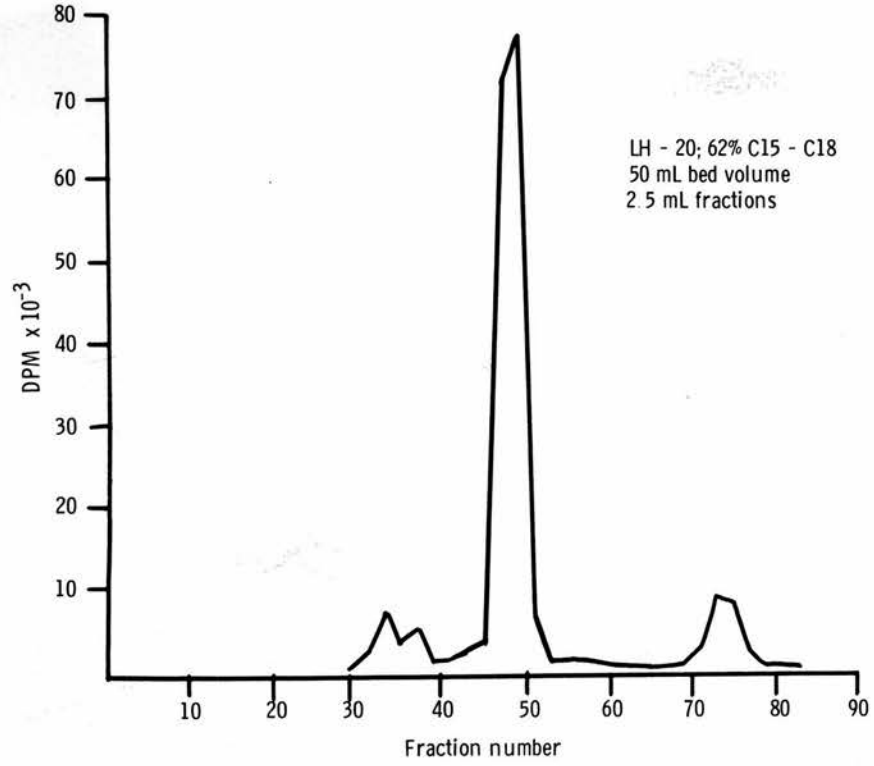

Figure 31. The elution profile from a lipophilic substituted (62% w/w C15 to C18 hydroxyalkyl side chain) LH-20 gel of fraction 11-14 obtained from the Lipidex 5000 column shown in figure 30, page 169. The eluting solvent was heptane, chloroform (90:10 v/v).

was identified as the corresponding 15 mono-O-methyl ether methyl ester of prostaglandin E_2. In both cases the material was identified as the n-butyloxime, trimethylsilyl ether derivative.

These compounds have as yet not been tested as substrates for the 9-keto reductase present in sheep blood.

SECTION III

DISTRIBUTION STUDIES ON THE REDUCTION OF PROSTAGLANDIN E_2 TO PROSTAGLANDIN $F_{2\alpha}$ BY TISSUE HOMOGENATES

Introduction

During the experiments recorded in Section II of this thesis, it was confirmed that sheep whole blood contained at least one enzyme that was capable of stereospecifically reducing the 9-keto group of PGE_2 to the corresponding 9α hydroxy group and thereby producing $PGF_{2\alpha}$. At the time of this work few reports had been made concerning the interconversion of PGE_2 to $PGF_{2\alpha}$. It was known that these two prostaglandins were not interconverted during their biosynthesis from arachidonic acid (Anggard and Samuelsson, 1965; Hamberg and Samuelsson, 1967). Therefore when these experiments with sheep whole blood began the major evidence for the possible interconversion of E and F prostaglandins was based on studies of the guinea pig urinary metabolites of PGE_2. The major metabolite (see figure 3 on page 24) was shown to have Fβ ring configuration which is the opposite configuration of the hydroxyl group at C-9 to the one normally produced by the prostaglandin synthetase. Therefore, if the enzyme present in sheep whole blood that stereospecifically reduced the 9-keto group into the Fα configuration was widely distributed, it could have major implications for the physiology, pharmacology and pathology of E prostaglandins. In many circumstances the E and Fα prostaglandins have qualitatively different actions (see general introduction). It was

therefore felt necessary to determine what the distribution of 9-keto reductase enzyme activity was in the tissues from a variety of animal species.

Materials and Methods

Kidney, heart and liver samples from guinea-pig, rat, dog, cat, cow, sheep, horse, pig and rabbit of both sexes were removed as soon as possible after death. If transport of the samples was required they were kept in crushed ice until used. Homogenates were prepared by chopping the tissues into small pieces approximately 1 cm^3, weighing and then homogenising in 3 volumes (by weight) of ice-cold 100 mM sodium phosphate buffer (NaH_2PO_4 2.7g; Na_2HPO_4 12.54g in 1 litre of distilled water pH 7.4) using a polytron homogeniser at maximum speed for 30-60 secs. The resulting homogenate was then centrifuged at 4°C for 30 minutes at 2400 x g and the supernatant decanted off and stored on ice until used.

Incubation and extraction procedure

The resulting supernatant was added to a 100 ml conical flask containing tritiated prostaglandin E_2 (Amersham, 5, 6, 8, 11, 12, 14, 15-^{3}H-labelled PGE_2 Sp. Ac. 36.4 Ci $mole^{-1}$) at a substrate concentration of 14 µM and a cofactor concentration of NAD and $NADH_2$ of 10 mM each. The samples were then incubated at 37°C for two hours (with agitation) in a water bath.

The products of the incubation were diluted to 150 ml with distilled water and acidified to pH4 with 10% aqueous acetic acid. After extraction twice with 2 volumes of either diethyl ether of diethyl ether/ethyl acetate (4:1 v/v), the pooled organic phases were washed twice with 50 ml of distilled water before being taken to dryness under vacuum and desiccated.

The resulting residue was dissolved in 50 ml of

67% aqueous ethanol and partitioned twice with an equal volume either benzene or toluene, before the aqueous phase was taken to dryness and desiccated. Where prostaglandin $F_{2\alpha}$ appeared to be a metabolite of prostaglandin E_2 (eg rabbit, horse and guinea-pig liver; horse heart) the experiment has been repeated at a substrate concentration of 140 µM and in addition 1-^{14}C PGE_1 has been compared in parallel at the same substrate concentration.

Preparation of methyl esters

The residue from the 67% ethanol phase was converted to methyl ester by the method described previously on page 61 . Where necessary (eg if material had precipitated out of solution) the resulting solution was filtered through a glass wool plug inserted into a Pasteur pipette. The original flask was then washed with excess methanol (5-10 ml) and this was also filtered. The pooled solution was taken to dryness under vacuum and desiccated.

Straight phase gel partition chromatography on Lipidex 5000

Glass columns (300-400 mm x 9-10 mm) were silanised and packed under free flow with Lipidex 5000 gel equilibrated in heptane-chloroform (80:20 v/v) as described on page 107 . Columns of 29-30 ml bed volume were used throughout and fractions of 10% bed volume were collected on an LKB Ultrorac fraction collecter until the 2000% bed volume point was reached. The flow rate was 6-12 ml hr^{-1}. The samples being chromatographed were dissolved in 100-200 µl of chloroform and then n-heptane was added

until the solvent composition was the same as the column eluant. The total loading volume (including washings) was never greater than 1.5 ml.

The elution profile of the column was obtained by liquid scintillation counting.

All columns were calibrated for authentic ^{3}H prostaglandins A_2, E_2 and $F_{2\alpha}$. Certain columns were calibrated for other prostaglandins and their metabolites by either liquid scintillation counting or by gas liquid chromatography. Columns which have begun to lose performance (as shown by peak broadening) have been purged by reverse flowing the column with a more polar solvent (eg methanol-chloroform-n-heptane 10:50:50 by volume) before re-equilibrating in the original solvent. These columns have been found to be re-usable and reproduceable for both authentic prostaglandins and biological samples.

From the elution samples it has been possible to bulk several fractions into corresponding zones. These have been taken to dryness and re-dissolved in 1.0 ml of methanol and an aliquot (50 - 100 µl) counted by liquid scintillation counting for the quantitative determination of the % of the total recovered radioactivity.

Thin-layer chromatography

Neutral silica gel (200 mm x 200 mm x 0.25 mm) on glass plates (A.G. Merck) were used. Marker standards containing ^{3}H-labelled prostaglandins A_2, E_2, 13, 14-dihydro E_1, $F_{2\alpha}$ and $F_{2\beta}$ methyl esters were spotted on the plate at the side of the samples from the Lipidex 5000 zones being chromatographed. The plates were then developed in closed equilibrated glass tanks using a modified form

of the G.C.M. solvent (Millar 1974). The solvent used was ethyl acetate-methanol-acetic acid (100:10:1 by volume).

Liquid Scintillation Counting

This was performed with a Mark II Nuclear Chicago liquid scintillation counter.

Aliquots of either the individual column fractions or the methanol solution containing the pooled fraction residue were counted in 5 ml of the toluene-ethoxyethanol based scintillant described on page 107 . Where dpm were required these were obtained using a PDP8I computer to correct for quenching by the automatic external standard channels ratio method and a quench-calibration curve constructed for the scintillation mixture.

Preparation of derivatives for Gas Liquid-Chromatography

An aliquot of the methanol residue from the appropriate zone of the Lipidex 5000 columns was taken to dryness in a capped Eppendorf tube and converted to the trimethylsilyl derivative using BSTFA by the method described on page 64 . The derivatised prostaglandin was injected into the gas chromatograph in 5-10 µl of BSTFA.

Radio Gas-Liquid Chromatography

A Pye series 104 gas chromatograph interfaced with a Panax radio detector was used for the simultaneous detection of mass and radioactivity. Only ^{14}C-labelled prostaglandins were analysed by this method due to the unreliability and insensitivity for ^{3}H-labelled prostaglandins. The gas chromatograph was equipped with a glass column (2-3 m x 4 mm i.d.) packed with 3% OV-1 on Supelcoport (100-120 mesh). The oven temperature

was 230-240°C, the carrier gas (argon) flow rate was 35-40 ml min^{-1} and the oxidation furnaces were at 650-680°C.

The instrument was calibrated for ''carbon values'' (mass and radioactivity) by the method described previously (Bergstrom, Ryhage, Samuelsson and Sjovall 1963). A log retention time versus carbon value for normal saturated fatty acid methyl esters (C_{16}-C_{24} in C_2 units) was constructed.

Combined Gas-Liquid Chromatography-Mass Spectrometry

Mass spectra were obtained using an LKB 9000 instrument equipped with a glass column (3 m x 4 mm i.d.) packed with 3% OV-1 on Supelcoport (100-120 mesh). The oven temperature was 240-260°C and the carrier gas (helium) flow rate was 30-35 ml min^{-1}. The instrument was calibrated for ''Carbon values '' and authentic prostaglandins as described above.

^{3}H-labelled prostaglandin E_2 quality control

The ^{3}H-labelled PGE_2 used in these experiments was checked at regular intervals prior to use. The initial criteria of radiochemical purity was determined by thin layer chromatography as described on page 34 . Where the material has been found to be contaminated (5% or greater) it has been purified by reversed phase partition chromatography as described previously on page 34 .

Solvents and Chemicals

All solvents were of analytical grade or re-distilled before use. The chloroform and n-heptane were re-distilled over anhydrous calcium chloride. Absolute

ethanol (1% by volume) was added to the re-distilled chloroform for stabilisation purposes.

The effect of Pseudopregnancy on the in vitro metabolism of Prostaglandin E_2 by rabbit liver homogenates

This work was performed using rabbit liver samples provided by Dr. N.L. Poyser and Miss F. Lytten, whose generosity is duly noted.

Normal New Zealand White rabbits were injected with 500 international units of human chorionic gonadotrophin (H.G.C.) between 3 and 4 pm in the afternoon. The following day was designated day 1 of pseudopregnancy. Control rabbits were injected with isotonic saline (0.9% w/v).

The livers from pairs of female rabbits on days o (control), 3, 7, 10, 13, 15, 16, 17, 18, 19 and 20 of pseudopregnancy and males (six) were removed after the rabbits had been lightly anaesthetised with Urethane (25% w/v solution of ethyl carbamate in isotonic saline; 7-8 ml kg^{-1} i.v.). From each liver a 20 g sample was obtained. This was homogenised in 100 mM sodium phosphate buffer and centrifuged as described on page 174 . An aliquot (20 ml) of the resulting supernatant was incubated with ^{3}H-labelled PGE_2 (14 µM; SpAc 3.64 Ci $mole^{-1}$) and a cofactor concentration for NAD and $NADH_2$ of 10 mM each. After 2 hours at 37°C in a water bath (with agitation) the samples were extracted as described on page 174 . After conversion to methyl esters the samples were purified by Lipidex 5000 straight phase gel partition chromatography. The glass column employed (290 mm x 25 mm) was packed with Lipidex 5000 (55 ml bed volume). Fractions of 5%

bed volume were collected and assayed for radioactivity as described on page 107 . The zones of radioactivity obtained from each sample were quantitatively assayed for radioactivity as described previously on page 107 .

Conclusive evidence of identification was obtained by combined gas chromatography-mass spectrometry on an LKB 9000 instrument. The derivatives employed and the methods used have been described previously on pages 61 - 64 respectively.

The effect of Pregnancy on the in vitro metabolism of prostaglandin E_2 by rabbit liver homogenates

Normal New Zealand White rabbits were used in these experiments. Pregnant rabbits of known gestation period were purchased from Animal Livestocks Ltd., Bradford. Adult rabbits were killed by a blow to the neck and exsanguination whereas the offspring were killed by exsanguination after diethyl ether anaesthetisation had been induced. Female rabbits were killed during the gestation period on days 25, 26 or 27, pregnancy being confirmed by autopsy examination.

From each adult rabbit 20 g samples of liver were removed. For the offspring whole livers (minus the gall bladder) from litter mates of the same sex were pooled. In all cases the liver samples were homogenised, centrifuged, incubated with a mixture of ^3H-labelled prostaglandin E_2 (14 μM Sp.Ac. 3.64 Ci mole^{-1}) NAD and $NADH_2$ (10 mM) and extracted as described on page 174 .

The products of the extraction were separated by thin layer chromatography as the free acids using

the multi-run development described on page 84 . After development the plates were scanned for radioactivity using a Panax thin layer plate scanner. The resulting zones of radioactivity were scraped from the plates into test tubes containing 5 ml of methanol as described on page 85 . The radioactivity was quantitatively assayed by liquid scintillation counting of an aliquot of the radioactivity from each zone (see page 85). Prostaglandins $F_{2\alpha}$ and E_2 were quantitatively assayed (as described on page 93) by multiple ion-detection gas-chromatography-mass spectrometry using a Finnigan 3000D quadrupole mass spectrometer interfaced with a Finnigan 9500 gas chromatograph equipped with glass columns (1.5 to 2 m x 2 mm i.d.) packed with 3% OV-1 on Supelcoport. The derivatives used were either the methyl or ethyl ester trimethylsilyl ether for $PGF_{2\alpha}$ and the corresponding iso-propyl oximes for PGE_2. The ions monitored for $PGF_{2\alpha}$ and PGE_2 and the corresponding 3,3,4,4-tetradeutero derivatives are shown below.

isotope derivative	d0 m/e		d4 m/e	
methyl ester TMS $PGF_{2\alpha}$	423	333	427	337
ethyl ester TMS $PGF_{2\alpha}$	437	347	441	351
methyl ester iso-Pr TMS PGE_2	295		299	
ethyl ester iso-Pr TMS PGE_2	309		313	

Conclusive evidence for the qualitative identification of $PGF_{2\alpha}$ was obtained by combined gas chromatography-mass

spectrometry. An LKB 9000 instrument was used and the derivative chosen was the methyl ester trimethylsilyl ether. The method used is described on page 98 .

The in vitro metabolism of prostaglandin E_2 by male human liver, heart and kidney homogenates

These samples were generously donated by Professor Currie of the Pathology Department, Edinburgh Royal Infirmary. The samples, taken during autopsy examination, were stored in ice during transport and used as soon as possible after removal. One batch of samples was taken from a male aged 19 years approximately 4 hours after death. The cause of death was cardiac failure following a prolonged illness diagnosed as Cushing's syndrome. The other male patient was a 62 year old male who died from cardiac failure of non-specified origin (natural causes). These samples were not obtained until 16 hours after death.

Tissue samples (10 g) were homogenised in 3 volumes of 100 mM sodium phosphate buffer as described on page 174 . The resulting homogenate was centrifuged at 2400 x g for 30 minutes at 4°C. An aliquot (15 ml) of the resulting supernatant was incubated for 2 hours at 37°C with ^{3}H-labelled prostaglandin E_2 (14 µM 36.4 Ci mole^{-1}) and co-factor concentration for NAD and $NADH_2$ of 10 mM each. The products of the incubation were extracted and converted to methyl esters as described on page 174 . The methyl esterified products were purified on a Lipidex 5000 column of 30 ml bed volume. The eluting solvent was a mixture of n-heptane-chloroform

(80:20 v/v) and the method used to detect and quantify the zones of radioactivity by liquid scintillation counting has been described on page 107 . Where prostaglandin $F_{2\alpha}$ (or more polar compounds with respect to PGE_2 methyl ester) appeared to be incubation products the residue obtained from the zone was converted to the trimethylsilyl ether (page 64) and subjected to qualitative analysis by combined gas chromatography-mass spectrometry using an LKB 9000 instrument.

The in vitro metabolism of prostaglandin E_2 by male and female human bronchiolar tissue

These samples were generously donated by the Pathology Department at the London Hospital, London and the experiments were performed at the Medical Products Research Laboratories, May and Baker Ltd., Dagenham. In all cases the samples were removed during surgical procedures (usually performed for the removal of lung carcinoma) and transported in ice until used. These samples (5-10 g) were homogenised in 3 volumes of 100 mM phosphate buffer and centrifuged at maximum speed on a bench centrifuge for 5 minutes at room temperature. The supernatant (in toto) was incubated with ^3H-labelled prostaglandin E_2 (3 µM; 0.5 µCi) and cofactor concentrations for NAD and $NADH_2$ of 10 mM each. After 2 hours at 37°C the products were diluted with 20 ml distilled water, acidified to pH 4 and 10% aqueous acetic acid and extracted as described on page 174 . The extraction products were then subjected to thin layer chromatography on neutral silica gel (50 mm x 200 mm x

0.1 on nylon) using the multi-run development described previously on page 84 . Authentic ^{3}H-labelled PGE_2 was run on identical plates in parallel. The plates were scanned for radioactivity corresponding with the peaks of the resulting scans were eluted with methanol as described previously. The residue from each zone was quantitatively assayed for radioactivity by liquid scintillation counting.

<u>The in vitro metabolism of prostaglandin E_2 by adult male chicken liver, heart and brain samples</u>

These samples were generously collected and dissected out from animals killed by decapitation by Mr. W.R.G. Stephen of the Department of Pharmacology, Edinburgh University.

Samples of liver (10 g; n=12) heart (5 g; n = 6) and brain (5 g; n = 3) were homogenised in 3 volumes of 100 mM sodium phosphate buffer as described previously (page 174). For brain and heart homogenates samples from different animals had to be pooled. The resulting homogenates were centrifuged (2400 x g 20 mins. at 4°C) and 10 ml of the resulting supernatants incubated with ^{3}H-labelled PGE_2 (28 µM; Sp.Ac. 3.64 Ci $mole^{-1}$) for 2 hours at 37°C. After extraction by the method described on page 174 the residue was purified (as the free acids) by thin layer chromatography using the multi-run development system described on page 84 . After development the plates were scanned for radioactivity and the resulting zones eluted with methanol by the methods described previously on page 85 .

The residue from each zone was re-dissolved in 1.0 ml of methanol and 4 x 50 ul aliquots were quantitatively assayed for radioactivity by liquid scintillation counting.

Aliquots from the prostaglandin $F_{2\alpha}$ zones were converted to :

a) Methyl ester trimethylsilyl ether (500 µl) and subjected to combined gas chromatography-mass spectrometry on an LKB 9000 instrument as described previously.

b) Ethyl ester trimethylsilyl ether (100 µl) and subjected to quantitative multiple-ion detection gas chromatography-mass spectrometry using a Finnigan 3000D instrument as described previously. The ions monitored for the protium $PGF_{2\alpha}$ were 347 + 437 and for the corresponding deuterum isotopes 351 and 441.

Changes in the in vitro metabolism of prostaglandin E_2 by male and female chickens with age

The chickens used in the experiments were all purchased from the Poultry Research Station, Edinburgh.

Male and female chickens (n = 3) were killed by decapitation following diethyl ether anaesthetisation on days 1, 8 and 22 after birth and the livers removed. For 1 day old chickens the livers from three animals of the same sex were pooled. In all cases 5 g of liver was homogenised in 3 volumes of 100 mM sodium phosphate buffer and treated as described previously. From the resulting homogenate 10 ml was removed and incubated with PGE_2 (14 µM; 36.4 Ci $mole^{-1}$) for 2 hours at 37°C. The resulting products were extracted into diethyl ether-ethyl

acetate (4:1 v/v) as described on page 107 and purified by multi-run thin layer chromatography as described on page 84 . The samples in the $PGF_{2\alpha}$ obtained after thin layer chromatography were subjected to qualitative and quantitative gas chromatography-mass spectrometry as described previously (page 93).

The effect of oestradiol and progesterone administered in vivo on the in vitro metabolism of prostaglandin E_2 by liver homogenates from ovariectomised guinea-pigs

Dr. N.L. Poyser and Mr. B. Naylor generously provided the liver samples for these experiments.

Virgin female guinea-pigs, which had cycled normally, were ovariectomised during the luteal phase. The guinea-pigs were randomly divided into four groups and four to eight weeks after surgery were injected with 0.5 ml arachis oil containing steroids as follows :

Group I - 0.5 ml vehicle only for 10 days (Control);

Group II - 0.5 ml vehicle for 7 days followed by oestradiol benzoate (10 µg daily for 3 days) in arachis oil;

Group III - 0.5 ml vehicle containing progesterone (2.5 mg daily) for 10 days;

Group IV - 0.5 ml vehicle containing progesterone (2.5 mg daily) for 7 days then 0.5 ml vehicle containing oestradiol benzoate (10 ug daily) and progesterone (2.5 mg daily) for 3 days.

Guinea-pigs were killed by a blow to the neck and exsanguination on the 11th day after the treatment started. Normally cycling non-ovariectomised guinea-pigs were also killed on day 7 (n = 4) and day 13 (n = 3) of the oestrous cycle (oestrous = day 1). The livers were removed and 10 g samples homogenised in 3 volumes of 100 mM sodium phosphate buffer as described on page 174. Aliquots (20 ml) of the supernatant obtained after

centrifugation at 4°C for 20 minutes at 2400 x g were incubated with prostaglandin E_2 (28 μM; 36.4 Ci mole^{-1}) as described on page 174 . The incubation products were extracted and purified by the multi-run thin layer chromatography system previously described. The plates were scanned for radioactivity using a Panax radio thin layer chromatography plate scanner. The zones of radioactivity were eluted with methanol and quantitatively assayed by the methods described on page 85 . The prostaglandin $F_{2\alpha}$ zones were qualitatively assayed as the methyl ester trimethylsilyl ether using an LKB 9000 combined gas chromatograph-mass spectrometer. Quantitative analysis of the $F_{2\alpha}$ zones by combined gas chromatography-mass spectrometry of an aliquot (10%) of the methanol residue was performed using a Finnigan 3000D mass spectrometer as described previously.

RESULTS

Distribution studies on the reduction of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ by tissue homogenates

In addition to confirming that guinea-pig liver homogenates convert prostaglandin E_1 or E_2 into the corresponding F_α derivatives, three other tissues, namely, the liver or horse and rabbit and the heart of horse, have also been found to be capable of converting PGE to PGF_α. At no time has any evidence been obtained for the conversion of PGE to PGF_β.

The separation of authentic ^3H-labelled prostaglandins A_2; E_2; $F_{2\alpha}$ and $F_{2\beta}$ methyl esters is shown in figure 32 on page 190. It can be seen that there is a good separation of these prostaglandins on the lipophilic LH-20 gel chromatography system employed. The separation of prostaglandins E_2; $F_{2\alpha}$ and $F_{2\beta}$ was essential because it has been demonstrated that in the guinea-pig, *in vivo*, PGE_2 is reduced to a urinary metabolite (see figure 3 page 24) with the $PGF_{2\beta}$ ring configuration (Hamberg and Israelsson, 1970). *In vitro*, however, it has been demonstrated that guinea-pig liver homogenates produce $PGF_{2\alpha}$ as one of the metabolites of PGE_2 (Hamberg and Samuelsson, 1969). The possibility therefore existed that the 9-keto group of PGE_2 could be reduced to either the 9α or 9β alcohol configuration by some of the tissues being studied. In addition, the use of these columns has enabled the detection of approximately 1% conversion of the PGE_2 to $PGF_{2\alpha}$.

Figure 32 on page 190 also shows an example

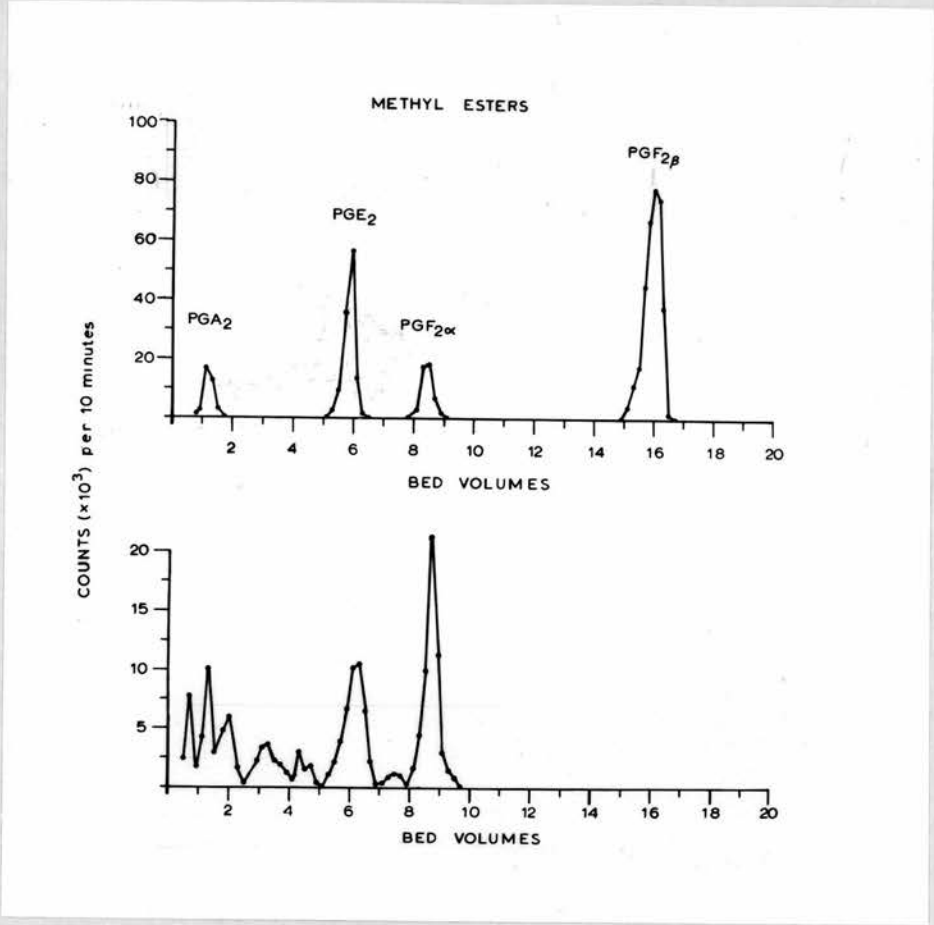

Figure 32. The straight phase separation on a Lipidex 5000 gel partition column of authentic ^{3}H-labelled prostaglandins A_2, E_2, $F_{2\alpha}$ and $F_{2\beta}$ methyl esters (upper) and the metabolites produced by incubating ^{3}H PGE_2 with a homogenate of rabbit liver. The experimental details are given on page 174.

of the elution profile obtained when a rabbit liver homogenate (after conversion to methyl esters) is chromatographed on the same column as the authentic standards immediately after these standards had been chromatographed. It can be seen that rabbit liver homogenates produce several metabolites when incubated with PGE_2, at least one of which is more polar than PGE_2 and has chromatographic properties identical with $PGF_{2\alpha}$ in this system.

The majority of these metabolites have only been provisionally identified by their retention volumes on Lipidex 5000 and their relative Rf values on thin-layer chromatography. These values for authentic prostaglandin standards are given in Table 18 on page 193 . The 1 and 2 series prostaglandins did not separate on the thin-layer chromatography system employed.

The broad peak of radioactivity eluted between fractions 51 and 64 on the Lipidex 5000 column (see figure 32 on page 190) indicated that although this peak probably contained PGE_2 at least one other metabolite was present. Thin layer chromatography of these fractions confirmed that this was so. An example of the radio scans obtained is shown in figure 33 on page 192 . Using the modified G.C.M. solvent described on page 174 it was thus possible to show that fractions 51-55 mainly contained a material that was identical with authentic 13,14-dihydro PGE_1, methyl ester, together with a smaller amount of material identical with PGE_2 methyl ester. In contrast, fractions 56-64 contained material which was identical with PGE_2 methyl ester and a smaller portion that was identical

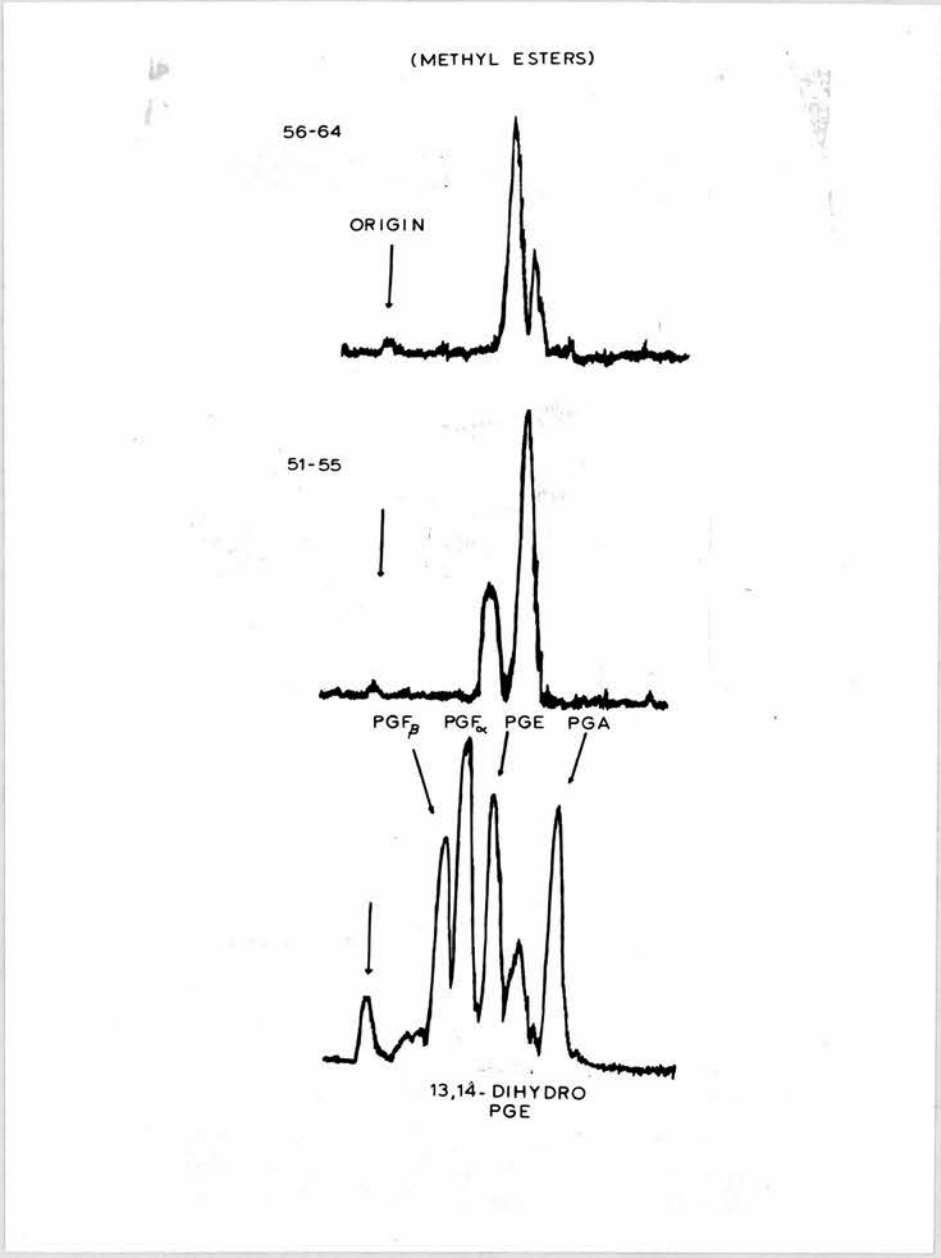

Figure 33. The separation on silica gel thin layer chromatography using the modified G.C.M. solvent (page 174) of fractions 56-64 (upper) and 51-55 (middle) obtained from the Lipidex 5000 column described in figure 32 page 190. The bottom trace shows the separation of authentic methyl esters of prostaglandins $F_{2\beta}$, $F_{2\alpha}$, PGE_2, 13,14-dihydro-PGE1 and PGA_2.

Prostaglandin	Mean Rf (methyl ester) in modified G.C.M. solvent	Mean % bed volume on Lipidex 5000
E_2	0.38	5.8
$F_{2\alpha}$	0.23	8.5
$F_{2\beta}$	0.16	15.7
A_2	0.61	1.4
15-oxo-E_2	0.55	2.9
15-oxo-$F_{2\alpha}$	0.49	3.9
15-oxo-13,14-dihydro E_2	0.58	1.7
15-oxo-13,14-dihydro $F_{2\alpha}$	0.50	1.9
13,14-dihydro E_1	0.48	-
13,14-dihydro $F_{1\alpha}$	0.39	-

Table 18. The chromatographic mobilities of some authentic methyl esters of prostaglandins and their metabolites on silica gel G using the modified GCM solvent (ethyl acetate, methanol acetic acid 100:10:1 by volume) and their corresponding retentions on Lipidex 5000 gel partition column chromatography when eluted with heptane, chloroform (80:20 v/v).

with 13,14-dihydro PGE_1.

The tissues so far studied have all been found to produce less polar compounds than PGE_2 and these have provisionally been identified by their mobilities on Lipidex 5000 and by thin layer chromatography. The two major metabolites produced by most tissues were provisionally identified by the above methods as 15-keto PGE_2 and 13,14-dihydro, 15-keto PGE_2; where prostaglandin $F_{2\alpha}$ has been found as a metabolite, the corresponding $PGF_{2\alpha}$ metabolites have also been identified.

Both the Lipidex 5000 gel column chromatography and the thin layer chromatography have been found to give comparable separations of the PGE_1 metabolites produced in parallel with the PGE_2 metabolites.

The tissues found to convert PGE_2 into $PGF_{2\alpha}$, namely, homogenates from guinea-pig, rabbit and horse liver and horse heart were incubated with 1-^{14}C PGE_1. These tissues produced several metabolites, one of which was provisionally identified by the above methods as $PGF_{1\alpha}$. An example of this is shown in figure 34 on page 195.

Here it can be seen from the upper trace that the methyl ester trimethylsilyl ether of both $PGF_{1\beta}$ and $PGF_{1\alpha}$ separate on the gas chromatography system employed. The lower half of figure 34 shows material produced by a rabbit liver homogenate and provisionally identified by Lipidex 5000 mobility and thin layer chromatography as $PGF_{1\alpha}$. It can be seen that when subjected to gas liquid chromatography as the methyl ester trimethylsilyl ether, this material has a retention time for both mass and radio-

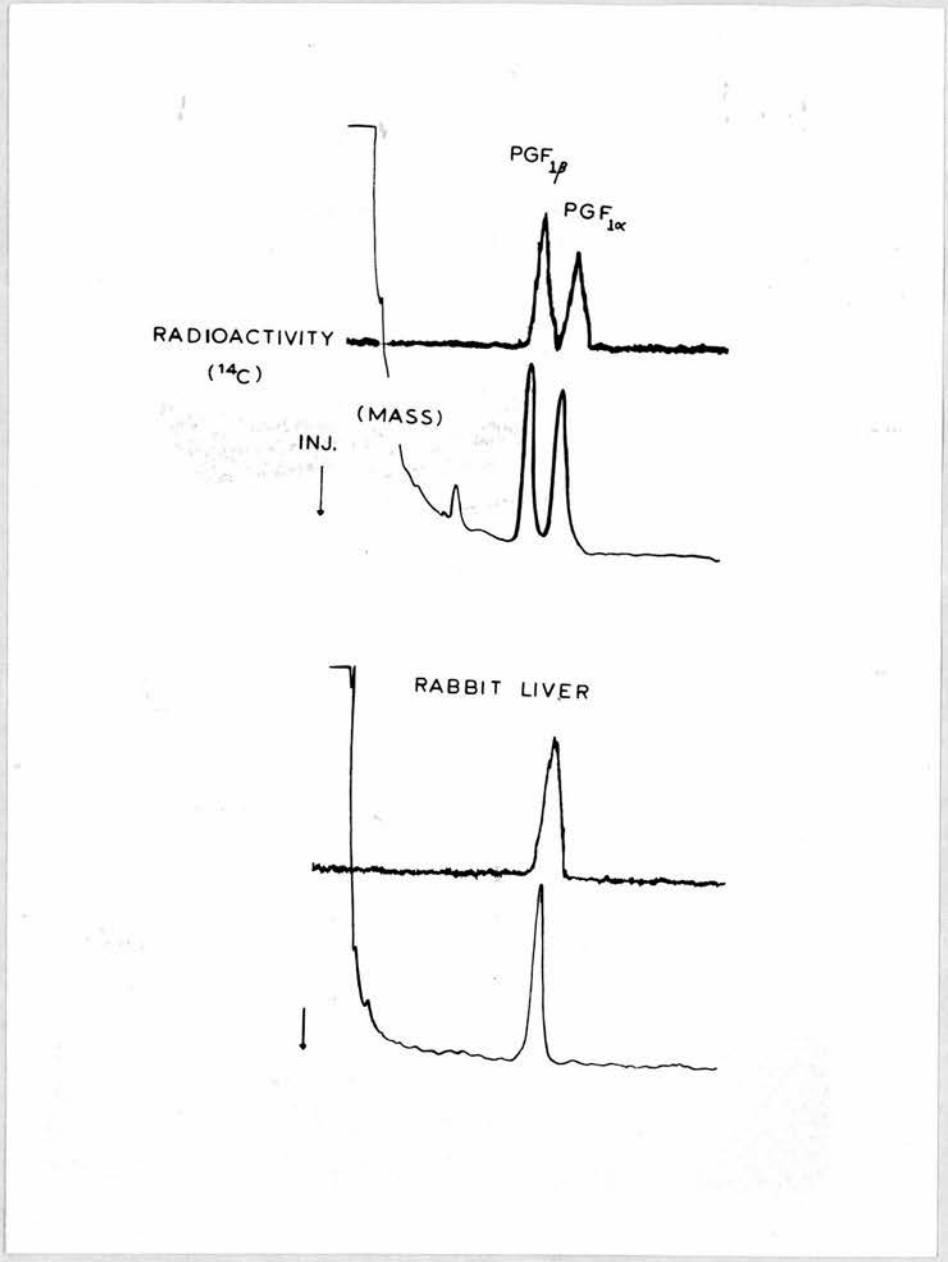

Figure 34. Radio gas liquid chromatography traces obtained for authentic ^{14}C labelled prostaglandins $F_{1\beta}$ and $F_{1\alpha}$ as the methyl ester trimethylsilyl ether (upper) and the corresponding trace obtained for the product with $PGF_{1\alpha}$ like mobility on Lipidex 5000 obtained from rabbit liver homogenate incubated with ^{14}C PGE_1.

activity (^{14}C) identical with that of authentic prostaglandin $F_{1\alpha}$ Me TMS.

Radio gas chromatography was not performed on any of the PGE_2 metabolites produced by any tissues, because the radio gas chromatography system was not sufficiently sensitive or reproducible at the time of these experiments.

Conclusive evidence that PGE_1 and PGE_2 were being converted into the corresponding $PGF\alpha$ compounds was obtained by combined gas chromatography-mass spectrometry. Examples of the tissues producing $PGF_{2\alpha}$ from PGE_2 are shown in figure 35 . These gas chromatography traces show that homogenates of horse, guinea-pig and rabbit liver and of horse heart produce material which, as the methyl ester trimethylsilyl ether, chromatographs identically with authentic prostaglandin $F_{2\alpha}$. Mass spectral scans taken at the crest of each gas chromatography peak were identical to that of authentic prostaglandin $F_{2\alpha}$. Prominent ions were at m/e 584 (M+); 569 (M-15); 513 (M-71); 494 (M-90); 423 (M-90+ 71); 404 (M-2 x 90); 333 (M-2 x 90 + 71) 217; 199; 191 and 173. These ions are characteristic of prostaglandin $F_{2\alpha}$ methyl ester trimethylsilyl ether. An example of the mass spectrum of this prostaglandin has been given previously in figure 13 on page 126 . Similar results were obtained with the $PGF_{1\alpha}$ zones produced from incubating the tissues with the 1-^{14}C PGE_1. The corresponding $PGF_{1\beta}$ and $PGF_{2\beta}$ compounds are separated from the $PGF_{1\alpha}$ and $PGF_{2\alpha}$ by the gas chromatography systems used. No detectable levels of these 9β epimers were found.

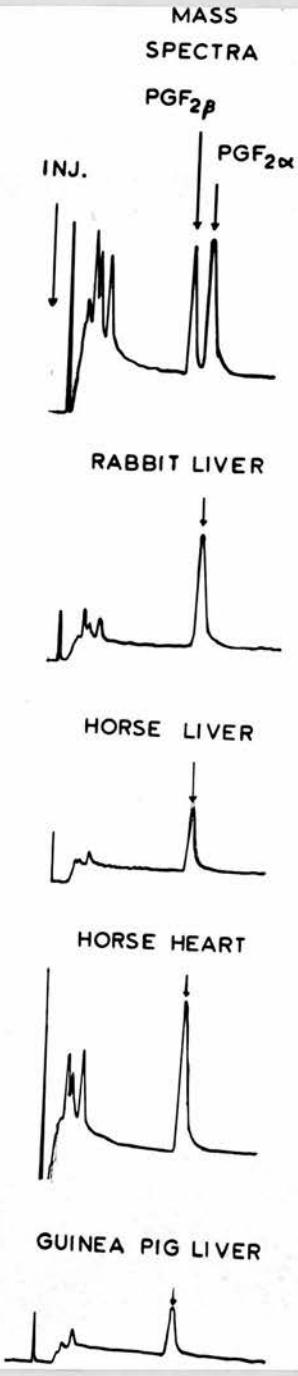

Figure 35. Gas liquid chromatograph traces (total ion current) obtained using an LKB 9000 GC-MS for the analysis of the methyl ester trimethylsilyl derivative of authentic $PGF_{2\beta}$ $PGF_{2\alpha}$ and the $PGF_{2\alpha}$ zones obtained from Lipidex 5000 columns used to purify the products of incubating ^{3}H PGE_2 with rabbit, horse and guinea-pig liver and horse heart homogenates.

It has been observed during these experiments that rabbit and guinea-pig liver homogenates appeared to contain different levels of 9-keto reductase enzyme activity as determined by the recovered $PGF_{2\alpha}$. In the case of the rabbit the female levels were consistently lower than the corresponding male levels. The range of conversion to $PGF_{2\alpha}$ was from 25% to 48%. The possibility that the differences being monitored were due to hormonal influences was considered a possibility and therefore the effect of pseudo-pregnancy and pregnancy on the conversion of PGE_2 to $PGF_{2\alpha}$ by rabbit liver homogenates was studied.

<u>The effect of pseudo-pregnancy on the in vitro metabolism of prostaglandin E_2 by rabbit liver homogenates</u>

The results obtained in these experiments are presented in Table 19 on page 200 and in Figure 36 on page 199 .

It can be clearly seen that on average the female liver homogenate samples possess less prostaglandin metabolising capability than the corresponding male liver homogenates. Furthermore, the levels of prostaglandin metabolism vary throughout the duration of pseudo-pregnancy. There are definite increases in the general metabolism of prostaglandin E_2 and more specifically in the conversion of PGE_2 to $PGF_{2\alpha}$ after day 15 of pseudo-pregnancy. The peak of activity for all prostaglandin metabolising enzymes appears to be at or around day 18 of pseudo-pregnancy. The peak of peripheral plasma progesterone levels occurred much earlier in the pseudo-pregnancy cycle (N.L. Poyser, personal communication) and thus it may be that this hormone acts

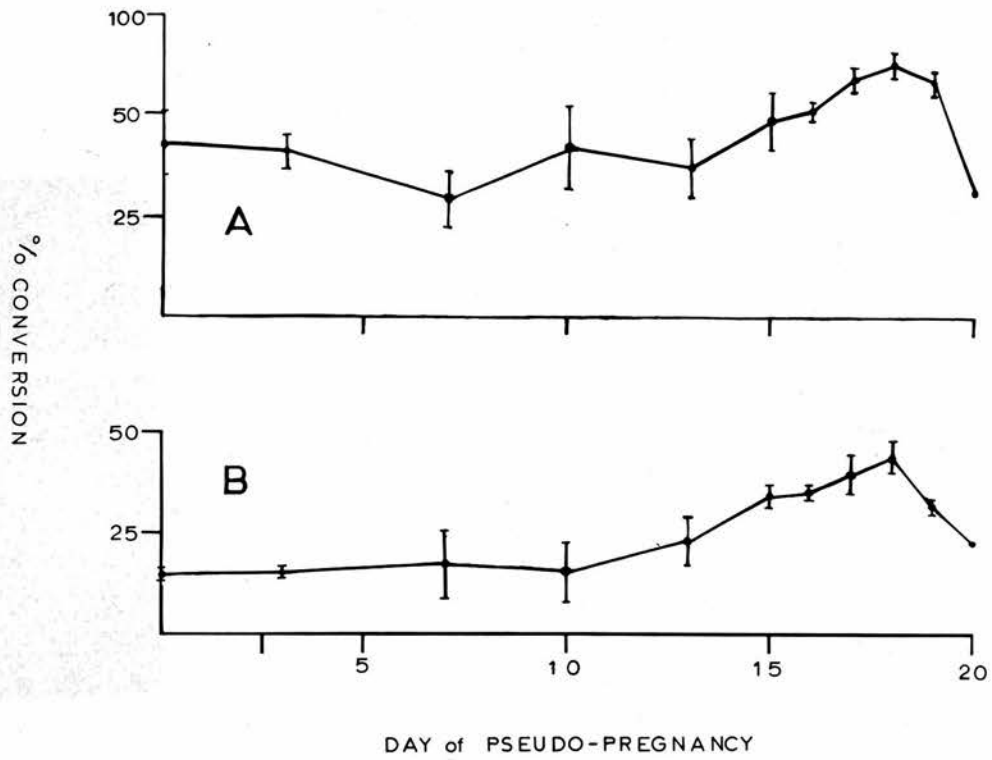

Figure 36. The effect of pseudo-pregnancy on the in vitro total metabolism (upper) and specific conversion to $PGF_{2\alpha}$ (lower) of prostaglandin E_2 by rabbit liver homogenates.

Table 19

The metabolism of PGE_2 by liver homogenates obtained during a pseudo-pregnancy.

Day of pseudo-pregnancy	% conversion of PGE_2 to to $PGF_{2\alpha}$	Average	% metabolism of PGE_2	Average
0 (normal female rabbit)	13.21 16.56	14.89	35.22 50.69	42.95
3	13.46 16.97	15.21	36.91 45.27	41.09
7	25.77 8.71	17.24	35.37 21.67	28.52
10	7.8 22.52	15.16	52.01 31.55	41.78
13	16.90 28.72	22.8	29.52 43.71	36.61
15	30.61 36.81	33.7	41.70 55.68	48.69
16	33.18 36.35	34.76	52.28 48.90	50.59
17	29.72 48.35	39.03	61.70 55.43	58.56
18	47.44 38.40	42.92	64.95 59.43	62.19
19	32.36 29.27	30.81	60.22 56.46	58.34
20	22.14	22.14	30.42	30.42
Male rabbits				
1	41.2		72.3	
2	44.2		64.1	
3	44.1	44.5	75.9	70.7
4	49.3		73.3	
5	35.7		58.9	
6	52.5		80.1	

The values quoted are expressed as a percentage of the total recovered radioactivity obtained from Lipidex 5000 columns.

on the protein synthesising enzymes present in the liver to stimulate the production of more prostaglandin metabolising enzymes. The mechanism by which this stimulation occurs is unknown and similarly whether it is specific for prostaglandin metabolising enzymes remains to be elucidated.

Similar increases (as will be shown) were found to occur during the gestation period of pregnant rabbits.

The effect of pregnancy on the in vitro metabolism of prostaglandin E_2 by rabbit liver homogenates

The results for the metabolism of prostaglandin E_2 by rabbit liver homogenates are shown in Table 20 on page 203 .

The levels of PGE_2 and $PGF_{2\alpha}$ calculated from the radioactive recoveries were also quantitatively assayed by combined gas chromatography-mass spectrometry and found to be in agreement by both of these methods. In all cases the $PGF_{2\alpha}$ produced was conclusively identified as the methyl ester trimethylsilyl ether derivative by combined G.C.M.S. The mass spectra produced were essentially as the one shown in Figure 38 on page 211 . Prominent ions were found at m/e 584 (M +); 569 (M-15); 513 (M-71); 494 (M-90); 423 (M-90 + 71); 404 (M-2 x 90); 217; 199; 191 and 173. These ions are characteristic of the mass spectrum of this prostaglandin $F_{2\alpha}$ derivative. The corresponding $PGF_{2\beta}$ was not identified during any of these experiments.

From the results it can be seen that the levels of prostaglandin metabolising enzymes are elevated at the end pregnancy relative to the non-pregnant rabbit. These elevated levels of enzyme activity apparently fall away

Table 20

The metabolism of PGE_2 by female adult and offspring rabbits during gestation and after birth.

Day of gestation	% conversion to $PGF_{2\alpha}$	% metabolism of PGE_2
25	35	52
26	39	56
27	40	64
non-pregnant	14 19	38 49
Day after parturition		
1	40	59
6	23	51
Day after birth		
1 (male)	7	21
(female)	6	19
6 (male)	11	24
(female)	8	30

The values quoted are expressed as a percentage of the total radioactivity recovered from thin layer chromatography plates.

after parturition in the maternal animal. The offspring by comparison have apparently much lower enzyme activities which increases after birth.

Again the mechanism of these changes in the apparent activities of prostaglandin metabolising enzymes is unknown, but may be due to the changes in the oestrogen and progesterone levels that occur during pregnancy.

The opportunity to study the effect of these two types of steroid hormones arose during experiments conducted by Dr. N.L. Poyser and Mr. B. Naylor. These experiments were designed to study the effect of progesterone and oestradiol administered *in vivo* to ovariectomised guinea-pigs on the *in vitro* production of $PGF_{2\alpha}$ by the guinea-pig uterus.

The effect of oestradiol and progesterone administered in vivo on the in vitro metabolism of PGE_2 by liver homogenates from ovariectomised guinea pigs

The results expressed in Table 21 on page 206 and Table 22 on page 207 show the percentage conversion to $PGF_{2\alpha}$ and the percentage total metabolism respectively of PGE_2 by guinea pig liver homogenates.

The conversion of PGE_2 to $PGF_{2\alpha}$ by liver homogenates from ovariectomised guinea pigs can be seen to be significantly increased with treatment by oestradiol ($P<0.05$) or oestradiol in combination with progesterone ($P<0.01$). Progesterone treatment alone did not cause a significant increase in the conversion of PGE_2 to $PGF_{2\alpha}$. Moreover, there also appears to be a significant difference ($p <$

0.05) between the conversion of PGE_2 to $PGF_{2\alpha}$ by ovariectomised (non-hormonal treated) and normal day 7 guinea pig liver homogenates. The statistical comparison of day 13 guinea pig liver homogenates with either of these two samples was not made due to insufficient data being available. However from the data available it would appear that there are higher levels of 9-keto reductase activity at day 13 than in either the normal day 7 or ovariectomised guinea pig liver homogenates.

When statistically analysed, the results presented in Table 22 on page 207 also show an identical analysis pattern. There are significant differences between the total metabolism of PGE_2 by liver homogenates from ovariectomised guinea pigs and identical ones treated with oestradiol benzoate ($p < 0.05$) or oestradiol benzoate and progesterone ($p < 0.01$) in vivo. Similarly, the day 7 guinea pigs have significantly higher levels of prostaglandin metabolising enzymes ($p < 0.05$) than the untreated ovariectomised guinea pigs.

It would thus appear from these results that both oestradiol and progesterone-like hormones can have marked influences on the degree of activity from the prostaglandin metabolising enzymes present in guinea pig liver samples. Whether these influences are common to all species remains to be elucidated, however, evidence has been presented that in lung tissue from the rabbit and sheep the activity of the prostaglandin metabolising enzymes is greater towards the end of pregnancy (Horton and Maule-Walker, 1975; Bedwani and Marley, 1975; Sun, 1975). Similar changes in both pregnant and pseudo-pregnant rabbits were reported

Table 21

The conversion of PGE_2 to $PGF_{2\alpha}$ by liver homogenates obtained from ovariectomised guinea pigs treated in vivo with oestradiol benzoate and progesterone.

Group	Sample	a	b	c	d	e	f	mean	SEM
	1	5.22	4.20	3.70	3.73	3.74	7.77	4.73	0.65
	2	6.93	8.24	8.63	5.44	6.47	7.91	7.27	0.49
	3	4.25	8.57	7.77	4.07	2.9	7.66	5.87	0.98
	4	8.49	-	10.31	8.02	7.42	7.71	8.39	0.51
normal day	7	6.32	7.28	8.62	6.84	-	-	7.27	0.49
normal day	13	10.14	8.54	9.30	-	-	-	9.33	0.46

The values quoted are the radioactivity recovered in the $PGF_{2\alpha}$ zone when expressed as a percentage of the total recovered radioactivity.

Table 22

The metabolism of PGE_2 by liver homogenates obtained from ovariectomised guinea pigs treated in vivo with oestradiol benzoate and progesterone.

Group	Sample	a	b	c	d	e	f	mean	SEM
	1	48.5	53	44	56	49	61	51.92	2.47
	2	70	63	61	72	70	54	65.00	2.83
	3	54	50	49	62	59	63	56.17	2.49
	4	77	-	72	78	81	79	77.40	1.50
normal day	7	60	58	49	54	-	-	55.25	2.43
normal day	13	72	79	85	-	-	-	78.67	3.76

The values quoted represent the percentage of the total recovered radioactivity minus the percentage in the PGE_2 zone.

Groups 1,2,3 and 4 are described on page 187 .

earlier in this section. It would obviously be interesting to know whether the levels of prostaglandin metabolising enzymes present in tissues fluctuate throughout the oestrous cycle. The different levels of prostaglandin synthetase in uterine tissue throughout the oestrous cycle of the guinea pig and sheep is well documented (see Poyser, 1973, 1975). There is also evidence to support the theory that the 15-hydroxy prostaglandin dehydrogenase enzyme has a very short half-life (Flower et al., 1975). Therefore, this and other prostaglandin synthsising and metabolising enzymes may be regulated throughout the oestrous cycle by the changes in oestrogen and progesterone hormone levels.

The metabolism of prostaglandin E_2 by male human liver, heart and kidney homogenates

One set of samples obtained approximately 16 hours after death were subjected to Lipidex 5000 chromatography (after conversion to methyl esters). In all cases the PGE_2 was found to be metabolised to less polar compounds, however, the metabolism was only 6%, 11% and 12% for the kidney, heart and liver samples. These samples were not further analysed. In the second example the tissues had been obtained within 4 hours of death and were found to metabolise exogenous PGE_2 (as determined by radioactive recoveries from Lipidex 5000 column eluants) by 39%, 31% and 46% respectively. All the tissues produced metabolites that were less polar than PGE_2 on Lipidex 5000 columns. These metabolites were not further characterised. The liver sample, however, was found to produce a metabolite (7% of total recovered radioactivity) that was more polar than either PGE_2 or $PGF_{2\alpha}$ (figure 37 on page 210). The possibility that this was a PGF compound was further investigated. The material was subjected to combined GCMS as the methyl ester trimethylsilyl ether and found to have a carbon value of 23.40 . Prostaglandin $F_{2\alpha}$ methyl ester trimethylsilyl ether had a carbon value of 23.55 when chromatographed on this system. The mass spectra of the derivatised metabolite and $PGF_{2\alpha}$ obtained are shown in Figure 38 on page 211 . The derivatised metabolite was found to have a molecular ion at m/e 582 which indicated the presence of an extra double bond relative to $PGF_{2\alpha}$ Me TMS (M^* 584). The base peak of the metabolite was found

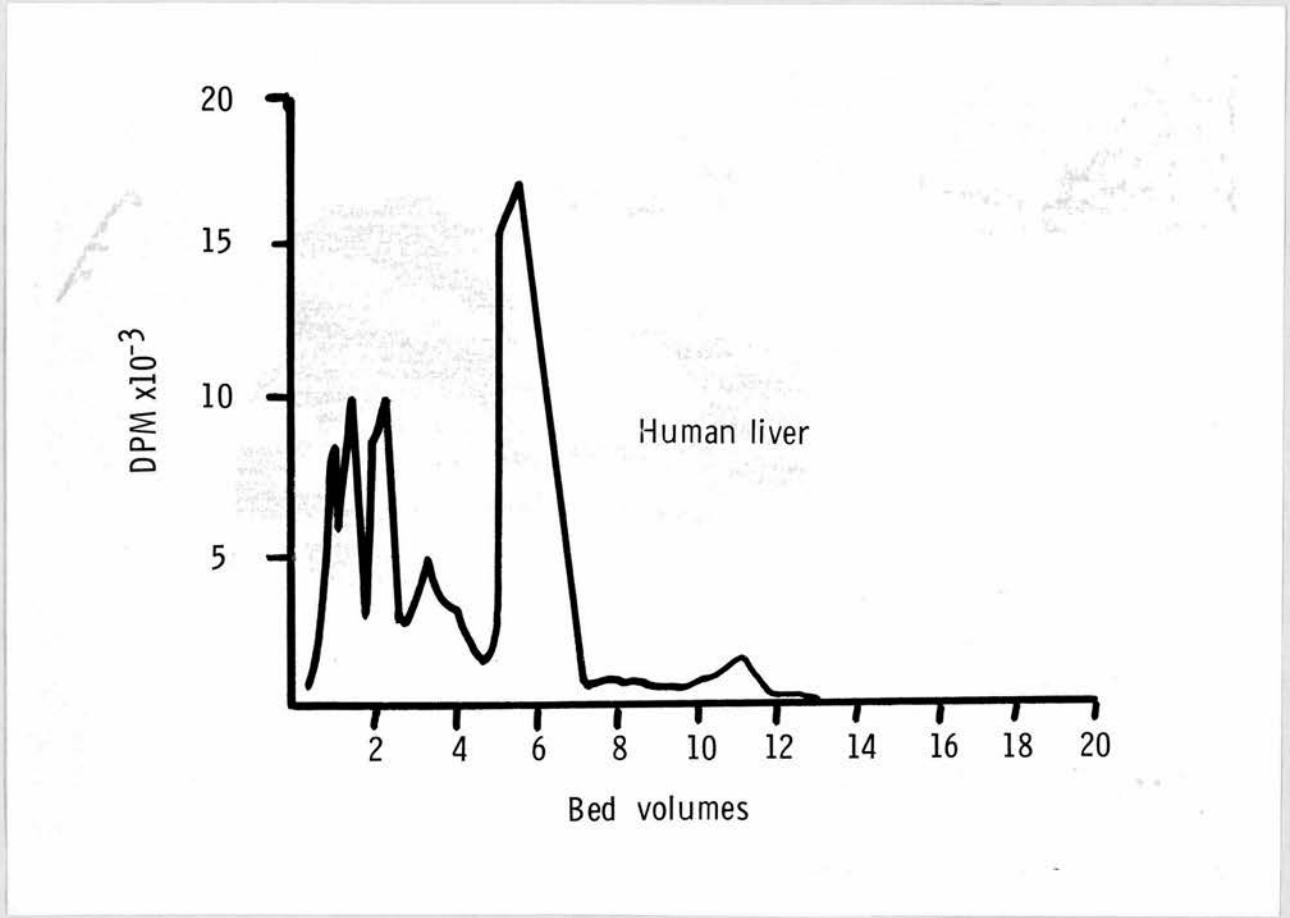

Figure 37. Lipidex 5000 profile obtained for the extract (after conversion to methyl esters) of the products obtained after incubating a human liver homogenate with ^{3}H PGE_2.

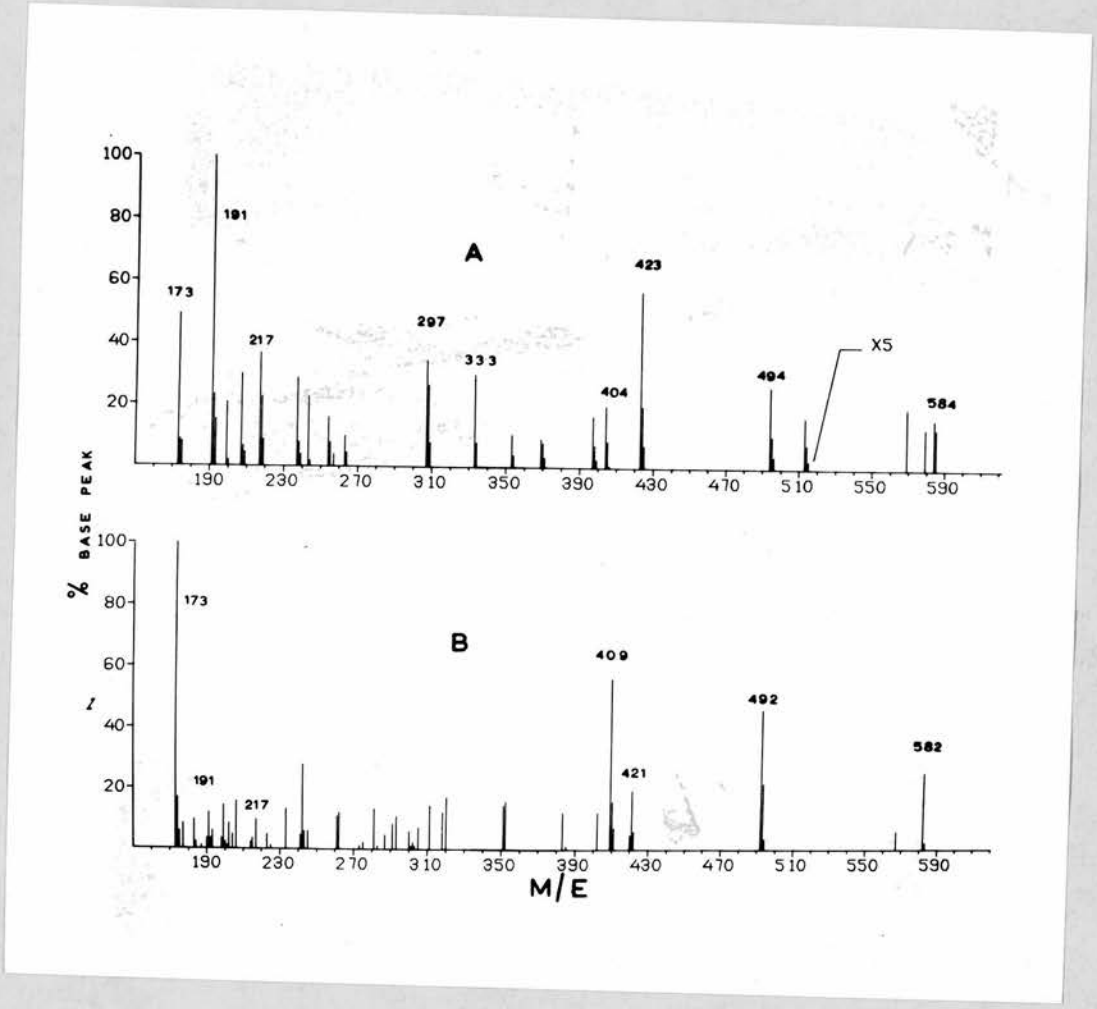

Figure 38. Mass spectra recorded at 70 eV using an LKB 9000 GC-MS for authentic prostaglandin $F_{2\alpha}$ methyl ester trimethylsilyl ether (upper) and the corresponding derivative of the material obtained from the Lipidex 5000 column (figure 37, page 210) with a polarity greater than PGE_2 methyl ester.

to be at m/e 173, whereas in $PGF_{2\alpha}$ Me TMS it was at m/e 191. This large ion at m/e 173 is characteristic of the sodium borohydride reduction products of 12-iso PGD_2 (R.L. Jones, personal communication) and indicated that the 13, 14-trans double bond had been transposed to the 12, 13 position in the metabolite (i.e. exo-cyclic to the ring). This was supported by the smaller m/e 199 ion relative to $PGF_{2\alpha}$. This ion also confirmed that the extra double bond present in the metabolite was not situated in the w-side chain, as in $PGF_{3\alpha}$ because the m/e value of the corresponding ion would have been at 197. Similarly the presence of an ion at m/e of 421 is probably due to the same fragmentation that results in the ion at m/e 423 in $PGF_{2\alpha}$ Me TMS, namely the loss of one trimethyl-silanol group (TMSi-OH) plus the C_{16}-C_{20} (C_5H_{11}) fragment from the w-side chain. The side chain appeared to be intact from the presence of ions at m/e of 141. It was therefore concluded that the extra double bond was in the cyclopentane ring. The most probable positions being either between C-8 and C-9 or between C-10 and C-11. This could have resulted from an enolisation of the 9-ketonic group. Another possibility was that the double bond was situated between C-8 and C-12 as in the B series prostaglandins, however, the evidence above suggested that the 13,14-trans double bond had been transposed to the 12,13 position would indicate that this was not possible. The fact that the hydrogen atom situated at C-8 is more labile than either of those at C-10 would suggest that the extra double bond was situated between C-8 and C-9. Thus the metabolite

was tentatively identified as 9,11,15-trihydroxy-5-cis-8(9),12(13) prostatrienoic acid.

Further mass spectral or radio gas chromatographic analysis, however, was not performed to confirm this structure, due to the lack of metabolite available for analysis.

It is known that man excretes a urinary metabolite of PGE_2 with a $PGF_{2\alpha}$ configuration (Hamberg and Wilson, 1973) thus, from the evidence quoted above, the enzyme responsible for this reduction may be located in the liver. However, one area where the conversion of PGE_2 to $PGF_{2\alpha}$ could be of marked physiological and pathological importance is in the bronchiolar tissue. Thus the metabolism of PGE_2 by human bronchiolar tissue was studied.

<u>The in vitro metabolism of prostaglandin E_2 by male and female human bronchiolar tissue</u>

An example of typical radio scan of the incubation products produced is shown in Figure 39 on page 214 . It can be clearly seen that no evidence for the conversion of PGE_2 to $PGF_{2\alpha}$ was obtained during these experiments. For both male and female human bronchiolar tissue homogenates the two major metabolites produced were always less polar than PGE_2 in the thin layer chromatography system employed. These metabolites were not conclusively identified, however, their mobilities on thin layer chromatography suggested that they were mainly the 15-keto PGE_2 and the 15-keto-13,14-dihydro PGE_2 respectively. It was thus concluded that using the incubation procedures described on page there was no evidence for a 9-keto reductase being present

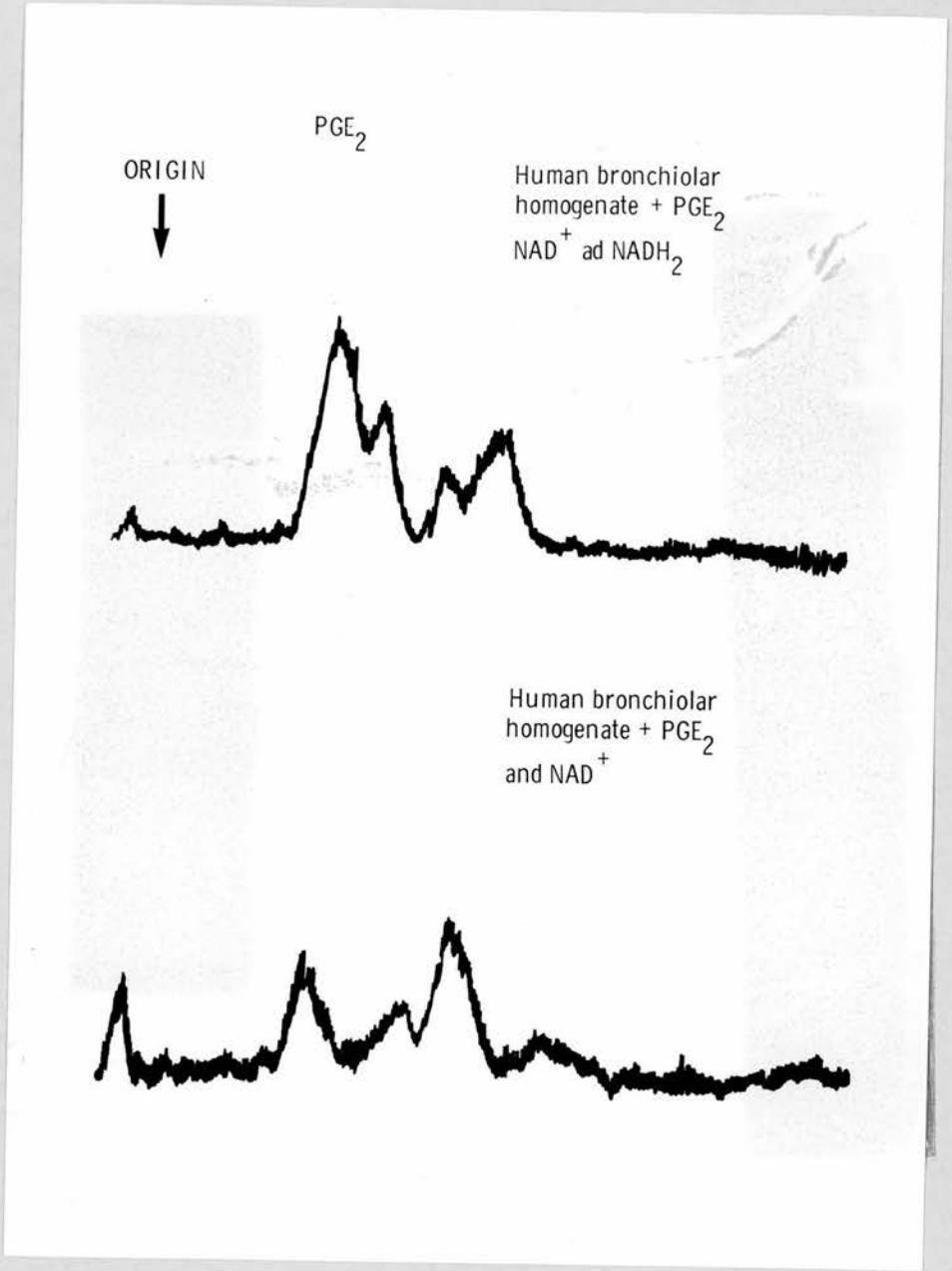

Figure 39. Thin layer chromatography radioscans obtained for two extracts of human bronchiolar homogenates incubated with ^{3}H PGE_2 in the presence (upper) and absence (lower) of $NADH_2$.

in human bronchiolar tissue.

In one experiment it was observed that the addition of $NADH_2$ to the incubation medium caused the appearance of a third metabolite. The polarity of this metabolite suggested that it was 13,14-dihydro PGE_2 and thus was probably produced by a reversal of the 15-hydroxy prostaglandin dehydrogenase. The reversal of this enzyme, present in bovine lung has previously been reported (Shaw et al., 1973).

<u>The in vitro metabolism of prostaglandin E_2 by male chicken liver, heart and brain samples</u>

When incubated with PGE_2 all the above samples produced a more polar metabolite that had a thin layer chromatographic behaviour identical with $PGF_{2\alpha}$. When the material from the $PGF_{2\alpha}$ zones of thin layer plates was subjected to combined gas chromatography-mass spectrometry as the methyl ester trimethyl-silyl ether, gas chromatography peaks and mass spectra identical with authentic prostaglandin $F_{2\alpha}$ were obtained. The mass spectra were essentially the same as those shown on page 211 . Prominent ions were present at m/e of 584 (M +); 569 (M-15); 494 (M-90); 423 (M-90+71); 404 (M-2 x 90); 397; 333 (M-2 x 90 + 71); 217; 199; 191 and 173. These m/e values are characteristic of $PGF_{2\alpha}$ Me TMS.

Due to the fact that the radio gas chromatography system previously employed for the identification of PGE_2 metabolites was no longer sufficiently sensitive for ^3H-labelled compounds, it was decided to confirm the levels of $PGF_{2\alpha}$ assayed by the radiochemical method by quantitative

gas chromatography-mass spectrometry. When quantitatively assayed as the ethyl ester trimethyl-silyl ether, the levels of $PGF_{2\alpha}$ calculated from the recovered ^{3}H label in the $PGF_{2\alpha}$ thin layer zone and the specific activity of the added ^{3}H-labelled PGE_2 substrate were found to be in good agreement when assayed by either method. It was therefore concluded that the majority of the $PGF_{2\alpha}$ being quantitatively assayed was being produced from the PGE_2 added to the homogenates and not by de novo synthesis. The levels of conversion to $PGF_{2\alpha}$ when assayed by the radio chemical method are shown in Table 23 on page 217 .

It was known from the studies with newly born rabbits that the levels of 9-keto reductase were lower immediately after birth than at adult age (see page 203). Consequently it was decided to measure the levels of 9-keto reductase present in the liver homogenates obtained from male and female chickens on days 1,8 and 22 after birth. The results are presented in Table 24 on page 218 .

It can be seen from these results that both male and female chicken liver homogenates contain more 9-keto reductase activity as the animal ages. However, as can be seen from the results expressed in Table 23 on page 217. the levels of 9-keto reductase present in male liver homogenates can vary quite considerably (6%-30%). Thus it may possibly be that as yet unknown factors other than age are affecting the levels of 9-keto reductase present in male chicken liver. The possibility that part of this fluctuating level may be due to the different levels of steroid hormones such as oestrogens, androgens or progesterones

Table 23

The conversion of PGE_2 to $PGF_{2\alpha}$ by male chicken liver, brain and heart homogenates *in vitro*

Sample		% conversion to $PGF_{2\alpha}$	
LIVER	1	15	
	2	23	
	3	6	
	4	9	
	5	14	
	6	30	Average conversion =
	7	12	14.54%
	8	16	
	9	17	
	10	9	
	11	13	
	12	14	
	13	11	
HEART	1	4	
	2	3	
	3	6	Average conversion =
	4	2	3.83%
	5	3	
	6	5	
BRAIN	1	9	
	2	5	Average conversion = 6.67%
	3	6	

The values quoted represent the recovered radioactivity in the $PGF_{2\alpha}$ zones of thin layer plates as a percentage of the total recovered radioactivity.

Table 24

The conversion of PGE_2 to $PGF_{2\alpha}$ by chicken liver homogenates obtained from male and female chickens of different ages.

AGE (days)	SEX	% conversion to $PGF_{2\alpha}$	AVERAGE
1	M	4	4.5%
	F	5	
8	M1	9	6.33%
	M2	3	
	M3	7	
8	F1	11	9.67%
	F2	12	
	F3	6	
22	M1	13	10.67%
	M2	7	
	M3	12	

The values quoted are the percentage of the total recovered radioactivity present in the $PGF_{2\alpha}$ zone of thin layer plates.

cannot as yet be discounted. Indeed, from the results obtained with the rabbit and guinea pig liver homogenates previously reported in this section of this thesis it may well be that one or more of these steroids do have an important physiological function relevant to prostaglandin metabolising enzymes.

GENERAL DISCUSSION

At the beginning of this investigation the primary objective was to develop a radioimmunoassay method specific for prostaglandin E_2. Radioimmunoassay methods were already well developed for prostaglandin B_2 and $F_{2\alpha}$ in the laboratories at the Pharmacology Department when the work described in this thesis began. It was therefore decided to perform the initial developmental methods that had previously led to the successful radioimmunoassays for PGB_2 and $PGF_{2\alpha}$.

The radioimmunoassay method that was initially envisaged was to have been used for the analysis of prostaglandin E_2 in a wide range of biological situations. In collaboration with the research personnel at the Pharmacology laboratories at May and Baker Ltd., Dagenham, it was proposed to initially study the uptake and metabolism of prostaglandin E_2 (as monitored by the decrease in PGE_2 levels in blood administered to guinea pigs by a variety of routes. These were to include the intravenous, aerosol and gastro-intestinal routes. However, alternative uses for the radioimmunoassay of prostaglandin E_2 would have included the analysis of prostaglandin E_2 produced and released from specific areas including the cat and dog C.N.S.; guinea pig uterus; rabbit kidney *in vitro* and *in vivo* and the isolated cat or dog spleen in response to a variety of stimuli. It was realised at the time that the use of a radioimmunoassay procedure, whilst offering advantages of sensitivity, specificity and efficiency relative to the classical bioassay procedures that were

being routinely used, could never offer the conclusive identification that was available from combined gas chromatography-mass spectrometry (GCMS). It was therefore apparent that confirmation for some of the results obtained with the radioimmunoassay of PGE_2 should ultimately be obtained with combined G.C.M.S.

The first description of an attempt to raise antibodies to an E series prostaglandin was made by Levine and Van Vunakis (1970). This has been followed in the intervening years by several reports of attempts to raise antibodies to E series prostaglandins (Jaffe, Smith, Newton and Parker, 1971; Yu, Chang and Burke, 1972; Yu and Burke, 1972). In all these examples quoted the immunogen used resulted in the production of anti-sera with some significant degree of cross-reactivity to either the corresponding prostaglandin A or B. The cause of this has been suggested as being due to the carbodiimide used to covalently link the E prostaglandin to the protein immunogen inducing a dehydration of E to the corresponding A prostaglandin (Levine, Gutierrez-Cernosak and Van Vunakis, 1971; 1973). The resulting A prostaglandin immunogen was then postulated to be isomerised to the corresponding B prostaglandin by the prostaglandin A isomerase present in either plasma or tissues (Jones and Cammock, 1973; Horton, Jones, Thompson and Poyser, 1971; Polet and Levine, 1971). The result would be that the antibody synthesising systems would be presented with prostaglandin B conjugated to protein and thus antibodies to this and not prostaglandin E would be produced. An attempt to detect the conversion of the PGE_2 during the

conjugation reaction with bovine serum albumin into the corresponding PGA_2 was described in Section I. The free prostaglandin that was recovered after dialysis of the conjugation reaction was found by U.V. spectrophotometric and thin layer chromatographic analysis to be identical with authentic PGE_2. No evidence was obtained for the conversion of the PGE_2 to either PGA_2 or PGB_2. This result is in good agreement with a similar attempt to monitor the carbodiimide conjugation step (Yu and Burke, 1972). These workers also failed to detect any carbodiimide catalysed dehydration of the PGE_1.

However, when rabbits were immunised with the resulting PGE_1 immunogen (conjugated with human serum albumin) four out of six rabbits produced antisera that had virtually complete cross reaction with E, A and B series prostaglandins (Yu and Burke, 1972). This indicated that the PGE_1 had been dehydrated and isomerised in vivo. The same result was obtained when, as reported in Section I, rabbits were immunised using the multiple intradermal technique with an apparently homologous PGE_2-BSA immunogen. The major antibodies produced were apparently a mixed population of PGB, PGA and PGE, the majority of which had specificities directed mainly towards PGB_2. The possibility cannot be discounted from the result presented in Section I that although the "free" prostaglandin was still PGE_2 the conjugated prostaglandin may have been either PGA_2 or PGB_2. Certainly the conjugation reaction, which is performed at slightly alkaline pH (~8) and results in a condensation

(i.e. dehydration) of the carboxylic acid group of PGE_2 to the carbodiimide, is favourable for the dehydration of the PGE_2 to PGA_2. Another alternative explanation is that although the injected immunogen still retained PGE_2 the duration in the animal (rabbit) prior to the antibody synthesising processes being operated, was sufficiently long to enable the combination of slightly alkaline body pH (ca. 7.4) and temperature (36-38°C) to induce a chemical dehydration and isomerisation of the PGE_2 to PGA_2 and PGB_2 respectively.

One other possibility is that there may be enzymes present in the rabbit (or other animal species) capable of dehydrating the PGE_2 to PGA_2, even when it is conjugated with a large molecular weight protein. Certainly evidence for the dehydration of PGE to PGA by the rat (Green, 1971) and various plasmas (Levine et al, 1975), has been presented. The resulting PGA_2-protein conjugated could then be isomerised to the PGB_2 derivative by the mechanism suggested by Levine et al (1972) or by simple chemical induced isomerisation.

In an attempt to minimise the duration of time between injection of the immunogen and the antibody process being activated, it was decided to attempt to successfully raise PGE_2 specific antibodies in the rabbit by injecting the immunogen directly into the popliteal lymph nodes. The use of intranodal immunisation procedures was pioneered by Boyd and Peart (1968) in an attempt to improve the effectiveness of feeble or scarce immunogens. The major disadvantages of this method are that the immunised animals are more susceptible to infection

because of the operating procedures and also that the irritant adjuvant emulsions tend to destroy the injected lymph node tissues. It was for these reasons that the multiple intradermal technique developed by Vaitukaitis, Robbins, Nieschlag and Ross (1971) and used initially in these experiments has become so popular as the route of immunisation. However, this method failed to successfully produce a suitable antiserum for PGE_2 and therefore the method of Boyd and Peart (1968) was attempted. Unfortunately the antisera produced were again principally directed towards PGB_2.

These results could support either the theory of enzyme or chemical breakdown of the PGE_2 conjugated to the BSA. Therefore it was decided to determine whether any PGE_2 dehydrase enzyme activity could be detected in the blood or lymph samples obtained from New Zealand white rabbits. No evidence for such an enzyme was obtained and therefore the reason for the inability to raise antibodies to PGE_2 in the rabbit must remain unanswered.

In an attempt to develop another form of radioimmunoassay suitable for the PGE_2 present in biological samples several alternatives were considered. The options available includedthe development of antibodies to a stable compound into which PGE_2 was readily convertible by simple chemical means. One such compound is PGB_2 which is produced from PGE_2 by treatment with alkaline media (Andersen, 1969). Antibodies have successfully been raised to this compound (Levine et al., 1971; Levine, 1974, Emslie et al., 1975).

However, in the absence of specific antibodies to prostaglandins A_2 and C_2, both of which are intermediate in the conversion of PGE_2 into PGB_2, it was decided that it would be extremely difficult to obtain accurate results by this method for the levels of PGE_2. Another chemical step known to produce quantitative conversions is the reduction of the 9-keto group in PGE_2 with sodium borohydride. The resulting products $PGF_{2\alpha}$ and $PGF_{2\beta}$, are epimeric at C-9. Antibodies had successfully been raised in our laboratories to $PGF_{2\alpha}$ (Emslie et al., 1975), however, it was decided that antibodies to $PGF_{2\beta}$ would be a better assay for PGE_2. The primary reasons for this choice were that by assaying a biological sample for $PGF_{2\alpha}$ before and after sodium borohydride, whilst giving an estimate of the PGE_2 present, would not be an accurate account for the contribution due to $PGF_{2\beta}$ produced concomitently or the $PGF_{2\alpha}$ and like compounds produced from both $PGF_{2\alpha}$ and PGE_2 metabolites, as the proposed $PGF_{2\beta}$ antibodies. For example only half the number of the potential compounds produced would have the $PGF_{2\beta}$ ring configuration when compared with the $PGF_{2\alpha}$ ring configuration. This is because the $PGF_{2\alpha}$ metabolites already have this ring configuration. Therefore the potential degree of interference with the assay by these compounds is in theory considerably lower. Certainly, $PGF_{2\beta}$ radioimmunoassay procedures have been successfully developed (Levine et al., 1975) and found to give comparable results for PGE_2 when compared with samples assayed with a $PGF_{2\alpha}$ antiserum.

Although in certain rabbits antisera were successfully developed for $PGF_{2\beta}$ in terms of the number of antibodies produced (as determined by the dilution curves) the cross-reactivities with $PGF_{2\alpha}$, the major potential interfering compounds were frequently greater than 5% of the $PGF_{2\beta}$ value causing a 10% fall from 60% binding. Similarly the difficulties encountered in obtaining a reproduceable and quantitative conversion of PGE_2 to $PGF_{2\alpha}$ and $PGF_{2\beta}$ at the levels probably due to be encountered in most biological systems (less than 1 ng per assay) make the value of this assay at present of limited use.

During the studies on the metabolism of PGE_2 by rabbit blood and lymph glands it was thought advisable to study the metabolism of PGE_2 by sheep whole blood. This animal, unlike the rabbit, does not appear to have a prostaglandin A isomerase within its plasma (Jones, Cammock and Horton, 1972). If this animal lacked this enzyme and the postulated PGE dehydrase then it may offer the ideal system for the successful raising of antibodies and the development of a radioimmunoassay specific for PGE_2. When incubated with sheep whole blood, however, a metabolite was produced that had thin layer chromatographic properties identical with authentic $PGF_{2\alpha}$. This was subsequently conclusively identified by radio gas chromatography and combined gas chromatography-mass spectrometry as being $PGF_{2\alpha}$. The reduction of the 9-keto group was found to be stereoselective unlike the comparable reduction by sodium borohydride which results

in both 9-hydroxy epimers being produced. The reduction process by sheep blood was found to be heat and pH labile, and the enzyme responsible to be located in the cellular fraction. The type of metabolism being described, namely reduction of the 9-keto, had only been described in two instances previously at the time of the discovery of this enzyme in sheep blood. One instance was the conversion of PGE_2 to $PGF_{2\alpha}$ by guinea pig liver homogenates (Hamberg and Israelsson, 1970; Samuelsson et at, 1970), whilst the other was conversion of the 9-keto group to a metabolite with the $PGF_{2\beta}$ ring structure in the guinea pig <u>in vivo</u> (Hamberg and Samuelsson, 1969).

The 9-keto reductase enzyme present in sheep whole blood was further characterised for substrate specificity in an attempt to define whether this enzyme was specific for the metabolism of E series prostaglandins. It was found that this enzyme had certain structural requirements for maximal activity. Certainly the substituents at C-11 were found to be critical because PGA_2 was not acceptable as a substrate. However, the interesting observation that PGD_2, a substrate with a ketonic function at C-11 and a α-hydroxyl at C-9, was an equally good substrate stimulated the desire to discover whether 15-keto PGE_2 or 15-keto PGE_1 were substrates. It was surprising to find that in these compounds not only were the ketonic functions at C-15 stereospecifically reduced to the 15(S) configuration but also that the 13,14 trans double bond could also be reduced. From the results obtained with these compounds as substrates it would appear that the enzyme reducing

the 9-keto group is different to that reducing the 15-keto group. However, the possibility that the two reduction processes may be being performed by a low substrate specific reductase cannot be discounted. Certainly reduction of the C-15 ketonic function of prostaglandins has been described (Samuelsson et al., 1971; Anggard and Samuelsson, 1964). In addition, reversibility of the 15-hydroxyprostaglandin dehydrogenase present in bovine lung is known to be a possibility (Shaw et al., 1973). This enzyme, however, does not appear to convert PGE_2 into $PGF_{2\alpha}$ when performing the reverse reaction. Similarly when sheep whole blood was incubated with $PGF_{2\alpha}$ and NAD+ no conversion to 15-keto $PGF_{2\alpha}$ was detectable, and yet these are conditions that would normally be suitable for the oxidation of the C-15 alcohol group (Anggard and Samuelsson, 1966; Nakano et al., 1969; Shio et al., 1969). The possibility that the reduction of the ketonic functions described above was being performed by a 15-hydroxyprostaglandin dehydrogenase working in reverse therefore seems minimal.

The metabolism of 15-keto PGE by either human or bovine blood was also studied and found in both examples to produce a major metabolite, namely 15-keto-13,14-dihydro-PGE. Whether the presence of this Δ13 prostaglandin reductase has any physiological significance for the inactivation of circulating 15-keto prostaglandins is unknown. Certainly both 15-keto PGE_2 and 15-keto $PGF_{2\alpha}$ rapidly lose most of their biological activity on the additional reduction of the 13,14-trans double bond

(R.L. Jones, personal communication). It could possibly be that this enzyme is present in the blood samples of many species to act as an additional protective mechanism for the cardiovascular system. Normally both PGE_2 and $PGF_{2\alpha}$ lose most of their biological activity (95%-98%) on a single passage through the lungs (Ferreira and Vane, 1967; Vane, 1969; Horton and Jones, 1969; McGiff et al., 1969).

The interconversion of E and F_α prostaglandins was considered to be of sufficient potential physiological importance to warrant further distribution studies in an attempt to ascertain whether this type of metabolism is a widespread phenomenon. Most previously reported metabolism studies had not reported the inter-conversion of PGE and PGF_α. Certainly it was known that these two prostaglandins were not interconverted during their biosynthesis (Anggard and Samuelsson, 1965; Hamberg and Samuelsson, 1967). However, from 1972 onwards, evidence has been accumulating for the presence in a variety of tissues obtained from several animal species of enzymes capable of interconverting E and F_α prostaglandins (Hamberg and Wilson, 1973; Leslie and Levine, 1974; Lee and Levine, 1973; 1975). Actively fermenting Baker's yeast has also been shown to convert PGE_2 to $PGF_{2\alpha}$ (Schneider and Murray, 1973). The potential importance of this type of enzyme as a possible modulator of a physiological or pathological role mediated via fluctuating levels of PGE and PGF_α has been suggested (Lee and Levine, 1974). This certainly could be a distinct possibility in

the organs where the E and F_{α} series prostaglandins have qualitatively and quantitatively different actions.

In the distribution studies described in Section III of this thesis, in addition to confirming the presence of 9-keto reductase activity present in guinea-pig liver homogenates (Hamberg and Israelsson, 1970; Samuelsson et al., 1971) several new examples were recorded. These were namely the horse, rabbit and chicken liver, horse and chicken heart and chicken brain. In addition, evidence for a metabolite of PGE_2 with a PGF like structure was obtained from studies with human liver homogenates. It was not established whether the enzymatic conversion of PGE_2 to $PGF_{2\alpha}$ is reversible during these experiments. Similarly, the failure to detect the conversion of PGE_2 to $PGF_{2\alpha}$ in all the tissues studied may indicate that this type of metabolism is not widely distributed. However, the possibility that the correct co-factors for the detection of this type of metabolism were not being used may exist. Certainly Levine and Lee (1975) maintain that the 9-keto reductase activities in pigeon and monkey tissues is stimulated by $NADPH_2$ but not by $NADH_2$. This could explain why no 9-keto reductase activity was detectable in rat heart, liver or kidney homogenates all of which has previously been described as containing relatively high levels of 9-keto reductase activity (Levine, 1973; Lee and Levine, 1974). Until more detailed comparisons of the two biochemical and subsequent analytical procedures are made it will not be possible to define if either or both results are correct.

The observations that pregnancy or pseudo-pregnancy in rabbits or *in vivo* treatment with Oestradiol & Progesterone in guinea pigs can quantitatively change the metabolism of prostaglandins is significant. It is known that during pregnancy the ability of lung tissue to inactivate circulating PGE and PGF_{α} and PGA increases in the rabbit and sheep (Horton and Maule-Walker, 1974; Bedwani and Marley, 1975). This probably explains why Oakes et al. (1972) claimed that the cardiovascular system of sheep was insensitive to prostaglandins. It had previously been reported that this animal was sensitive to E prostaglandins given intra-aortically but not intravenously (Horton, Main and Thompson, 1965).

The obvious importance of understanding what factors control the biosynthesis and metabolism of prostaglandins hopefully will ultimately reveal the processes by which these compounds function *in vivo*. The significance of the connection between prostaglandin metabolising capacity in other tissues, particularly the lung, and the changes in oestrogen and progesterone hormonal levels may explain how the cardiovascular system in particular is protected from relatively larger levels of prostaglandins reported to be circulating during pregnancy. More detailed studies with particular reference to a correlation between the prostaglandin metabolising capacity of other tissues and the changes in hormonal levels are obviously required.

APPENDIX

During the work reported in this thesis, many occasions have arisen where the conclusive identification of PGE_2 by combined GCMS was essential. Prostaglandins of the E series contain a β-keto ring system that is readily susceptible to dehydration on the gas chromatography systems employed and therefore it was essential to protect this β-keto system by oximation. The initial method of oximating PGE_2 was described by Green (1969). In collaboration with Drs. R.L. Jones and N.H. Wilson the analysis of PGE_2 and 15-keto PGE_2 by combined GCMS using a series of O-alkyl oximes was undertaken. There were three major reasons for wanting to study these oximes :

1) The oximation process results in the formation of two oxime isomers per ketonic function (Syn and anti isomers). This results in the mass of PGE_2 being split between the two oxime isomers and therefore reduces the efficiency of quantitative analysis.

2) The major ions for PGE_2 methyl ester, trimethylsilyl ether o-methyl oxime (second isomer) were at m/e 225 and 295. These ions are thus in the mass spectral region where many interfering biological compounds produce ions. Consequently the analysis of PGE_2 using this derivative can be markedly affected at the nanogram range.

3) The Me TMS derivative of $PGF_{2\alpha}$ does not separate well from the corresponding Me Mo TMS derivative of PGE_2. This is unfortunate because the analysis of PGE_2 and $PGF_{2\alpha}$ together reduces the amount of preparative work required for GCMS. However, when analysed together as the above derivatives the limit of detection for each was reduced relative to the analysis of each individually.

Using the method of Fujii, Wu and Yamada 1967 a series of O-substituted alkyl oximes were prepared. These included the o-iso-propyl, o-n-butyl and o-n-pentyl oximes. The carbon values for each isomer of PGE_2 when chromatographed as the methyl ester trimethylsilyl ether of these oximes is shown below.

PGE_2 oxime	Carbon value	
	1st isomer	2nd isomer
methyl	24.1	24.7
ethyl	24.45	25.1
Iso-propyl	24.6	25.2
n-butyl	26.1	26.95
n-pentyl	27.05	27.85
hydroxylamine	24.35	25.05
$PGF_{2\alpha}$	23.55	

It can be seen that the o-n-butyl and o-n-pentyl-oxime derivatives of PGE_2 are well separated from $PGF_{2\alpha}$ Me TMS. These have successfully been employed for

the analysis of PGE_2 and $PGF_{2\alpha}$ produced by the rabbit kidney by combined GCMS without prior chromatographic separation. Unfortunately the desired preferential formation of one oxime isomer only was not obtained, indeed the ratio of the two oxime isomers remained fairly constant throughout the series.

When the mass spectra of the second isomer for each PGE_2 oxime were studied the ions at m/e 225 and 295 were found to consistently be the two major ions produced. The ion at m/e 225 was proposed to result from the loss of C-1 to C7 (141) and C-15 to C-20 (173) in the methyl ester methoxime TMS derivative of PGE_2 (Green, 1969). However, this could not be the major fragmentation mode because the ion m/e 225 is the base peak in all the oxime isomers of PGE_2 studied. In addition W-dihomo-PGE_2 has the base peak at m/e 253 which indicates that the W-side chain remains intact in this ion. Similarly, when the 15-O methyl ether methoxime TMS derivative of PGE_2 was studied the ions corresponding with m/e 225 and 295 (PGE_2 Me Mo TMS) were found to be at m/e 167 and 237 respectively. This result also indicated that the ion at m/e 295 in PGE_2 could not have resulted entirely from the loss of the α-chain and the oxime as previously predicted (Green, 1969). The results of studying different substituents on the m/e values of these two ions are shown in Figure 40 on page 235 . It was thus concluded from these results that the major mode of fragmentation resulting in the formation of m/e 225 and 295 in PGE_2 Me Mo TMS is as depicted in figure 41 .

on page 236.

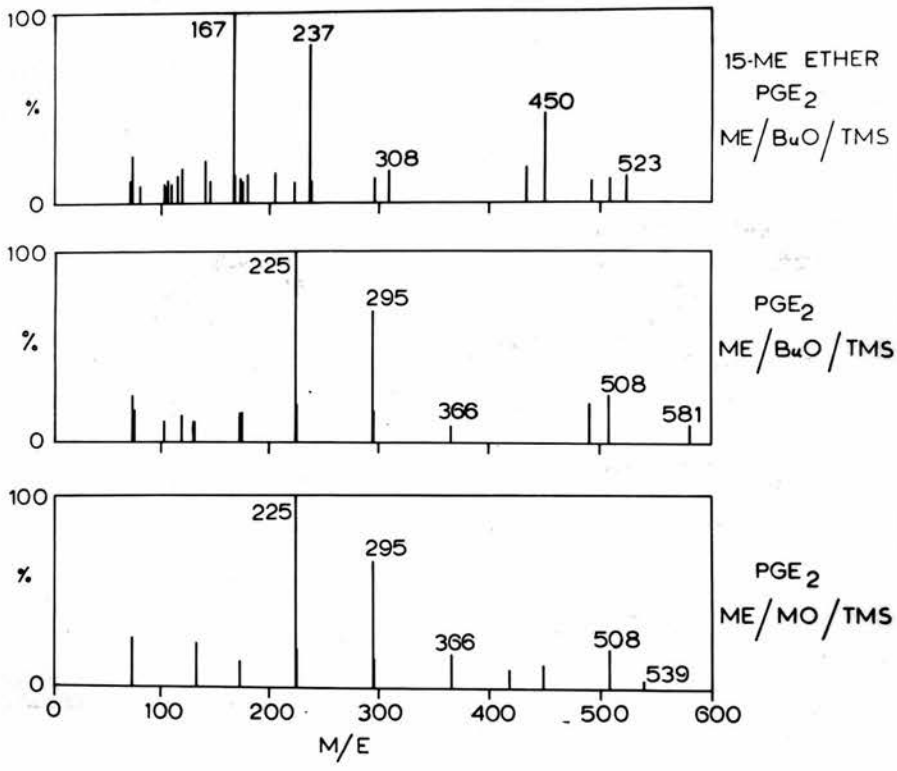

Figure 40. Mass spectra obtained using an LKB 9000 GC-MS at 70 eV of the major isomer (second) eluted from the gas liquid chromatography column (3m x 4mm 3% OV-1 on Supelcoport) for the following derivatives of prostaglandin E_2 methyl ester :- 1) 9-O-n-butyloxime, 11 trimethylsilyl ether, 15 methyl ether (upper); 2) 9-O-n-butyloxime, 11, 15 bis trimethylsilyl ether; 3) 9-O-methyloxime, 11, 15 bis trimethylsilyl ether.

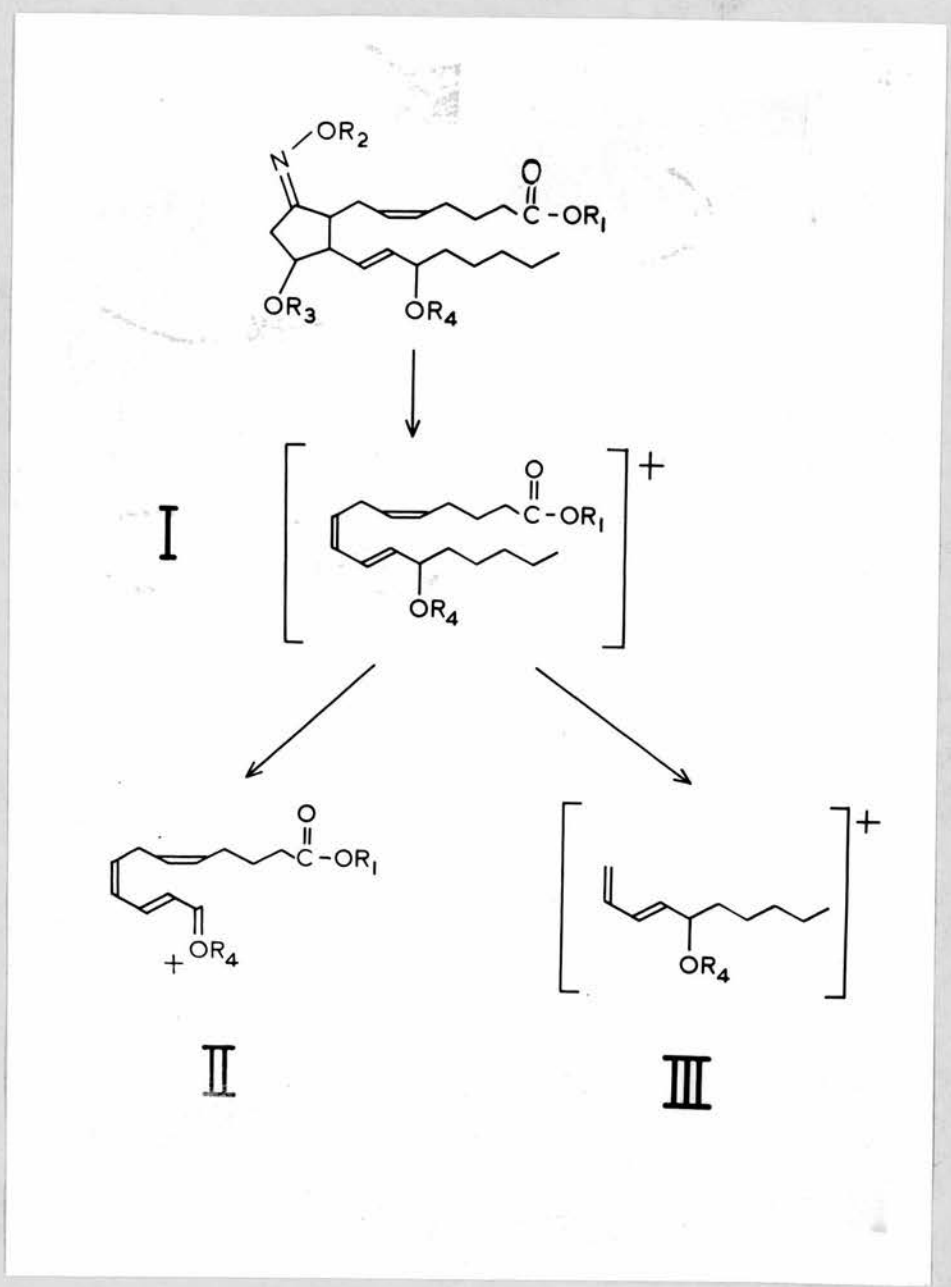

Figure 41. The proposed mode of fragmentation (at 70 eV) for the major isomer (second) of prostaglandin E_2 o-alkyl oximes, resulting in the two major ion intensities present above m/e 150 in the mass spectrum of such compounds.

These ions are therefore postulated to be derived from C8 - C9 and C11 - C12 fissions with additional fissions respectively at C7 - C8 and C15 - C16.

When the bis-o-alkyloximes were studied (e.g. 15-keto PGE_2) similar fragmentation modes were found to occur in the 3rd and 4th oxime isomers (on GLC retention). These isomers are predicted as being isomeric at C-15. The postulated fragmentation mode for 15-keto PGE_2 oxime derivatives is shown in figure 42 on page 238 .

To date the proposed fragmentation modes for the o-alkyloxime trimethylsilyl ether derivatives of PGE_2 and 15-keto PGE_2 have been found to be applicable to all the 2 series E prostaglandins so far studied. One important group that appears to be essential is the presence of a functional leaving group at C-11. In neither 11-deoxy, nor 11-methyl PGE_2 is the above fragmentation mode of any major significance.

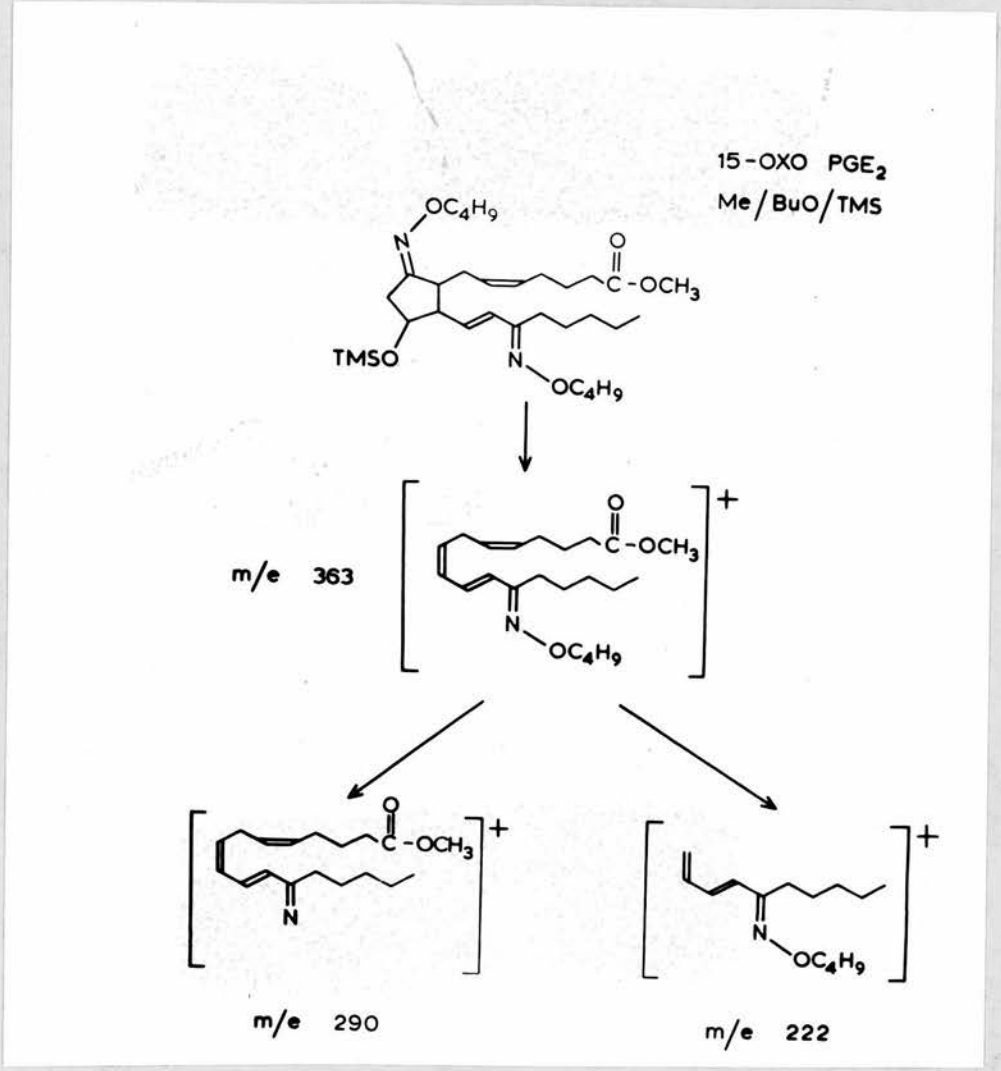

Figure 42. The proposed mode of fragmentation at 70 eV for the major isomers (third and fourth) of 15-keto prostaglandin E_2 bis O-alkyl oximes resulting in the two major ion intensities present above m/e 150 in the mass spectrum of such compounds.

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THE ENZYMATIC CONVERSION OF PROSTAGLANDIN D_2 TO PROSTAGLANDIN $F_{2\alpha}$

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ABSTRACT

Prostaglandins E_2 and D_2 were both converted to prostaglandin $F_{2\alpha}$ (9α,11α) by an enzyme present in sheep blood. Neither the 9ß,11α epimer nor the 9α,11ß epimer was produced from PGE_2 or PGD_2 respectively. The rate of reduction was measured using isotope dilution (D_4 $PGF_{2\alpha}$) and multiple-ion detection gas chromatography-mass spectrometry.

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The reduction of prostaglandin E_2 (PGE_2) to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by an enzyme present in sheep blood has recently been reported[1,2]. Few other examples of this type of conversion have been described[3-7]. These two prostaglandins and a third primary prostaglandin, prostaglandin D_2 (PGD_2) are enzymatically produced from the same 9,11-cyclic endoperoxide[8-10]. Although these three primary prostaglandins (PGD_2, PGE_2 and $PGF_{2\alpha}$) are formed from a common precursor and are not precursors of each other, the results reported here indicate that some interconversion of these three primary prostaglandins is possible. If the enzyme concerned is widely distributed, it may be of importance in the physiological role of prostaglandins since these three compounds have many qualitatively and quantitatively different pharmacological actions[11].

Freshly citrated sheep blood was treated as described previously[1] to produce the six different stock enzyme preparations (A to F) that were used to compare PGE_2 and PGD_2 as substrates for the enzyme producing $PGF_{2\alpha}$. These were incubated at 37°C and a substrate concentration of 5 $\mu g/ml^{-1}$. In addition blank samples were also incubated without any substrate. Aliquots (0.2 ml) were sampled from each preparation at 1, 5, 10, 15, 30. 60 and 90 minutes and extracted into ethyl acetate after the addition of 1 µg of 3, 3, 4, 4, tetradeutero $PGF_{2\alpha}$. The residue obtained from the ethyl acetate was converted to the methyl ester trimethylsilyl ether as described previously[12] and quantitatively assayed by multiple ion detection (MID), gas chromatography-mass spectrometry as reported earlier[13].

The mean results for six experiments are shown in figure 1.

It can be seen that both PGD_2 and PGE_2 produce $PGF_{2\alpha}$ in a time dependent fashion when incubated with sheep blood. Although the enzyme concerned had been shown to produce only the 9α hydroxy epimer ($PGF_{2\alpha}$) from PGE_2, confirmation that PGD_2 was converted to $PGF_{2\alpha}$ only, required more detailed chromatographic and mass spectrometric analysis since the 9α,11α and the 9α,11ß PGF_2 epimers did not separate on the gas liquid chromatography system employed.

The prostaglandin residue obtained at the end of the incubation period (approximately 100 min) from samples D, E and F converted to methyl esters and subjected to straight phase gel chromatography on a lipophilic substituted LH-20 gel using the method described previously[14], co-chromatographed with $PGF_{2\alpha}$ (and not its 11ß-epimer). The chromatographic separation of the 9α,11ß PGF_2 and the 9α,11α PGF_2 epimers gave greater than 98% purity with respect to each other when authentic prostaglandin methyl esters were chromatographed (Figure 2). Further evidence of identifi-

cation of the end product obtained from PGD_2 was obtained by using the cyclic n-butyl boronate ether described previously[15]. The n-butyl boronate derivative is produced only when the hydroxyl groups at the 9 and 11 positions are in the cis configuration. An example of the gas liquid chromatography result obtained with this derivative is shown in figure 3. This evidence combined with the mass spectral evidence confirmed that the end product of the reduction of PGD_2 is $PGF_{2\alpha}$. No evidence was obtained that suggested any of the 11ß epimer was produced.

The experiments described here were designed to compare the reduction of PGE_2 and PGD_2 by an enzyme present in sheep blood and in addition to identify conclusively the reduced products. The enzyme concerned is believed to be prostaglandin specific[1,2] however, further substrate specificity studies are required to confirm this and to characterise the enzyme in more detail.

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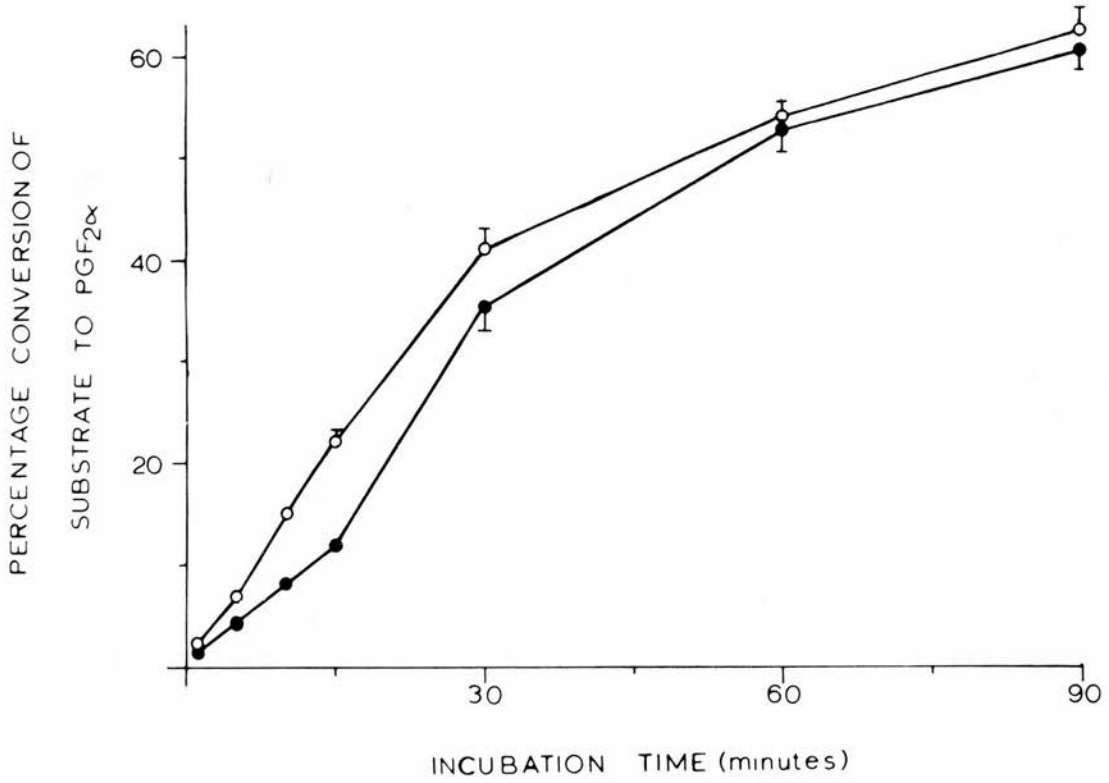


Figure 1: Time course of formation of $PGF_{2\alpha}$ from PGD_2 (O—O) and PGE_2 (●—●). The points represent the mean value + S.E.M. obtained from triplicate determinations of each point in six experiments. Incubation temperature was 37°C and substrate concentration 5 μg ml^{-1}.

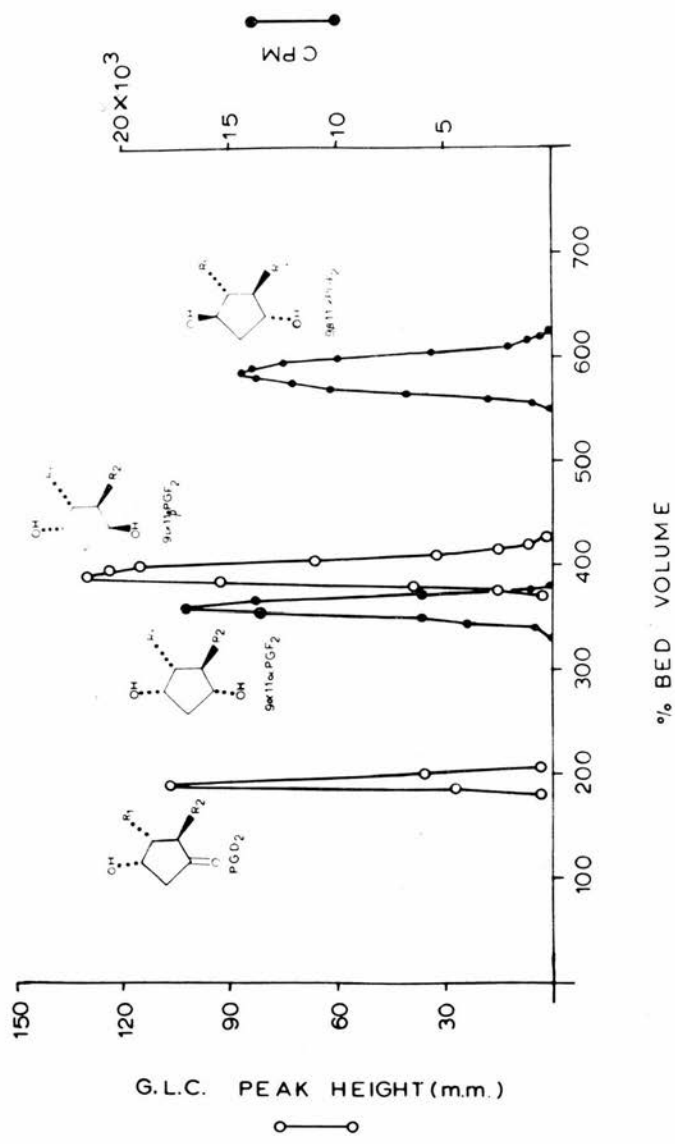


Figure 2: Elution profiles of authentic prostaglandin methyl esters on a N1114-51%-LH20 gel column of 85 ml bed volume eluted with heptane:chloroform (70:30). N1114-51%-LH20 is Sephadex LH20 with hydroxalkyl (C_{14}-C_{14} hydrocarbon chains) group content of 51% (W/W). The flow rate was 6-8 ml.hr^{-1} and fractions of 4.25 ml (5% bed volume) were collected.

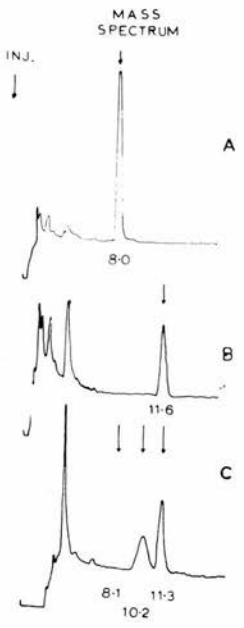
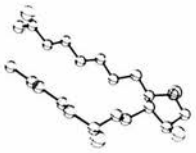


Figure 3: Gas liquid chromatograms of authentic 9α11$\alpha$$PGF_2$ and 9α11$\beta$$PGF_{2\alpha}$ and also the 9α11$\alpha$$PGF_2$ zone from 1 liquid gel chromatography of sample E. These were prepared as the methyl ester (diazomethane) n-butyl boronate (n-butylboronic acid in 2,2 dimethoxy propane) trimethyl silyl ether (BSTFA). The n-butyl boronate on forms the 9,11 cyclic ether when the 9,11 hydroxyl are cis to the ring consequently 9α11$\beta$$PGF_2$ under these circumstances produces the methyl ester trimethyl silyl ether derivative only.

A) Authentic 9α11$\beta$$PGF_2$. The mass spectrum of this G.L.C. peak was characteristic of the Me TMS derivative.

B) Authentic 9α11$\alpha$$PGF_2$. The mass spectrum of this peak was characteristic of the Me NBB TMS derivative.

C) 9α11$\alpha$$PGF_2$ zone from the 51% LH20 gel column. Three mass spectral scans at 8.1, 10.2 and 11.3 minutes showed that only the Me NBB TMS derivative of 9α11$\alpha$$PGF_2$ was present (11.3).



ANNOUNCEMENT

The Vail Conference on Prostaglandins

The fourth annual conference on Recent Advances in Prostaglandins Research will take place in Vail, Colorado, March 9-13th, 1975. The tentative program includes sessions on the following topics: Analogue Chemistry; Prostaglandins and Animal Reproduction; The Nervous System; and the Renal-Cardiovascular System.

As in previous meetings, participation and attendence will be by invitation only. If readers of the Journal are interested in attending the meeting, please write to the Editors of the Journal, stating the following: area of research interest; and intention to present new information.

Attendence will be limited to approximately 100 scientists insuring that an air of informality with a free exchange of ideas (even a few arguments) will prevail.

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DISTRIBUTION STUDIES ON THE REDUCTION OF PROSTAGLANDIN E_2 TO PROSTAGLANDIN $F_{2\alpha}$ BY TISSUE HOMOGENATES

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Summary

Liver homogenates from guinea-pig, rabbit and horse, and heart homogenates from the horse have been found to convert prostaglandins E_1 and E_2 to prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$, respectively. The end products have been provisionally characterised by thin-layer chromatography, and conclusive identification was carried out by gas-liquid chromatography-mass spectrometry.

Introduction

The reduction of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ by an enzyme present in the cellular fraction of sheep blood has recently been reported [1,2]. This type of metabolism, namely reduction of the 9-oxo group has been conclusively described in only a few instances. The first example was the demonstration that prostaglandin $F_{2\alpha}$ is produced from prostaglandin E_2 when incubated with guinea-pig liver homogenates in vitro [3].

Many of the pharmacological actions of prostaglandin E_2 and prostaglandin $F_{2\alpha}$, differ both qualitatively and quantitatively an example being that prostaglandin E_2 is a bronchodilator whereas prostaglandin $F_{2\alpha}$ is a bronchoconstrictor [5]. Although these two prostaglandins are both formed enzymatically from the same precursor (arachidonic acid), there is no interconversion of prostaglandin E and prostaglandin F_α in their biosynthesis [6,7]. The discovery of an enzyme in sheep blood which reduces prostaglandin E to prostaglandin F_α raised the possibility that this conversion may be widespread in occurrence and of biological importance. It was thus felt necessary to ascertain whether the conversion of prostaglandin E_2 into prostaglandin $F_{2\alpha}$ was a widely distributed phenomenon.

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Materials and Methods

Preparation of tissue homogenates. Tissues from guinea-pig, rat, dog, cat, cow, sheep, horse, pig and rabbit of both sexes, were removed as soon as possible after death (kept on ice if transport was required) and chopped into small pieces approx. 1 cm^3. These were then homogenised in 3 volumes (by weight) of ice-cold 0.1 M sodium phosphate buffer (NaH_2PO_4, 2.7 g; Na_2HPO_4 12.54 g in 1 l of distilled water) using a Polytron homogeniser at maximum speed. The resulting homogenate was then centrifuged at 4° C for 30 min at 2400 × *g* and the supernatant was carefully decanted and stored on ice until used.

Incubation and extraction procedure. The resulting supernatant was added to a 100 ml conical flask containing tritiated prostaglandin E_2 (Radiochemical Centre, England, [5,6,8,11,12,14,15-^{3}H] prostaglandin E_2 spec. act., 36.4 Ci/mol) at a substrate concentration of 14 μM and a cofactor concentration of NAD and NADH of 10 mM each. The samples were then incubated at 37° C for 2 h (with agitation) in a water bath.

The products of the incubation were diluted to 150 ml with distilled water and acidified to pH 4 with 10% acetic acid. After extraction twice with 2 volumes of either diethyl ether or diethyl ether/ethyl acetate (4 : 1, v/v), the pooled organic phases were washed twice with 50 ml of distilled water before being taken to dryness under vacuum and desiccated.

The resulting residue was dissolved in 50 ml of 67% by volume aqueous ethanol and partitioned twice with one equal volume of either benzene or toluene, before the aqueous phase was taken to dryness and desiccated. Where prostaglandin $F_{2\alpha}$ appeared to be a metabolite of prostaglandin E_2, the experiment was repeated at a 10-fold increase of substrate concentration (140 μM) and in addition [2-^{14}C] prostaglandin E_1 was compared in parallel at the same substrate concentration.

Preparation of methyl esters. The residue from the 67% ethanol phase was dissolved in excess diazomethane in diethyl ether/methanol (9 : 1, v/v) and left at room temperature for 15 min to allow methyl esters to form. Where necessary (e.g. if material had precipitated out of solution) the resulting solution was filtered through a glass wool plug inserted in a Pasteur pipette. The original flask was then washed with excess methanol (5—10 ml) and this was also filtered. The pooled solution was then taken to dryness under vacuum and desiccated.

Straight phase gel chromatography. Glass columns (300—400 × 10 mm) were silanized by treatment for a minimum of 2 h at room temperature with a 5% dichlorodimethylsilane (Sigma) solution in toluene (v/v). These were then washed thoroughly with chloroform and methanol to remove excess silanizing agent and plugged at the base with glass wool. These columns were then packed with a lipophilic Sephadex LH-20 gel (Lipidex 5000) as described previously by Brash and Jones [8]. This gel has been substituted with hydroxyalkyl groups (C_{1-4} hydrocarbon chain lengths) to 50% by weight. Prior to packing, the gel had previously been equilibrated with a heptane/chloroform (80 : 20, v/v) mixture and was further equilibrated in situ before being used. A constant pressure head at the top of the column was maintained and a flow rate of 6—12

ml/h was obtained. Room temperature was maintained at 20 ± 1°C. The sample to be chromatographed (as the methyl esters) was dissolved in 100—200 μl of chloroform and then heptane was added until the solvent composition was the same as the column eluant. The total loading volume (including washings) was never greater than 1.5 ml.

The column effluent was led via narrow bore Teflon tubing to the drop-counting head of an LKB Ultrorac fraction collector. Fractions, equivalent to 10% bed volume, were collected until the 2000% bed volume point was reached.

The elution profile of the column was obtained by liquid scintillation counting of an aliquot of each fraction. These columns have been found to be re-usable and reproducible both for authentic standard prostaglandins and also biological extracts. Columns which had begun to lose performance (as shown by peak broadening) were purged by reverse flowing the column with a more polar solvent (e.g. methanol/chloroform/heptane, 10 : 50 : 50, by vol.) before re-equilibrating in the original solvent.

From the elution profile it was possible to bulk several fractions into corresponding zones. These were taken to dryness and re-dissolved at 1.0 ml of methanol and an aliquot counted by liquid scintillation counting to determine the percent of the total recovered radioactivity.

Thin-layer chromatography. Neutral silica gel (200 mm × 200 mm × 0.25 mm) on glass plates (Merck) was used. Marker standards containing [^{3}H]-prostaglandins $F_{2\beta}$, $F_{2\alpha}$, E_2, 13,14-dihydro E_1 and A_2 methyl esters were spotted on the plate at the side of the samples being chromatographed. The plate was then developed in a closed equilibrated glass tank using a modified form of a thin-layer solvent described previously [9]. The solvent was ethyl acetate/methanol/acetic acid (110 : 5 : 1, by vol.). This system was found to give a good resolution of many prostaglandins and their metabolites.

Liquid scintillation counting. This was performed with a Mark II Nuclear Chicago Liquid scintillation counter.

Aliquots of either the individual column fractions or the methanol solution containing the pooled fraction residue were counted in 5 ml of a toluene, ethoxyethanol-based scintillant mixture (toluene/ethoxyethanol, 1500; 900, v/v; DMPOPOP/PPO/napthalene, 0.9 : 10.5 : 112.5, by weight). Where dpm were required these were obtained by correcting for quenching using the automatic external standard channels ratio method and a quench-calibration curve constructed for the scintillation mixture.

Radio gas chromatography. A Pye series 104 gas chromatograph interfaced with a Panax radio detector enabled the simultaneous recording of mass and radioactivity. The gas chromatograph stationary phase was 3% OV-1 on Supelcoport 100—120 mesh. The oven temperature was 230—240°C, the carrier gas (argon) flow rate was 35—40 ml/min and the oxidation furnaces were at 650—680°C.

Carbon values of compounds were obtained from a log retention time versus carbon value for normal saturated fatty acid methyl esters as described previously [10].

Combined gas-liquid chromatography — mass spectrometry. Mass spectra were obtained using an LKB 9000 combined gas chromatograph — mass spec-

trometer, fitted with a glass column packed with 3% OV-1 on Supelcoport (100—120 mesh). The oven temperature was 240—260°C and the carrier gas (helium) flow rate was 30—35 ml/min.

Preparation of derivatives for gas-liquid chromatography — mass spectrometry. An aliquot of the methanol residue from the appropriate zone of the Lipidex 5000 columns was taken to dryness and desiccated. Trimethylsilyl ethers were prepared by treating the residue with *N*, *N*-bis (trimethylsilyl)-trifluoroacetamide by the method described previously [11]. The prostaglandin derivatives were injected into the gas chromatograph in 5—10 µl of this reagent.

Purification of [^{3}H] prostaglandin E_2. An aliquot (approx. 0.5 µCi) of the tritiated prostaglandin E_2 free acid (The Radiochemical Centre, Amersham, England: [5,6,8,11,12,14,15-^{3}H] prostaglandin E_2, spec. act. 167 Ci/mmol) was found by thin-layer chromatography to be contaminated by a less polar compound that had a chromatographic behaviour identical to that of unlabeled prostaglandin A_2 free acid.

The total [^{3}H] prostaglandin E_2 (1.5 mCi, 3.5 µg) was then purified by reversed phase partition chromatography on a graded LH-20 column (essentially as described previously [1]). The glass column used (300 × 10 mm) was packed with graded LH-20 (50 ± 5 µM diameter size particle) previously equilibrated in the solvent mixture (methanol/water/chloroform/butanol/acetic acid, 50 : 50 : 5 : 5 : 0.1, by vol.) and allowed to flow at a constant pressure head for 12 h before use. The column bed volume was 20 ml, the flow rate 7—8 ml/h and the room temperature 23 ± 1°C. Effluent fractions of 2.0 ml volume were collected as described previously and a 5 µl aliquot counted. The chromatographic step yielded 1.0 mCi of tritiated prostaglandin E_2 that was greater than 99% pure by thin-layer chromatography.

[2-^{14}C] Prostaglandin E_1 (New England Nuclear, spec. act. 40 Ci · mol^{-1}) was found to be greater than 99% pure when tested in the above thin-layer chromatography systems and thus was not further purified.

Solvents and chemicals. All solvents were of analytical grade or redistilled before use. The chloroform was redistilled over anhydrous $CaCl_2$ and 1% (by vol.) of absolute ethanol was added to the redistillate to stabilize it.

Results

The liver of horse, rabbit and guinea-pig and horse heart have been found in these studies to be capable of converting prostaglandin E to prostaglandin F_α. The average degree of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ conversion was 14; 42; 9 and 12%, respectively, as determined by the recoveries from the Lipidex 5000 columns. At no time has any evidence been obtained for the conversion of prostaglandin E to prostaglandin F_β.

Separation of prostaglandin metabolites by Lipidex 5000

The separation of methyl esters of authentic tritiated prostaglandins A_2, E_2, $F_{2\alpha}$ and $F_{2\beta}$ is shown in Fig. 1A. There was good separation of these prostaglandins on the lipophilic LH-20 gel chromatography system employed.

Fig. 1B shows an example of the elution profile obtained when a rabbit liver homogenate (after conversion to methyl esters) is chromatographed on the

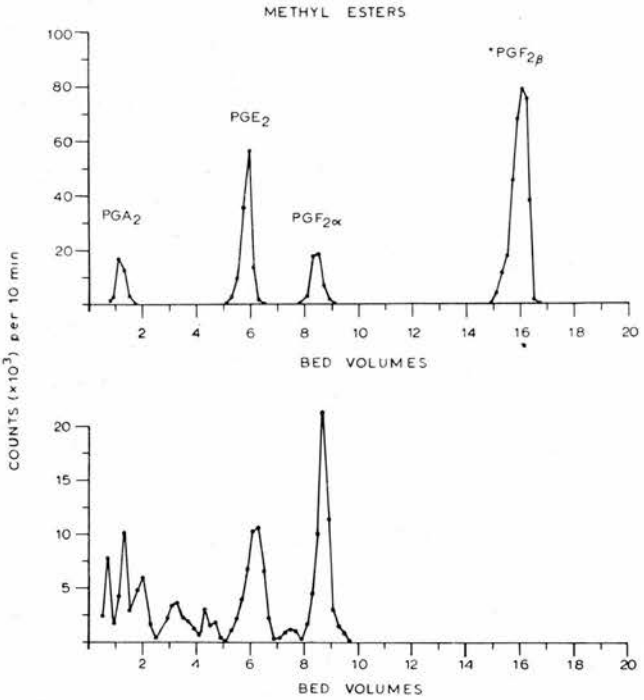

Fig. 1. (A) The upper diagram represents the chromatographic separation on Lipidex 5000 of authentic ^{3}H-labelled prostaglandins A_2, E_2, $F_{2\alpha}$ and $F_{2\beta}$ methyl esters. (B) The lower trace represents the elution profile on Lipidex 5000 (after conversion to methyl esters) of an extract from rabbit liver incubated for 2 h at 37° C with prostaglandin E_2.

same column as the authentic standards shown in Fig. 1A immediately after these standards had been chromatographed. It can be seen from Fig. 1B that rabbit liver homogenates produce several metabolites when incubated with prostaglandin E_2 at least one of which is more polar than prostaglandin E_2 and has chromatographic properties identical with prostaglandin $F_{2\alpha}$ in this system.

The majority of these metabolites have only been provisionally identified

TABLE I

Prostaglandin	Mean R_F (methyl ester) modified GMC solvent	Mean percent bed volume
E_2	0.38	5.8
$F_{2\alpha}$	0.23	8.5
$F_{2\beta}$	0.16	15.7
A_2	0.61	1.4
15-oxo E_2	0.55	2.9
15-oxo $F_{2\alpha}$	0.49	3.9
13,14-dihydro, 15-oxo E_2	0.58	1.7
13,14-dihydro, 15-oxo $F_{2\alpha}$	0.50	1.9
13,14-dihydro E_1	0.48	—
13,14-dihydro $F_{1\alpha}$	0.39	—

Abbreviation: GMC, gas-liquid chromatography-mass spectrometry.

by their retention volumes on Lipidex 5000 and their R_F values on thin-layer chromatography. These values for authentic prostaglandin standards are given in Table I. The separation of prostaglandins E_2, $F_{2\alpha}$ and $F_{2\beta}$ was essential because it has been shown by Hamberg and Samuelsson [4] that in the guinea-pig in vivo, prostaglandin E_2 is reduced to the β isomer as indicated by a urinary metabolite with the prostaglandin $F_{2\beta}$ configuration. In vitro, however, it has been shown that guinea-pig liver homogenates produce prostaglandin $F_{2\alpha}$ as one of the metabolites of prostaglandin E_2 [3]. The possibility, therefore, existed that the 9-oxo group of prostaglandin E_2 could be reduced to either the 9α or 9β alcohol by some of the tissues being studied. The use of these columns have enabled the detection of approx. 1% metabolism of prostaglandin E_2 to be detected.

Thin-layer chromatography

It was suspected that the peak corresponding to fractions 51—64 on the Lipidex 5000 column (shown in Fig. 1B) contained at least two compounds.

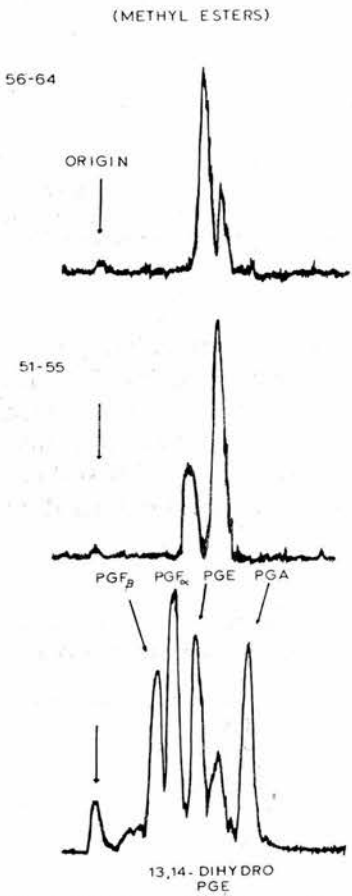

Fig. 2. Thin-layer chromatographic separation. (A) The lower trace represents the separation of authentic ^{3}H-labelled prostaglandins F_β, F_α, E, 13,14-dihydroE and prostaglandin A methyl esters. (B) The middle and upper trace represent the separation of fractions 51—55 and 56—64, respectively, obtained from the Lipidex 5000 elution profile represented in Fig. 1.

Although this peak overlaps with the peak corresponding to authentic prostaglandin E_2 it is much broader. Thin-layer chromatography of these fractions confirmed this was so, (Fig. 2). Fractions 51—55 mainly contained a material that was identical with authentic 13,14-dihydro-prostaglandin E_1 methyl ester, together with a smaller amount of material identical with prostaglandin E_2 methyl ester. In contrast fractions 56—64 contained material most of which was identical with prostaglandin E_2 methyl ester and a smaller portion that was identical with 13,14-dihydro-prostaglandin E_1 methyl ester. The 1 and 2 series prostaglandins do not separate on the thin-layer chromatography systems employed.

The tissues so far studied have all been found to produce less polar compounds than prostaglandin E_2 and these have provisionally been identified by their mobilities on Lipidex 5000 and by thin-layer chromatography. The two major metabolites produced by most tissues were provisionally identified by the above methods as 15-oxo-prostaglandin E_2 and 13,14-dihydro,15-oxo-prostaglandin E_2; where prostaglandin $F_{2\alpha}$ has been found as a metabolite the corresponding prostaglandin $F_{2\alpha}$ metabolites have also been identified.

Both the Lipidex 5000 gel column chromatography and the thin-layer chromatography have been found to give comparable separations of the prostaglandin E_1 metabolites produced in parallel with the prostaglandin E_2 metabolites.

Radio gas chromatography

The tissues found to convert prostaglandin E_2 into prostaglandin $F_{2\alpha}$, namely, homogenates from guinea-pig, rabbit and horse liver and horse heart were incubated with [2-^{14}C]prostaglandin E_1. These tissues produced several metabolites, one of which was provisionally identified by the above methods as prostaglandin $F_{1\alpha}$. An example of this is shown in Fig. 3. Here it can be seen from the upper trace that the methyl ester trimethylsilyl ether of both prostaglandins $F_{1\beta}$ and $F_{1\alpha}$ separate on the gas chromatography system employed. The lower half of Fig. 3 shows material produced by a rabbit liver homogenate and provisionally identified by Lipidex 5000 mobility and thin-layer chromatography as prostaglandin $F_{1\alpha}$. It can be seen that when subjected to gas-liquid chromatography as the methyl ester trimethylsilyl ether, this material has a retention time for both mass and radioactivity (^{14}C) identical with that of authentic prostaglandin $F_{1\alpha}$ methyl ester trimethylsilyl ether.

Radio gas chromatography was not performed on any of the prostaglandin E_2 metabolites produced by any tissues because ^{14}C-labelled prostaglandin E_2 was not available and the radio gas chromatography system employed was not sufficiently sensitive to detect the levels of ^{3}H-labelled metabolites produced.

Mass spectrometry

Conclusive evidence that prostaglandins E_1 and E_2 were being converted into the corresponding prostaglandin F_{α} compounds was obtained by combined gas chromatography-mass spectrometry. Homogenates of horse, guinea-pig and rabbit liver and of horse heart produced material which, as the methyl ester trimethylsilyl ether, chromatographs identically with authentic prostaglandin $F_{2\alpha}$. Mass spectral scans taken at the crest of each gas chromatography

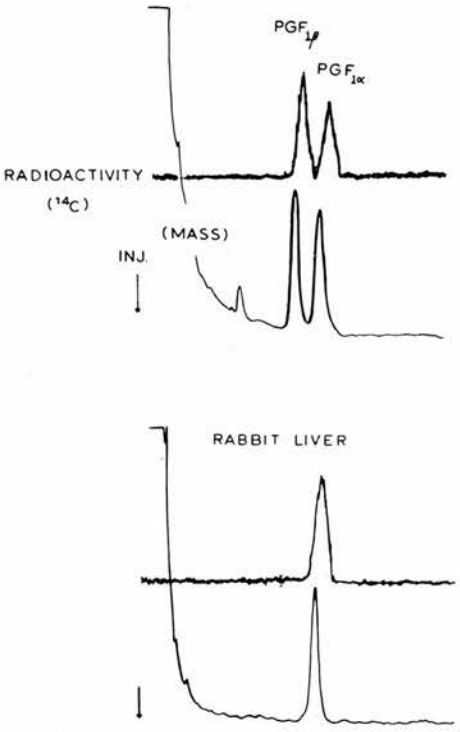

Fig. 3. Radio gas chromatographic separation. (A) The upper trace represents the separation of authentic ^{14}C-labelled prostaglandins $F_{1\beta}$ and $F_{1\alpha}$ as their methyl ester trimethylsilyl derivatives. (B) The lower trace represents the material eluted in the prostaglandin $F_{1\alpha}$ zone from Lipidex 5000 of an extract of rabbit liver incubated for 2 h at 37°C with prostaglandin E_1. Again the derivative is the methyl ester trimethylsilyl derivative.

peak were identical to that of authentic prostaglandin $F_{2\alpha}$. Prominent ions were at *m/e* 584 (M^+); 569 (M—15); 513 (M—71); 494 (M—90); 423 (M—90 + 71); 404 (M—2 × 90); 333 (M—2 × 90 + 71) 217; 199; 191; and 173. These ions are characteristic of prostaglandin $F_{2\alpha}$ methyl ester trimethylsilyl ether. Similar results were obtained with the prostaglandin $F_{1\alpha}$ zones produced from incubating the tissues with [2-^{14}C] prostaglandin E_1.

The corresponding prostaglandin $F_{1\beta}$ and $F_{2\beta}$ compounds are separated from the prostaglandin $F_{1\alpha}$ and $F_{2\alpha}$ by the gas-liquid chromatography systems used. No detectable levels of these β epimers were found.

Discussion

The purpose of the experiments described in this paper was to establish whether the conversion of prostaglandin E_2 to the corresponding prostaglandin F_α previously found to occur in a variety of systems [1—3,12—16] was a widely distributed phenomenon.

In the present studies in addition to confirming the presence of an enzyme in guinea-pig liver that reduces prostaglandin E_2 to prostaglandin $F_{2\alpha}$ three other conclusive examples have been described. These are the liver of horse and

rabbit and the horse heart. In these tissues both prostaglandins E_1 and E_2 have been found to be equally good substrates for the enzymes that reduce prostaglandin E to prostaglandin F_α.

It is not yet established whether the enzymatic conversion of prostaglandin E to F_α is reversible. However, much evidence is accumulating for the conversion of the prostaglandin E into the corresponding prostaglandin F_α in certain tissues.

The failure of the experiments described in this paper to detect this type of prostaglandin E metabolism in all tissues investigated, may indicate that this is not a widely distributed type of metabolism. Alternatively, it may be that an excess of other prostaglandin E-metabolising enzymes makes difficult the detection of any reduction of the 9-ketonic substituent. Further studies are being conducted to obtain a prostaglandin E_2 analogue that is a good substrate for 9-oxo-reducing enzymes but that is not metabolised by other prostaglandin-metabolizing enzymes. Such a substrate would have obvious advantages in the study of the conversion of prostaglandin E_2 into prostaglandin $F_{2\alpha}$. One such analogue at present under investigation is the 15-methyl ether of prostaglandin E_2.

Other factors such as age, sex and pregnancy that may affect the conversion of prostaglandin E_2 into prostaglandin $F_{2\alpha}$ by rabbit and guinea-pig liver homogenates are at present under investigation.

The possibility that the reduction of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ may be of a modulatory nature as suggested previously [15] obviously requires further elucidation. This and further distribution studies of the 9-oxo reductase are at present being conducted in an attempt to elucidate its possible physiological, biochemical or pathological function.

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