# PROSTAGLANDINS OF UROGENITAL ORIGIN

by

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# CONTENTS

## INTRODUCTION

Prostaglandins were discovered by the Swedish
scientist Ulf von Euler, who observed pharmacological
effects with semen and prostatic/vesicular extracts
and established beyond doubt that the active principle,
which he named prostaglandin, belongs to a completely
new group of naturally-occurring substances (von Euler,
1934, 1935, 1936, 1939). Others had observed this
biological activity previously but had not pursued
the investigation much further than reporting the
phenomenon.

It was another two decades before the active
principles were isolated by Bergström and Sjövall
(1960a, b). Isolation of two prostaglandins ($E_1$
and $F_{1\alpha}$), assigned the empirical formulae $C_{20}H_{34}O_5$
and $C_{20}H_{36}O_5$ respectively, was followed by the
elucidation of their full chemical structures (Bergström,
Ryhage, Samuelsson and Sjövall, 1963). This work pro-
vided a tremendous impetus to biological research and
the field has advanced rapidly in the past 15 years.

The biosynthesis of prostaglandins from arachidonic
acid, and from its precursor, dihomo-gamma-linolenic
acid, was discovered independently by van Dorp,
Beerthius, Nugteren and Vonkeman (1964) and by Bergström

Danielsson and Samuelsson (1964) (Fig. 1). Inhibition of prostaglandin biosynthesis by aspirin, indomethacin and pharmacologically-related drugs was subsequently reported by Vane and his colleagues (Vane, 1971; Ferreira, Moncada and Vane, 1971; Smith and Willis, 1971).

Meanwhile the prostaglandin field developed on three main lines: (1) the discovery of their pharmacological actions on numerous biological systems including the kidney and the uterus (see Horton, 1965, 1969, 1972; Bergström, Carlson and Weeks, 1968; Ramwell, 1973, 1975, 1977; Karim, 1975, 1976a, b): (2) the discovery of their presence (and biosynthesis) in virtually every animal tissue investigated (see Bergström and Samuelsson, 1965; Bergström and Bernhard, 1973; Samuelsson and Paoletti, 1976), and (3) the discovery of their release from organs in response to a variety of chemical and physical stimuli (see Ramwell and Shaw, 1967, 1970; Vane, 1969; Horton, 1972). All these findings tended to suggest that prostaglandins might have local regulatory functions in different biological systems. For the most part any role as a circulating hormone seemed to be ruled out by the observation that as much as 90 to 95% of infused prostaglandins $E_2$ and $F_{2\alpha}$ is removed by a single passage through the pulmonary circulation (Ferreira and Vane, 1967), although this argument may not apply to prostaglandins of the A series which are not removed in this way (Horton and Jones, 1969).
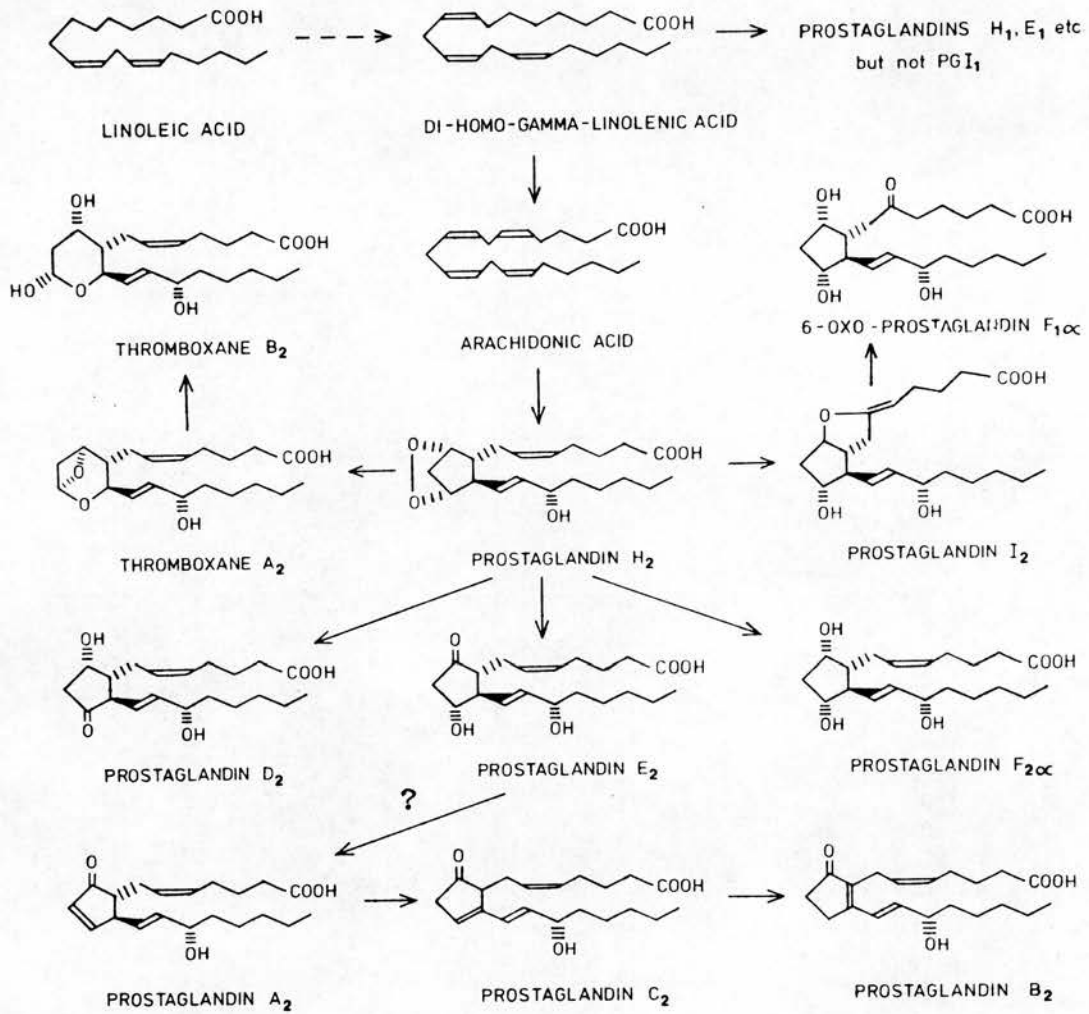
Figure 1    Biosynthesis of Prostaglandins

Interest has long focussed upon the possible physiological and pathophysiological roles of prostaglandins. In this study three of these have been investigated. All relate to organs of the urogenital tract.

Adrenergic Nerve Terminals    As early as 1938 von Euler had shown that in the rabbit, pressor responses to adrenaline are reduced following prostaglandin administration (von Euler, 1938). When pure prostaglandin $E_1$ became available, this observation was confirmed (Holmes, Horton and Main, 1963). Similar effects were observed, however, on vascular responses to noradrenaline, vasopressin and angiotensin. It was therefore postulated that $PGE_1$ acts upon a point in the final common path for the actions of all these vasoconstrictor drugs. A similar conclusion was reached simultaneously by Steinberg, Vaughan, Nestel and Bergström (1963) from their work on the rat epididymal fat pad in vitro. At the Nobel Symposium on Prostaglandins in 1966 it was proposed, from this and other evidence, that the mode of action of $PGE_1$ is to inhibit adenylate cyclase (Steinberg and Vaughan, 1967;  Butcher, Pike and Sutherland, 1967). At the same symposium Ramwell and Shaw (1967) reported that adrenergic nerve stimulation releases a prostaglandin-like substance from the rat epididymal fat pad in vitro. Thus a physiological mechanism was proposed in which the actions of noradrenaline released on nerve

stimulation are partly inhibited by simultaneously released prostaglandins of the E series, that is by a post-synaptic effect.

All these experiments were *in vitro* on saline-perfused tissues. We therefore decided to investigate these phenomena in another adrenergically-innervated organ but *in vivo*. Electrical stimulation of the blood-perfused dog spleen resulted in an enormous output of prostaglandin $E_2$ (Davies, Horton and Withrington, 1968) though other lipids including a prostaglandin F-like substance were also released. The post-synaptic hypothesis was then investigated in the same preparation by infusing prostaglandins intra-arterially. Little or no effect on splenic responses to either noradrenaline or electrical stimulation using either prostaglandin $E_1$ or prostaglandin $E_2$ could be detected (Davies and Withrington, 1968). The hypothesis of Ramwell and Shaw did not appear to extend to the dog spleen. However, encouraged by our observations on release, Hedqvist (1969) studied the effect of prostaglandin $E_2$ on the perfused cat spleen. He too detected little effect on the responses to noradrenaline but the responses to electrical stimulation of the nerve were greatly diminished during prostaglandin $E_2$ infusion. He demonstrated that this was a pre-synaptic action resulting in a reduction of transmitter outflow. This in turn led to the hypothesis that adrenergic nerve stimulation releases not only noradrenaline but also (as in the dog spleen) prostaglandin $E_2$ and that this prostaglandin

exerts a physiological role by reducing the amount of transmitter released from adrenergic neurones on continued electrical stimulation (as in the cat spleen) (Hedqvist, 1971, 1973).

The arguments for and against Hedqvist's hypothesis have been presented previously (Horton, 1973, 1976a). The chief weaknesses in the evidence have been the lack of conclusive identification of the prostaglandin actually released (notably from the cat spleen) and the necessity to use evidence from different organs and species in order to present a unified hypothesis. The need to overcome both these criticisms was the reason for the investigation presented in part 1 of the Experimental Section of this thesis.

## Human Seminal Prostaglandins

Prostaglandins were first discovered in human semen where they occur in higher concentrations than in any other known mammalian tissue or tissue fluid. From the earliest times it has been suggested that they might have some role in reproduction and possibly be essential for full fertility of the human male (von Euler, 1939, 1966; Asplund, 1947; Eliasson, 1959; Hawkins and Labrum, 1961). Evidence in support of this contention has been presented by Bygdeman, Fredricsson, Svanborg and Samuelsson (1970) who showed that the seminal prostaglandin E concentration of men in infertile marriages with no abnormal clinical or laboratory

findings was lower (18 μg/ml) than that of men of proven recent fertility (54 μg/ml), a difference that was highly significant statistically.

The mechanism by which prostaglandin E compounds aid conception is unknown, there have been many speculations but few positive results of relevance. Interactions of prostaglandins with sperm, effects on seminal vesicle emptying and effects on the female reproductive tract following sexual intercourse have all been invoked. Certainly the possibility that biologically active substances such as the prostaglandins may be produced in one individual of the species (the male) and exert their physiological action upon target organs in a second individual (the female) is interesting as a biological mechanism. It would place the seminal prostaglandins in a category somewhat analogous to pheromones (Horton, Main and Thompson, 1963). Von Euler (1966) coined the name 'exohormone' for substances of this type.

The discovery of biosynthesis inhibition by drugs such as aspirin, referred to above, provided a readily available tool for investigators wishing to study the physiological roles of prostaglandins in animals and man. In part 2 of the Experimental Section of this thesis the levels of seminal prostaglandins were measured in human volunteers before, during and after the administration of aspirin for a three-day period, to see how far it was possible to induce changes similar

to those observed in the apparently healthy but
infertile males referred to above.

## Prostaglandins and the Uterine Luteolytic Hormone

One of the many unexpected biological properties
of the prostaglandins was reported by Pharriss and
Wyngarden (1969) who first showed that prostaglandin $F_{2\alpha}$,
unlike other natural prostaglandins, is a potent luteo-
lytic agent in pseudopregnant rats.    Given parenterally
in high doses this compound reduces the duration of
pseudopregnancy and lowers the level of progestogens
produced by the corpora lutea.    It was soon confirmed
that this luteolytic effect is seen in many other
species under conditions where functional corpora lutea
are present - these included rabbit, guinea-pig, monkey,
cow, horse and pig (see Horton and Poyser, 1976).
In the last three, considerable veterinary application
has been made of this observation using both natural
prostaglandin $F_{2\alpha}$ and synthetic analogues.

Considerable evidence had accumulated over the
years for the existence of a luteolytic hormone pro-
duced by the uterus and acting upon the corpus luteum
within the ovary.    All the evidence in guinea-pigs and
sheep tended to point to some local mechanism, the
hormone reaching its target via a vascular route other
than the systemic circulation.    All attempts to isolate
and characterise luteolysin were foiled by the small
amounts of material available relative to the amounts

required for assay during the purification procedure.
At the time of the discovery of the luteolytic action
of prostaglandin $F_{2\alpha}$, it was generally believed that
luteolysin was a peptide or protein.

Several procedures have been shown to stimulate
premature luteolysis, for example the presence of
beads in the lumen of the uterus and the injection of
oestrogen early in the cycle. There was also evidence
that these acted by releasing luteolytic hormone from
the uterus. This was the starting point for the
investigation in guinea-pigs described in part 3 of
the Experimental Section of this thesis. The historical
background and more recent developments have been the
subject of several reviews (Horton and Poyser, 1976;
Anderson, Bland and Melampey, 1969; Denamur and Netter,
1973; Rowson, 1970; McCracken, Carlson, Glew, Goding,
Baird, Green and Samuelsson, 1972; Pharriss, Tillson
and Erickson, 1972).

## Nomenclature and Abbreviations

The basic 20-carbon skeleton of the prostaglandins
has been named prostane, the corresponding monocarboxylic
acid being prostanoic acid. The full chemical names of
all natural prostaglandins are derived by reference to
this basic skeleton. Since these full names are long
and tedious to use, trivial names have been retained for
most prostaglandins and these have been abbreviated fur-
ther throughout the text of this thesis. The structural

formulae of the 2 series of prostaglandins showing their route of derivation from arachidonic acid are illustrated in Figure 1. Prostaglandins of the 1 series lack the 5,6-cis double bond whereas those of the 3 series have an additional 17,18-cis double bond. The full chemical names of the chief prostaglandins dealt with in the study, together with the abbreviations used in this thesis are given in Table 1. Prostaglandin nomenclature was the subject of an excellent paper by Nelson (1974).

TABLE 1: Names and designations of some prostaglandins

| Trivial name | Abbreviation (used in this thesis) | Chemical name |
|---|---|---|
| Prostaglandin $E_1$ | $PGE_1$ | $11\alpha,15S$-dihydroxy-9-oxo-13-<u>trans</u>-prostenoic acid |
| Prostaglandin $E_2$ | $PGE_2$ | $11\alpha,15S$-dihydroxy-9-oxo-5-<u>cis</u>-13-<u>trans</u>-prostadienoic acid |
| Prostaglandin $F_{1\alpha}$ | $PGF_{1\alpha}$ | $9\alpha,11\alpha,15S$-trihydroxy-13-<u>trans</u>-prostenoic acid |
| Prostaglandin $F_{2\alpha}$ | $PGF_{2\alpha}$ | $9\alpha,11\alpha,15S$-trihydroxy-5-<u>cis</u>-13-<u>trans</u>-prostadienoic acid |
| Prostaglandin $A_1$ | $PGA_1$ | $15S$-hydroxy-9-oxo-10,13-<u>trans</u>-prostadienoic acid |
| Prostaglandin $B_1$ | $PGB_1$ | $15S$-hydroxy-9-oxo-8(12),13-<u>trans</u>-prostadienoic acid |
| Prostaglandin $A_2$ | $PGA_2$ | $15S$-hydroxy-9-oxo-5-<u>cis</u>-10,13-<u>trans</u>-prostatrienoic acid |
| Prostaglandin $B_2$ | $PGB_2$ | $15S$-hydroxy-9-oxo-5-<u>cis</u>-8(12),13-<u>trans</u>-prostatrienoic acid |

## PROSTAGLANDINS   FROM   THE   KIDNEY

### A study of the output of Prostaglandins from the Rabbit Kidney in vivo

Following the discovery that electrical stimulation of the splenic nerve in the dog causes the release of prostaglandin $E_2$ into the splenic venous blood (Davies et al, 1968), evidence accumulated in support of a functional role for this prostaglandin as a modulator of transmitter output at adrenergic nerve endings (see Hedqvist, 1973). One criticism of this hypothesis is that the various pieces of evidence supporting it have been obtained in different species and/or organs (Horton, 1973).

It was therefore decided to test the hypothesis in three different ways using the same biological system. The rabbit kidney was chosen because this organ is rich in prostaglandins (Lee, Crowshaw, Takman, Attrep and Gougoutas, 1967; Daniels, Hinman, Leach and Muirhead, 1967) and like the spleen has a rich and readily accessible adrenergic innervation.

This study has shown that (1) renal nerve stimulation increases the output of prostaglandins into the venous blood and that (2) indomethacin-induced blockade of prostaglandin biosynthesis not only reduces or abolishes this release but also enhances the renal vasoconstrictor response to renal nerve stimulation. The third piece of evidence was obtained by complementary experimental

studies arranged with and carried out by colleagues in Stockholm. They showed, also in the rabbit kidney, that $PGE_2$ infused intra-arterially reduces transmitter output in response to electrical stimulation of the renal nerve (Frame and Hedqvist, 1975).

## METHODS

Surgical Procedures    In each of six experiments, three male litter-mate rabbits weighing between 1.5 and 3.0 kg were used, two being blood donors for the third (experimental) animal. All animals were anaesthetized with ethyl carbamate (urethane) in a dose of 1.75 g/kg injected slowly as a 25% aqueous solution into a marginal ear vein. The abdominal aorta of each donor rabbit in turn was exposed and cannulated through a mid-line incision. Heparin (1000 i.u./kg) was then injected intravenously and blood was collected from the aortic cannula into polyethylene tubes.

The trachea, right external jugular vein, right common carotid artery and right femoral vein of the experimental rabbit were then cannulated in that sequence. Carotid arterial pressure was monitored throughout the experiment with a Statham pressure transducer (P23DC) and displayed on a Grass polygraph. A rectal thermometer was inserted and the body temperature maintained at 37°.*

The abdomen was opened via a mid-line incision and the left renal vein and nerve were exposed and dissected free of connective tissue. Heparin (1000 i.u./kg body weight) was then injected intravenously and the left renal

*Temperatures throughout are quoted in degrees Centigrade

vein was cannulated. Renal venous blood was diverted to the femoral vein via polyethylene tubing connecting the renal and femoral cannulae. A Y-piece with a side-arm was inserted for the collection of renal blood samples; this side-arm was clamped off between collections of renal venous blood. Heparinized (donor) blood (10 ml) was then slowly infused by a Watson-Marlow pump into the right external jugular vein of the rabbit, in order to compensate for the extra-corporeal blood in the renal-femoral vein circuit. The renal nerve was cut 2 cm from the kidney and wound around platinum electrodes which were connected to a Grass stimulator (SD5). The nerve and the kidney were bathed in a pool of liquid paraffin.

All cannulae inserted into blood vessels in these experiments were filled with a saline* containing heparin (50 i.u./ml), except for the carotid artery and abdominal aorta cannulae, which were filled with a more concentrated heparin solution (500 i.u./ml). After surgery, a period of 30 minutes was allowed to elapse before collecting blood samples.

<u>Collection of Blood Samples</u>  The time periods for renal venous blood sample collections and the volumes of the samples are given in Table 2. During the collection of all blood samples, the right femoral venous cannula was clamped and the blood directed from the kidney into an

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*'saline' is used throughout the text to mean 0.9% $^w\!/\!v$ sodium chloride solution in distilled water

ice-cold measuring cylinder. During the collection
of all samples, heparinized (donor) blood at 37º was
infused into the right external jugular vein. Blood was
infused at a slightly faster rate than it was being
removed. The blood pressure, therefore, remained fairly
constant.

A control sample of renal venous blood was initially
collected. This was followed by a 5 minute period during
which blood was directed from the renal vein into the
femoral vein.

In experiments 1 and 2 (Table 2), a test sample was
then collected. During this period, the renal nerve was
stimulated at 10 volts, frequency 10 Hz and duration
0.5 milliseconds. In experiment 2, the renal nerve was
stimulated for a further 20 minutes and a second test
sample (Test 2) was collected in the last 5 minutes of
this 20 minute stimulation period. A 20 minute interval
was then allowed during which renal venous blood was once
more directed into the femoral vein. This period allowed
time for the kidney to revert to 'pre-test' resting con-
ditions before a final post-test sample was collected.
In experiments 3, 4, 5 and 6, the renal nerve was stimu-
lated (as above) for 10 minutes before a test sample was
collected. A 15 minute period was then allowed during
which renal venous blood was directed into the femoral
vein.

After collection of the test sample in experiment 3, 2 ml of polyethylene glycol 200 (Analar) and 1 ml of saline were mixed and slowly injected into the right external jugular vein. A 30 minute period was then allowed before collecting a control polyethylene glycol sample (Control Glycol). After collecting this sample, the renal nerve was stimulated (as above) for 10 minutes, before collecting a test polyethylene glycol sample (Test Glycol). A 15 minute period was then allowed during which renal venous blood was directed into the femoral vein.

In experiment 3, after collection of the test glycol sample, and in experiments 4, 5 and 6, after the collection of the test sample, indomethacin (10 mg/kg) dissolved in 2 ml of polyethylene glycol and 1 ml saline was slowly injected into the right external jugular vein. In experiment 4, a 10 minute period, but in experiments 3, 5 and 6, a 30 minute period was allowed before collecting a control indomethacin sample (Control Ind.). After the collection of this sample, the renal nerve was stimulated for 10 minutes before collecting a test indomethacin sample (Test Ind.).

In experiment 6 (Table 2), a 20 ml blood sample was collected from the abdominal aorta before collection of the initial renal venous control blood sample.

After collection of a blood sample, 0.1 μCi each of $(5,6-^3H_2)$-PGA$_1$, $(5,6-^3H_2)$-PGE$_1$ and $(9-^3H_2)$-PGF$_{2\alpha}$ was added. This radioactivity represented 100 ng PGA$_1$,

150 ng PGE$_1$ and 3.3 ng PGF$_{2\alpha}$. Two volumes of saline at 0° were then added to the blood samples. The samples were kept at 0° until extraction.

TABLE 2

| Experiment number | Samples collected | Collection time (min) | Volume of blood sample (ml) |
|---|---|---|---|
| 1 | Control | 5 | 39 |
| | Test | 5 | 30 |
| 2 | Control | 5 | 46 |
| | Test 1 | 5 | 49.5 |
| | Test 2 | 5 | 48 |
| | Post test | 5 | 46 |
| 3 | Control | 4 | 55 |
| | Test | 4 | 46 |
| | Control Glycol | 4 | 42 |
| | Test Glycol | 4 | 36 |
| | Control Ind. | 4 | 40 |
| | Test Ind. | 4 | 25 |
| 4 | Control | 7 | 41 |
| | Test | 7 | 36.5 |
| | Control Ind. | 7 | 26 |
| | Test Ind. | 7 | 23 |
| 5 | Control | 7 | 33 |
| | Test | 7 | 36 |
| | Control Ind. | 7 | 41 |
| | Test Ind. | 7 | 30 |
| 6 | Control | 5 | 50 |
| | Test | 5 | 40 |
| | Control Ind. | 5 | 22 |
| | Test Ind. | 5 | 16 |

Samples collected before (Control) and during (Test) renal nerve stimulation; after polyethylene glycol 200 and saline, samples collected before (Control Glycol) and during (Test Glycol) nerve stimulation; after indomethacin, samples collected before (Control Ind.) and during (Test Ind.) nerve stimulation

Extraction Procedure    The diluted blood samples were centrifuged for 10 minutes at 1000 g and $4^0$.  The plasma/saline was separated from the cells and stored at $0^0$, while the cells were washed twice with an equal volume of saline at $0^0$.  The saline was removed from the cells by centrifugation and added to the diluted plasma.  The pooled plasma/saline samples were adjusted to pH 4.5 with glacial acetic acid, then extracted by the methods illustrated in Figure 2.

Silicic acid Column Chromatography    Silicic acid, 4.5 g (Sigma, Sil-R, 100 mesh) in 1 x 10 cm glass columns, was activated by heating at $110^0$ for one hour.  After cooling it was suspended in 10% ethyl acetate in toluene and poured into the column.  The dried residue from the solvent extraction procedure was dissolved in 1 ml of 10% ethyl acetate in toluene and applied to the column with a Pasteur pipette.  The residue flask was then washed with five successive 2 ml portions of 10% ethyl acetate in toluene and these separate washings were applied dropwise to the column.  Thus, 10 ml of the first 80 ml of 10% ethyl acetate in toluene was used for transferring the extract residue from the flask to the column.

The columns were run under reduced pressure at a rate of approximately 1 ml/min.  Prostaglandins were eluted by stepwise increases in concentration of ethyl acetate in toluene, namely, 10% (80 ml);  20% (80 ml); 40% (80 ml);  65% (150 ml);  80% (150 ml);  100% ethyl acetate (80 ml);  and finally methanol 100% (80 ml).
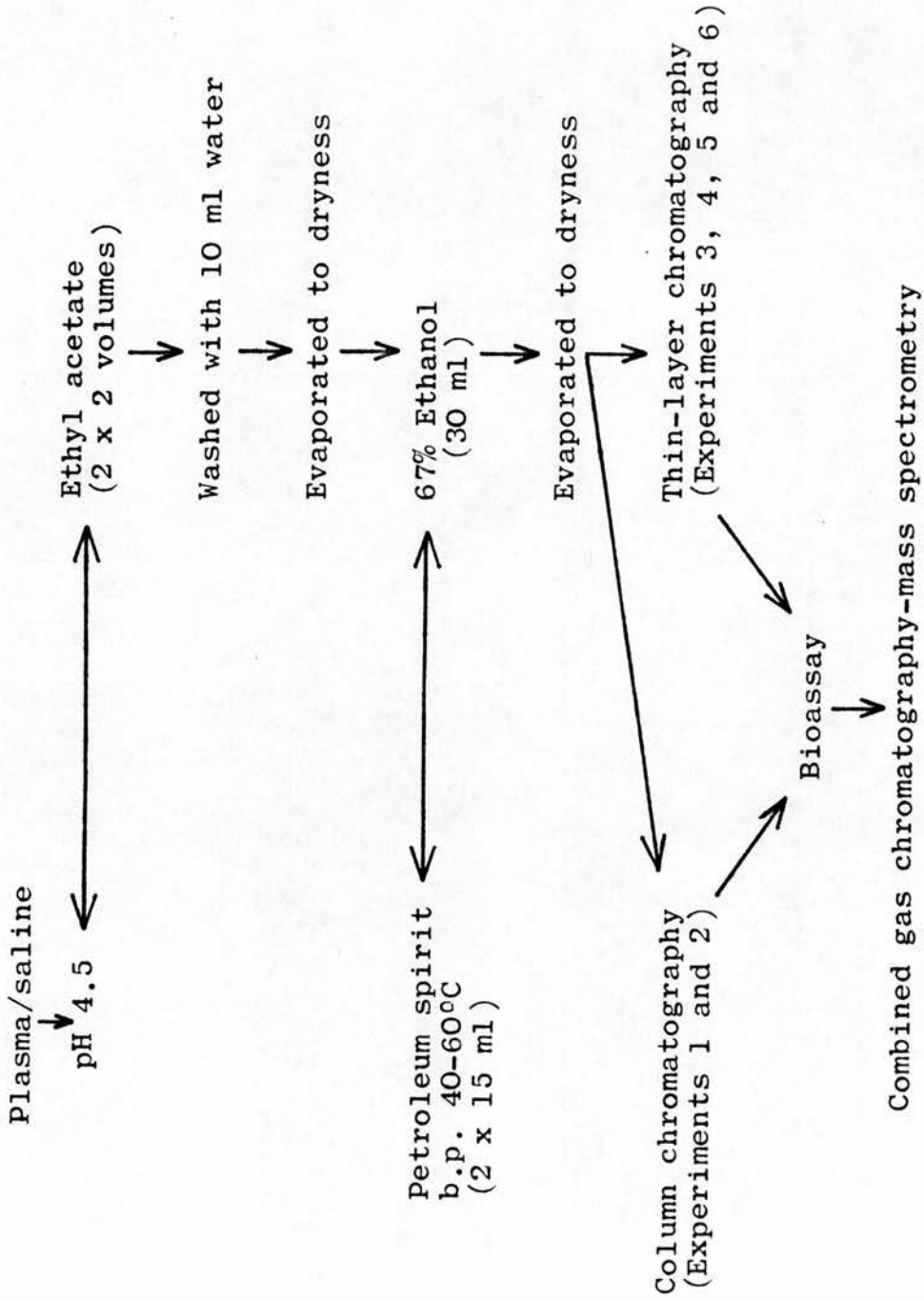
Figure 2   Procedure for extraction of prostaglandins from renal venous plasma

The order of elution of prostaglandins from the silicic acid, as indicated by the radioactive tracer prostaglandins, was as follows:  $PGA_1$ in the 40% ethyl acetate fraction, $PGE_1$ in the 65% and $PGF_{2\alpha}$ in the 80%. Any PGC or PGB in the extract would co-chromatograph with $PGA_1$.

All eluates from the columns were evaporated to dryness and re-dissolved in a known volume of methanol for liquid scintillation counting.  After this procedure the samples were again evaporated to dryness.  The 10% and 20% eluate fractions were dissolved in 1 ml of 0.9 saline and two-fifths of the 40% fraction was dissolved in 0.4 ml saline.  These were assayed in terms of $PGA_2$ on the kitten blood pressure preparation (Horton and Jones, 1969).  The remainder of the 40% eluate fraction was analysed by combined gas liquid chromatography-mass spectrometry (GLC/MS).  The other eluates were dissolved in 1 ml water for biological assay on the rat fundal strip.

These assay procedures were chosen because the rat fundus is very sensitive to both PGE and PGF but relatively insensitive to PGA and PGB whereas the kitten blood pressure is very sensitive to PGA (Horton and Jones, 1969) and PGC (Jones, 1972), but less sensitive to PGB, PGE and PGF.  Since neither assay was sensitive to PGB compounds, these could only be detected by GLC/MS.

Thin-layer Chromatography  Grooved glass plates (Chromalay, May & Baker) of dimensions 20 x 5 cm were used.  The plates were coated with a 0.75 mm layer of silica gel G (Merck).  The silica gel, 30 g, was

suspended in 57 ml of freshly re-distilled water for spreading the plates. The plates were allowed to dry overnight at room temperature and then used without further activation. Each extract, containing tracer $PGA_1$, $PGE_1$ and $PGF_{2\alpha}$ was dissolved in 0.5 ml methanol and applied, as a band, using an Agla micrometer syringe, to the groove of the plate. The extract flask was washed twice with 0.2 ml methanol and these aliquots were also applied to the plate.

The plates were developed in the following solvent system: toluene: dioxan: acetic acid (50:30:1) and a 16 cm run was used. All solvents were freshly re-distilled. Using this solvent system, $PGA_1$ has an Rf value of about 0.5 whereas PGE and PGF have lower Rf values. This solvent system allows the plate to be developed twice without prostaglandins of the A series running too far up the plate. It does not, however, separate prostaglandins of the A, B and C series. During the first 16 cm run, the prostaglandins were eluted from the groove and thus separated from pigments and more polar lipids which either remained in the groove or very close to the origin. The tracer prostaglandins were located on the plate by scanning with a Panax thin-layer chromatogram scanner (RTLS-1A). The silica gel in the groove and for approximately 1 cm beyond the origin was scraped off, so as to leave a further 1 cm length of silica gel intact on the plate below the start of the PGF zone. The plate was then developed again in the same solvent system up to the 16 cm line. The new locations of the tracer prostaglandins were determined by scanning again. The zones of silica gel

corresponding to the positions of the radioactive
tracers were scraped off and transferred to glass test
tubes which had been rinsed in methanol. The prosta-
glandins were eluted from the silica gel with two portions
of 5 ml methanol. The methanol was evaporated to dryness
and the residues were re-dissolved in an accurately known
volume of methanol in preparation for liquid scintil-
lation counting. All other silica gel zones on each
plate were also extracted and their radioactivity measured
to confirm that no prostaglandin had been left on other
parts of the plate.

After scintillation counting, the PGE, PGF and PGA
(C and B) eluates were evaporated to dryness. The PGE
and PGF residues were dissolved in water* (1 ml) for bio-
assay on the rat fundus, whereas 40% of the PGA eluate
was dissolved in saline for bioassay on the kitten blood
pressure and 60% was retained for analysis by GLC/MS.

<u>Liquid Scintillation Counting</u>   Radioactivity was
measured with a Nuclear Chicago Mark II liquid scintil-
lation counter, using the channels ratio method. Samples
were dissolved in a known volume of either methanol or
or ethyl acetate. Aliquots (50 or 100 mcl) were pipetted
into vials to each of which was added scintillation fluid
of the following composition:   toluene 2.5 l;   2,5-
diphenyoxazole (PPO) 10.65 g;   1,4-di-(2-(4-methyl-5-
phenyloxazolyl)-benzene) 0.275 g.   Counting efficiencies
for samples in organic solvents ranged between 40 and
50%.

* 'Water' throughout means glass distilled
unless otherwise stated.

Further Purification of PGF Fractions    After thin-layer
chromatography and biological assay, samples containing
PGF were purified further, prior to combined GLC/MS analysis.
To each sample in aqueous solution, 15 ml of pH 8.0 phosphate
buffer was added. The buffered extract was then extracted
by the method outlined in Figure 3.    The pH 8.0 buffer was
prepared by adding 189.4 ml of 0.5M disodium phosphate to
5.3 ml of 1M sodium dihydrogen phosphate and making up to
1 litre with water.

Conversion of Prostaglandins of the A, C or E series to PGB
After biological assay, prostaglandins of the A, C or E series,
in the remainder of the extracts were converted to the corres-
ponding PGB compound for identification by GLC/MS. The extracts
were evaporated to dryness and 1 ml of 0.1N KOH in methanol was
added. After one hour at room temperature (approximately 18°),
20 ml of water was added to each sample. This solution was
acidified to pH 4.0, using a pH meter, by the dropwise addition
of glacial acetic acid. It was then partitioned twice against
an equal volume of ethyl acetate. After separation the com-
bined ethyl acetate phases were washed twice with 5 ml water
and then evaporated to dryness.

Gas Liquid Chromatography-Mass Spectrometry

The methyl ester/trimethylsilyl ether (Me/TMS), methyl
ester/trifluoroacetate (Me/TFA) and trimethylsilyl ester/
trimethylsilyl ether (TMS/TMS) derivatives were prepared
for combined GLC/MS on a micro-scale in 0.5 ml stoppered
test tubes, using the methods described by Thompson, Los
and Horton (1970).

pH 8 phosphate buffer
(15 ml)

$\downarrow$ Adjusted to pH 4
with acetic acid

pH 4

Ethyl acetate
(2 x 15 ml)

Ethyl acetate
(2 x 30 ml)

$\downarrow$ Washed with 2 x 5 ml water

$\downarrow$ Evaporated to dryness

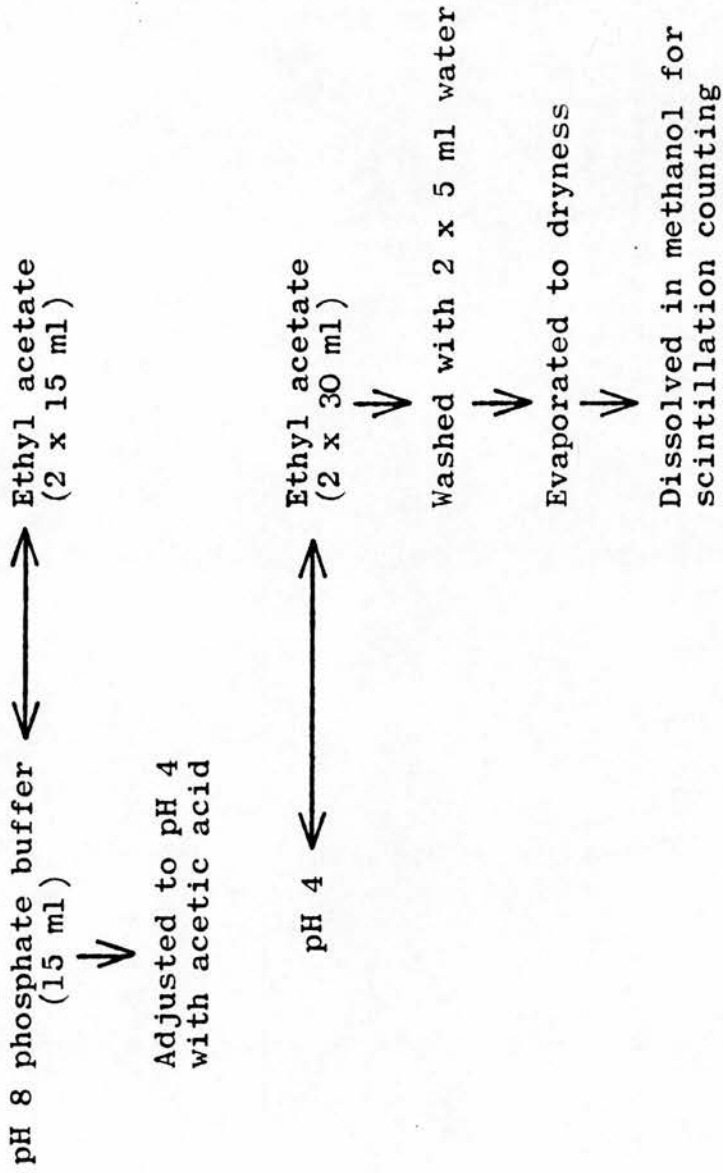$\downarrow$ Dissolved in methanol for
scintillation counting

Figure 3   Outline of additional purification procedure for PGF fractions after
thin-layer chromatography

Methyl esters were prepared by reacting the sample with a freshly prepared solution of diazomethane in diethyl ether-methanol (9:1) for 20 minutes. Trimethylsilyl ether derivatives were formed by the addition of 13 μl of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) to the methyl ester (Me/TMS) or the free acid (TMS/TMS) as the dry residue. PGB derivatives required 15 minutes at 60° and PGF derivatives 3 hours at room temperature (approximately 18°) for completion of the reaction. Without removal of the BSTFA, approximately 12 μl were injected on to the gas chromatographic column. Trifluoroacetates were prepared by reacting the methyl esters for 2 hours with 200 μl trifluoroacetic anhydride which was then removed in a vacuum desiccator. The Me/TFA derivatives were dissolved in 25 μl hexane and 10 μl fractions (400 ng) were injected on to the GLC column.

Analyses were performed on an LKB 9000 gas liquid chromatograph-mass spectrometer using methods previously described (Thompson et al., 1970; Davis, Horton, Jones and Quilliam, 1971). The GLC column (1.5 metres long and 1.5 mm internal diameter) was packed with 3% OV1 on Supasorb AW 100-200 mesh pre-treated with dimethylchlorosilane in carbon tetrachloride. Column temperature was either 200° or 190°. The carrier gas, helium, flowed at 20 ml/minute. All mass spectra were recorded at an electron voltage of 27.5. Mass spectra were recorded at the retention time corresponding to the peak of the GLC peak of standard prostaglandins similarly derivatized.

Biological Assays    Smooth muscle preparations in vitro
were suspended in a 4 ml organ bath and longitudinal con-
tractions were recorded isometrically with a force-displace-
ment transducer (Grass FT.03) on a Servoscribe potentio-
metric pen recorder.  A dose cycle of 4 to 7 minutes with a
contact time of 1 to 2 minutes was used for all preparations.
All estimates of content were made by bracketting with pure
prostaglandins.

Rat fundal strip    Fundal strips from rats weighing 100 -
300 g were prepared as described by Vane (1957) and sus-
pended in Tyrode's solution vigorously gassed with oxygen
at 37°.

Rabbit jejunum    Proximal jejunum from rabbits weighing 1
to 3 kg was suspended in Tyrode's solution gassed with air
at 37° (Horton and Main, 1965).

Kitten Blood Pressure Preparation    Kittens weighing between
0.5 and 0.7 kg were anaesthetized with pentobarbitone sodium
(40 mg/kg) and the anaesthesia maintained with a slow inter-
mittent intravenous pentobarbitone sodium infusion (5 mg/ml).
The blood pressure was recorded from a common carotid artery
using a Statham transducer and a Grass polygraph.  Assay
samples were administered in saline by rapid intravenous
injection into the cannulated external jugular vein and
washed in with 0.2 ml saline (Horton and Jones, 1969).

Estimates of Recovery        Recovery of the added tracer
prostaglandins after extraction and chromatography was
estimated by liquid scintillation counting.   The following
recoveries were found:    PGA$_1$   55-89%;   PGE$_1$   42-70%;
PGF$_{2\alpha}$  60-90%.   These radio-active recoveries for PGE$_1$
and PGF$_{2\alpha}$ agreed well with the recovery of added unlabelled
prostaglandins as estimated by bio-assay, namely PGE$_1$ (54-67%),
PGF$_{2\alpha}$ (53-60%).   However, recoveries of PGA$_1$ as measured by
bio-assay on the kitten blood pressure, namely 5-14%, were
far lower than those obtained by scintillation counting.
The recovery of PGA$_1$ from rabbit blood was therefore investi-
gated further.

PGA$_1$ was added to four 40 ml samples of rabbit aortic
blood at 37$^o$, containing heparin (20 i.u./ml), to give a
final concentration of 100 ng/ml in two samples and 200 ng/ml
in the other two samples.   These concentrations of PGA$_1$
included 5 ng/ml of tracer PGA$_1$ (0.2 μCi per sample).

The blood samples were extracted, as previously des-
cribed.   After thin layer chromatography, the material with
the chromatographic behaviour of tracer PGA$_1$ was eluted and
the percentage recovery estimated both by scintillation
counting and by assay on the kitten blood pressure.   The
results (Table 3) suggest that PGA$_1$ is at least partly
converted to a biologically inactive product during the
extraction procedure.

After bioassay, the Me/TMS derivatives of the remaining
prostaglandin-like material in the extracts of the four
samples was prepared for GLC/MS.   Mass spectra taken at the

TABLE 3 Recovery of PGA$_1$ added to rabbit blood and estimated by both biological assay and liquid scintillation counting

| Sample number | Added PGA$_1$ concentration (ng/ml) | % Recovery by biological assay | % Recovery by scintillation counting |
|---|---|---|---|
| 1 | 100 | 17 | 75 |
| 2 | 100 | 19 | 63 |
| 3 | 200 | 19 | 64 |
| 4 | 200 | 20 | 53 |

retention times of pure $PGA_1$ (7.9 minutes) and $PGB_1$ (11.9 minutes), confirmed that the extracts of all samples contained $PGB_1$ but only extract No. 1 contained any $PGA_1$. Since the final extracts still contained many other substances, quantitation by GLC/MS was not possible and the percentage of $PGA_1$ converted to $PGB_1$ could not be calculated by this method.

Since $PGA_1$ is very rapidly converted enzymatically to $PGC_1$ in the presence of rabbit blood even at $0^o$ (Jones, 1970) and since $PGC_1$ is then isomerized non-enzymatically but more slowly in aqueous solution to $PGB_1$ (Jones, 1972), it is probable that $PGC_1$ was also present in these blood extracts. Since $PGC_1$ is active in lowering kitten blood pressure (Jones, 1972) but would not be detected (except possibly as $PGB_1$) on GLC/MS, the possibility of such enzymatic loss of $PGA_1$ could explain the discrepancy in the results. The liquid scintillation counting procedure would not distinguish between radioactivity present in $PGA_1$, $PGB_1$ or $PGC_1$.

## RESULTS

### Output of prostaglandins into renal venous blood

Prostaglandins $E_2$ and $F_{2\alpha}$ were detected in renal venous blood samples of all six experimental rabbits under resting conditions. When the renal nerve was stimulated electrically, this output increased in all cases (Table 4). There was a less consistent and less pronounced increase in the output of a PGA compound also.

Intravenously injected indomethacin in four experiments reduced the resting output in response to renal nerve

TABLE 4 Amounts of prostaglandin (ng PG equivalent released/min) detected after column or thin-layer chromatography in renal venous blood samples

| Experiment number | Samples collected | Collection time (min) | Volume of blood sample (ml) | PGA | $PGE_2$ | $PGF_{2\alpha}$ |
|---|---|---|---|---|---|---|
| 1 | Control | 5 | 39 | <26 | 237 | 133 |
| | Test | 5 | 30 | 54 | 610 | 281 |
| 2 | Control | 5 | 46 | <7 | 303 | 216 |
| | Test 1 | 5 | 49.5 | 17 | 461 | 327 |
| | Test 2 | 5 | 48 | 52 | 678 | 327 |
| | Post-test | 5 | 46 | <11 | 356 | 238 |
| 3 | Control | 4 | 55 | 99 | 903 | 475 |
| | Test | 4 | 46 | 129 | 1700 | 949 |
| | Control Glycol | 4 | 42 | 39 | 168 | 240 |
| | Test Glycol | 4 | 36 | 49 | 322 | 272 |
| | Control Ind. | 4 | 40 | 11 | 15 | 23 |
| | Test Ind. | 4 | 25 | <10 | 14 | 21 |
| 4 | Control | 7 | 41 | 12 | 424 | 170 |
| | Test | 7 | 36.5 | 33 | 787 | 430 |
| | Control Ind. | 7 | 26 | 33 | 121 | 160 |
| | Test Ind. | 7 | 23 | 16 | 41 | 81 |
| 5 | Control | 7 | 33 | <12 | 30 | 72 |
| | Test | 7 | 36 | <12 | 54 | 180 |
| | Control Ind. | 7 | 41 | <12 | 14 | 10 |
| | Test Ind. | 7 | 30 | <12 | 15 | 9 |
| 6 | Control | 5 | 50 | 38 | 541 | 357 |
| | Test | 5 | 40 | 46 | 618 | 431 |
| | Control Ind. | 5 | 22 | <16 | <1 | <23 |
| | Test Ind. | 5 | 16 | <16 | 3 | 23 |

Samples collected before (Control) and during (Test) renal nerve stimulation; after polyethylene glycol 200 and 0.9% saline, samples collected before (Control Glycol) and during (Test Glycol) nerve stimulation; after indomethacin, samples collected before (Control Ind.) and during (Test Ind.) nerve stimulation. PGA (C or B)-like eluates assayed on kitten blood pressure preparation in terms of $PGA_1$, PGE and PGF-like eluates assayed on rat fundus in terms of $PGE_2$ and $PGF_{2\alpha}$ respectively. Amounts (ng) of tracer $PGE_1$ and $PGF_{2\alpha}$ recovered in each sample were deducted from total amounts of PG detected. PGE and PGF concentrations were then corrected for recovery. The estimates of PGA were not corrected for recovery.

stimulation. Injection of the solvent vehicle alone (polyethylene glycol 200 and saline) did not abolish nerve-stimulated output.

## Identification of prostaglandins in renal venous blood

In every experiment prostaglandins were identified on the basis of GLC/MS supported by their partition, chromatographic and biological properties. The results obtained in one experiment (No. 3) are presented in detail below; similar results were obtained in the other experiments.

### Identification of $PGF_{2\alpha}$

In experiment 3 (Table 4) the material in control and test samples with chromatographic behaviour corresponding to $PGF_{2\alpha}$ was assayed biologically on the rat fundus. After phosphate buffer partition, each sample was divided into two parts for the preparation of Me/TMS and Me/TFA derivatives prior to GLC/MS.

Mass spectra taken at the appropriate retention times showed that both control and test samples contained $PGF_{2\alpha}$. $PGF_{1\alpha}$ could not be detected. The mass spectral data ($^m/e$ peaks greater than 300) are shown in Figure 4. The small amounts of tracer (3.3 ng) added could not have been detected by the GLC/MS method.

The Me/TMS derivative of pure $PGF_{2\alpha}$ had prominent mass spectral peaks at 584 $[M^+]$, 569 $[M - 15]$, 513 $[M - 71]$, 494 $[M - 90]$, 423 $[M - (90 + 71)]$, 404 $[M - (2 \times 90)]$,
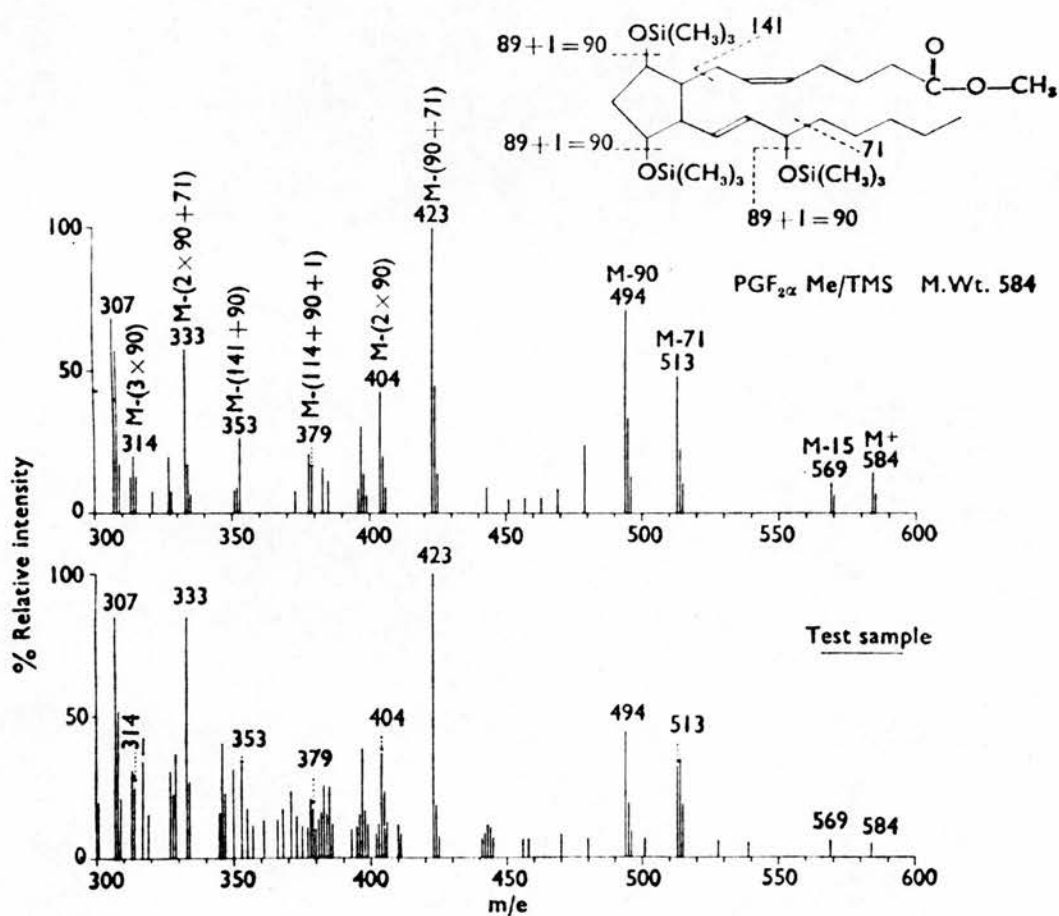
Figure 4    Mass spectra of the Me/TMS derivatives of
standard $PGF_{2\alpha}$ (upper) and of half the PGF
fraction from the test sample in experiment 3
(lower) - material extracted from renal
venous blood collected during renal nerve
stimulation

379 $[M - (114 + 90 + 1)]$, 353 $[M - (141 + 90)]$, 333 $[M - (2 \times 90 + 71)]$, 314 $[M - (3 \times 90)]$ and 307. The presence of these 11 peaks in approximately the same ratios in the mass spectra of both the control and test extracts confirmed that the PGF-like material detected by bioassay was $PGF_{2\alpha}$.

Further confirmation was obtained with the Me/TFA derivatives. That of authentic $PGF_{2\alpha}$ showed prominent peaks at 542 $[M - 114]$, 511 $[M - (31 + 114)]$, 441 $[M - (101 + 114)]$, 428 $[M - (2 \times 114)]$ and 314 $[M - (3 \times 114)]$. The same five $^m/e$ peaks in similar ratios were present in both control and test samples (Fig. 5).

Identification of $PGE_2$    Material from both control and test samples with the chromatographic behaviour of $PGE_2$ contracted the isolated rat fundus.

After conversion to $PGB_2$ and derivatization to Me/TMS, authentic $PGE_2$ showed prominent mass spectral peaks identical to the Me/TMS of authentic $PGB_2$, namely: 420 $[M^+]$, 405 $[M - 15]$, 389 $[M - 31]$, 349 $[M - 71]$, 330 $[M - 90]$, 321 $[M - (71 + 28)]$, 279 $[M - 141]$, 247 $[M - 173]$, 221 $[M - 199]$ and 199. These characteristic peaks were also found in the control and test samples similarly derivatized, the mass spectra being taken at 8.9 minutes corresponding to the retention time of authentic $PGB_2$, Me/TMS. $PGB_1$, Me/TMS had the same retention time but $^m/e$ peaks corresponding to this compound (2 a.m.u. greater than those of $PGB_2$) could not be detected (Fig. 6).
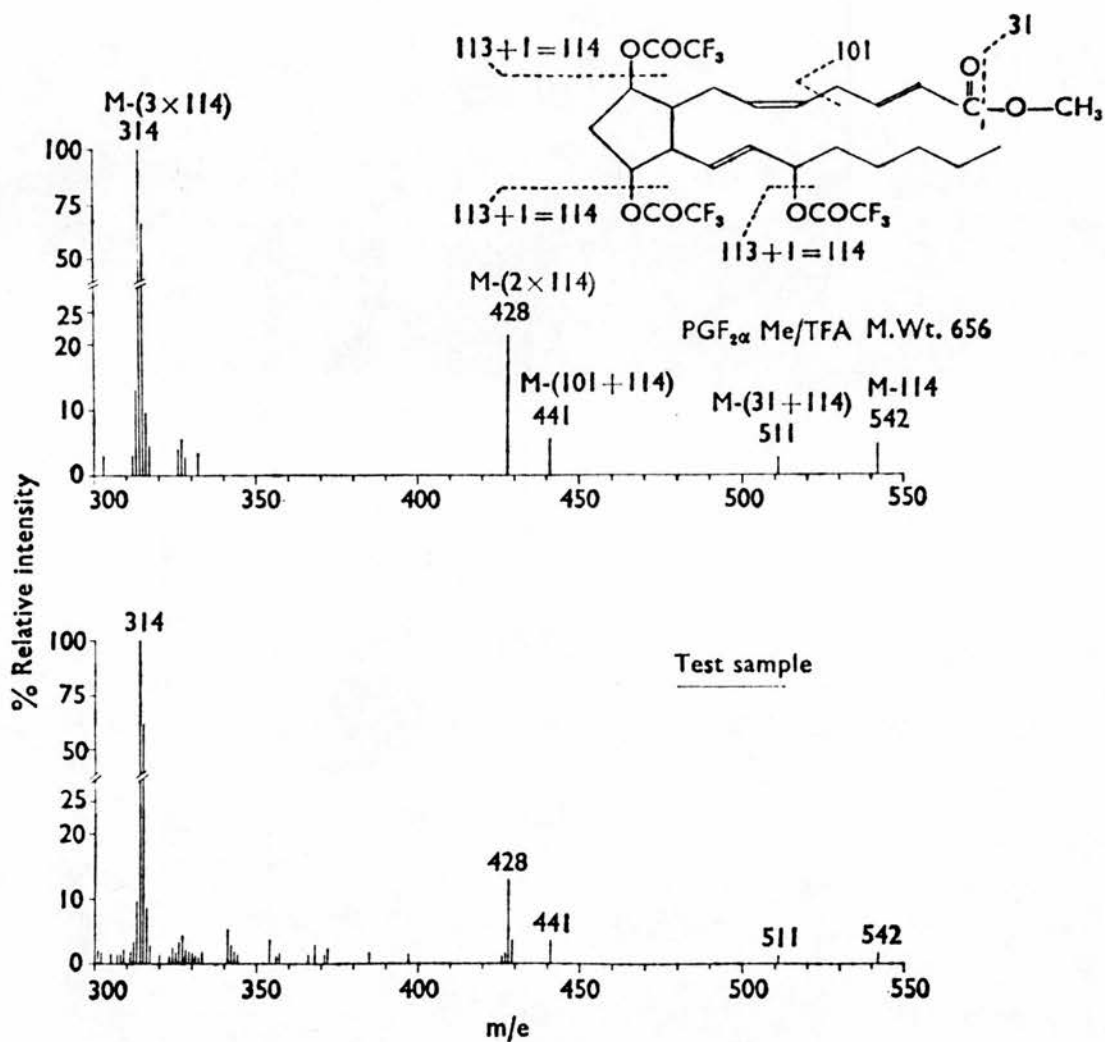
Figure 5   Mass spectra of the Me/TFA derivatives of
standard PGF$_{2\alpha}$ (upper) and of half the PGF
fraction from the test sample in experiment 3
(lower) – material extracted from renal
venous blood collected during renal nerve
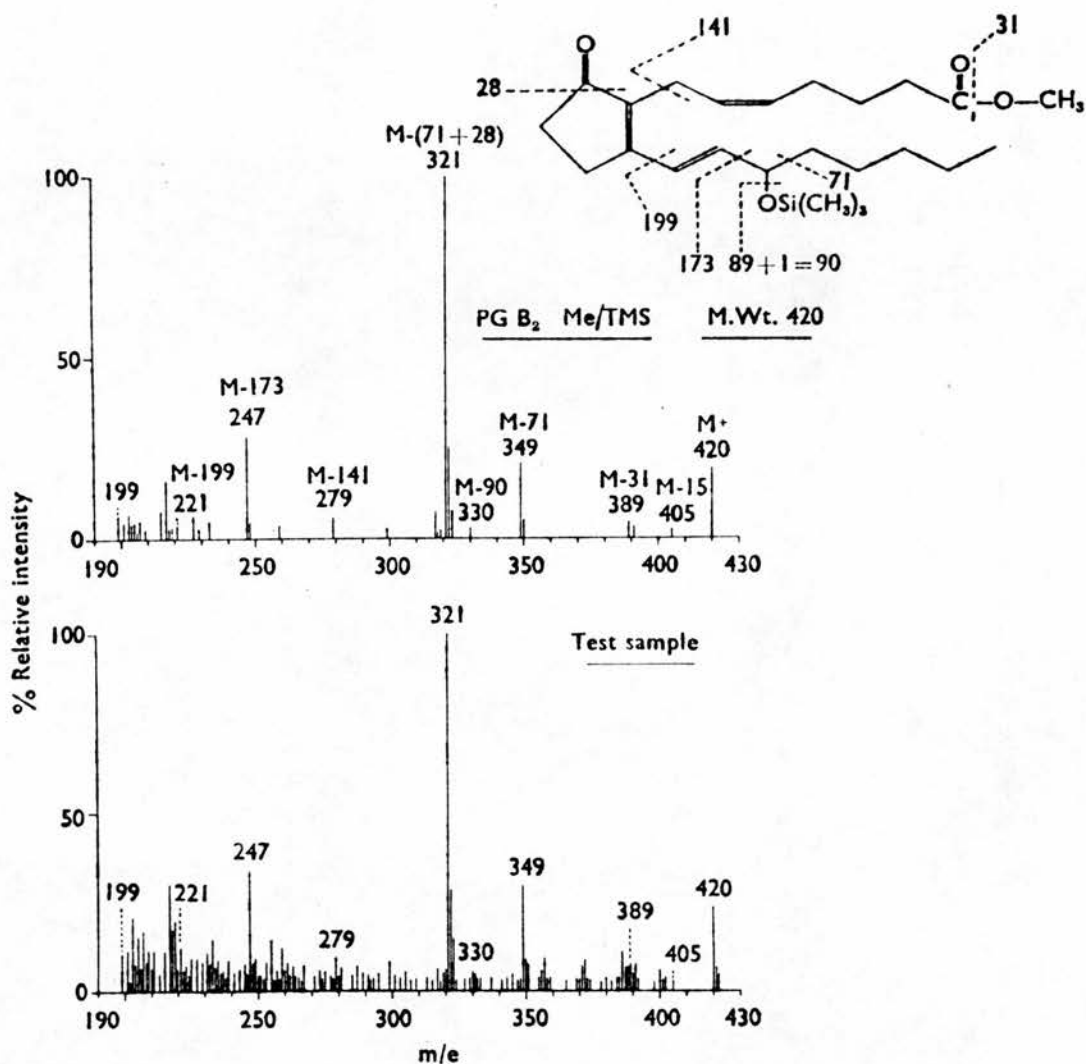stimulation

Figure 6    Mass spectra of the Me/TMS derivatives of
            standard PGB$_2$ (upper) and of the PGE fraction
            (after conversion to PGB) from the test sample
            in experiment 3 (lower) - material extracted
            from renal venous blood collected during
            renal nerve stimulation

This evidence confirmed that the original extracts contained predominantly $PGE_2$ not $PGE_1$. In other instances the TMS/TMS derivatives were prepared and again the results indicated the presence of $PGE_2$ but no (or negligible) $PGE_1$.

## Identification of $PGA_2$

Material which co-chromatographed with tritiated $PGA_1$ in both control and test samples of experiment 3, lowered the kitten blood pressure on intravenous injection. This effect could have been due to a PGA, to smaller quantities of the corresponding PGC, to very high amounts of PGB or to a mixture (Table 5).

The Me/TMS derivatives of $PGB_2$ and $PGB_1$ both had a GLC retention time of 10.0 minutes. Neither test nor control samples contained any detectable amounts of either of these prostaglandins. However, the amount of PGA or PGC present calculated from the bioassay results would not yield sufficient PGB to be detected by the GLC/MS method used. Only if the biological activity in the sample had been wholly or largely attributable to the presence of a PGB, would this compound have been readily detected by GLC/MS. Since it was not, the original compound present in the extracts must have been a PGA or a PGC or both, the concentration being low.

TABLE 5     Amounts (ng) of PGA-like activity assayed on the kitten blood pressure in control and test samples (experiment 3) expressed in terms of $PGA_1$, $PGA_2$, $PGC_1$, $PGB_1$ and $PGB_2$

| | Prostaglandin | | | | |
| --- | --- | --- | --- | --- | --- |
| | $A_1$ | $A_2$ | $C_1$ | $B_1$ | $B_2$ |
| Control | 238 | 59 | 159 | 9528 | 2858 |
| Test | 309 | 77 | 206 | 12360 | 3708 |

The effect of polyethylene glycol 200 on prostaglandin output

In experiment 3, following the injection of a mixture of 2 ml of polyethylene glycol 200 and 1 ml saline intravenously, systolic blood pressure rose by 22 mm Hg and diastolic by 12 mm Hg. This effect lasted 10 minutes. Although there was a fall in output of prostaglandin of all three series following glycol injection, the resting output of prostaglandins was not abolished nor was their release in response to nerve stimulation prevented (Table 4). Both $PGE_2$ and $PGF_{2\alpha}$ were identified by GLC/MS in control and test samples of experiment 3 following polyethylene glycol administration.

## The effect of indomethacin on prostaglandin output

Following the intravenous injection of indomethacin, systolic blood pressure rose by 10 to 40 mm Hg and diastolic by 16 to 28 mm Hg. These effects lasted longer than an hour in all four experiments (3, 4, 5 and 6). In experiments 4 and 6 total renal blood flow also decreased. Indomethacin reduced the resting output of renal venous $PGE_2$ and $PGF_{2\alpha}$ in all four experiments and virtually abolished the release of prostaglandins in response to renal nerve stimulation (Table 4). The resting levels were lowered 10 minutes after indomethacin (experiment 4) but greatly lowered 30 minutes after indomethacin (experiments 3, 5 and 6). Therefore only in experiment 4 was sufficient $PGE_2$ and $PGF_{2\alpha}$ released spontaneously after indomethacin to be identified by GLC/MS. By pooling resting samples from the three other experiments collected 30 minutes after indomethacin, the total amount of $PGF_{2\alpha}$ present was just sufficient to be identified by GLC/MS. $PGE_2$, however, could not be detected by GLC/MS in these pooled post-indomethacin samples.

## Concentrations of prostaglandins in arterial blood

In experiment 6, a 20 ml aortic blood sample was collected before the first control sample of renal venous blood. The levels of PGA ($< 2$ ng/ml $PGA_1$ equivalent) and PGF ($< 3.5$ ng/ml $PGF_{2\alpha}$ equivalent) were undetectable by the methods used above. However, a low concentration of $PGE_2$ (about 1.5 ng/ml) was detected by bioassay on the rat fundus. The identification of this arterial $PGE_2$ rests

upon its chromatographic behaviour and its biological
activity - there was insufficient material for GLC/MS.
It is apparent, therefore, that the amounts of prosta-
glandins of all three series found in renal venous blood
in the absence of nerve stimulation greatly exceed those
present in the arterial blood supplying the kidney.   In
experiment 6 under resting conditions, the concentration
of $PGE_2$ in the renal vein was more than 30 times that
found in the renal artery.   In other rabbits not used
in these experiments the arterial levels of prostaglandins
were almost invariably below the limits of detection.

## DISCUSSION

In all six experiments $PGE_2$, $PGF_{2\alpha}$ and a PGA - like
compound were found in renal venous blood collected under
resting conditions.   The amounts exceeded those found in
arterial blood;   hence it may be concluded that the
prostaglandins found in renal venous blood are of renal
origin;   they appear to be released 'spontaneously'.   In
all instances their output was increased by stimulating
the renal nerve electrically and in all instances the $PGE_2$
and $PGF_{2\alpha}$ have been identified by their GLC/MS behaviour
in addition to their biological, chromatographic and par-
tition properties.

The precise nature of the PGA - like material remains
in doubt.   Moreover, if it was $PGA_2$ (or its product $PGC_2$)
it could have arisen from $PGE_2$ artefactually during the
extraction procedure (Lee et al., 1967).   It is known

that the use of an organic acid in preference to a mineral acid during the extraction procedure reduces the likelihood of dehydration of $PGE_2$ (Andersen, 1969). Acetic acid was used in this investigation for acidification of extracts, furthermore the acid was always removed by washing with water as quickly as possible during the separative procedure. This should have minimized the amount of chemical degradation of $PGE_2$ but other techniques would be needed to exclude this possibility altogether.

Handling of organs may cause prostaglandin release (Piper and Vane, 1971) but in this study the kidney was not touched and 30 minutes was allowed after the surgical procedure was completed before collecting blood samples. It seems unlikely, therefore, that the 'spontaneous' release could be attributed to manipulation of renal tissue.

Both $PGE_2$ and $PGF_{2\alpha}$ output increased during nerve stimulation in all experiments. This confirms previous findings in the dog spleen (Davies et al., 1968) where adrenergic nerve stimulation also increases the output of both these prostaglandins. However the dog spleen does not release detectable amounts of prostaglandins in the absence of stimulation, despite much greater inevitable manipulation and handling during the surgical procedures than in the present study.

In all four experiments in which the effect of indomethacin was tested, spontaneous release of prostaglandins was reduced by the drug whilst the increased output in response to nerve stimulation was abolished.

Moreover, this abolition of prostaglandin output was
accompanied by significant physiological changes, the
arterial blood pressure was raised and renal blood flow
reduced. These changes are compatible with the postu-
lated role for prostaglandins at adrenergic nerve endings
reducing transmitter output in response to nerve stimu-
lation.

Frame and Hedqvist (1975) also working on the rabbit
kidney have shown that the intra-arterial infusion of $PGE_2$
reduces the output of noradrenaline in response to nerve
stimulation. This evidence taken with the findings here
that $PGE_2$ is released from the kidney on nerve stimulation
and that blockade of its synthesis leads to vascular changes
typical of enhanced sympathetic nerve activity strongly
suggests that $PGE_2$ does have a role at adrenergic nerve
endings in the kidney as a modulator of transmitter out-
put. Support for the hypothesis originally formulated by
Hedqvist (1971) is stronger from this study than from
previous investigations because all the observations have
been made on the same preparation. Moreover, the nature
of the prostaglandins released is based in this study on
far less equivocal data obtained by mass spectrometric
analysis.

What the hypothesis in its present form does not
account for is the release of other prostaglandins, notably
$PGF_{2\alpha}$ and the PGA-like compound. There is currently no
convincing evidence to suggest that these prostaglandins
have a physiological role at adrenergic nerve terminals,
although it is claimed that $PGF_{2\alpha}$ may enhance transmitter

release (Kadowitz, 1972). However, the concommitant release of several prostaglandins does not itself negate the evidence regarding a role for $PGE_2$, though it may mean that the hypothesis will need modification in the light of future results.

# PROSTAGLANDINS FROM THE MALE REPRODUCTIVE TRACT

## A Study of the Daily Output of Prostaglandins in Human Semen and the Effects of Treatment with High Doses of Aspirin

Human semen contains several different prostaglandins and is the richest known vertebrate souce of these substances. Inhibition of the biosynthesis of prostaglandins by aspirin was discovered in 1971 (Ferreira et al., 1971; Smith and Willis, 1971; Vane, 1971); subsequently Collier and Flower (1971) reported that male volunteers treated with aspirin had reduced seminal levels of prostaglandins as measured by biological assay in terms of $PGE_2$ and $PGF_{2\alpha}$.

The object of the present investigation was to determine how completely prostaglandin synthesis in man can be inhibited by high doses of aspirin (3.6 and 7.2 g/day). The prostaglandins were measured by physico-chemical methods and bioassay, estimates of recoveries being obtained by the use of a radioactive tracer. Fructose levels were also measured.

## METHODS

Human Subjects   Two healthy adult males aged 25 (subject A) and 42 (subject B) each collected ejaculates manually between 0800 and 0900 hours every 24 hours, each over two periods of 9 to 12 days. The first sample in each series was discarded. After 2 to 5 control collections aspirin (soluble aspirin tablets B.P.) was taken orally in divided doses for 3 days. Dose levels of 3.6 and 7.2 g/day were studied in each subject.

<u>Extraction of Prostaglandins</u>    Seminal fluid was collected
into a tared flask containing 0.2 μCi 5,6-$^3$H-PGE$_1$ (specific
activity 210 Ci/mol) in 2 ml saline.  The flask was re-weighed
immediately following collection and stored at 4$^o$ for up to
one hour.  Saline (20 ml) was then added and the diluted
ejaculate was centrifuged at 1200 g for 5 minutes.  The
supernatant was poured off, the pellet re-suspended in a
further 20 ml saline and the centrifugation repeated.  The
combined supernatants were made up to 50 ml with saline and
5 ml of this was removed to a separate vessel and stored
at -20$^o$ for fructose assay.   The remainder of the sample
was acidified to pH 4 with 10% citric acid solution and
extracted twice with 2 volumes of diethyl ether.   The com-
bined ether phases were washed with 20 ml water and then
concentrated <u>in</u> <u>vacuo</u> to about 20 ml.   The concentrate
was extracted twice with equal volumes of 0.1 M tris-HCl
buffer (pH 9.0).  The combined buffer phases were acidified
to pH 4 and extracted twice with equal volumes of diethyl
ether.  The combined ether phases were washed with 5 ml
water and evaporated to dryness.

The residue was dissolved in a small volume of toluene:
methanol (1:1) and applied to a previously prepared silica
gel chromatography plate (Merck).   The plate was developed
to a height of 15 cm in the A VII solvent system of Hamberg
and Samuelsson (1966).  The zone containing the labelled PGE$_1$
was detected with a Panax thin layer chromatogram scanner
(RTLS - 1A).  The silica gel from two zones, one containing
the prostaglandins E (Rf 0.31 to 0.44) and the other the

prostaglandins F and the 19-hydroxy-prostaglandins (as
A and B) (Rf 0.16 to 0.31) was removed from the plate.
The prostaglandins were eluted from the silica with methanol.

Estimation of Prostaglandins    Spectroscopic assays of the
prostaglandins were performed with a Pye Unicam SP800
ultra-violet spectrophotometer.  After appropriate dilution,
2 ml of the chromatogram eluate were placed in a cell of
10 mm path length and a spectrum was recorded between 250
and 325 nm.  N-potassium hydroxide in methanol (0.2 ml)
was added to the cell and the spectra were recorded at 5
minute intervals until the extinction change at 278 nm had
reached a maximum.  The $\mathcal{E}_{278}$ for the conversion of PGE$_1$
to PGB$_1$ is 27,200 (Andersen, 1969).

Gas chromatographic-mass spectrometric analysis of
the methyl ester-trimethylsilyl ether derivatives of the
prostaglandins B was carried out by the method of Thompson
et al.,(1970).   The amount of radioactivity in the PGE
eluates was determined with a Nuclear-Chicago Mk II liquid
scintillation counter.  The prostaglandin F eluates were
assayed biologically in terms of PGF$_{2\alpha}$ on the isolated
rabbit jejunum (Horton and Main, 1965).

Estimation of Fructose    The fructose content of the seminal
fluid was measured using the resorcinol/HCl reaction as
described by Mann (1948).  The extinction of the chromophore
at 400 nm was measured spectrophotometrically.  Glucose-free
D(-)-fructose (B.D.H.) was used to produce a calibration
curve.

Statistical Analysis   For statistical analysis by Student's t test, the mean of all pre-treatment and the second and third post-treatment values (mean controls) was compared with the mean of the second and third treatment values (mean test).

## RESULTS

### Weight of ejaculates collected by 24 hour intervals

In both subjects there were daily fluctuations in the weight of ejaculates but no obvious trend could be detected in these variations over the 8 to 11 day periods of the study (Tables 6 and 7).   The weights of the ejaculates collected during aspirin treatment at the two dose levels were not significantly different (P >0.1) from those collected during the control periods in four experiments (two in each subject) (Table 8).

### Seminal fructose

In subject A, the mean fructose levels during the control periods and during the aspirin (7.2 g/day) administration were 2.32 and 2.50 mg/g respectively.   The difference was not statistically significant (P >0.1).   The levels in subject B were lower, 1.06 and 1.38 mg/g during the control periods and 1.31 and 1.30 mg/g during the treatment with low and high dosage of aspirin.   These differences were also not significant (Table 8).

TABLE 6   Effect of aspirin on semen weight, fructose concentration and prostaglandin concentrations in Subject A

| Parameter | Pre-treatment day | | | | | Treatment day | | | Post-treatment day | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 1 | 2 | 3 |
| Semen weight (g) | – | 3.60 | 2.70 | 3.16 | 2.31 | 2.59 | 2.45 | 2.77 | 1.99 | 2.23 | 2.52 |
| | | | | 2.62 | 4.23 | 2.82 | 3.16 | 2.55 | 2.66 | 2.27 | |
| Fructose concentration (mg/g) | – | 2.15 | 2.69 | 1.74 | 1.61 | 2.89 | 2.64 | 2.35 | 2.71 | 3.41 | – |
| Prostaglandin E concentration (µg/g) | – | 119 | 155 | 245 | 206 | – | 71.0 | 103 | 163 | 221 | 191 |
| | | | | 164 | 187 | 43.5 | 26.6 | 29.2 | 41.9 | 134 | – |
| 19-Hydroxy-prostaglandins concentration (µg/g) | – | 5.7 | 7.2 | 21.2 | 3.2 | – | <4.7 | <1.2 | <5.6 | 9.5 | 8.1 |
| | | | | 7.6 | 12.9 | <3.7 | <1.5 | <1.0 | <1.0 | <3.7 | – |
| Prostaglandin F concentration (µg/g) | – | 2.4 | 3.7 | 3.8 | 2.1 | 1.1 | 0.25 | 0.16 | 0.11 | 2.4 | – |

The upper and lower values refer to dosage levels of 3.6 and 7.2 g aspirin/day respectively

TABLE 7 Effect of aspirin on semen weight, fructose concentration and prostaglandin concentrations in Subject B

| Parameter | Pre-treatment day | | | | | Treatment day | | | Post-treatment day | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 1 | 2 | 3 |
| Semen weight (g) | 1.60 | 1.17 2.11 | 1.67 1.60 | 1.54 1.83 | 2.27 1.53 | 1.97 1.69 | 2.24 1.69 | 1.43 2.23 | 1.78 1.89 | 2.37 1.23 | 1.77 1.65 |
| Fructose concentration (mg/g) | 0.94 | 1.37 1.47 | 0.97 1.19 | 0.94 1.26 | 1.08 1.24 | 0.88 1.17 | 1.42 1.32 | 1.19 1.28 | 1.04 1.32 | 0.83 1.40 | 0.89 1.74 |
| Prostaglandin E concentration (μg/g) | 63.9 | 33.7 52.0 | 35.6 33.6 | 38.0 44.1 | 54.0 31.2 | 19.7 12.7 | 19.4 8.0 | 26.5 10.8 | 41.1 15.0 | 60.1 37.4 | 53.1 58.6 |
| 19-Hydroxyprostaglandins concentration (μg/g) | 11.4 - | 11.9 8.0 | 7.5 7.4 | 5.3 6.2 | 10.4 1.7 | 4.2 2.5 | 1.7 2.0 | 2.6 1.6 | 4.9 2.4 | 6.9 5.8 | 7.7 10.6 |

The upper and lower values refer to dosage levels of 3.6 and 7.2 g aspirin/day respectively

## Prostaglandins E

By converting PGE compounds to their PGB derivatives and measuring the resultant absorption at 278 nm, an estimate of total PGE is obtained. This method does not provide a separate estimate of $PGE_1$ and $PGE_2$. In one experiment, the PGB formed by this method in a control sample showed a single peak as its methyl ester-trimethylsilyl ether derivative on gas chromatography at a retention time corresponding to that found for authentic $PGB_1$ (which does not separate from $PGB_2$ on the gas chromatographic system used). A mass spectrum taken during the GLC peak exhibited prominent m/e peaks in pairs at a.m.u. values of 422 and 420 $[M^+]$, 391 and 389 $[M - 31]$, 351 and 349 $[M - 71]$, and 323 and 321 $[M - (71 + 28)]$. This corresponds to a mixture of $PGB_1$ and $PGB_2$ (see Fig. 6), the heights of the pairs of peaks differing by 2 a.m.u. indicating approximately equal amounts of $PGE_1$ and $PGE_2$ in the original sample. The mean recovery determined by the tritium-labelled internal standard was 42% ($\pm$ 12). Concentrations of PGE shown in the Tables are corrected for recovery.

The levels of PGE fluctuated during the control period but no obvious trend in these variations could be detected. During treatment with aspirin in both subjects, the levels of PGE fell by 52% and 60% at the 3.6 g/day level and by 78% and 82% at the 7.2 g/day level. The effect was maximal after 48 hours of treatment, but after 72 hours of treatment the PGE levels were slightly but consistently higher in all four experiments. A return to pre-treatment levels of

PGE had occurred by 48 hours after cessation of aspirin treatment.

## 19-Hydroxy-prostaglandins

In both subjects, ejaculates collected at 24 hour intervals contained far lower concentrations of the 19-hydroxy-prostaglandins than of PGE. The levels of these 19-hydroxy compounds (estimated in terms of 19-hydroxy-PGB) were not corrected for recovery since there was no available source of isotopically-labelled internal standard. The levels of the 19-hydroxy compounds fell to a minimum after 48 hours of aspirin treatment, but then started to rise despite continued treatment.

## Prostaglandins F

In ejaculates collected at 24 hour intervals, the levels of PGF were low as measured biologically on the isolated rabbit jejunum after thin layer chromatography and after treatment of the sample with dilute KOH (which does not inactivate PGF). There was a difference between the two subjects. Subject A had a control level equivalent to 2.9 g $PGF_{2\alpha}$/g semen which fell to 0.21 g/g during treatment with aspirin at 7.2 g/day, whereas subject B had less than 0.5 μg/g even during the control periods and the contractions were qualitatively different from those to $PGF_{2\alpha}$. Contractions of the jejunum to the extract were quick in onset and short-lived whereas those to $PGF_{2\alpha}$ were more gradual in onset and longer-lasting. The nature of this substance was not determined but its presence made estimates of PGF levels in subject B unreliable.

TABLE 8 Effects of aspirin on semen weight, fructose concentration and prostaglandin concentrations in Subject A and Subject B

| | Subject | Aspirin dosage (g/day) | Mean control ± S.E. | Mean test ± S.E. | % change | Statistical significance (P value) |
|---|---|---|---|---|---|---|
| Semen weight (g) | A | 3.6 | 2.55 ± 0.21 | 2.61 ± 0.16 | +2 | >0.1 |
| | A | 7.2 | 3.08 ± 0.36 | 2.86 ± 0.30 | -7 | >0.1 |
| | B | 3.6 | 1.77 ± 0.16 | 1.83 ± 0.41 | +3 | >0.1 |
| | B | 7.2 | 1.66 ± 0.12 | 1.96 ± 0.27 | +18 | >0.1 |
| Fructose concentration (mg/g) | A | 3.6 | 2.32 ± 0.33 | - | - | - |
| | A | 7.2 | 1.00 ± 0.07 | 2.50 ± 0.14 | +8 | >0.1 |
| | B | 3.6 | 1.38 ± 0.08 | 1.31 ± 0.11 | +31 | >0.05 |
| | B | 7.2 | | 1.30 ± 0.02 | -6 | >0.1 |
| Prostaglandin E concentration ($\mu g/g$) | A | 3.6 | 216 ± 12 | 87.0 ± 16.0 | -60 | <0.01 |
| | A | 7.2 | 152 ± 12 | 27.9 ± 1.3 | -82 | <0.01 |
| | B | 3.6 | 48.3 ± 4.7 | 23.0 ± 3.6 | -52 | <0.05 |
| | B | 7.2 | 42.8 ± 7.0 | 9.4 ± 0.8 | -78 | <0.01 |
| Prostaglandin F concentration ($\mu g/g$) | A | 7.2 | 2.89 ± 0.36 | 0.21 ± 0.04 | -93 | <0.01 |

## Symptoms experienced during aspirin treatment

At the lower dose level of aspirin (3.6 g/day), no
side effects were reported by either subject. At 7.2 g/day,
subject A experienced tinnitus, deafness and slight hyper-
pnoea 24 hours after the start of treatment. After 48 hours
the symptoms were most marked. There was nausea but no
vomiting and the faeces were dark in colour. In view of
the possibility of a severe gastro-intestinal haemorrhage,
aspirin treatment in this subject was stopped after 64 hours.
The severe symptoms persisted for a further 20 hours, were
slight after 56 hours and were absent 80 hours after the
cessation of aspirin therapy.

At the higher dose level, subject B reported trouble-
some tinnitus and deafness throughout the treatment period,
but the treatment was not curtailed. These symptoms were
still marked 16 hours after the end of the treatment.
Abdominal discomfort, heartburn and epistaxis occurred
during the last day of treatment.

## DISCUSSION

In a previous investigation, Collier and Flower (1971)
showed that human seminal prostaglandin levels, estimated
by bioassay, are reduced during a 7-day course of aspirin
treatment (2.4 g/day). In the present investigation both
PGE and 19-hydroxy-prostaglandin, which together comprise
about 90% of the total prostaglandin content of human semen,
have been measured using an absorptiometric method
(Bygdeman and Samuelsson, 1966) which is more accurate than

bioassay. The use of a radioactive internal standard enabled corrections for recoveries to be made (with respect to PGE).

With these physico-chemical methods of determination, it has been possible to show that aspirin administration reduces the levels of the three groups of prostaglandins in human semen. However, even at near toxic doses used in this study, the PGE content was never reduced to zero, although 19-hydroxy prostaglandin levels became undetectable and the PGF levels were reduced by over 90% in one subject. On the other hand, this effect of aspirin appears to be selective since neither the quantity of semen nor the fructose levels were affected even by the higher dose.

The results provide evidence that in human subjects, aspirin in high therapeutic doses reduces the levels of all prostaglandins in the semen. If seminal prostaglandins are important for fertility, these drug-induced low levels may be associated with diminished fertility. It seems unlikely, in the light of toxic effects experienced by both subjects, that high aspirin dosage could be maintained for sufficiently long periods in man to put this suggestion to the test. It is possible that a retrospective study amongst male patients on a high aspirin regime therapeutically might reveal some interference with fertility, but no evidence has so far been produced.

It is not clear whether prostaglandins of the E series or their hydroxylated derivatives (Kelly and Taylor, 1976) are likely to be the more important physiological constituents

of human semen. Both are present in exceptionally high concentrations though in certain primates it is the 19-hydroxylated derivatives only that are found. The biosynthetic route of the 19-hydroxy compounds is not known but this study showed that their production is reduced by aspirin and therefore that they are likely to be indirect products of the enzyme, fatty acid cyclo-oxygenase.

PROSTAGLANDINS   FROM   THE   FEMALE   REPRODUCTIVE   TRACT

A Study of the Output of Prostaglandins by the
Guinea-Pig Uterus under Various Conditions and the
Role of Prostaglandin $F_{2\alpha}$ as the Uterine Luteolytic Hormone

Hysterectomy in the guinea-pig is followed by great length-
ening of the oestrous cycle (Loeb, 1923). This is a local
effect since removal of only one horn results in ipsilateral
retention of functional corpora lutea. Work on both guinea-
pigs and sheep prior to 1970 provided much evidence to support
the hypothesis that the uterus in these species secretes a
substance which is luteolytic (Anderson et al., 1969)

Premature luteolysis can be induced by inserting beads
into the uterine lumen of guinea-pigs (Donovan and Traczyk,
1962) or by administration of oestrogen early in the
cycle (Bland and Donovan, 1970). These two stimuli appear
to exert their luteolytic effects by causing premature
release of the luteolytic hormone, and were used as the
starting point in this study which concerned the identifi-
cation of the luteolytic hormone. The reports of the
luteolytic activity of $PGF_{2\alpha}$ by Pharriss and Wyngarden (1969)
and Blatchley and Donovan (1969) considerably simplified
this task since the question which then had to be answered
was: 'Is the luteolytic hormone in the guinea-pig identical
with $PGF_{2\alpha}$?'.

The truth of this hypothesis has been subjected to various kinds of test reported here. It appears that the guinea-pig does release $PGF_{2\alpha}$ from its uterus under physiological conditions and this contributes to the control of oestrous cycle length.

## METHODS

Distension of guinea-pig uterus in vitro    Female guinea-pigs weighing about 500 g were smeared daily by a vaginal lavage technique and killed on day 3 or 4 of the oestrous cycle (day 1 being the day before the post-ovulatory influx of leucocytes, when cornification of the vagina was maximal). The two uterine horns were removed and separated. One horn was distended by the intraluminal insertion of a piece of polyethylene tubing (3 mm wide and 30 mm long). The other horn was treated similarly except that the polyethylene tubing was removed immediately after insertion. Both horns were incubated separately at $37^0$ in 8 ml of Tyrode's solution for 3 hours and gassed with 5% $CO_2$ in oxygen. The two incubated solutions were assayed on the rat fundal strip (see p. 26) in terms of $PGE_1$ and of $PGF_{2\alpha}$.

The pooled test and control samples were then extracted by the procedure outlined in Figure 2 (p.19) and re-assayed in terms of prostaglandins.

Silicic acid column chromatography, formation of Me/TMS and Me/TFA derivatives and combined GLC/MS were performed as described previously (p. 23 and 25).

<u>Effect of oestrogen treatment</u>    Guinea-pigs were injected subcutaneously with 10 µg of oestradiol benzoate (Oestroform, British Drug Houses) on days 4, 5 and 6 of the oestrous cycle. Blood was collected from them on day 7. Groups of 5 animals were injected and the same number of untreated day 7 guinea-pigs were used as controls. Plasma from 4 or 5 treated guinea-pigs was pooled in each experiment to give approximately 30 ml. Plasma from untreated controls was similarly pooled. This comparison was made in eight separate experiments.

<u>Investigation of guinea-pigs during the oestrous cycle</u>

Adult female guinea-pigs of mixed stock weighing 500 to 800 g were used. They were housed under constant conditions of light and temperature, fed a proprietary diet (RGP, Dixon & Sons Ltd, Ware) and given drinking water to which ascorbic acid (1 g/l) and citric acid (2 g/l) were added as an anti-scorbutic measure, <u>ad</u> <u>libitum</u>.

<u>Blood collection</u>    Animals were anaesthetized with pento-barbitone sodium (Nembutal, Abbott Laboratories Ltd) 400 mg/kg injected intraperitoneally. A large ventral mid-line abdominal incision was made and the alimentary tract was displaced and kept damp in a polyethylene bag. The utero-ovarian vein on one side (usually the left) was exposed, care being taken to avoid manipulation of the uterus, and the fat surrounding the vein was handled as little as poss-ible. The vein can be very small and frail and constricts to a mere thread unless great care is taken. For this

reason no attempt was made to separate the artery and the vein which are very closely applied to each other. After placing a ligature round the vein as far cranially as possible, 5000 i.u. heparin (Pularin, Evans Medical Ltd) were injected into a vein in the leg. The ligature round the utero-ovarian vein was tightened and a needle attached to a silastic tubing (internal diameter 0.025 in., external diameter 0.047 in.) was inserted into the vein with the aid of a dissecting microscope. Blood was allowed to flow under negative pressure into an ice-cooled tube for 60 to 90 minutes before the animal was killed, by which time about 10 to 16 ml had been collected. Oestrogen pre-treatment did not appear to alter the volume of blood collected during this period. When the collection was finished and a haematocrit taken, the blood was centrifuged at $4^0$ and the plasma stored at $-20^0$ until extracted and assayed.

Schedule of blood collection from cyclic guinea-pigs   Blood was collected from 5 animals each on days 3, 9, 10, 11, 12, 13, 14 and 15 of the cycle. A small aliquot of plasma from each guinea-pig was kept for progesterone analysis, the remainder being pooled for each of the groups so that 30 to 40 ml plasma per day was available for prostaglandin analysis.

Estimation of progesterone levels by protein binding

0.1 ml plasma from the utero-ovarian vein was diluted 15
times with water and 0.1 ml of the diluted solution assayed
by a rapid competitive protein binding method based on that
described by Johansson (1969). Progesterone was extracted
by shaking with 2.5 ml petroleum ether (Mallinckrodt 4980,
b.p. 30-60° Lot XKH) for one minute. The petroleum extract
was dried and the protein binding reaction carried out using
corticosteroid-binding globulin (obtained on day 21 of the
menstrual cycle from a human volunteer who was regularly
taking a combined oestrogen-progestogen contraceptive pill)
labelled with ($^3$H)-corticosterone (31.7 Ci/mmol, Radio-
chemical Centre, Amersham). The progesterone extracted
from the samples displaced the labelled corticosterone
in proportion to the amount of progesterone present. A
standard curve was run in parallel. After separation of
the free and bound steroid by the use of the adsorbent
Florisil (VMA Purifier, Sigma), 0.5 ml aliquots were added
to scintillation fluid and counted with an efficiency for
tritium of 40%. Samples were assayed in duplicate on
two separate occasions and if the mean value of each assay
differed by more than 10%, a third assay was carried out.
The coefficient of variation in the measurement range of
0.0 to 2.0 ng on the standard curve was less than 15%.
The mean percentage recovery of ($^3$H)-progesterone from the
plasma samples over a three-month period was 91.5 $\pm$ 0.8%.
The method measured total progestins but the contribution
from 17$\alpha$-hydroxy-progesterone was limited by its low

extraction from plasma (30%) whilst contribution from
20α-dihydroprogesterone was reduced by its low affinity
to corticosteroid-binding protein.    Moreover, it has been
reported that insignificant amounts of these two substances
are produced by the guinea-pig (Challis, Heap and Illingworth,
1971).    Known amounts of progesterone added to plasma from
castrated male guinea-pigs were almost wholly recovered
over the range 0.0 to 5.0 ng progesterone.    The limit of
sensitivity of the method when measuring progesterone in
utero-ovarian venous plasma is 12 ng/ml plasma.

Estimation of progesterone by radioimmunoassay    Blood (2 ml)
was collected by cardiac puncture from guinea-pigs treated
with indomethacin or actively immunized or control animals
three times weekly (Monday, Wednesday and Friday) throughout
the investigation.    The animals stood up well to this regime
and remained healthy.    Eventually they died of pericardial
haemorrhage following cardiac puncture.    The blood was
drawn into a syringe containing 10 i.u. heparin in 0.1 ml
saline.    The plasma was separated by centrifugation and
stored at -20° until assayed.    Progesterone levels were
determined by radioimmunoassay.    The antibody which had
the cross-reactivities shown in Table 9, was kindly supplied
by Drs Dighe and Hunter whose method of assay was followed
(Dighe and Hunter, 1974), 0.2 ml of each plasma sample
being extracted in duplicate by light petroleum (b.p. 40-60°).
Recoveries of progesterone were determined by their method
alone.    Tritiated progesterone (specific activity 81 Ci/mmol)

TABLE  9   Cross-reactivity of progesterone antiserum
as determined by K. K. Dighe using the
method of Dighe and Hunter (1974)

|  | %<br>Cross-reactivity |
|---|---|
| Progesterone | 100.0 |
| 11α-OH-Progesterone | 29.0 |
| 11β-OH-Progesterone | 12.0 |
| 17α-OH-Progesterone | 1.0 |
| 11-Desoxycorticosterone | 1.0 |
| 11-Deoxycortisol | 0.2 |
| Cortisol | 0.01 |
| Pregnenolone | 0.2 |
| Cholesterol | 0.002 |
| Androstenedione | 0.03 |
| 20α-Hydroxypregn-4-en-3-one | 0.9 |
| 20β-Hydroxypregn-4-en-3-one | 0.05 |
| 5α-Pregnane-3,20-dione | 12.0 |
| 5β-Pregnane-3,20-dione | 7.0 |

was supplied by the Radiochemical Centre, Amersham.
The accuracy and precision of the assay were determined
by adding known amounts of progesterone to 0.2 ml male
guinea-pig plasma and estimating the amount recovered.
For quality control purposes, 0.1 or 0.2 ml of pooled
plasma from guinea-pigs in the luteal phase was incorpor-
ated into each assay.   From the results obtained the co-
efficient of variation between assays was calculated.   The
coefficient of variation within assays was calculated by
the method of Dighe and Hunter (1974).

Estimation of prostaglandin levels   Prostaglandins were
extracted from plasma, separated by silicic acid chromato-
graphy, bioassayed on the rat fundal strip and analysed by
GLC/MS using methods described above (pp. 18-25).

Prostaglandin recovery experiments   The percentage recov-
eries of $PGF_{2\alpha}$ and $PGE_2$ over concentration ranges from
10 ng/ml blood upwards were estimated as follows.   Blood
was collected from three guinea-pigs and heparinized.
Sufficient prostaglandin was added to give the required con-
centration and after mixing, the blood was incubated at $37^\circ$
for 1 hour before centrifugation.   The plasma samples were
extracted, chromatographed and bioassayed as described above.
The percentage recoveries thus calculated were for $PGF_{2\alpha}$
(10, 20 and 330 ng/ml) 48, 44 and 45% respectively and for
$PGE_2$ (10, 30 and 50 ng/ml) 43, 46 and 48% respectively.

Derivatization of prostaglandin E    The sample was first
esterified with diazomethane as described above (p. 25).
The methoxime was then prepared by the addition of 150 μl
of a freshly prepared solution of 1% methoxyamine hydro-
chloride in pyridine.    After 12 hours, the sample was
evaporated to dryness in vacuo, and the TMS formed by the
addition of 25 μl BSTFA.    On GLC two peaks were obtained
corresponding to the two stereoisomers of the methoximes.

    In other experiments PGE was first converted to PGB
(as described above, p. 23).    The PGB was purified by solvent
extraction and thin layer chromatography in the toluene:
dioxan:acetic acid system already described.    The Me/TMS
derivative of this purified PGB was then prepared for GLC/MS.

Derivatization of prostaglandin F    Me/TMS and Me/TFA deriv-
atives were prepared as above (p.25 ).

Indomethacin Injections    Indomethacin dissolved in 0.5 ml
polyethylene glycol was injected subcutaneously at 12 hourly
intervals from the 7th day of the cycle until the 1st day of
the next cycle, at two dose levels (4 and 20 mg/day).
Control guinea-pigs were similarly injected with 0.5 ml
polyethylene glycol.

Intra-uterine implants of indomethacin    Implants (15 mm
long, 2 mm maximum diameter, weighing 25 mg) were prepared
by the incorporation of indomethacin powder into paraffin
wax (m.p. 43-45°) in the ratio of 1:2 parts by weight.    For
control purposes similar implants containing paraffin wax
alone were prepared.

Active immunization of guinea-pigs    The test guinea-pigs were injected intradermally at multiple sites on the back with a bovine serum albumin-$PGF_{2\alpha}$ conjugate (Dighe, Emslie, Henderson and Simon, 1975) emulsified in Freund's adjuvant. The total volume injected in each animal was 1.0 ml, containing the equivalent of 100 µg of $PGF_{2\alpha}$. Pertussis vaccine (0.1 ml) was injected subcutaneously at 2 sites. The 5 control animals received identical treatment except that the $PGF_{2\alpha}$ and the bovine serum albumin were not conjugated.    Booster injections (1.0 ml) in Freund's incomplete adjuvant were given subcutaneously 4 weeks later at 4 sites, 2 suprascapular and 2 in the femoral region.

Determination of antibody levels    Blood for antibody level determination was collected from each animal by cardiac puncture 4 to 6 months after the initial immunization.  The titre in each serum was estimated using 10 pg $^3H$-$PGF_{2\alpha}$ (specific activity 160 Ci/mmol).  The reaction volume was 1 ml and the 'titre' was expressed as the dilution of serum which bound 50% of $^3H$-$PGF_{2\alpha}$.  Sera were considered not to have a titre if at a dilution of 1 in 500, the level of $^3H$-$PGF_{2\alpha}$ bound was similar to that bound in the non-specific binding tests (about 10%).

Hysterectomy    For comparative purposes, three guinea-pigs were hysterectomized under sodium pentobarbitone anaesthesia and blood for plasma progesterone determinations was collected from two of these by cardiac puncture three times weekly.

## RESULTS

Foreign Bodies in the Lumen of Guinea-pig Uterus in vitro

In the first series of experiments the amount of smooth muscle stimulating activity in the Tyrode's solution bathing the horn containing polyethylene tubing exceeded that in the incubate from the control in 8 out of 9 tests (Table 10). None of the three autacoid antagonists, methysergide, mepyramine or atropine, abolished the biological activity of the incubate on the fundus indicating that 5-hydroxytryptamine, histamine and acetylcholine, responses to which were abolished, were not present. Moreover, the biological activity remained after incubation with chymotrypsin (Horton, 1959), showing that it was not attributable to a smooth muscle stimulating peptide such as bradykinin, substance P, angiotensin or oxytocin.

In a further control experiment with one pair of uterine horns, one horn was incubated in the presence of a piece of polyethylene tubing (not inserted into the lumen) and the other horn was incubated alone. There was no difference between the amount of smooth muscle stimulating activity in the incubation media of the two horns (equivalent to 7.5 ng $PGF_{2\alpha}$/ml), indicating that extraluminal polyethylene tubing did not affect output. Finally, two pieces of polyethylene were incubated separately in the absence of uterine tissue; no prostaglandin could be detected in the incubation medium of either. It follows therefore that the difference between test and control samples found above must be attributable

TABLE 10     Assay of the nine pairs of incubated samples obtained in the second experiment on the rat fundal strip

| | Activity    ng of $PGE_1$/ml | |
|---|---|---|
| Sample | Test (distended horn) | Control (non-distended horn) |
| 1 | 12 | 7.5 |
| 2 | 10 | 2.5 |
| 3 | 15 | n.d. (<3) |
| 4 | 10 | 10 |
| 5 | 14 | n.d. (<3) |
| 6 | 16 | n.d. (<3) |
| 7 | 18 | 7 |
| 8 | 14 | 7 |
| 9 | 20 | n.d. (<3) |

n.d.     No detectable activity

to the presence of polyethylene tubing within the uterine lumen.   This must stimulate increased release of a smooth muscle stimulating substance from the uterus.

A further series of experiments were designed to identify the active principle.   Pooled incubation fluid from 35 pairs of guinea-pig uteri were solvent extracted;   the active principle behaved like a polar acidic lipid on partition. On silicic acid chromatography the activity in both test and control samples was eluted with 80% ethyl acetate in benzene (Fig. 7).   This corresponds to the chromatographic behaviour of authentic $PGF_{2\alpha}$.   In terms of $PGF_{2\alpha}$ the extracts in total contained 1 µg $PGF_{2\alpha}$ (test) and 100 ng (control).

Quantitative parallel biological assays provide strong evidence of identification (Chang and Gaddum, 1933).   Assay of the 'PGF' chromatographic fractions on the rat fundus, rabbit jejunum, guinea-pig ileum and the ascending colon of the Mongolian gird (Meriones unguiculatus) showed good agreement when assayed in terms of $PGF_{2\alpha}$ (Table 11).   The greatest index of discrimination (Gaddum, 1955) between the unknown and $PGF_{2\alpha}$ was only 2 whereas between the unknown and the closely related $PGE_1$, it was 297.   Moreover, on the rabbit jejunum contractile responses to $PGE_1$ and $PGF_{2\alpha}$ were qualitatively different (as described by Horton and Main, 1965).   The responses to the test sample resembled those to $PGF_{2\alpha}$.   This combination of physico-chemical, chromatographic and biological evidence showed that the active principle was prostaglandin-like.   From the parallel assay results a PGE could not be the active principle released by uterine distension.

ELUANT:- ETHYL ACETATE IN BENZENE
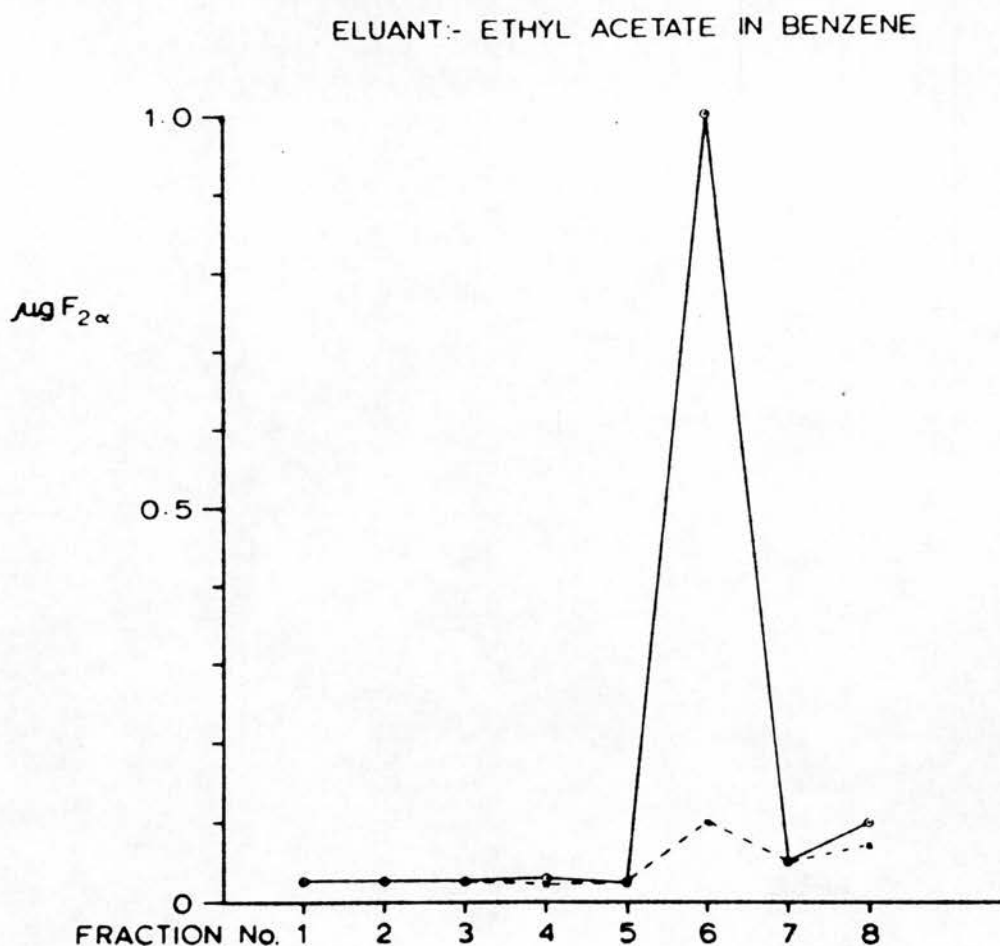


Figure 7    Silicic acid chromatography of extracted
            incubation fluid from distended (continuous
            line) and non-distended (interrupted line)
            horns of guinea-pig uterus in vitro.
            Fractions of column effluent were assayed
            in the isolated rat fundus strip preparation
            Ordinate:  biological activity in terms of
            $\mu g$ equivalent of $PGF_{2\alpha}$.  Abscissa:  fraction
            numbers (1 = 30% ethyl acetate in benzene;
            2 = 40%;  3, 4 and 5 = 60%;  6 = 80%; 7 = 100%;
            8 = 100% methanol)

TABLE 11 Results of the parallel assay of the 80% ethyl acetate in benzene fraction obtained from silicic acid chromatography of incubate extracts from test and control uteri

## Total Biological Activity

(in microgram equivalents of $PGF_{2\alpha}$ or $PGE_1$)

|  | Test | | Control | |
| --- | --- | --- | --- | --- |
|  | $PGF_{2\alpha}$ | $PGE_1$ | $PGF_{2\alpha}$ | $PGE_1$ |
| Rat fundus | 1.0 | 1.5 | 0.1 | 0.15 |
| Rabbit jenunum | 1.0 – 2.0 | 10 – 20 | 0.06 – 0.13 | 0.6 – 1.3 |
| Jird colon | 1.2 – 1.4 | 0.6 – 0.7 | 0.09 – 0.1 | 0.04 – 0.05 |
| Guinea-pig ileum | 1.3 – 2.0 | 0.07 – 0.1 | not tested | |

The evidence strongly suggests that the substance is $PGF_{2\alpha}$ but it is insufficient alone to prove that this is so (see Horton and Main, 1967, for the presentation of this argument).

The next experiments were designed to obtain conclusive evidence . The previous experiment was repeated with a further 35 guinea-pigs and the $PGF_{2\alpha}$-like material from the silicic acid column (about 700 ng in the test sample and 80 ng in the control sample as estimated by bioassay) was subjected to combined GLC/MS. The Me/TMS and Me/TFA derivatives of the test samples gave gas chromatographic peaks at 19 and 6 minutes respectively corresponding exactly to the retention times of the same derivatives of authentic $PGF_{2\alpha}$. Estimated by the size of the GLC peak, the amount of $PGF_{2\alpha}$ in the test samples agreed well with the figure obtained by biological assay. The control sample gave no detectable GLC peak at these times, presumably due to insufficient material being present.

Mass spectra of these two derivatives of test and authentic $PGF_{2\alpha}$ samples were recorded at the maximum of the. GLC peaks and at the equivalent retention time (for control samples which showed no GLC peaks). Mass spectra showing the major $^m$/e peaks above 300 a.m.u. are illustrated in Figure 8.

The spectrum of authentic $PGF_{2\alpha}$ (Me/TMS) had eight abundant $^m$/e peaks 584 $[M^+]$, 569 $[M - 15]$, 513 $[M - 71]$, 494 $[M - 90]$, 423 $[M - (90 + 71)]$, 404 $[M - (90 \times 2)]$, 333 $[M - (2 \times 90 + 71)]$ and 307. These eight peaks were seen in the mass spectra of the test (distended) sample
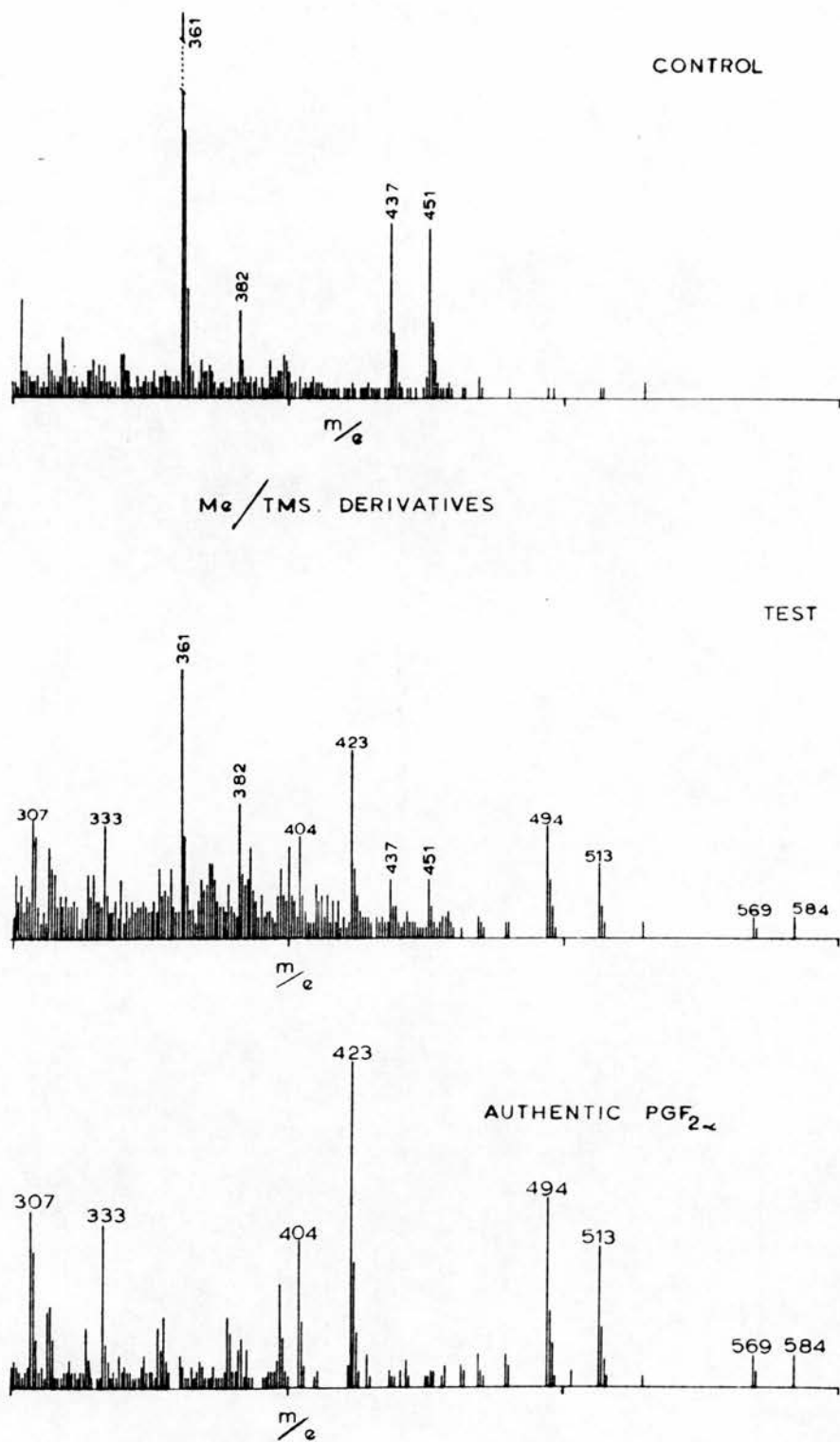
Figure 8    Mass spectra of the Me/TMS derivatives of
            control incubate (upper), test incubate
            (middle) and standard $PGF_{2\alpha}$ (lower).  The
            control and test samples were PGF fractions
            from incubate extracts of non-distended and
            distended horms of guinea-pig uterus in vitro
            respectively.  Ordinate:  relative abundance.
            Abscissa:  $^m/e$.  Peaks characteristic of
            $PGF_{2\alpha}$ Me/TMS numbered horizontally, other
            major peaks vertically.

but not in the control (non-distended) sample. Moreover, the heights of the peaks in the test samples are in similar ratio to those in the authentic $PGF_{2\alpha}$ spectrum. Four other conspicuous peaks were recorded in the test sample (451, 437, 382 and 361). These were not present in the authentic $PGF_{2\alpha}$ (Me/TMS) spectrum but were found in similar abundance in control samples. These peaks can be attributed to the presence of interfering substances which were common to both test and control and are not due to the presence of a prostaglandin.

Similar results were obtained with the Me/TFA derivatives (Fig. 9). The $PGF_{2\alpha}$ (Me/TFA) and test sample had three conspicuous $^m/e$ peaks in common at 542 [M - 14], 426 [M - (2 x 114)] and 314 [M - (3 x 114)] whereas test and controls had a 326 peak in common which was not present in the $PGF_{2\alpha}$ (Me/TFA) spectrum.

It was concluded from this evidence that the test sample contained $PGF_{2\alpha}$, but that any $PGF_{2\alpha}$ present in the control was below the level of detectability.

On the basis of GLC behaviour neither $PGF_{1\alpha}$ nor $5\alpha,7\alpha$-dihydroxy-11-oxo-tetranorprostanoic acid (isolated from guinea-pig urine where it is the principal $PGF_{2\alpha}$ metabolite) could be detected in either the test or control samples.

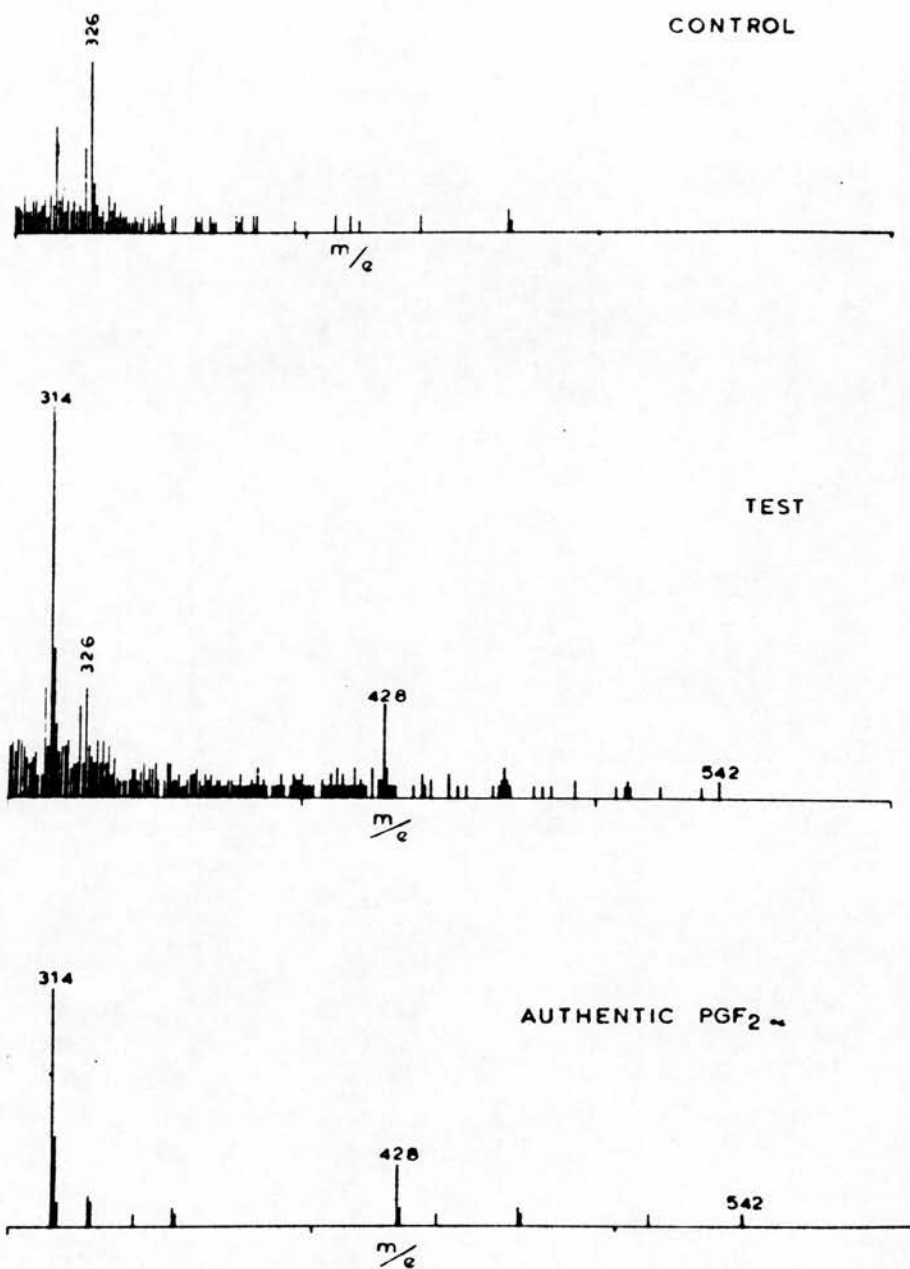Figure 9    Mass spectra of the Me/TFA derivatives of
            control incubate (upper), test incubate
            (middle) and standard $PGF_{2\alpha}$ (lower).  The
            control and test samples were PGF fractions
            from incubate extracts of non-distended and
            distended horns of guinea-pig uterus in vitro
            respectively.  Ordinate:   relative abundance.
            Abscissa: $^m/e$.  Peaks characteristic of
            $PGF_{2\alpha}$ Me/TFA numbered horizontally, other
            major peaks vertically.

Treatment with oestrogen   Biological assay on the rat
fundal strip of fractions eluted from the silicic acid
column showed the presence of a PGF-like substance in all
seven of the plasma extracts from intact guinea-pigs treated
with oestrogen on days 4 to 6 of the oestrous cycle (Table 12).
None of the (seven) controls contained any PGF-like activity
nor did the oestrogen-treated hysterectomized group.  Bio-
logical assay on the rat fundal strip of fractions eluted
from silicic acid was used to detect PGF-like activity.
In none of the samples (test or control) was any biological
activity detected in other chromatographic fractions.  All
values shown in Table 12 have been corrected for 45%
recovery.

Two of the test samples were subjected to GLC/MS after
derivatization.  On GLC the Me/TMS gave a peak at the
retention time corresponding to authentic $PGF_{2\alpha}$ (Me/TMS).
Mass spectra (Fig. 10) showed all the principal $^m$/e peaks
of $PGF_{2\alpha}$ found in previous experiments described previously.
These GLC/MS data confirmed that the substance detected by
biological assay was $PGF_{2\alpha}$.

The guinea-pig metabolises $PGF_{2\alpha}$ to $5\alpha, 7\alpha$-dihydroxy-
11-oxo-tetraprostanoic acid which co-chromatographs with
$PGF_{2\alpha}$ on silicic acid (Horton and Los, unpublished), but
separates from $PGF_{2\alpha}$ on GLC, its Me/TMS derivative having
a retention time of 5.3 minutes ($PGF_{2\alpha}$ Me/TMS 16.6 minutes).
The metabolite isolated from the urine of guinea-pigs
injected with $PGF_{2\alpha}$ (during a study of its luteolytic
activity) was purified and converted to the Me/TMS.  Its

TABLE 12 Concentration of $PGF_{2\alpha}$ in the utero-ovarian
venous blood collected on day 7 of the
oestrous cycle of oestrogen-treated and
control guinea-pigs

| Experiment No | Treated | Control |
|---|---|---|
| 1 | 22 | <3.0 |
| 2 | 27 | <3.8 |
| 3 | 12 | <6.7 |
| 4 | 3.2 | <5.2 |
| 5 * | 10 | <2.2 |
| 6 | 5.2 | <1.9 |
| 7 *† | 12 | <1.9 |

* Confirmation of identification of $PGF_{2\alpha}$ by GLC/MS

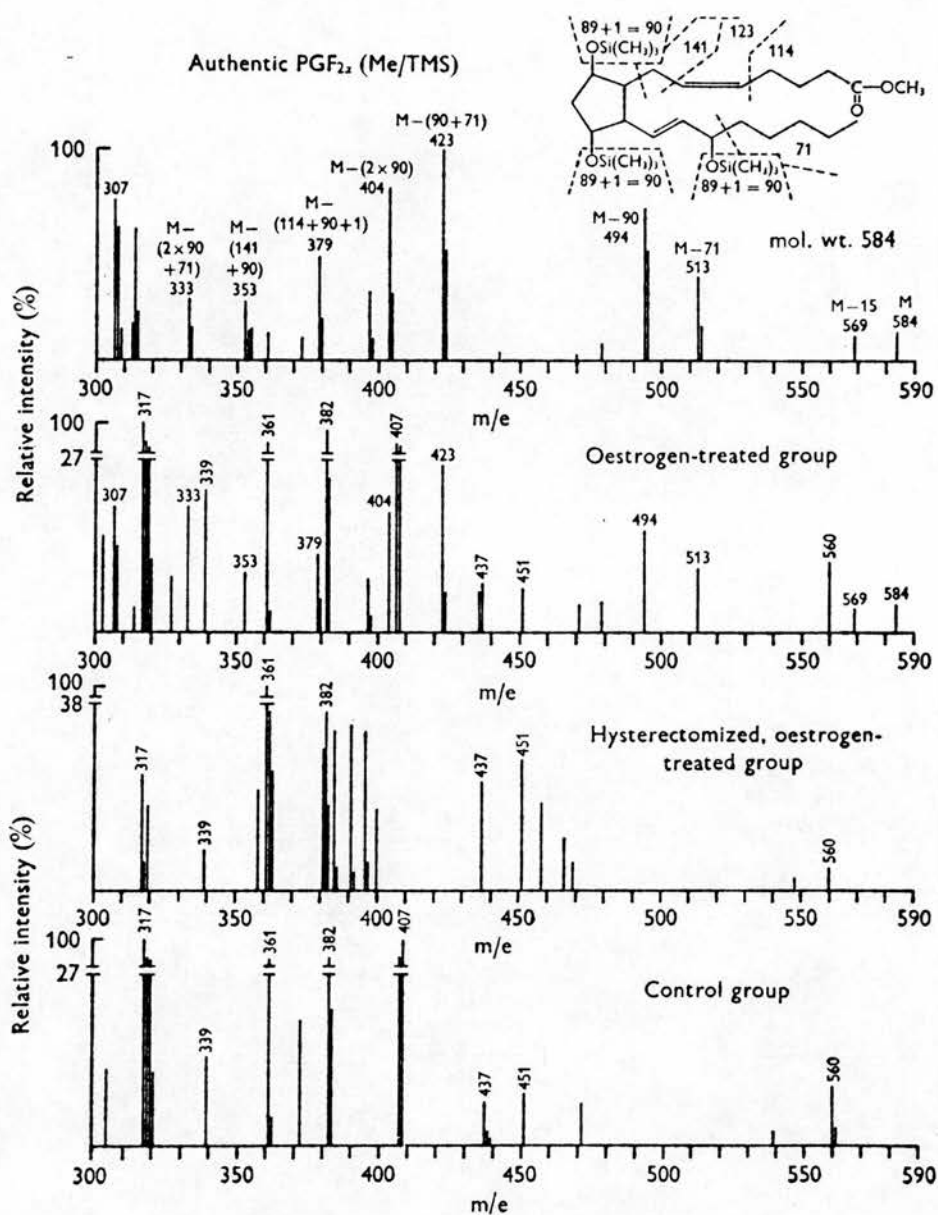† Hysterectomized oestrogen-treated animals
    <1.8 ng $PGF_{2\alpha}$/ml

Figure 10  Mass spectra of the Me/TMS derivatives of material extracted from the utero-ovarian venous blood of intact guinea-pigs treated with oestrogen, of hysterectomized guinea-pigs treated with oestrogen and intact non-treated control animals, and (top) standard $PGF_{2\alpha}$.
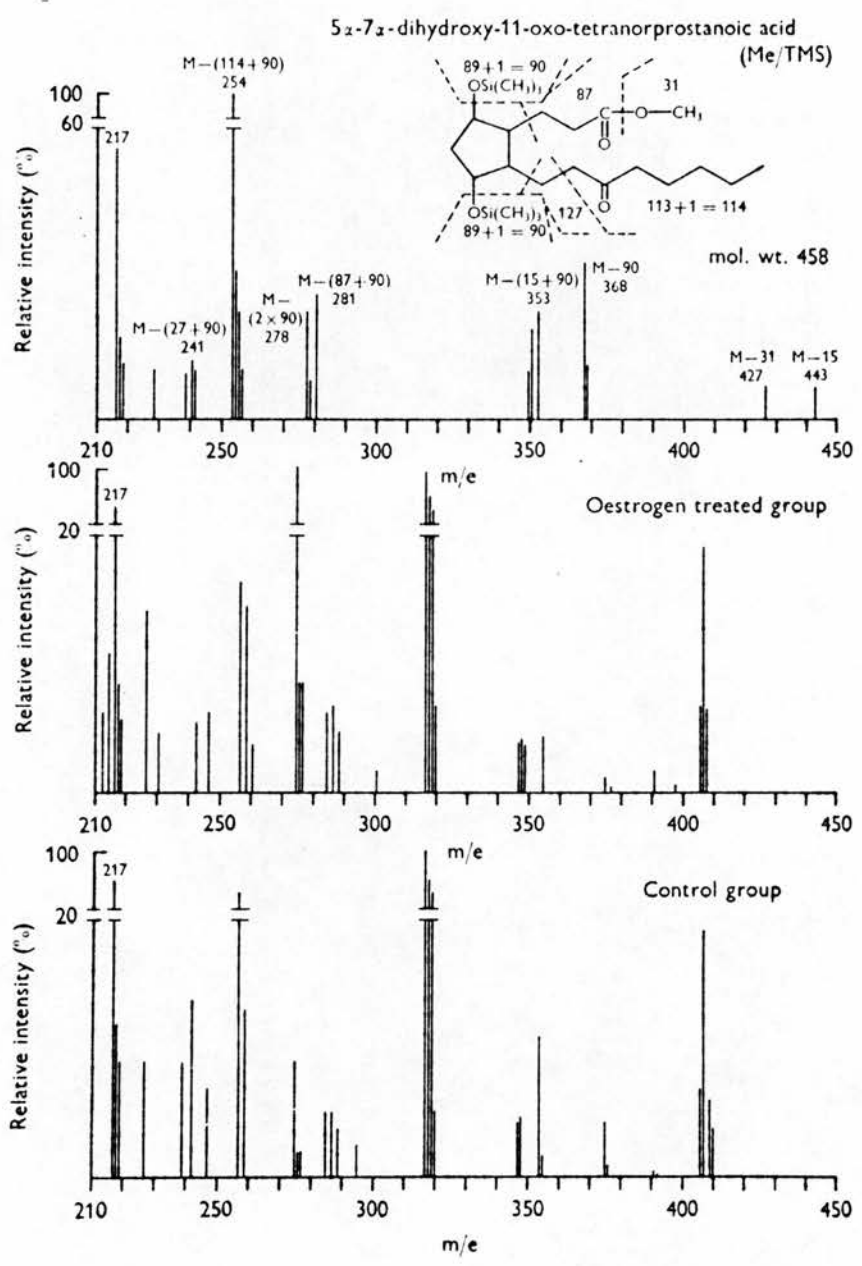
Figure 11  Mass spectra of the Me/TMS derivatives of
material extracted from the utero-ovarian
venous blood of oestrogen-treated and non-
treated (control) guinea-pigs and of
authentic 5α,7α-dihydroxy-11-oxo-tetranor
prostanoic acid.

mass spectrum (Fig. 11) had the following peaks:
443 $[M - 15]$, 427 $[M - 31]$, 368 $[M - 90]$, 353 $[M - (15 + 90)]$, 281 $[M - (87 + 90)]$, 278 $[M - (2 \times 90)]$, 254 $[M - (114 + 90)]$, 241 $[M - (127 + 90)]$, and 217. The molecular ion (458 a.m.u. was not seen. Of these peaks only the 217 was detected in any of the blood extracts analysed (Fig. 11). It is evident, therefore, that this metabolite is not found in guinea-pig utero-ovarian venous blood after oestrogen treatment.

## Plasma Levels of Prostaglandins and Progesterone in the Normal Oestrous Cycle of Guinea-pigs

Identification of $PGF_{2\alpha}$    GLC/MS analysis provided conclusive evidence for the presence of $PGF_{2\alpha}$ in utero-ovarian blood samples taken from guinea-pigs towards the end of the oestrous cycle. Under the GLC conditions used in these experiments the Me/TMS and Me/TFA derivatives of standard $PGF_{2\alpha}$ had retention times of 16.6 and 4.9 minutes respectively. Mass spectra were taken at these times of the material in the column effluent following injection on the GLC of the Me/TMS and Me/TFA derivatives of the plasma extracts derived from day 15 guinea-pigs. The major peaks (above a.m.u.) of $^m/e$ values 584 $[M^+]$, 569 $[M - 15]$, 513 $[M - 71]$, 494 $[M - 90]$, 423 $[M - (90 + 71)]$, 404 $[M - (2 \times 90)]$, 379 $[M - (114 + 90 + 1)]$, 353 $[M - (141 + 90)]$, 333 $[M - (2 \times 90 + 71)]$ and 307. These peaks were present in approximately the same ratios in the Me/TMS derivative of the plasma extract derived from day 15 guinea-pigs. Similarly the Me/TFA deriv- ative of oestradiol material and standard $PGF_{2\alpha}$ had peaks in common at $^m/e$ values of 542 $[M - 114]$, 511 $[M - (31 + 114)]$, 441 $[M - (101 + 114)]$, 428 $[M - (2 \times 114)]$, and 314 $[M - (3 \times 114)$ again in similar ratios. These GLC/MS results are identical to

to those shown above in Figures 8, 9 and 10. This is strong evidence for the presence of $PGF_{2\alpha}$ in utero-ovarian blood collected from guinea-pigs on day 15 of the cycle. Similar evidence with respect to the Me/TMS derivatives alone was found for the presence of $PGF_{2\alpha}$ in blood collected on days 13 and 14 of the cycle. In samples collected on days 3, 9, 10, 11 and 12 no pattern of mass spectral peaks characteristic of $PGF_{2\alpha}$ could be discerned. The much lower concentrations likely to be present (as shown by biological assay) necessitated the use of the mass spectrometer at a much higher amplification. This increased the size of peaks from interfering substances and obscured any signs of the $PGF_{2\alpha}$ spectrum.

No GLC/MS evidence for the presence of $PGF_{1\alpha}$ in any of the samples was obtained.

Identification of $PGE_2$    Identification of $PGE_2$ proved more difficult. The Me/MO/TMS derivatives of $PGE_1$, $PGE_2$ and the silicic acid PGE eluates from day 14 and day 15 blood samples were compared by GLC/MS. The pure prostaglandins had retention times of 18.4 ($PGE_1$) and 14.8 ($PGE_2$) for the first isomer and 22.3 and 18.4 minutes respectively for the second. So many interfering peaks were present in the derivatized test sample, however, that it was impossible to discern the expected prostaglandin $^m$/e peaks. No definite conclusion could be drawn from this experiment. Analysis by GLC/MS of day 15 extracts, after chemical conversion of PGE to PGB prior to derivatization, was more successful. On this GLC system the Me/TMS derivatives of $PGB_1$ and $PGB_2$ each had a retention time of 17.2 minutes. The mass spectrum of

PGB$_1$ (Me/TMS) had prominent peaks at 422 $\left[\text{M}^+\right]$, 351 $\left[\text{M} - 71\right]$, and 323 $\left[\text{M} - (71 + 28)\right]$. Corresponding peaks for PGB$_2$ (Me/TMS) were 420, 349 and 321. The difference of 2 a.m.u. is due to the additional 5,6 double bond in PGB$_2$.

A mass spectrum of derivatized day 15 plasma extract taken at 17.2 minutes contained peaks at $^m$/e values of 420, 349 and 321 in the ratio of 4:1:1. This ratio is characteristic of PGB$_2$ (Me/TMS) (see Fig. 6) However, these three peaks, although clearly visible, were smaller than several other peaks in the spectrum due to the presence of other substances in the extract. It was significant, however, that there were no peaks at $^m$/e values of 422, 351 and 323 - the peaks characteristic of PGB$_1$ (Me/TMS). On the basis of this evidence it can be seen that PGB$_2$ but not PGB$_1$ was present in the final extract. This must have arisen from PGE$_2$ in the original plasma extract, since any PGA$_2$ or PGB$_2$ in the original plasma would have been separated from the PGE$_2$ by silicic acid chromatography (see p. 20) prior to the chemical conversion of PGE$_2$ to PGB$_2$. It is concluded that the PGE-like material extracted from utero-ovarian venous blood on day 15 as detected and measured by biological assay can be identified as PGE$_2$ on the basis of mass spectral data.

## Levels of prostaglandins in the utero-ovarian blood of guinea-pigs during the oestrous cycle

The concentrations of $PGF_{2\alpha}$ and $PGE_2$ in pooled samples of blood collected from animals on days 3, 7 and 9 to 15 of the oestrous cycle were determined by biological assay and the results corrected for recovery are shown in Table 13 and Figure 12. The mean values for day 7 were taken from the assays performed on control animals described above (Table 12, p. 75).

The level of $PGF_{2\alpha}$ on day 3 was 14 ng/ml. This had fallen to below detectable limits (<3 ng/ml) on day 7, but on days 9 and 10 low levels (5 and 4 ng/ml) were present. The concentration then rose to 17 ng/ml on day 11 and remained around that level until day 15 when it again rose sharply to 61 ng/ml.

$PGE_2$ was not detectable in blood collected on days 3, 7, 9, 10, 11 and 12. It was just detectable (1 ng/ml) on day 13 and then rose sharply to 5 ng/ml (day 14) and 55 ng/ml (day 15).

Losses in derivative formation and during GLC/MS (particularly with $PGE_2$) probably accounted for the somewhat lower levels as measured by GLC/MS compared with bioassay.
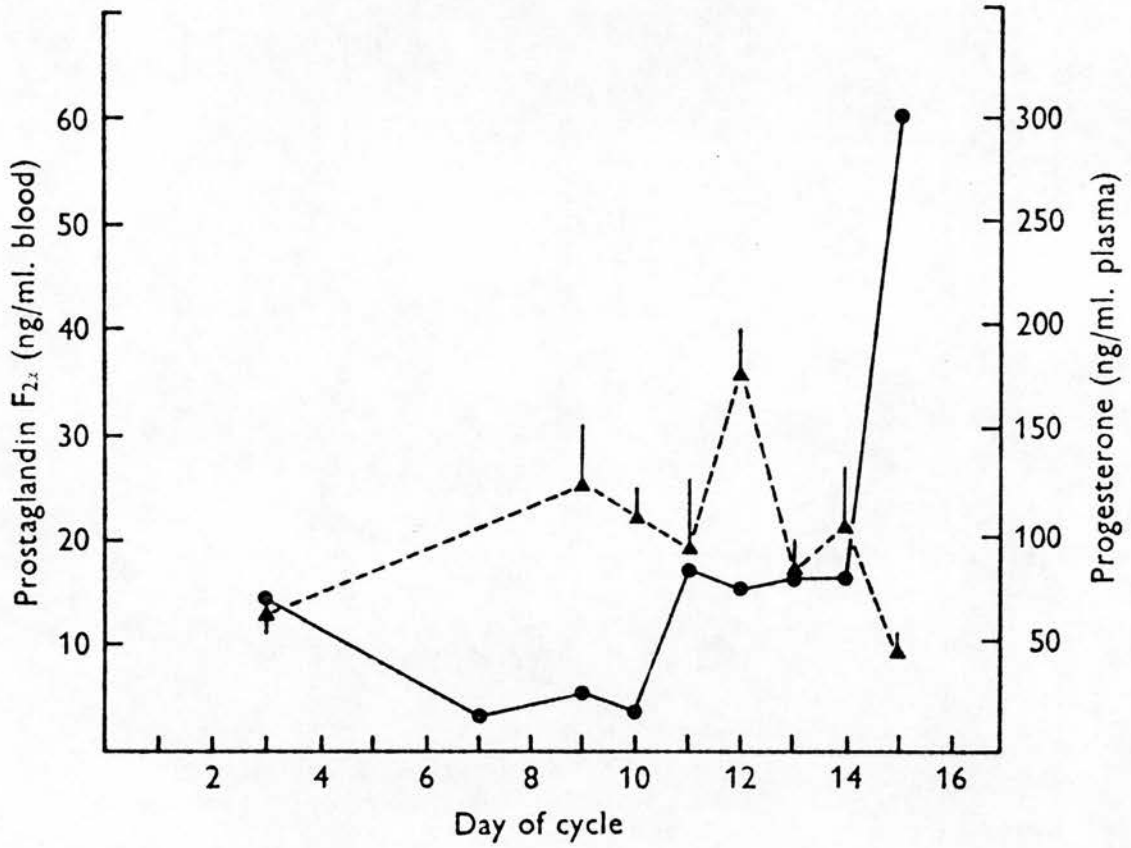
Figure 12    The relationship between the concentrations
            of PGF$_{2\alpha}$ (——) and progestin (---; mean $\pm$
            s.e. of the mean) in utero-ovarian venous
            blood of guinea-pigs at different stages of
            the oestrous cycle.

TABLE 13 : Concentrations of $PGF_{2\alpha}$ and $PGE_2$ in the
utero-ovarian venous blood of guinea-pigs
during the oestrous cycle

| Day of cycle | Level of $PGF_{2\alpha}$ (ng/ml) | Level of $PGE_2$ (ng/ml) |
|---|---|---|
| 3 | 14.1 | <0.7 |
| 7 | <3.3 | - |
| 9 | 5.2 | <0.8 |
| 10 | 3.5 | <0.8 |
| 11 | 17.2 | <0.8 |
| 12 | 16.2 | <0.8 |
| 13 | 15.7 | 1.1 |
| 14 | 16.0 | 5.4 |
| 15 | 60.7 | 54.9 |

TABLE 14     Total plasma progestin levels (ng/ml) in utero-ovarian venous blood of guinea-pigs during the oestrous cycle (base line value, i.e. mean for guinea-pigs with no corpora lutea in appropriate ovary = 40.0 ng/ml)

| Day of cycle | Mean concentration total progestins (ng/ml plasma ± S.E. of mean) |
|---|---|
| 3 | 65.9 ± 12.3 |
| 9 | 126.0 ± 31.0 |
| 10 | 108.2 ± 15.2 |
| 11 | 94.9 ± 37.9 |
| 12 | 177.2 ± 31.8 |
| 13 | 81.1 ± 22.1 |
| 14 | 105.9 ± 30.0 |
| 15 | 45.1 ± 10.0 |

## Levels of progesterone in utero-ovarian venous blood of guinea-pigs during the oestrous cycle

The mean levels of plasma progesterone ($\pm$ standard error of the mean) found in the utero-ovarian vein of guinea-pigs throughout the oestrous cycle are given in Table 14. From a low value on day 3 (66 ng/ml), the level rose and between days 9 and 14 values of 80 - 180 ng/ml were recorded. A fall to 45 ng/ml occurred between days 14 and 15 which represents near basal levels because the concentration of progesterone in the utero-ovarian venous plasma of guinea-pigs having no corpora lutea in the ipsilateral ovary was 40 ng/ml.

## Effect of indomethacin on the length of the oestrous cycle

Subcutaneous injections of indomethacin    The mean length of the oestrous cycle in guinea-pigs injected subcutaneously with indomethacin (20 mg/day) from the 7th day of the cycle until the onset of the next cycle was 19.3 $\pm$ 0.5 days (n = 12). This result was significantly greater (P <0.001) than the length of cycles of the same guinea-pigs before and after indomethacin treatment.    It was also significantly greater than the cycles of control guinea-pigs injected with vehicle alone, and of animals injected with a lower dose of indomethacin (4 mg/day).    These results summarized in Table 15, indicate that indomethacin at the higher dose, but not at the lower dose, has a significant effect in increasing the length of the oestrous cycle.    However, at the 20 mg/day level, the indomethacin caused toxic effects, two of the animals died after 11 days of subcutaneous injections.

TABLE 15 Effect of indomethacin on oestrous cycle length in guinea-pigs following subcutaneous injections

| Dose of indomethacin (mg/day, sc) | Oestrous Cycle Length (days) | | |
| --- | --- | --- | --- |
| | Before treatment | During treatment | After treatment |
| 20 | 16.7 $\pm$ 0.3 (n = 12) | 19.3 $\pm$ 0.5* (n = 12) | 16.0 $\pm$ 0.2 (n = 12) |
| 4 | 17.8 $\pm$ 0.5 (n = 4) | 17.3 $\pm$ 0.3 (n = 4) | 17.0 $\pm$ 0.4 (n = 4) |
| Control | 16.9 $\pm$ 0.4 (n = 8) | 16.1 $\pm$ 0.3 (n = 8) | 15.8 $\pm$ 0.3 (n = 8) |

* Significantly lengthened (P <0.001)

n = Number of oestrous cycles per group

<u>Intra-uterine indomethacin</u>    In six guinea-pigs, implants of paraffin wax containing indomethacin were placed in each uterine horn, whilst in the six control animals paraffin wax alone was inserted.    The results are shown in Table 16. The length of the three oestrous cycles before operation in each of the 12 guinea-pigs was estimated.    The mean lengths of $16.4 \pm 0.2$ (n = 18) and $16.6 \pm 0.2$ (n = 18) days for the two groups were not significantly different.    In the six guinea-pigs receiving paraffin wax implants without indomethacin the mean length of the cycles during which the operations were performed was $19.5 \pm 1.0$ days and the mean length of the three subsequent cycles was $14.8 \pm 0.2$ days. In contrast, in each of the guinea-pigs receiving intra-uterine indomethacin implants, the cycles in which the operations were performed were all in excess of 47 days. Three of these guinea-pigs were killed on the 47th day for assessment of luteal function (see below).    The remaining three came into oestrus after cycle lengths of 75, 70 and 51 days.    The first of these three had a subsequent cycle of 49 days and was then killed on the 8th day of the next cycle.    The second was killed on the 9th day of the first post-operative cycle and the third had two post-operative cycles of 17 days before being killed on the 9th day of the next cycle.

In two further guinea-pigs implanted with intra-uterine indomethacin, 5 cycles of $15.8 \pm 1.0$ and $15.4 \pm 1.0$ days each were observed before implantation.    Subsequently, the first of these guinea-pigs had cycle lengths of 72 and

TABLE 16   Effect of intra-uterine implants of indomethacin on the length of oestrous cycles in guinea-pigs

| Implant | Length of Oestrous Cycle (days) | | |
| --- | --- | --- | --- |
| | Before implantation | Cycle in which implants were inserted | After implantation |
| Indomethacin and paraffin wax | 16.4 ± 0.2 (n = 18) | 75  70  51 <br> >47  >47  >47 | see text |
| Paraffin wax (control) | 16.6 ± 0.2 (n = 18) | 19.5 ± 1.0 (n = 6) | 14.8 ± 0.2 (n = 18) |

over 62 days and the second had cycles of 30, 24, 15, 29,
16 and more than 41 days. Elevated plasma progesterone
levels were maintained throughout the elongated cycles
and in some cases the levels were higher than normal
(Fig. 13).

Assessment of luteal function   The six control animals
which received paraffin wax implants without indomethacin
were allowed to come into oestrus four times following the
operation. During the next cycle three were killed on the
8th day and three were killed on the 14th or 15th day of
the cycles. These times corresponded to low and high
uterine output of prostaglandin $F_{2\alpha}$ as shown above. Of the
six animals with indomethacin implants, three were killed
37 days after the operation (on the 47th day of the cycle)
whilst three were killed on the 8th or 9th day of a sub-
sequent cycle. Luteal function was assessed independently
and blindly by two workers using histological measurement
and blood progesterone measurement respectively. The
results are shown in Table 17.

The plasma progesterone levels of three guinea-pigs
treated with indomethacin and killed on day 8 or 9 were
2.4, 3.1 and 3.6 ng/ml whereas the levels of indomethacin-
treated guinea-pigs which had still not come into oestrus
when they were killed on the 47th day of the cycle were 3.8,
8.8 and 8.9 ng/ml. The mean sizes of the corpora lutea
were 3.8, 2.9, 3.1 and 4.3, 4.3 and 6.0 mm$^3$ respectively.
In the last two guinea-pigs corpora lutea resembled those
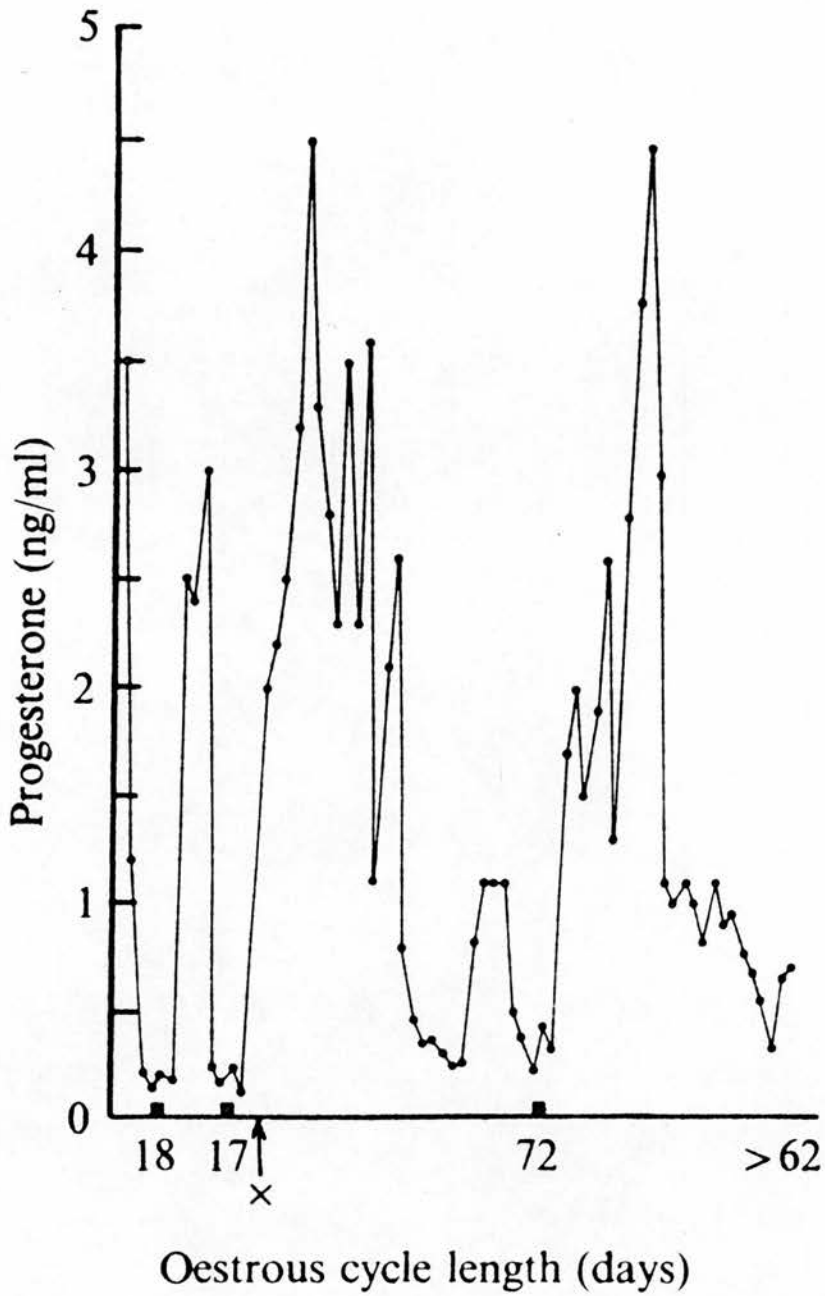seen in hysterectomized guinea-pigs. In the control animals

Figure 13  Effect of intra-uterine indomethacin
(33 mg/horn) implants inserted at X on
the progesterone levels (ordinate) of
guinea-pigs. Beginning of each cycle
shown by vaginal smears is indicated by
a black square;  the numbers on the
abscissa refer to the length of each
cycle.

TABLE 17 Tests of luteal function in guinea-pigs with intra-uterine implants

| Guinea-pig | Day of cycle killed | Plasma progesterone (ng/ml) | Size of corpora lutea mm$^3$ |
|---|---|---|---|
| **Indomethacin implants** | | | |
| 1 | 8 | 2.37 | 3.77 |
| 2 | 9 | 3.14 | 2.88 |
| 3 | 9 | 3.60 | 3.11 |
| 4 | 47 | 8.77 | 4.28 |
| 5 | 47 | 3.78 | 4.25 |
| 6 | 47 | 8.90 | 6.00 |
| **Paraffin wax implants** | | | |
| 7 | 8 | 5.51 | 3.07 |
| 8 | 8 | 4.55 | 2.00 |
| 9 | 8 | 6.84 | 2.40 |
| 10 | 15 | 0.36 | 0.95 |
| 11 | 14 | 0.27 | 0.79 |
| 12 | 14 | 0.62 | 0.55 |

on day 8, plasma progesterone levels ranged from 4.6 to 6.8 ng/ml and the corpora lutea sizes from 2.0 to 3.1 mm$^3$. In those killed at the end of the cycle, plasma progesterone levels were within the range of 0.3 to 0.6 ng/ml. The corpora lutea sizes ranged from 0.6 to 1.0 mm$^3$ and had clearly regressed.

These results show that elongation of the cycle following intra-uterine implantation of indomethacin is associated with retention of functioning corpora lutea.

Autopsy examination of the uterus    Uteri from both control and treated guinea-pigs were hypertrophied and contained intraluminal fluid in amounts ranging from 1.5 to 63 ml The fluid was slightly alkaline (pH 8).    There was no difference macroscopically or microscopically between indomethacin-treated and control animals.

Effect of active immunization against PGF$_{2\alpha}$ on length of the oestrous cycle

The results on cycle length following active immunization against PGF$_{2\alpha}$ are summarized in Figure 14.    In the control animals injected with the mixture of bovine serum albumin and PGF$_{2\alpha}$ no significant change in length of oestrous cycle occurred (before injection, $16.3 \pm 0.3$ days, $n = 29$;    after injection, $16.4 \pm 0.2$ days, $n = 50$).    In guinea-pigs injected with the conjugate of bovine serum albumin and PGF$_{2\alpha}$, the cycle lengths before injection ($16.5 \pm 0.2$ days) were not significantly different from the controls.    In three,
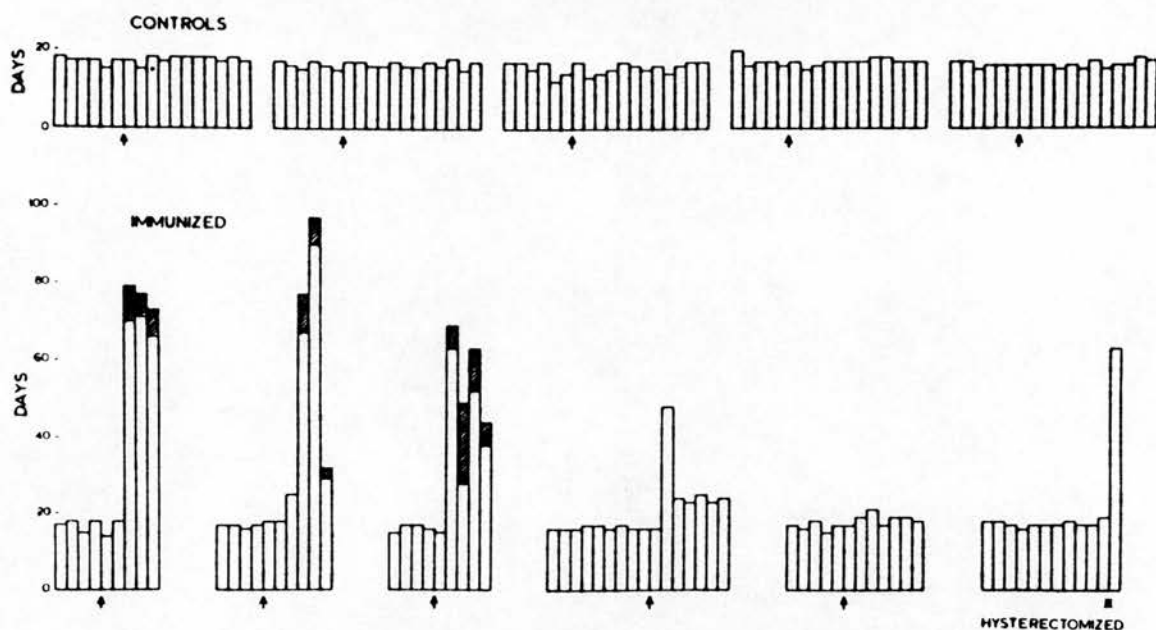
Figure 14  Effect of active immunization against $PGF_{2\alpha}$ on
oestrous cycle length in guinea-pigs.
Ordinate:  oestrous cycle length in days.
Abscissa:  numbers of successive cycles recorded;
arrows indicate time of injection.
Upper row:  5 control animals injected with a
mixture (non-conjugated) of $PGF_{2\alpha}$ and bovine
serum albumin.  Lower row:  5 animals injected
with a conjugate of $PGF_{2\alpha}$ with bovine serum
albumin and a sixth animal which was hysterect-
omized (but not immunized).

oestrous cycles of normal length followed the initial injection. The length of their cycles then greatly prolonged, approximating to that produced by hysterectomy (see below). In the fourth guinea-pig, one cycle of normal length was followed by a cycle of 48 days; thereafter the cycles were 23 to 25 days in duration. All these increases in length were significant (P <0.01). In the fifth animal, there was a small but significant increase (P <0.05) from 16.7 $\pm$ 0.4 to 18.8 $\pm$ 0.5 days.

The vaginal histological changes were less abrupt and less definite in the animals with prolonged cycles than in animals with cycles of normal duration. This is represented by the hatched areas in the columns in Figure 14. In spite of this lack of precision about the exact day of vaginal cornification and leucocytic influx, there is no doubt that the oestrous cycles were prolonged.

When this study was completed the five control guinea-pigs were themselves immunized against $PGF_{2\alpha}$. All five showed elongated cycles (Table 18).

Serum levels of $PGF_{2\alpha}$ antibody    There was some correlation between the titre level and the degree of elongation of oestrous cycle in the seven immunized guinea-pigs in which titres were measured. The results are summarized in Table 19. In four guinea-pigs with cycles longer than 70 days after immunization there was a 50% binding at dilutions ranging from 1 in 1300 to 1 in 3200. In the three guinea-pigs with cycles in which maximum elongation of any cycle was 48, 28 and 19 days, the binding at 1 in 500 dilution was 31, 26 and 10% respectively. In the 5 control animals (prior to

TABLE 18 Length of oestrous cycles (in days) of guinea-pigs before and after immunization against $PGF_2\alpha$.

| Guinea-pig | Control Cycle Length | Post-immunization cycles | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| R | 16.3 ± 0.2 (n = 18) | 17 | 19 | 25 | 96 | 30 | 73 | >50 | | | |
| W | 16.1 ± 0.2 (n = 18) | 19 | 19 | 73 | | | | | | | |
| WL | 17.2 ± 0.2 (n = 17) | 20 | >31 | | | | | | | | |
| G | 15.4 ± 0.4 (n = 18) | 15 | 18 | >39 | | | | | | | |
| P | 17.0 ± 0.3 (n = 17) | 17 | 18 | 28 | 28 | 24 | 28 | 21 | 25 | 24 | 22 |

n = number of control (pre-immunization) cycles

TABLE  19    Antibody titres and length of longest
             post-immunized cycles in guinea-pigs
             immunized against $PGF_{2\alpha}$

| Guinea-pig | Titre: 50% binding at a dilution of | Longest cycle recorded (days) |
|---|---|---|
| 1 | 1 in 3200 | 80 |
| 2 | 1 in 2800 | 100 |
| 3 | 1 in 1300 | 78 |
| 4 | (31% at 1 in 500) | 48 |
| 5 | (10% at 1 in 500) | 19 |
| R | 1 in 1800 | 96 |
| P | (26% at 1 in 500) | 28 |

their subsequent immunization), the binding of tritiated $PGF_{2\alpha}$ at a serum dilution of 1 in 500 ranged from 7 to 12%. These figures are not regarded as different from the non-specific binding level of 10%.

Plasma progesterone levels in immunized guinea-pigs    In five guinea-pigs plasma progesterone levels were measured before and after immunization three times weekly.  This blood sampling technique did not affect cycle length in other guinea-pigs nor in these guinea-pigs prior to immunization.  These five guinea-pigs had 17 or 18 normal cycles prior to immunization.  After immunization against $PGF_{2\alpha}$ all had elongated cycles and in all five progesterone levels were maintained at luteal levels or higher throughout the lengthened cycles. One of these is illustrated in Figure 15.

Plasma progesterone levels in hysterectomized guinea-pigs Three guinea-pigs were hysterectomized.  The first (data shown in Fig. 14) had the expected elongated cycle following the operation but no blood samples were collected.  Cardiac blood was collected from the other two guinea-pigs before and after hysterectomy.  Unfortunately the first of these died on the 19th day of the post-operative cycle but abnormally high levels of progesterone were found on days 13, 16 and 18 and functional corpora lutea were still evident at the time of death.  The second of these guinea-pigs (Fig. 16) had cycles of 117 and 127 during which high levels of plasma progesterone were maintained.  This clearly demonstrates that removal of the uterus results in maintenance of functional corpora lutea in the ovaries throughout these abnormally long cycles.
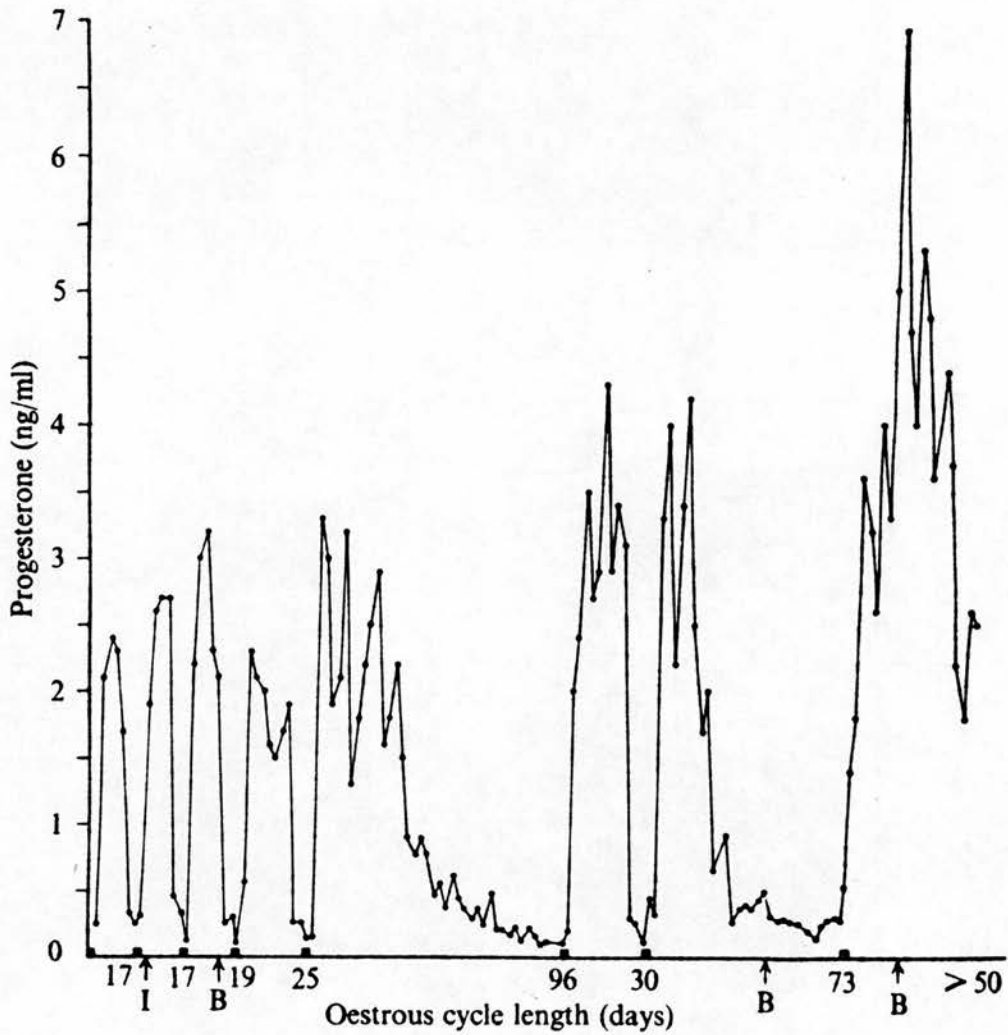
Figure 15    Plasma levels of progesterone in a guinea-pig
immunized against $PGF_{2\alpha}$.   At I, the animal
was immunized and at B, booster injections
were given.   The beginning of each cycle is
indicated by a black square, the numbers on
the abscissa refer to the length of each
cycle.

Figure 16    Effect of hysterectomy on plasma progesterone
            levels in the guinea-pig.  Ordinate:  plasma
            concentration.  Abscissa:   length of two
            successive cycles following the operation –
            the beginning of each cycle is indicated by a
            black square, the numbers refer to the length
            of each cycle.
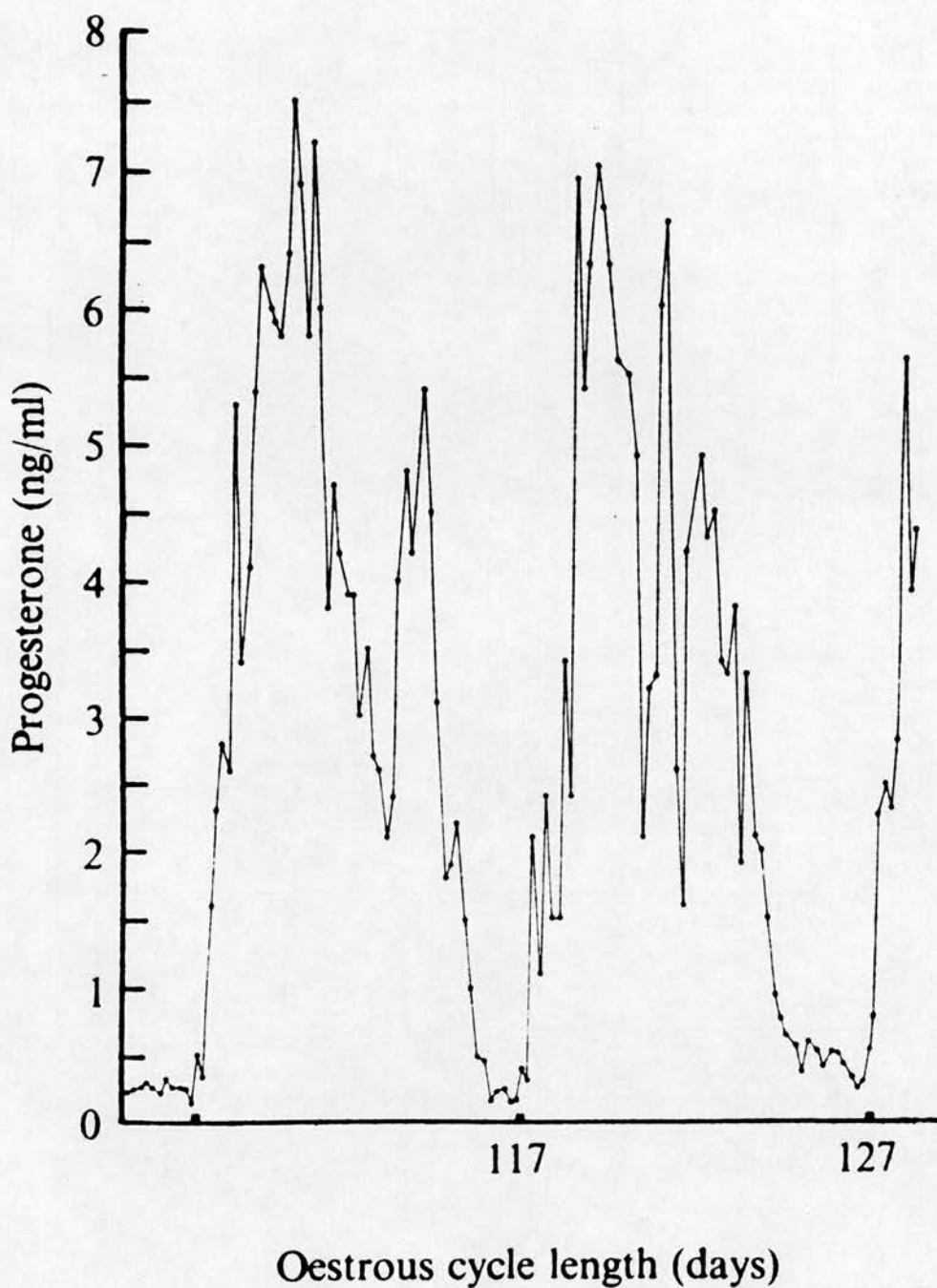
## DISCUSSION

### Guinea-pig uterus distension in vitro

Distension of the guinea-pig uterus with beads in vivo
results in premature luteolysis (Donovan and Traczyk, 1962).
The evidence from the present experiments, in vitro, shows
that distension with polyethylene tubing causes the guinea-
pig uterus to release $PGF_{2\alpha}$ in about 10 times the amounts
released in absence of distension. The evidence of identifi-
cation is based upon partition properties and chromatographic
behaviour which suggest that the active principle is a
prostaglandin-like lipid, quantitative biological assay which
exludes $PGE_1$ (and $PGE_2$) but is suggestive of $PGF_{2\alpha}$ and,
most conclusively, GLC/MS which shows the presence of $PGF_{2\alpha}$
by GLC peaks and by characteristic MS in the samples collected
from uterine horns which had been distended. The evidence
excludes $PGE_1$, $PGF_{1\alpha}$ and the main guinea-pig urinary metabolite
of $PGF_{2\alpha}$ but it does not exclude the additional presence of
all other prostaglandins. However, the close quantiative
agreement between the estimates of $PGF_{2\alpha}$ concentration by
biological assay and by gas chromatography indicates that
$PGF_{2\alpha}$ can account for all the biologically-active material
released from the uterus as originally detected by the use
of the rat fundal strip.

$PGF_{2\alpha}$ is luteolytic in many species (Pharriss and Wyngarden,
1969; Kirton, Pharriss and Forbes 1970) including the guinea-
pig (Blatchley and Donovan, 1969). The amounts of $PGF_{2\alpha}$
needed to produce this luteolytic effect are far in excess of
the amounts that were released from the uterus in the experiments

reported here. It is probable that this discrepancy can be attributed to the difficulty in injecting $PGF_{2\alpha}$ in a way which mimics the presumed physiological mode and rate of release. For example, 95-99% of any $PGF_{2\alpha}$ injected parenterally and hence passing through the pulmonary circulation, will be taken up and metabolised (Ferreira and Vane, 1967) before it can reach a target organ, whilst further metabolism occurs in the liver and elsewhere.

The evidence regarding the role of $PGF_{2\alpha}$ in physiological luteolysis can at this point be summarized as follows: distension of the uterus is known to induce luteolysis. $PGF_{2\alpha}$ is known to be luteolytic, the present study has shown that distension of the uterus releases $PGF_{2\alpha}$. This provides good preliminary support for the hypothesis that the uterine luteolytic hormone in the guinea-pig is $PGF_{2\alpha}$. Since this work was completed, in vivo experiments have shown that insertion of beads into the uterus of the guinea-pig increases the output of $PGF_{2\alpha}$ in the utero-ovarian venous blood, decreases the size of the corpora lutea and reduces the level of progesterone (Blatchley, Donovan and Poyser, 1976).

Oestrogen treatment    It is well established that oestrogen treatment of guinea-pigs early in the oestrous cycle causes premature luteolysis. This could be due to an action on one or more of several sites, notably the pituitary (Anderson et al., 1969). However, the demonstration that this luteolytic action is abolished by hysterectomy (Bland and Donovan, 1970)

reaffirms the importance of the uterus in this mechanism. It seemed probable that oestrogen might be acting upon the uterus to cause premature release of the uterine luteolytic hormone in a similar manner to that occurring after the insertion of foreign bodies within the lumen as discussed above. Knowing that such distension causes the release of $PGF_{2\alpha}$ it was logical to look for similar release in response to oestrogen.

In this series of experiments the biological preparation used and the protocol were exactly the same as those used to demonstrate the luteolytic effects of oestrogen. Under these conditions an increased output of $PGF_{2\alpha}$ in the utero-ovarian vein in response to oestrogen has been demonstrated. That the $PGF_{2\alpha}$ arises from the uterus and not elsewhere, particularly the ovary, is shown by the absence of any $PGF_{2\alpha}$ in the plasma sample collected from hysterectomized animals after treatment with oestrogen. That $PGF_{2\alpha}$ is released in response to oestrogen is shown by its presence in all seven test samples and its undetectability in the seven controls. In one control sample the limit of detectability by bioassay was high, otherwise each test was higher than its corresponding control (Table 12). Identification again has been obtained by GLC/MS analysis which conclusively demonstrates the presence of $PGF_{2\alpha}$.

The mechanism of the luteolytic action of oestrogen can therefore now be explained as follows: the oestrogen stimulates the uterus to produce and release increased quantities of $PGF_{2\alpha}$, which in turn reaches the ovary to exert

its luteolytic action by a route and a mechanism as yet unknown. It seemed possible that a similar mechanism could be involved physiologically in the normal oestrous cycle of the guinea-pig if $PGF_{2\alpha}$ production by the uterus increases under the influence of oestrogen secretion from the growing follicles in the ovary. This possibility was therefore examined next.

Oestrous cycle    Measurement of $PGF_{2\alpha}$ levels in uterus venous blood collected on different days of the oestrous cycle revealed a cyclical variation in the amount of $PGF_{2\alpha}$ released. The moderately high levels of $PGF_{2\alpha}$ on day 3 probably reflect the tail of the peak rise at the end of the cycle. Thereafter the levels remained low but detectable until day 11 when they rose to around 17 ng/ml at which they remained until between day 14 and day 15. While $PGF_{2\alpha}$ levels were low progesterone levels were high, ranging from 86 to 170 ng/ml, but by day 15 the progesterone concentration had halved and was close to basal levels whereas the $PGF_{2\alpha}$ concentration had almost quadrupled and was 60 ng/ml. It is not known how much of this $PGF_{2\alpha}$ would have reached the corpora lutea.

The level of progesterone in utero-ovarian venous blood was about forty times greater than that found in the systemic blood at the same stage of the cycle (Blatchley, personal communication). The large variation between individual guinea-pigs, indicated by the large standard error, can be explained, to some extent, by the different amounts of luteal tissue in the ovary on the side from which blood was collected.

The demonstration of an _in vivo_ release of $PGF_{2\alpha}$ during the oestrous cycle and prior to the onset of physiological luteolysis is further persuasive evidence in support of the hypothesis that this substance is the uterine luteolytic hormone in guinea-pigs.

Effect of indomethacin    The plasma half-life of indomethacin in the guinea-pig following intravenous injection is short (20 minutes) and the uterus takes up very small amounts (Hucker, Zacchei, Cox, Brodie and Cantwell, 1966).    These facts may account for the high dose levels (20 mg/day) required to cause a significant increase in the length of the oestrous cycle when indomethacin was injected subcutaneously.    Certain tissues, notably the small intestine, concentrate indomethacin above plasma levels and it is significant that in this study two animals died with gastro-intestinal perforations after 11 days of subcutaneous indomethacin treatment.

The increase in cycle length following indomethacin injections at the 20 mg/day level is compatible with the hypothesis that indomethacin blocks formation of luteolysin in the uterus and thus prolongs the life of the corpora lutea. Similar results were obtained by Marley (1973) following daily oral administration of indomethacin (40 mg/kg) beginning on day 11 of the cycle.    It is probable, however, that prostaglandin synthesis was also blocked at many other sites in the body and that these could have contributed to the overall picture.    It was therefore important to try to localize the site of action of indomethacin and for this purpose a

method was devised with the aim of producing a high concentration of indomethacin near the site of formation of luteolysin, namely the endometrium. The indomethacin was mixed with paraffin wax to form a slow release formulation. Although the total amount of indomethacin placed in each uterine horn was high (33 mg), subsequent analysis of the implants removed at autopsy indicated that the quantities of indomethacin released in each uterine horn ranged from 0.2 to 0.6 mg/day. This dose released in the neighbourhood of the endometrium over a prolonged period was sufficient to produce a considerable increase in the length of the oestrous cycle.

In three of the guinea-pigs treated with intra-uterine indomethacin, vaginal smears showed that oestrus had still not occurred up to the 47th day when the animals were killed. The plasma progesterone levels at this time were high (see Table 17), the normal peak of plasma progesterone levels being about 3 ng/ml. The corpora lutea were larger than normal resembling those produced by hysterectomy (Rowlands, 1961). In the other three treated guinea-pigs, cycles greatly in excess of normal were observed, their length being similar to that seen in hysterectomized guinea-pigs (Loeb, 1923 and see below). When these animals were killed during a subsequent cycle, the progesterone levels and corpora lutea size were normal for the time of the cycle (8 or 9 days) (Challis, Heap and Illingworth, 1971; Bland and Donovan, 1965).

The three control animals killed at the end of the cycle had progesterone levels within the normal range for that time.

The corpora lutea were typically small and had regressed.
The control animals killed at mid-cycle, however, had
elevated plasma progesterone levels but the corpora lutea
were within the normal range.  This may be associated with
the finding that these guinea-pigs were exhibiting shorter
oestrous cycles.  In the six control animals, the cycle
in which the paraffin wax implants were inserted was
slightly but significantly prolonged (P <0.001) whereas the
subsequent cycles were slightly but significantly shortened
(P <0.001).  These results are identical to those of
Ginther (1969) and are probably attributable to the uterine
ligation.

It is now well established that indomethacin blocks the
biosynthesis and release of prostaglandins (Vane, 1971).  If
the effects observed here following intra-uterine adminis-
tration of indomethacin are attributable to this mechanism,
further evidence is provided in support of the hypothesis
that the uterus exerts its luteolytic influence upon the
ovary by the formation and release of PGF$_{2\alpha}$.

Active immunization  Immunization of adult female guinea-
pigs using a conjugate of PGF$_{2\alpha}$ with bovine serum albumin
was followed by an increase in length of oestrous cycles in
9 out of 10 animals.  These changes were associated with
elevated plasma progesterone levels lasting for the duration
of the elongated cycles in 5 of the guinea-pigs in which
measurements were made.  These results complement those with
indomethacin and the effects like those of intra-uterine
indomethacin resemble the changes seen after hysterectomy

(compare Figs. 13, 15 and 16). The mechanism may be envis-
aged as follows: $PGF_{2\alpha}$ is synthesised by the uterus towards
the end of the cycle and released into the uterine vein.
There it is neutralised by $PGF_{2\alpha}$ antibodies in the plasma
thus preventing free $PGF_{2\alpha}$ from reaching its target in the
ovaries in sufficient concentration. Hence the corpora
lutea do not receive the stimulus to regress at the normal
time and cycles are prolonged with continued luteal function.

Another interpretation of these results is that immuniz-
ation has interfered with other actions of $PGF_{2\alpha}$ elsewhere.
For example, there is evidence in some species that prosta-
glandins are involved in the release of gonadotrophins
(Armstrong and Grinwich, 1972; Behrman, Orczyk and Greep,
1972; Carlson, Barcikowski and McCracken, 1973; Harms,
Ojeda and McCann, 1974; Sato, Taya, Jyujyo, Hirono and
Igarashi, 1974) and in ovulation (Armstrong and Grinwich,
1972; Grinwich, Kennedy and Armstrong, 1972; O'Grady,
Caldwell, Auletta and Speroff, 1972; Orczyk and Behrman,
1972; Tsafriri, Lindner, Zor and Lamprecht, 1972; LeMaire,
Yang, Behrman and Marsh, 1973; Armstrong, Grinwich, Moon
and Zamecnik, 1974). If $PGF_{2\alpha}$ is physiologically involved
in gonadotrophin release, blockade of this release would pre-
sumably inhibit all cyclical behaviour (ovulation and lutein-
ization with its corresponding increase in progesterone levels)
after the first set of corpora lutea had regressed. This was
not seen in any of the immunized guinea-pigs in this study.

It is not known whether ovulation was blocked in the immunized animals. It is possible that progesterone levels were maintained by secretions from luteinized non-ovulated follicles, as reported by others using indomethacin (Armstrong and Grinwich, 1972; Behrman et al., 1972; Grinwich et al., 1972; O'Grady et al., 1972). It is not known whether luteinized non-ovulated follicles would be susceptible to the luteolytic actions of $PGF_{2\alpha}$.

Yet another explanation of the effects of immunization could be that it interfered with histological changes in the vaginal epithelium thus masking otherwise normal cyclical activity. This possibility is ruled out, however, by the plasma progesterone levels which clearly reflect a continuously prolonged period of luteal function. This evidence in turn may be criticized on the grounds that the radioimmunoassay method may not have been specific for progesterone. Cross-reactivity studies (Table 9) with a variety of related steroids indicated, however, that the most likely interfering compounds can be ruled out.

The most reasonable explanation therefore of these results from active immunization is that $PGF_{2\alpha}$ after its release from the uterus into the uterine vein is being bound by the circulating antibody before it can exert its normal luteolytic action. In conjunction with the preceding evidence it can be regarded as yet further support for the hypothesis that the uterine luteolytic hormone in guinea-pigs can be identified as $PGF_{2\alpha}$.

## Conclusion

The original work of Loeb (1923) clearly showed that removal of the uterus in the guinea-pig results in a greatly elongated oestrous cycle. This has been amply confirmed by others as well as by the present study which has also provided convincing biochemical evidence of the retention of functional corpora lutea. It is also apparent in the guinea-pig that ovarian cyclical activity is controlled by the ipsilateral uterine horn and that the factor responsible is humoral, probably a substance originating in the endometrium.

If this postulated uterine luteolytic hormone is physiologically important for the control of oestrous cycle length, it must be released from the uterus towards the end of the cycle to initiate regression of the corpora lutea. Two stimuli believed to release the luteolytic substance are the presence of foreign bodies in the uterine lumen and the administration of oestrogen early in the cycle.

Since prostaglandin $F_{2\alpha}$ possesses luteolytic activity (Pharriss and Wyngarden, 1969; Bland and Donovan, 1969), and since this prostaglandin is synthesised by an enzyme system which is widely distributed in tissues, it seemed reasonable to propose as a working hypothesis that the uterine luteolytic hormone in the guinea-pig is identical to this prostaglandin.

The cumulative evidence obtained from this study all tends to confirm that this hypothesis is true. Foreign bodies in the uterine lumen and pre-treatment with oestrogen both caused $PGF_{2\alpha}$ release, whilst uterine venous blood from

the 11th day of the cycle onwards contains increasing amounts of $PGF_{2\alpha}$. Indomethacin blocks the biosynthesis of all prostaglandins and when injected subcutaneously, or more especially when implanted into the uterine lumen, this drug mimics the effects of hysterectomy. A similar interference with the normal luteolytic mechanism follows active immunization of female guinea-pigs against $PGF_{2\alpha}$.

Thus $PGF_{2\alpha}$, a natural luteolytic substance, is released from the uterus at the times when the uterus is thought to release a luteolytic substance, whilst the blockade of the synthesis or actions of $PGF_{2\alpha}$ also blocks the normal luteolytic mechanism in guinea-pigs.

This cumulative evidence clearly points to the identical nature of $PGF_{2\alpha}$ and the uterine luteolytic hormone. It does not however provide any evidence as to how the $PGF_{2\alpha}$ reaches the ovary from the uterus. Its presence in the uterine vein suggests a vascular route but the vascular association of the ovarian artery with the utero-ovarian vein, which is seen in the sheep, is not immediately apparent macroscopically in the guinea-pig. Further work is needed on this facet of the problem.

## GENERAL DISCUSSION

### Discovery of Prostaglandins in the Urogenital Tract

Prostaglandins were first discovered in the urogenital tract, hence their name. Von Euler discovered them in human seminal plasma and in extracts of sheep prostate and vesicular glands. At first it was believed that the prostaglandins were from the prostate which comprised the bulk of this combined tissue but in fact they are made in far greater abundance by the sheep vesicular glands. Evidence that the seminal vesicles produce a substance of the prostaglandin type had previously come from von Euler's discovery of vesiglandin in monkey seminal vesicles. Both prostaglandin and vesiglandin lowered arterial blood pressure when injected into anaesthetized animals but only prostaglandin contracted isolated non-vascular smooth muscle preparations. The subsequent discovery that prostaglandins of the A series also lower blood pressure (Lee et al., 1967; Horton and Jones, 1969) but lack appreciable smooth muscle stimulating activity raised the possibility that vesiglandin was likely to be a prostaglandin A (see Horton, 1972). It seemed unlikely that this vesiglandin could have arisen artefactually from a PGE during von Euler's extraction procedure because he has used the identical methodology to demonstrate the presence of 'prostaglandin' in sheep prostate/vesicular glands and this was later shown to be a mixture of PGE and PGF.

More recently Kelly and Taylor (1976) discovered that
semen of primates contains prostaglandins of the 19-hydroxy-
PGE group.   These prostaglandins (also found in human semen)
are so far believed to be peculiar to the male reproductive
tract.   Under von Euler's extraction conditions these
would have been very readily dehydrated to the correspond-
ing 19-hydroxy-PGA or 19-hydroxy-PGB compounds (the form
in which these compounds were first reported in human semen -
Hamberg and Samuelsson, 1966).   It has been suggested by
Kelly, Taylor, Hearn, Short, Martin and Marston (1976)
that von Euler's vesiglandin was a 19-hydroxy-PGE.   It seems
more likely, however, that a 19-hydroxy-PGE was the starting
material in von Euler's extracts but that what he extracted,
detected biologically and called vesiglandin was the corres-
ponding 19-hydroxy-PGA.   Certainly, 19-hydroxy-PGA compounds,
like vesiglandin, do lower arterial blood pressure (Horton
and Jones, 1969).

The now classical work of Bergström and Sjövall (1960a, b)
on the ram seminal vesicles and on human semen (Bergström,
Krabisch and Sjövall, 1960) amply confirmed all von Euler's
work, clearly established the existence of at least two new
natural substances and demonstrated that they possessed a
chemical structure completely novel amongst natural products,
at least in the Animal Kingdom (Bergström et al., 1963).
Final proof of the true origin of prostaglandins from
seminal vesicles was furnished by the demonstration of
their biosynthesis in remarkably high yield from arachidonic
acid by an enzyme complex in ram seminal vesicles (van Dorp,
et al., 1964;   Bergström et al., 1964)

Even before the isolation of prostaglandins by Bergström and Sjövall, there was evidence for the presence of lipid substances of the prostaglandin type in several non-reproductive organs. These had been given trivial descriptive names, for example, irin extracted from rabbit iris (Ambache, 1957, 1959), darmstoff from the gut (Vogt, 1949), medullin from the kidney (Lee, Covino, Takman and Smith, 1965) and the menstrual stimulant from human endometrium (Pickles, 1957). Shortly after the announcement of the chemical structures of the first prostaglandins isolated from organs of the male reproductive tract, Pickles and Raphael reported that certain tissues of the female reproductive tract, namely human endometrium and menstrual fluid, also contain prostaglandins - $PGE_2$ and $PGF_{2\alpha}$ (Eglinton, Raphael, Smith, Hall and Pickles, 1963). This finding raised not only the important question of the physiological role of prostaglandins in the uterus but also the biological significance of the wider distribution of prostaglandins, until then believed to be derived only from reproductive organs of the male.

Almost simultaneously $PGF_{2\alpha}$ was isolated from the lungs (Bergström, Dressler, Krabisch, Ryhage and Sjövall, 1962) and very soon many organs were shown to contain prostaglandins (Bergström, 1967). Already medullin, a new compound of unknown structure, had been demonstrated in the medulla of the rabbit kidney (Lee, et al., 1965). The active principle, like vesiglandin, lowered arterial blood pressure but had little effect on non-vascular smooth muscle. In the light of the results of Bergström

and his school further investigation revealed in the medullary extracts the presence of three prostaglandins - $PGE_2$, $PGF_{2\alpha}$ and $PGA_2$ (the original medullin) (Lee, et al., 1967; Daniels, et al., 1967). $PGA_2$ may conceivably have been formed artefactually from $PGE_2$.

Thus three of the principal organs of the urogenital tract were shown at an early stage to contain (and subsequently shown also to synthesize) prostaglandins of the E, F and possibly A series. However, in view of their wide distribution throughout the body and of the equally wide distribution of the enzyme system, prostaglandin synthase, it could not be argued that prostaglandins had a role peculiar to the urogenital tract. Likewise it could not be argued that their urogenital distribution in some way reflected the common embryological origin of tissues of the urinary and reproductive tracts. It seemed probable that prostaglandins might be implicated in biochemical mechanisms common to many different cell types (just as cyclic 3'5'-adenosine monophosphate has been shown to be) and that this mechanism might be expressed by quite different physiological functions in different cells.

It was therefore decided to examine separately the role of prostaglandins in three systems of the urogenital tract - namely, the kidney, the male reproductive tract and the female reproductive tract.

## Concepts of Chemical Mediation

Although claims about the therapeutic efficacy of various animal tissue extracts have been made from the earliest medical times, it was not until the nineteenth century that research into biological activity of such extracts began in earnest, thus heralding the dawn of the Science of Endocrinology. Some of the earliest experiments were on endocrine organs, notably the adrenals, thyroid, gonads and pituitary, leading to the eventual isolation of adrenaline, thyroxine and other classical hormones. Extracts of non-endocrine tissue also proved fruitful, indeed it was the discovery of secretin, extracted from the small intestine by Bayliss and Starling (1902) that led to their introduction of the term hormone, for a substance which is secreted by one organ or tissue, circulates in the blood and acts upon a target organ elsewhere in the body, in the case of secretin, the exocrine cells of the pancreas.

A further boost to interest in this field of the pharmacology of natural products - drugs of animal origin - came from the work of Loewi and of Dale and his school (see Dale, 1965). In particular must be mentioned their demonstration of acetylcholine as the chemical transmitter at nerve endings. This introduced a completely new concept namely that neurones secrete chemicals which transmit their message onwards. During this work on the search for chemical transmitters, von Euler came to work in Dale's laboratory and in collaboration with Gaddum discovered substance P (Gaddum and von Euler, 1931), a peptide which has now been shown to be a transmitter in the central

nervous system. It was after his return to Stockholm
that von Euler, continuing in this theme, made the funda-
mental discovery which led to his recognition of the prosta-
glandins as a new group of natural products.

In both the hormone and transmitter fields, the sequence
of events and line of reasoning which have led to the recog-
nition of the physiological role for a natural substance
have been similar. Often the existence of the natural
substance as a normal constituent of some animal tissues
has been detected first by its pharmacological activity on
a biological preparation. The biological actions of the new
substance are then observed to include one or more that
mimic the effects of some physiological response (for example,
the injection of noradrenaline mimics the effect of stimu-
lating the adrenergic nervous system; the injection of
cortisol mimics the effect of stimulating the adrenal cortex
with corticotrophin). Measurement of the release of the
natural product from an organ in response to such a physio-
logical stimulus provides the next line of evidence.
Finally, if the natural product is implicated as a mediator,
blockade of its action or of its formation and release will
abolish the physiological response to stimulation: con-
versely, blockade of an enzyme system which inactivates the
mediator will potentiate the responses. In all these lines
of evidence quantitative considerations are paramount.

In general, a similar approach has been used in the
investigations reported in this thesis except that the
release of a prostaglandin has been demonstrated in each
case before the pharmacological one of relevance to the

system in question has been reported - in this study the latter have been reported by workers elsewhere, namely the action of $PGE_2$ on adrenergic nerve terminals in the rabbit kidney (Frame and Hedqvist, 1975) and the luteolytic action of $PGF_{2\alpha}$ first in the rat (Pharriss and Wyngarden, 1969) then in other species including the guinea-pig (Blatchley and Donovan, 1969). Experiments on release in response to a physiological stimulus (e.g. renal nerve stimulation, hormonal changes of the oestrous cycle) have three aspects - first detection, usually by means of biological activity, then identification by GLC/MS and lastly measurement. The validity of the methods used is discussed below. Finally, the effect of blocking prostaglandin release by inhibiting fatty acid cyclo-oxygenase with aspirin or indomethacin and the effect of immunization in the investigation of luteolysin was tested to determine the effects on the physiological response of interfering with the presumed normal action of the mediator. The lack of selective prostaglandin antagonists ruled out the possibility of testing the effect of blocking the actions of prostaglandins at the receptor level.

Validity of the Methodology

(a) Pharmacological experiments    Pharmacological observations reported by others were crucial to the argument that prosta-glandins $E_2$ and $F_{2\alpha}$ have physiological roles at adrenergic nerve endings and in luteolysis respectively. The inhibitory effect of $PGE_2$ on noradrenaline release from adrenergic neurones in the rabbit kidney was demonstrated by infusing

intra-arterially in the isolated kidney perfused with a saline solution. Such a preparation is necessarily subjected to abnormalities of osmolarity and of oxygen tension and thus must be increasingly unphysiological the longer the experiment proceeds. Evidence about the viability or otherwise of the tissue might have been obtained had measurements of renal function been made. Certainly in other (unpublished) experiments the author has found that both the dog spleen and guinea-pig lungs may become grossly oedematous after more than 2 hours perfusion with various saline solutions such as Krebs' and McEwen's solutions.

It is not easy to make valid comparisons between the amount of $PGE_2$ required to produce pharmacological effects and the amount released in response to nerve stimulation. The critical concentration is that present in the interstitial fluid around the sympathetic nerve endings. Intra-arterial infusion may be an inefficient way of applying exogenous $PGE_2$ to the target organ. On the other hand, the saline-perfused kidney may well have been more permeable to the passage of exogenous $PGE_2$ through the vascular wall. In the original experiments of Davies and Withrington (1968) on the dog spleen, prostaglandins were infused into the blood-perfused organ - it may well be that their negative results can be attributed to the lack of such increased permeability under the more physiological conditions used for that preparation. It may be argued that the less physiological conditions pertaining in the rabbit kidney experiments of Frame and Hedqvist permitted a physiological

response to be observed because the abnormal permeability
of the vasculature allowed the $PGE_2$ to reach its target
despite an unphysiological route of administration.

The route of administration and the dose used to
demonstrate the luteolytic actions of $PGF_{2\alpha}$ in rats and
guinea-pigs are even more removed from the physiological
mode of release. Milligram doses of $PGF_{2\alpha}$ were injected
intra-peritoneally. On the other hand, the experiment was
in vivo and the luteolysis was observed under otherwise
physiological conditions. It would be useful to see whether
intra-uterine implantation of $PGF_{2\alpha}$ in some slow release
preparation (analogous to that used in the indomethacin
experiments described in this thesis) would mimic more
closely the physiological release and produce effects on the
ovary with very much smaller doses than those that had to
be given systemically.

Each of these two pharmacological responses was obtained
in the same preparation and species as were used for the
studies involving release and its blockade. This greatly
strengthens the hypothesis concerned with their physiological
roles.

The pharmacological effects of seminal prostaglandins
have been subjected to nearly as much speculation as experi-
ment. Certainly they have effects upon various smooth
muscle preparations of both the male and female reproductive
tracts (Eliasson, 1959).

(b) <u>Identification of Prostaglandins Released</u>    In all
instances the biological fluids have been extracted by
methods designed to separate polar acidic lipids like the
prostaglandins from natural substances of other chemical
types - bases, non-lipids, neutral lipids etc.    The par-
tition procedure, by its selective extraction, itself pro-
vided some evidence of identification.    Chromatographic
evidence from silicic acid columns or silica gel plates
provided further evidence - if the extracted material
differed in its chromatographic behaviour from a known
prostaglandin it was strong negative evidence of identifi-
cation.    Thus the material extracted from incubates of
distended uterine horns could not be $PGE_2$ because its
chromatographic behaviour on silicic acid was clearly
different.    It could be $PGF_{2\alpha}$ but it could also be some
other acidic lipid that happened to share the chromato-
graphic behaviour of $PGF_{2\alpha}$ (e.g. the urinary metabolite of
$PGF_{2\alpha}$ in guinea-pigs).

Similarly, strong negative evidence can be obtained
from quantitative parallel biological assays (Chang and
Gaddum, 1933).    Disagreement between results on different
preparations shows that the test material and the pure
standard are not the same.    Agreement between different
assays provides some evidence that the two substances are
identical - the greater the number of assays the stronger
the evidence (Gaddum, 1955);    but again the positive evi-
dence is never absolutely conclusive (Horton and Main, 1967).
In both physico-chemical investigations and in bioassay the
presence of impurities in the extracts may confuse the pic-
ture;    chromatographic behaviour, for example, may be altered

dramatically in the presence of interfering substances. This problem may be overcome by adding radioactive tracer prostaglandins to the biological material before extraction as was done routinely in the present investigations on the kidney and the seminal fluid.

The most unequivocal means available for the identification of submicrogram quantities of the prostaglandins is combined GLC/MS. This was used throughout this study. Both GLC behaviour (retention time) and mass spectral data contribute to the evidence of identification. Provided that sufficient material is available for analysis, the chief difficulties are to distinguish between stereoisomers (e.g. $PGF_{2\alpha}$ and $PGF_{2\beta}$) and between closely similar substances (e.g. $PGF_{2\alpha}$ and $PGF_{1\alpha}$). GLC separation can be achieved provided that the column conditions are suitable - mass spectral differences may be convincing where compounds differ by as little as 2 a.m.u. provided that the instrument is accurately calibrated. Mass spectral differences between two stereoisomers are often minimal in which case distinction between them has to rest upon other evidence (for example GLC, biological assay, TLC separation or the preparation of a suitable derivative (e.g. the butylboronide of $PGF_{2\alpha}$).

(c) <u>Measurement</u>  Prostaglandins in this study were measured by three different techniques - spectrophotometry, biological assay and GLC/MS. Provided that amounts are adequate (as in semen) and provided that the prostaglandin yields a suitable chromophore (e.g. $PGE_2$) spectrometry gives accurate

results.   The addition of tracer provides a built-in
method for correcting the results for recovery.   It
will be noted that recoveries in all three investigations
were lower than many people would regard as ideal.   This
problem was studied at considerable length but despite
modifications to the scheme of extraction these recoveries
were the best that could be achieved.   They were, however,
consistent and fell within a reasonably narrow range.   It
may be noted that these recoveries do raise doubts about
the value of those investigations reported in the literature
in which no attempt at all has been made to check or correct
for this source of error.

In the kidney and uterus work, the final assays were made
biologically on material which had been highly purified -
extensive partition followed by chromatography.   Virtually
all the biologically-active material present in these final
extracts possessed physico-chemical properties like the
prostaglandins and usually it could be defined even more
narrowly on the basis of chromatographic separation as
PGE-like, PGF-like etc.

With the introduction of radioimmunoassay which has
numerous advantages, such as sensitivity, speed and precision,
it has become fashionable to dismiss biological assay as
out-moded and non-specific.   The critics point out that the
bioassays frequently respond to natural substances which are
chemically very diverse (e.g. amines, peptides and nucleo-
tides as well as prostaglandins).   This criticism may
be justifiable if the biological assay is done using crude

biological extracts but in all the experiments described
here such non-prostaglandin substances of natural origin
are discarded during the partition or separated by the
chromatography, both of which precede the biological assay.
In fact, biological assay often has advantages of selec-
tivity, not necessarily possessed by radioimmunoassay. A
very slight change in chemical structure may be sufficient
to diminish or qualitatively change the pharmacological
activity of a molecule, whereas cross-reactivity with the
antibody used in the radioimmune method may be scarcely
affected. Certainly, radioimmunoassay has many advantages
over biological assay, superior specificity is not necessarily
one of them.

Quantitation using conventional GLC/MS as in this study
depends primarily upon the size of the GLC peak which measures
total ion current. This has the advantage that a full mass
spectrum can be taken at the top of the peak to provide con-
clusive identification of the molecule being measured.
The method has the disadvantage that the amount which can
be detected by the total ion current size restricts the
sensitivity of the method. Multiple ion detection is a
variation of the technique which could not be used with
the GLC/MS equipment available at the time of this study.
It provides evidence of identification based upon one or
more of the abundant MS peaks the size of which is measured
in terms of a deuterated prostaglandin used as an internal
carrier. The disadvantage of the method is that the identi-
fication is less than conclusive unless a full mass spectrum
is obtained. In all the investigations described in this
study full mass spectra were taken.

The advantages and disadvantages of these different methods of measurement have been discussed more fully elsewhere (Horton, 1976b).

(d) <u>Inhibition of prostaglandin biosynthesis or action</u>   The effects of aspirin and indomethacin as inhibitors of the enzyme, fatty acid cyclo-oxygenase, a component of the prostaglandin synthase complex is now undisputed (Vane, 1971 and many subsequent papers).   The effects of aspirin on such synthesis in man are clearly apparent from the work described here on seminal prostaglandin levels.   Nevertheless, the possibility that these drugs may have actions on other systems, including other enzymes, must be considered. Responses which follow the administration of these drugs may not necessarily be attributable therefore to blockade of prostaglandin synthesis.

In the experiments on renal adrenergic nerve endings clear-cut evidence of the blockade of prostaglandin release hence biosynthesis (Davies, <u>et al</u>., 1968;   Piper and Vane, 1971) was obtained.   The vascular changes which ensued may well have resulted from such blockade but other explanations cannot be ruled out.   In the luteolysin study, the effects on indomethacin were clear-cut, closely resembling those which follow hysterectomy.   No direct evidence was obtained that indomethacin was in fact inhibiting the biosynthesis of $PGF_{2\alpha}$ in the uterus, though it is well documented from <u>in vitro</u> studies that such an inhibition was highly likely to have occurred.

Similarly the effects following active immunization against $PGF_{2\alpha}$ were clear cut, again resembling those following hysterectomy, but there was no conclusive evidence that the presumed blockade of $PGF_{2\alpha}$ was occurring in the local circulation between the uterus and the ipsilateral ovary. Other possibilities have been considered above (p.107).

## How Conclusive is the Experimental Evidence Presented for Physiological Roles of the Prostaglandins?

(a) Prostaglandin $E_2$ as a modulator of adrenergic nerve transmission

Taking into account the various snags and alternative explanations discussed already, it may be argued that the evidence is still not conclusive. There can certainly be no doubt that prostaglandins are released from the kidney in increased amounts during stimulation of the renal nerves which are adrenergic, that this release is abolished by indomethacin and that two of these prostaglandins ($PGE_2$ and $PGF_{2\alpha}$) have been identified unequivocally in the appropriate extracts. There is also no doubt that in the saline-perfused rabbit kidney intra-arterial infusion of $PGE_2$ reduces transmitter output in response to stimulation of the renal nerves, though it may be argued that the evidence is weakened by the use of a saline-perfused preparation and by the route of administration which is certainly very different from the physiological route – but it was the best that could be devised. The vascular changes following indomethacin are certainly compatible with the enhancement of sympathetic activity which would result if the

influence of an inhibitory modulator, such as $PGE_2$, were removed. More precise measurements of the vascular changes in the kidney are needed to strengthen this particular piece of evidence.

As already discussed, the hypothesis, true as it may be, for $PGE_2$ in the rabbit kidney, does not necessarily extend to all adrenergic nerve endings. The discrepancy between the output experiments in the dog and cat spleen (Davies, et al., 1968; Bedwani and Millar, 1975) and between the pharmacological experiments on the dog and cat spleen (Davies and Withrington, 1968; Hedqvist, 1971) have been highlighted previously (Horton, 1973). Particularly relevant are the findings of Bedwani and Millar (1975) who confirmed the output of prostaglandins from the dog spleen on nerve stimulation but were unable to obtain any convincing evidence for the release of prostaglandins from the cat spleen. Dubocovitch and Langer (1975) were able to demonstrate prostaglandin release only after the cat spleen had been saline-perfused for several hours. Moreover, abolition of this output by indomethacin did not affect responses to nerve stimulation nor the amount of noradrenaline overflow, although, like Hedqvist, they were able to demonstrate that exogenous $PGE_2$ inhibits transmitter release.

Unfortunately, as it stands, the hypothesis does not explain all the observations, namely the release of $PGF_{2\alpha}$ and of a PGA-like compound simultaneously with $PGE_2$ in response to nerve stimulation. It may be argued that the output of these substances need not detract from the evidence supporting $PGE_2$ as a modulator, but the hypothesis may need future

modification to account for the possible role of these
other prostaglandins at adrenergic nerve terminals.

It seems reasonable to conclude that $PGE_2$ has a physio-
logical role as an inhibitor of noradrenaline output during
adrenergic nerve stimulation in the rabbit kidney but that
this function does not necessarily extend generally to all
adrenergic nerves and relates only to a function for $PGE_2$.

(b)  Role of Prostaglandins in Human Semen

The presence in semen of high concentrations of pharma-
cologically-active substances like the prostaglandins suggests
but by no means proves that seminal prostaglandins have a
physiological role.  It is easy to speculate that they may
be involved with the control of sperm transport in the male
and/or female reproductive tracts by an action on smooth
muscle organs such as the vas deferens or the oviduct.  The
only real evidence of a function comes from comparisons of
fertile and infertile males in which infertility cannot be
accounted for by other factors (low sperm count etc.).
Such evidence was first put forward by Hawkins and Labrum
(1961) and more convincingly (because the methodology of
estimation had improved) by Bygdeman et al., 1970.  Both
claimed that low prostaglandin levels were associated with
such cases of unexplained infertility.

The present study has shown that aspirin in high dosage,
such as might be used in the treatment of acute rheumatic
episodes, does greatly reduce prostaglandin levels in human
seminal plasma.  In theory a long-term trial could be

mounted in which male volunteers would be given aspirin whilst the effects on fertility were monitored. In practice, it would be difficult and possibly hazardous to conduct such a trial - volunteers would be hard to obtain for such a long-term study, the effects of aspirin on sperm (and hence offspring should fertilization occur) are unknown and most significantly, from the evidence of this short-term study, the effects of the doses of aspirin required might have potentially lethal effects over a longer period.

If a less toxic inhibitor of prostaglandin synthase or one which more selectively blocked the enzyme in the male reproductive tract were forthcoming, it is possible that such a trial might be feasible and, on a more speculative note, that such a drug might be used as a contraceptive agent in the human male.

(c) <u>The Role of $PGF_{2\alpha}$ as the Guinea-pig Luteolytic Hormone</u>

The accumulation of evidence in favour of the hypothesis that the guinea-pig uterus produces $PGF_{2\alpha}$ towards the end of the oestrous cycle and secretes this to act upon the corpora lutea causing their regression and so termination of the cycle, has been summarized above (p.110). The deficiencies in this hypothesis are easily detectable. First, there is, as yet, no explanation as to how (or indeed whether) the $PGF_{2\alpha}$ released into the uterine venous blood reaches the ipsilateral

ovary to act upon the corpora lutea there. It may be a
vascular route, although examination of the blood vessels
under a dissecting microscope failed to reveal any evidence
for a relationship between the ovarian artery and the utero-
ovarian vein as is evident in the sheep (McCracken, 1971; McCracken,
Baird and Goding, 1971). It might eventually prove feasible
to cannulate the ovarian artery so as to collect samples of
blood supplying that organ, for $PGF_{2\alpha}$ determinations. Alter-
natively, the local route may be via the lymph, the lumen of
the reproductive tract or a so-far undetected vascular con-
nection between uterus and ovary. Although the biochemical
mode of action of $PGF_{2\alpha}$ as a luteolytic substance is still
not clearly understood (see Horton and Poyser, 1976), this
need not detract from the hypothesis, provided that $PGF_{2\alpha}$
really does have a luteolytic action within the concentration
range likely to be found in the ovary under physiological
conditions.

Although the evidence from the immunization experiments
does not conclusively prove that the site of neutralization
of the endogenous $PGF_{2\alpha}$ occurred in utero-ovarian circulation
and although direct evidence as to the mode of action of
intra-uterine indomethacin is also lacking, the accumulation
of evidence concerning a physiological role for $PGF_{2\alpha}$ as
the uterine luteolytic hormone in the guinea-pig is considered
to be very compelling. Just how general this phenomenon is
within the Animal Kingdom has been discussed by Horton and
Poyser (1976). Certainly there is excellent evidence for
such a role for $PGF_{2\alpha}$ in the sheep (McCracken, et al., 1972);

there is also evidence in other domestic farm animals as
well as in the pseudopregnant rat and rabbit. Whether
local control mechanisms of this nature are found at other
sites requires further consideration - for example sub-
stances released from the spleen or the gastro-intestinal
tract may act as local hormones on the liver whilst sub-
stances released peripherally may exert a humoral effect
on the frog kidney via the renal portal veins.

Other functions of prostaglandins in the urogenital tract

Finally, brief consideration will be given to other pos-
sible functions of prostaglandins in organs of the urogenital
tract. In the kidney a prostaglandin may be implicated in
the mechanism of autoregulation (Herbaczynska-Cedro and
Vane, 1973), whilst a role as the renal antihypertensive
lipid has long been debated (see Horton and Ungar, 1974).
Since prostaglandins have both natriuretic and diuretic
activity, one of them could be implicated in the mode of
action of the postulated natriuretic hormone described by
de Wardener (de Wardener, Mills, Clapham and Hayter, 1961)
or possibly be identical with the natriuretic hormone itself.

Prostaglandins interact with the renin-angiotensin
system and have been involved in control of renin release
(Flamenbaum and Kleinman, 1977; McGiff and Malik, 1976).
A similar inter-relationship between renal prostaglandins
and erythropoeitin may be envisaged.

None of these other renal functions is necessarily related to the proposed function at adrenergic nerve terminals investigated here, although an inter-relationship between sympathetic nervous system activity and other facets of renal function must not be excluded.

The seminal prostaglandins present an even more difficult problem although their many pharmacological actions on reproductive organs all seem logically to point to some influence on fertility. Actions of prostaglandins on both smooth muscle and glands of female reproductive tract have been reported. Moreover, prostaglandins act upon uterine smooth muscle, the cervix and the oviduct (see Horton, 1969). Some of these potential targets in the female reproductive tract could be reached by local diffusion of prostaglandins from the ejaculate deposited in the posterior fornix. Other targets, notably the oviduct, could probably only be influenced by seminal prostaglandins if some means of transporting them so far is available. It is established (Horton, Main and Thompson, 1965) that $PGE_1$ is absorbed from the vagina in rabbits in amounts sufficient to affect oviduct smooth muscle and similar vaginal absorption has been reported in human female volunteers (Eliasson and Posse, 1965). Such mechanisms are of considerable biological interest and it would be of interest to know whether prostaglandins act as exohormones or even pheromones in an analogous way in other species.

Uterine prostaglandins formed in the endometrium may well have other functions, notably at the time of implantation (Phillips and Poyser, 1979) and in the pregnant animal (formed

by the uterus or uterine contents) as an oxytocic stimulus in parturition (see Karim and Hillier, 1975; Flint and Hillier, 1975).

It seems clear that the role of prostaglandins in ovulation is a local effect brought about by the ovarian prostaglandins themselves. Finally, a role of locally produced prostaglandins in controlling oviduct tone (Horton and Main, 1963) is likely to involve interesting inter-relationships with oestrogens and progestogens which control the hormonal status of that tissue and with the adrenergic terminals of neurones in the hypogastric nerve, stimulation of which causes pronounced changes in oviduct tone.

It may well be that further research on the oviduct is needed to solve these intriguing inter-relationships between the adrenergic nervous system, the ovarian hormones and the prostaglandins which have been studied separately in the research work reported in this thesis.

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1.  The separation, identification and estimation of
    prostaglandins in nanogram quantities by combined
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2.  Identification of prostaglandin $F_{2\alpha}$ released by
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4.  The release of prostaglandins and progestin into
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5.  Effects of aspirin on prostaglandin and fructose
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6.  Elongation of the oestrous cycle in the guinea-pig
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7.  Elongation of the oestrous cycle in guinea-pigs
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The thesis has been written by me. The research was
done in collaboration with members of my research team
but in every case, I made a substantial contribution
not only to the planning and design of the experiments,
but also to their performance in the laboratory.