

PROSTAGLANDINS AND THE PREGNANT ANIMAL

by

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SUMMARY

There is now substantial evidence that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the uterine luteolysin in the guinea-pig. There is also evidence to suggest that ovarian oestradiol- 17β , in the presence of progesterone, may be the physiological stimulus to increased production and release of $PGF_{2\alpha}$ from the uterus on days 14 and 15 of the oestrous cycle, this being the time of luteal regression.

In the pregnant guinea-pig luteal regression at day 15 must be prevented in some way since a functional corpus luteum is essential for the maintenance of pregnancy until days 20-25 post-coitum. Early pregnancy in the guinea-pig survives successfully in the absence of the maternal pituitary, therefore the developing conceptus must itself be capable of maintaining the pregnancy by either a direct luteotrophic action on the corpus luteum, or an anti-luteolytic action or quite possibly by a combination of these actions. Grafting of young conceptuses to the spleen of non-pregnant guinea-pigs causes elongation of the oestrous cycle and extension of luteal life-span, suggesting that the conceptus secretes a systemically active factor having one or more of the actions just considered. Hysterectomy of non-pregnant guinea-pigs results in maintained corpora lutea with a capacity to secrete progesterone equivalent to that of corpora lutea in day 10-15 pregnant animals, the critical time when luteal regression would be occurring in non-pregnant guinea-pigs. It appears, therefore, that negation of the luteolytic actions of the uterus by the conceptus (that is an anti-luteolytic action) could be sufficient to maintain the corpora lutea at the level normally found for days 10-15 of pregnancy. It is this hypothesis, that the young guinea-pig conceptus secretes a systemically active anti-luteolytic factor which is investigated in this thesis.

PGF_{2α} production and release from the uterus was found to be greatly reduced in the pregnant guinea-pig compared to the non-pregnant guinea-pig while metabolism of PGF_{2α} measured under the same conditions was low for both non-pregnant and pregnant uteri. It is concluded that the ability of the uterus to synthesise PGF_{2α} is reduced in early pregnancy. This reduction could not be overcome by the addition of excess arachidonic acid, the precursor of PGF_{2α}, nor could significant reduction be achieved in vitro by incubating the non-pregnant uterus in the presence of conceptuses. Reduction of precursor levels or direct inhibition of the synthetase complex seem unlikely therefore as possible mechanisms of action for an anti-luteolytic factor. The inhibition may, however, be exerted at an earlier point in the control of PGF_{2α} production.

Utero-ovarian venous plasma levels of oestradiol-17β were low in the early pregnant guinea-pig, in contrast to the high levels seen at a similar time in the non-pregnant animal. This inhibition of ovarian oestradiol-17β secretion could form part of the mechanism of the anti-luteolytic action of the guinea-pig conceptus. Administration of exogenous oestradiol benzoate to early pregnant guinea-pigs caused abortion, associated with increased uterine in vitro PGF_{2α} production. In some aborting animals where cervical dilation had not yet occurred the corpora lutea appeared less structurally regressed than in those animals where cervical dilation was complete. These results are discussed.

In unilaterally pregnant guinea-pigs luteal progesterone secretion was bilaterally maintained supporting the hypothesis that the anti-luteolytic factor is systemically active. However, uterine in vitro

PGF_{2α} production and utero-ovarian venous plasma levels of PGF_{2α} and oestradiol-17β were higher on the non-pregnant side than the pregnant side. Spacial separation of the non-pregnant horn from the pregnant horn, by transection, resulted in an increase in the amount of PGF_{2α} produced in vitro by the non-pregnant horn, while that of the pregnant horn remained low. These findings are discussed in view of ovarian oestradiol-17β being the possible physiological stimulus to uterine PGF_{2α} production and release. A local component is proposed for the anti-luteolytic actions of the guinea-pig conceptus, in addition to the major systemically active component already considered.

Intra-uterine infusion of embryonic homogenate to non-pregnant guinea-pigs caused extension of the oestrous cycle but was without effect on luteal life-span, an action identical to that of exogenous progesterone. Intra-peritoneal injections or intermittent intra-uterine injections of embryonic homogenate to non-pregnant guinea-pigs were without effect on either oestrous cycle length or luteal life-span. The significance of these results is discussed.

The results presented in this thesis support the hypothesis that the young guinea-pig conceptus secretes an anti-luteolytic factor in early pregnancy, having both systemic and local components to its actions. An inhibition of ovarian oestradiol-17β secretion may be part of its mechanism of action. These results are discussed in relation to events of early pregnancy seen in other species. The role of oestrogen as the possible physiological stimulus to uterine PGF_{2α} production and release is considered in the light of the local component to the anti-luteolytic actions of the guinea-pig conceptus.

INTRODUCTION

The guinea-pig is a polyoestrous, spontaneously ovulating hystricomorph rodent. The oestrous cycle is of 16-18 days duration and oestrus is evident from the opening of the vaginal membrane and a predominance of cornified epithelial cells in the vaginal smear. The observation that hysterectomy extends the oestrous cycle length to over 60 days (Loeb, 1923) was the first indication of the importance of the uterus in the control of the oestrous cycle. If only one horn of the uterus is removed, only the corpus luteum adjacent to the remaining horn regresses, (Fischer, 1965). Loeb (1927) observed similar effects to hysterectomy with uterine auto-transplantation, although others found only slightly extended cycle length (Anderson, Butcher & Melampey, 1961; Butcher, Chu & Melampey, 1962a). However, this may be a function of the distance of the transplantation site from the ovaries, extension of cycle length reducing as the two become closer. Bland (1970) consistently observed cycles extended to 21 days with uterine auto-transplantation to the abdominal wall. This extension, although less than that observed by Loeb (1927), is none-the-less significant. It is apparent, therefore, that the uterus exerts a local control over corpus luteum function. This control seems to be independent of the pituitary, since hypophysectomy on day 10 does not prevent luteal regression at the expected time, (Perry & Rowlands, 1962) and hypophysectomy in the hysterectomised guinea-pig is without effect on luteal

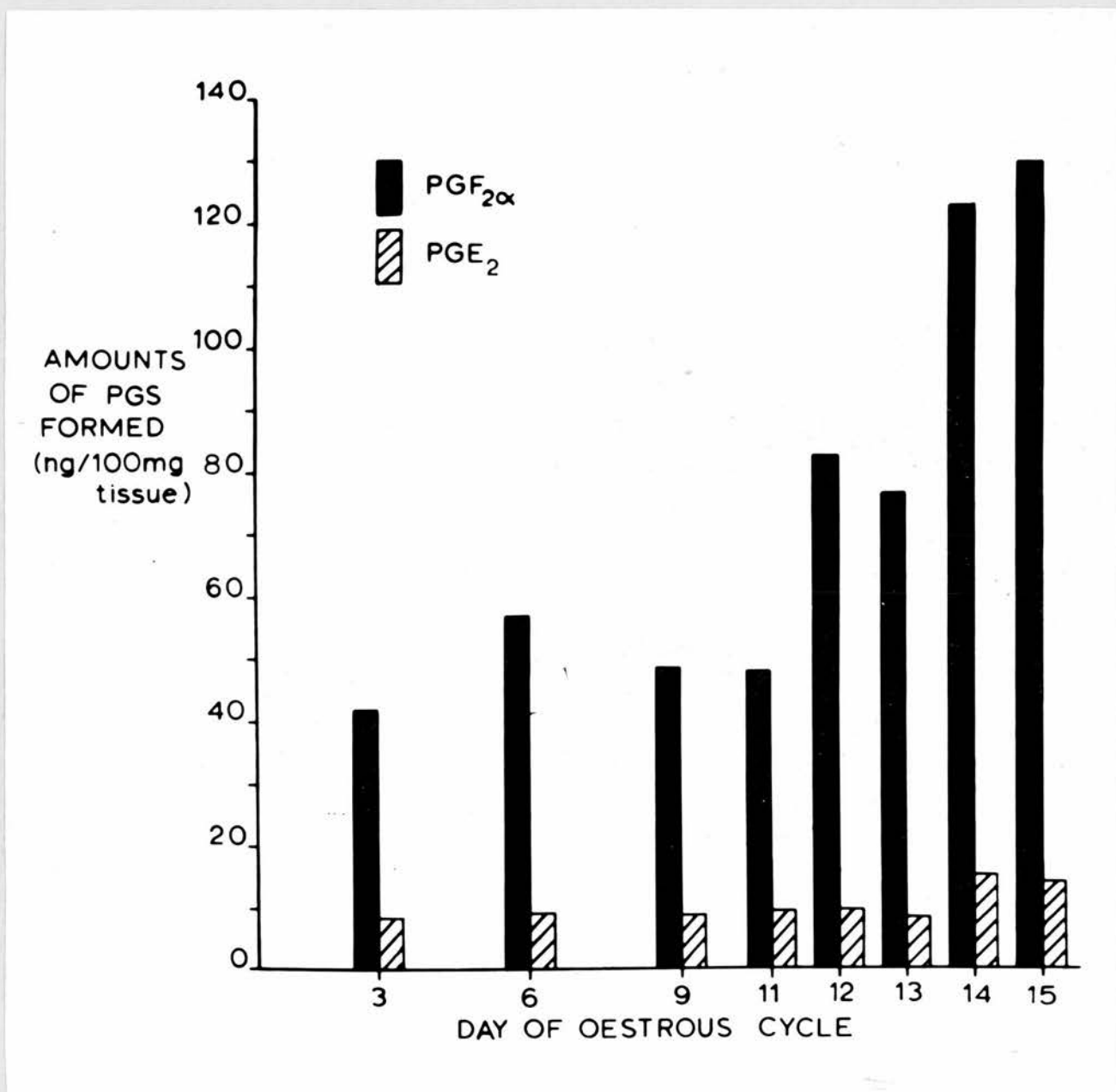
function, the corpora lutea being maintained (Rowlands, 1962). This evidence all supports the suggestion made by Loeb in 1927, that the uterus secretes a luteolysin which acts on the ovaries in a local manner. Since endometrial destruction gives results similar to hysterectomy, the endometrium may well be the source of this luteolysin (Butcher, Chu & Melampy, 1962b).

The guinea-pig uterus shows a cyclical variation in the amount of prostaglandins $F_{2\alpha}$ and E_2 ($PGF_{2\alpha}$ and PGE_2) produced when homogenised and incubated in vitro, (Poyser, 1972). (See Figure 1). There is a large increase in its ability to produce $PGF_{2\alpha}$ especially on days 14 and 15 of the oestrous cycle, at the time of luteal regression. The levels of prostaglandins in the uterus were, however, low with only small variations during the cycle (Poyser, 1972). This suggests that prostaglandins are not stored but are released as they are produced. This system of homogenisation and incubation appears to be a good model for the measurement of production of prostaglandins, although the ability of this system to metabolise prostaglandins has not yet been investigated.

The guinea-pig uterus has been shown to produce far more $PGF_{2\alpha}$ than PGE_2 (Poyser, 1972) and of these two prostaglandins only $PGF_{2\alpha}$ has been shown to be luteolytic in the hysterectomised guinea-pig (Blatchley & Donovan, 1969, Blatchley and Donovan, 1972). Utero-ovarian venous plasma levels of $PGF_{2\alpha}$ and PGE_2

Figure 1.

The production of prostaglandins by the guinea-pig uterus, in vitro, at different stages of the oestrous cycle. (Poyser 1972).



also show a cyclical variation, levels of $\text{PGF}_{2\alpha}$ again being greater than those of PGE_2 . An increase in the levels is seen at day 11, followed by a large increase on days 14 and 15 of the oestrous cycle, at the time of luteal regression. (Blatchley, Donovan, Horton & Poyser, 1972; Earthy, Bishop & Flack, 1975). (See Figure 2).

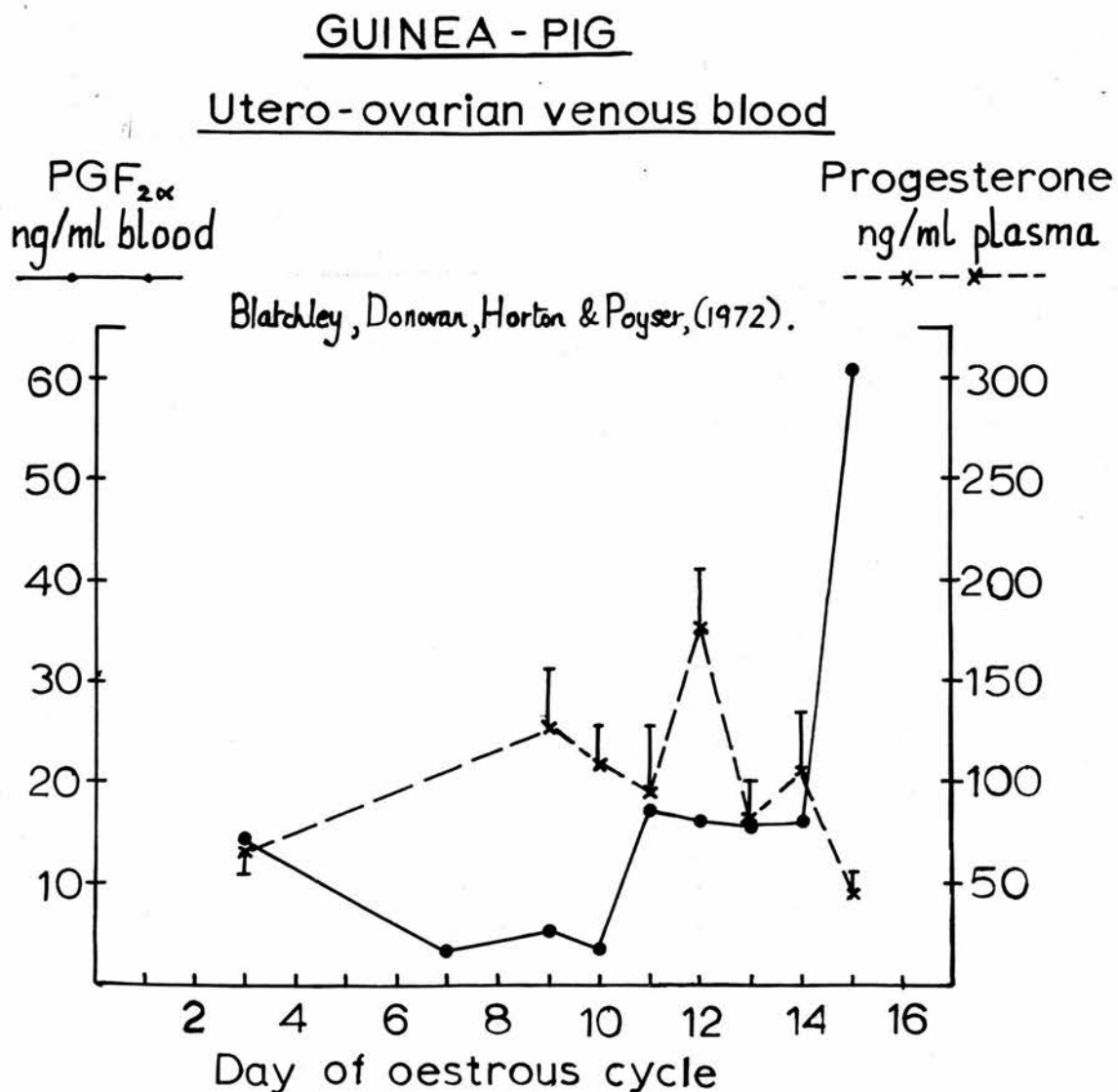
Systemic administration of indomethacin, an inhibitor of prostaglandin synthesis only prolonged oestrous cycle length when given in toxic doses (Marley, 1972). However, when placed in the uterus indomethacin caused significant increases in oestrous cycle length with functional luteal maintenance. (Poyser, 1973; Marley, 1973; Horton & Poyser, 1973; Poyser & Horton, 1975). Active immunisation of guinea-pigs against $\text{PGF}_{2\alpha}$ also prolongs the functional life of the corpus luteum to times approaching that seen with hysterectomy, (Horton & Poyser, 1974; Poyser & Horton, 1975).

Considering all this evidence, it appears almost indisputable that $\text{PGF}_{2\alpha}$, released from the uterus, is the luteolysin in the guinea-pig.

There are many other species where hysterectomy prevents the normal regression of corpora lutea. Such an effect is seen in the cycling sheep, cow (Wiltbank & Casida, 1956) and pig (Spies, Zimmerman, Self & Casida, 1960) and in the pseudopregnant rat

Figure 2.

The levels of Progesterone and $\text{PGF}_{2\alpha}$ in the utero-ovarian venous blood of guinea-pigs at different stages of the oestrous cycle.

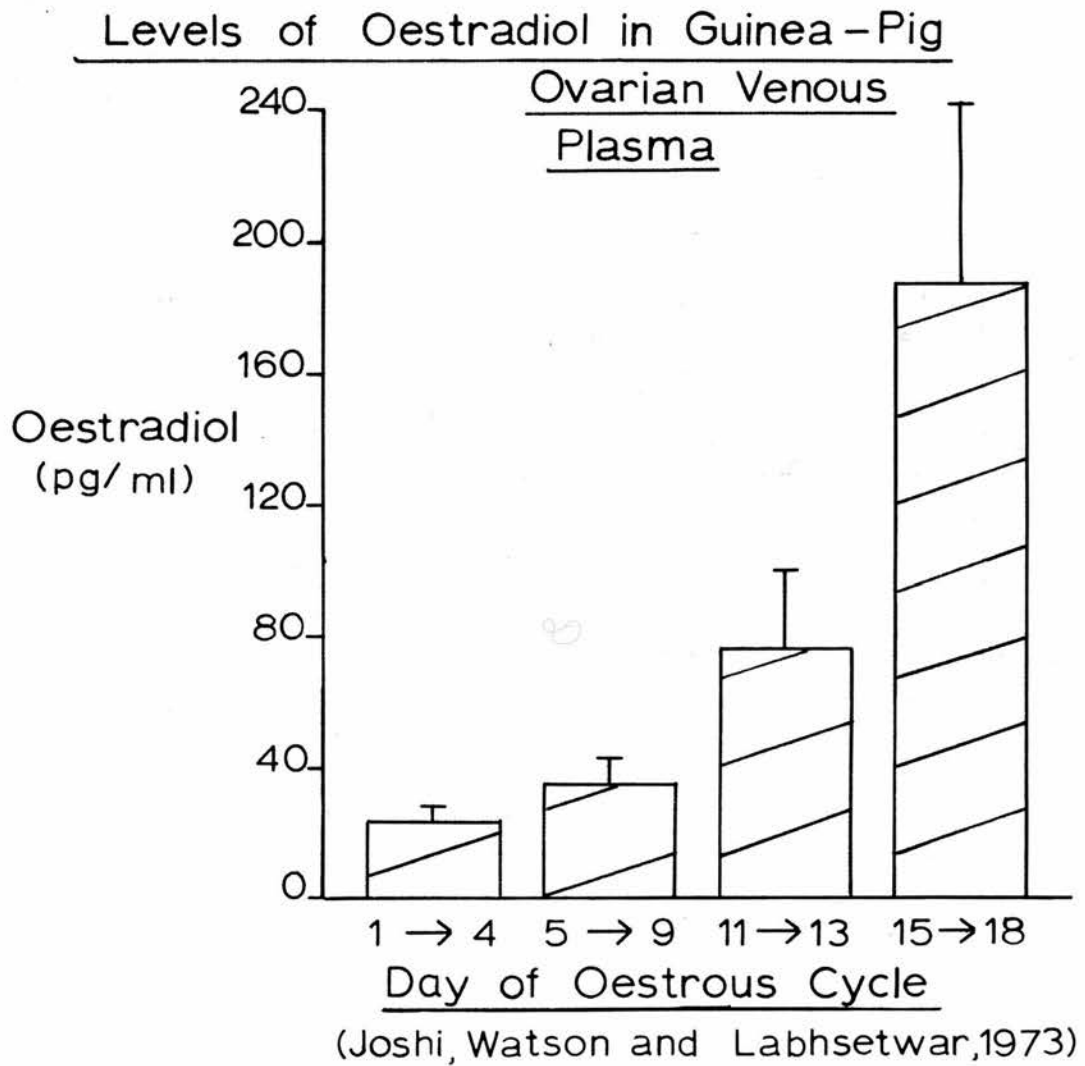


(Bradbury, 1937), rabbit (Asdell & Hammond, 1933) and hamster (Caldwell, Mazer & Wright, 1967). Extensive work has been carried out in the sheep, of a similar nature to that just described for guinea-pigs, and it is now almost beyond doubt that the luteolysin in this species is also $\text{PGF}_{2\alpha}$. (McCracken, Carlson, Glew, Goding, Baird, Green & Samuelsson, 1972 (a); McCracken, Carlson, Green & Samuelsson, 1972 (b); Goding, Cain, Cerini, Cerini, Chamley & Cumming, 1972; Goding, 1973, 1974). This species has an oestrous cycle with very similar characteristics to that of the guinea-pig. The main difference is seen in the spontaneous luteal regression that hypophysectomy causes in hysterectomised sheep. This is thought to be due to withdrawal of a pituitary luteotrophin (Anderson, Bland & Melampy, 1969).

In the guinea-pig much work has been carried out to determine the nature of the stimulus for the increased uterine production of $\text{PGF}_{2\alpha}$ at the appropriate time. Recently it has been shown that ovarian venous plasma levels of oestradiol-17 β show a cyclical variation very similar to that observed for uterine in vitro $\text{PGF}_{2\alpha}$ production and utero-ovarian venous plasma $\text{PGF}_{2\alpha}$ levels (Joshi, Watson & Labhsetwar, 1973). (See Figure 3). Oestrogen treatment can produce paradoxical results, luteolytic and luteotrophic actions being seen, but this is largely the result of using large doses which cause circulating

Figure 3.

The levels of oestradiol in the ovarian venous plasma of guinea-pigs at different stages of the oestrous cycle.



levels of oestrogen to be far higher than those found in physiological situations, (Illingworth & Perry, 1973). There are, however, several reports of oestrogen stimulating $\text{PGF}_{2\alpha}$ release. Thus it has been shown that systemic administration of oestradiol benzoate early in the cycle, or to ovariectomised animals, causes release of $\text{PGF}_{2\alpha}$ into the uterine venous blood (Blatchley, Donovan, Horton, Poyser, Thompson & Los, 1971; Blatchley et al., 1972; Blatchley & Poyser, 1974). In the ovariectomised guinea-pigs the effect of oestradiol was enhanced by the concurrent administration of progesterone. Recently it has been shown that the in vitro uterine $\text{PGF}_{2\alpha}$ production of ovariectomised guinea-pigs is enhanced by the systemic administration of oestradiol benzoate or by the presence of oestradiol 17β in the incubating medium (Naylor & Poyser, 1975). It appears, therefore, that the increasing levels of oestrogen around day 15 of the oestrous cycle, in the presence of progesterone, probably form the physiological stimulus for the release of $\text{PGF}_{2\alpha}$ from the uterus at the end of the oestrous cycle.

Therefore, in the non-pregnant guinea-pig the end of the oestrous cycle and luteal regression followed by oestrus appear to be brought about by the increased release of $\text{PGF}_{2\alpha}$ from the uterus, the stimulus for which is probably ovarian oestradiol, a possible source being the developing follicle.

Gestation in the guinea-pig lasts about 67 days, with

structural luteal maintenance until just before or around parturition (Rowlands, 1956). Implantation on day 6 to 7 post-coitum (p.c.) is not prevented by hypophysectomy on day 2 or 3 p.c., (Heap, Perry & Rowlands, 1967), although it is prevented by ovariectomy prior to day 2 p.c. Ovariectomy on days 3 to 7 p.c. does not prevent implantation (Deanesly, 1960 a, b). Early pregnancy in the guinea-pig appears to be generally independent of maternal pituitary support. Thus in animals hypophysectomised shortly after mating, if viable conceptuses are present, the size and progesterone content of the corpora lutea at day 28 p.c. closely resemble those of intact pregnant animals, (Heap et al., 1967). In contrast, in guinea-pigs ovariectomised shortly after mating (day 3 - 7 p.c.), pregnancy is always terminated on or around day 16, approximately the normal time of luteal regression in non-pregnant animals, (Loeb & Hesselberg, 1917; Deanesly, 1963, 1972). Normal embryos show rapid growth and differentiation in the day 14 - 16 p.c. period (Harman & Prickett, 1932; Scott, 1936). If, however, ovariectomy is not carried out until day 28 p.c. or in some animals day 20 p.c., pregnancy will continue successfully to term (Herrick, 1928; Courrier, Kehl & Raynaud, 1929). It is apparent, therefore, that ovarian support of some kind is essential for implantation and for the maintenance of pregnancy from days 16 to 28 p.c. approximately. Deanesly (1972) has

shown that embryonic, placental and decidual degeneration first become evident around day 12 p.c. in ovariectomised animals. She also demonstrated that administration of exogenous progesterone, preferably early after ovariectomy, but not later than day 15 p.c., can prevent decidual degeneration or check its spread once it has begun and thus allow the continuance of pregnancy. It is evident, therefore, that continuing ovarian progesterone support, that is a functional corpus luteum, is essential for the maintenance of pregnancy from days 12 to \wedge 20 - 28 p.c. From this time placental progesterone support is adequate to maintain pregnancy in the absence of the ovaries, (Courrier et al., 1929; Deanesly, 1963). It follows from these observations that luteal regression, normally occurring around days 14 to 16 of the oestrous cycle, must be prevented in some way in the pregnant guinea-pig. Maternal luteotrophic support can be ruled out since, as already described, pregnancy is independent of the maternal pituitary, (Heap et al., 1967),

Surgical removal of conceptuses even as late as day 15 p.c. results in the recurrence of oestrus at or soon after (within 4 to 5 days) the time at which it would have occurred had not fertile mating taken place (Bland & Donovan, 1969). A similar effect is seen throughout gestation, (Klein, 1939; Bland & Donovan, 1966) although the later effect is almost certainly connected with the withdrawal of placental progesterone support.

During the days 12 - 25 p.c. the conceptus appears to be continuously overcoming the lytic effect of the uterus either by an anti-luteolytic action or by a direct luteotrophic action on the corpus luteum.

The effect of pregnancy has been likened to the effect of hysterectomy in that the uterine luteolytic factor has been removed, (Heap, Illingworth & Perry, 1973). Yet there appears also to be a direct luteotrophic action evident as a slightly increased production of progesterone by the corpus luteum from about day 15 of pregnancy when compared with that of hysterectomised animals, (Illingworth, Heap & Perry, 1970; Challis, Heap & Illingworth, 1971). However, in quoting their results these workers made no allowance for body weight. Since the conditions compared such as hypophysectomy, hysterectomy and pregnancy cause substantial changes in body weight, this is an important consideration. This is none-the-less an important observation in need of further investigation earlier in pregnancy. It has become evident that the luteotrophic or anti-luteolytic actions of the conceptus must come into play by day 12 p.c., by which time in the oestrous cycle plasma progesterone is falling (Challis et al., 1971). The direct luteotrophic action just considered is, however, more marked later in pregnancy being only slight around day 15p.c. Unless a significant increase in the luteal production rate of progesterone

can be demonstrated earlier in pregnancy this direct luteotrophic action would not appear to be sufficient to be wholly responsible for the maintenance of pregnancy through days 12 to 28 p.c.

The high plasma progesterone concentrations of pregnancy can not be explained by increased production alone, but are directly related to a rapid fall in the metabolic clearance rate (MCR) of progesterone (Illingworth et al., 1970). The decrease in MCR has been shown to be inversely proportional to an increased concentration of a high-affinity progesterone-binding globulin (PBG) in the circulating plasma of pregnant guinea-pigs (Illingworth, Ackland, Heap & Weir, 1973). The physico-chemical properties of PBG differ from those of corticosteroid-binding globulin (Heap, 1969; Milgrom, Atger & Baulieu, 1970a; McLaughlin, Harding & Westphal, 1972) and from the uterine cytosol receptor (Milgrom, Atger & Baulieu, 1970b; McLaughlin et al., 1972; Heap & Illingworth, 1973). PBG has a relatively high specificity for progesterone and has been found in seven species of hystricomorph rodents, but not in the pig, sheep, ferret or rat, all of which belong to other orders of mammals, (Illingworth et al., 1973). PBG is first detected in maternal plasma on day 15 of pregnancy. It is at about this time that the allantois makes contact with the chorion and the definitive allanto-chorial placenta becomes established (Duval, 1892). The first point of contact is the central region where the sub-placenta

becomes differentiated, a structure considered unique to the hystricomorph rodents, (Mossman, 1937; Perrotta, 1959) and a possible site of a specialised function such as placental luteotrophin production, (Davies, Dempsey & Amoroso, 1961). However, PBG secretion can only be considered an assistance to a functional corpus luteum especially since it only appears on day 15 p.c., which would be too late to save a regressing corpus luteum. However, if the only action of the conceptus was to be a luteotrophic one, maintaining progesterone levels, progesterone must itself be able to prevent the actions or release of the factors causing luteolysis. (In this case the administration of sufficient amounts of progesterone to non-pregnant guinea-pigs should prevent luteal regression. In fact, although recurrence of oestrus is prevented in progesterone-treated non-pregnant guinea-pigs, luteal regression still occurs at the normal time (Deanesly, 1968). These animals were treated from day 10 of the oestrous cycle. If exogenous progesterone is administered early in the oestrous cycle to non-pregnant animals there is a small but significant reduction in oestrous cycle length, (Woody, First & Pope, 1967) and Aldred, Sammelwitz & Nalbandov, (1961) observed a reduction in corpus luteum size after exogenous progesterone.

It becomes essential to propose either some additional direct luteotrophic action or an anti-luteolytic action for either the conceptus (foetus and foetal membranes) or maternal decidua.

The experimental induction of maternal decidua in non-pregnant guinea-pigs does not prolong luteal function (Dempsey, 1938). Thus such a role for the maternal decidua may be excluded.

From this point on there has been relatively little work carried out for the guinea-pig and what has been done presents a rather confusing picture. However, a large amount of work has been done in the sheep. The pattern of events during the oestrous cycle in this species is remarkably similar to the guinea-pig, PGF_{2x} being the uterine luteolysin, as has already been pointed out (McCracken, et al., 1972 a, b; 1974). The events of pregnancy in the sheep are also similar to those in the guinea-pig, survival of early pregnancy being dependent upon the prevention of luteal regression around day 16 p.c. (Moor, 1968). Gestation is longer in the sheep (147 days) and ovarian dependence exists until about day 50 p.c. (Denamur & Martinet, 1961). However, an anti-luteolytic action of the conceptus has been proposed for this species and in support of this it has been shown that day 14 embryonic homogenates will prolong the functional life-span of the corpus luteum if infused into the uterus of non-pregnant ewes from day 12 of the oestrous cycle (Rowson & Moor, 1967). A single infusion on day 10 caused only a slight extension of the luteal life-span and intra-muscular injections had no effect at all. Thus in the sheep this anti-luteolytic action of the conceptus appears to need a continuing, local, type of

action. No comparable experiments have been carried out in guinea-pigs.

In sheep, transfer of embryos to non-pregnant ewes up to day 12 of the oestrous cycle (host and donor cycles synchronised) consistently results in normal pregnancy although transfer on days 13 or 14 has little or no effect on the recurrence of the next oestrus (Moor & Rowson, 1964, 1966 a,b). This indicates that events leading to luteal regression must be halted by day 12 if pregnancy is to continue, and it also shows that in sheep the day 12 conceptus has this ability. Thus far the observations agree with our tentative conclusions for the guinea-pig based on the evidence so far considered. In the guinea-pig, however, the transfer of day 6 blastocysts to the uterus of unmated cycling animals resulted in pregnancy in only three of six animals where host and donor cycles were synchronised. Normal development was never observed following the transfer of 9 to 11 day implanted conceptuses to the uterus of non-pregnant animals (Bland & Donovan, 1969). Yet the same workers found that 9 to 10 day implanted conceptuses transferred to the spleen of non-pregnant guinea-pigs developed in eight of thirteen animals, although recurrence of oestrus was not prevented. These results suggest that day 6 blastocysts, but not day 9 to 11 implanted conceptuses, were able to overcome the lytic effect of the uterus. However, implantation is early in the guinea-pig

(day $6\frac{1}{2}$ - $7\frac{1}{2}$ p.c., Deanesly, 1960) and by day 9 the uterus may no longer be receptive to implantation. It is hard to explain survival of the ectopic pregnancies developing from the splenic transplants, given the recurrence of oestrus and, therefore, presumably the absence of a functional corpus luteum. However, ectopic implantation in the guinea-pig in general is without effect on oestrous cycle length (Loeb, 1914, 1915; Bland & Donovan, 1965) and successful development has been observed after the transfer of day 12 implanted conceptuses to the spleen or testis of male guinea-pigs (Bland & Donovan, 1965).

In extreme contrast to the results just presented Bland & Donovan (1969) observed that grafts developing from 11 - 12 day implanted conceptuses in the spleen of non-pregnant guinea-pigs, maintained luteal function and delayed oestrous beyond day 20 in thirteen of twenty four animals. This would suggest that in these animals a systemically active anti-luteolysin or luteotrophin was produced by some part of the graft. Luteal enlargement was not as great in these animals as in normal pregnancy. This may have been due to the action on the corpus luteum having to be achieved entirely systemically. In animals pregnant in an auto-transplanted uterus there is a similar picture of luteal maintenance with reduced luteal enlargement (Bland, 1972). This may again indicate the loss of a local component of the anti-luteolytic or luteotrophic action. In unilaterally

pregnant guinea-pigs Deanesly (1967), observed bilateral luteal maintenance. This result is open to two interpretations. It could indicate either the secretion of a systemically active anti-luteolytic or luteotrophic factor by the conceptus, or conversely some local action may be present allowing the contents of the gravid horn to directly affect the non-gravid horn. However, since there is very little, if any, luminal contact between the two uterine horns in the guinea-pig it is difficult to envisage a local type of action so that the systemic action appears more likely. Oxenreider & Day (1967) disagreed with the observations of Deanesly (1967) but on close examination their data do not support their arguments.

It is evident that the work in this field in the guinea-pigs needs much clarification. Tentatively it may be suggested that in the guinea-pig the day 11 to 12 p.c. implanted conceptus possesses anti-luteolytic or luteotrophic activity which may be exerted systemically. Since removal of conceptuses up to and beyond day 15 p.c. results in the rapid recurrence of oestrous (Bland & Donovan, 1969) there appears to be an active negation of the luteolytic effect of the uterus in early pregnancy, the lytic effect remaining ready to be triggered-off if the antagonism is removed. In this context at least, the action of the conceptus appears to be anti-luteolytic although almost certainly having a luteotrophic component. The mechanism of maintenance of ectopic

pregnancies is not, at this time, understood.

It is the aim of this thesis to try to elucidate the nature of events occurring in early pregnancy in the guinea-pig. Initial experiments will be directed towards an attempt to distinguish between or identify anti-luteolytic or luteotrophic actions of the conceptus. In the presence of a luteotrophic action of the conceptus inhibition of uterine production of $\text{PGF}_{2\alpha}$, the luteolysin, would be unnecessary. An anti-luteolytic action could be achieved either by direct inhibition of uterine $\text{PGF}_{2\alpha}$ production, by inhibition of an earlier step controlling $\text{PGF}_{2\alpha}$ production or by antagonising the luteolytic actions of $\text{PGF}_{2\alpha}$.

It is hoped that the work to be described will yield information not only relevant to the corpus luteum of pregnancy, but also to general relationships between the ovarian steroid hormones, $\text{PGF}_{2\alpha}$ and luteal function.

APPENDIX TO THE INTRODUCTIONTHE PROSTAGLANDINS

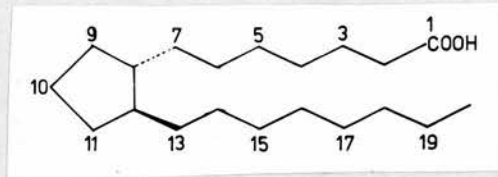
The prostaglandins are C-20 fatty acids, the parent compound being prostanic acid (see Fig. 4) which, although not naturally occurring, forms the basis for the nomenclature of the prostaglandins.

The different groups of prostaglandins each bear a different ring structure (see Fig. 4). The three series of prostaglandins differ in their degree of saturation. The "one" series have a trans-double bond at C-13,14. The "two" series have both the trans-double bond at C-13,14 and a cis-double bond at C-5,6. The "three" series of prostaglandins have the two double bonds of the "two" series and also a third double bond at C-13,14. There is also another group of prostaglandins with a hydroxyl group at C-19, the 19-hydroxy prostaglandins.

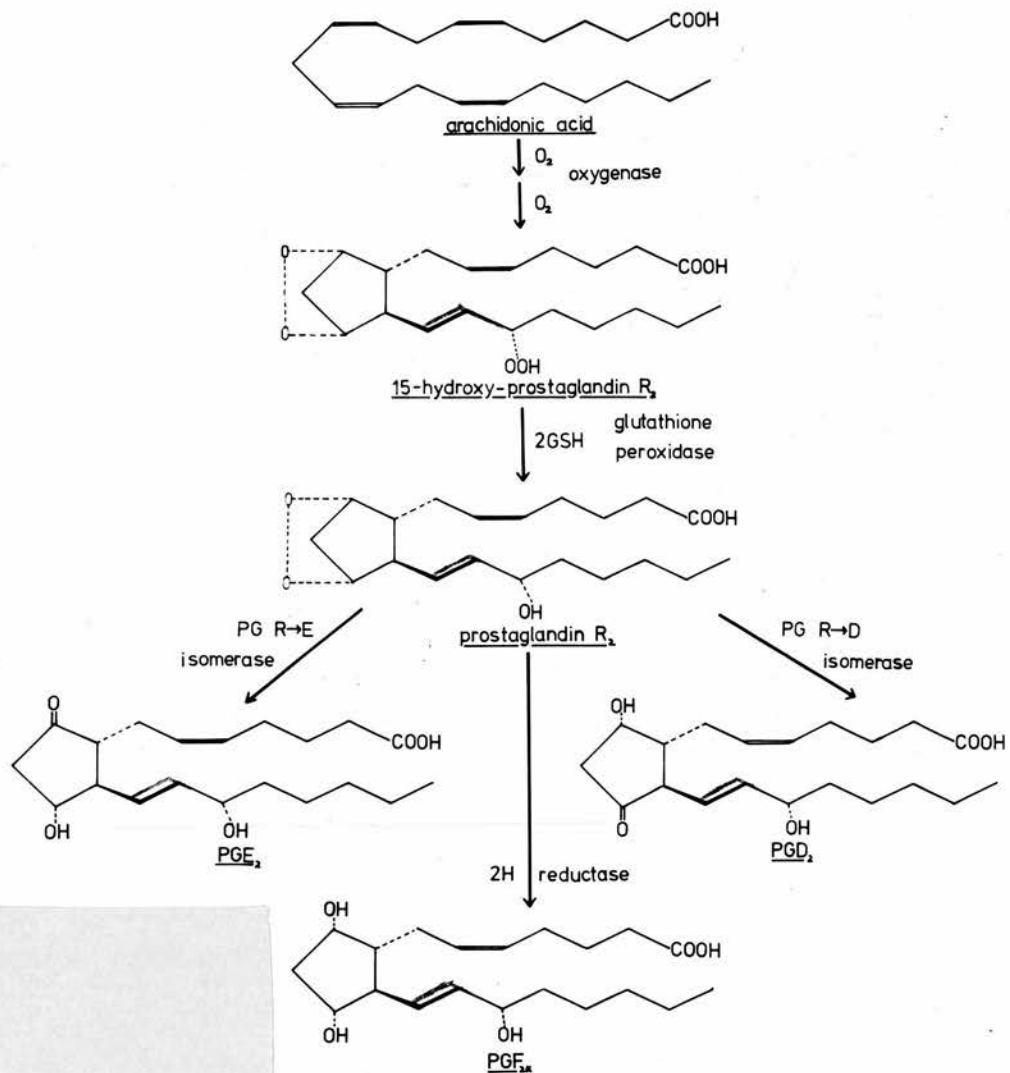
It is largely the prostaglandins of the "two" series that are considered in this thesis. The main stages in the biosynthesis of the prostaglandins E, F and D are shown in Fig. 4. The interconversion of these prostaglandins and their conversion to some of the other prostaglandins is shown in Fig. 5. Prostaglandins of the F group may be α at the C-9 hydroxyl, or β , but only the α form is naturally occurring in the "two" series.

Figure 4

(a) Prostanic acid.

(b) Some stages in the biosynthesis of PGE_2 , $\text{PGF}_{2\alpha}$ and PGD_2 .

(Nugteren and Hazelhof, 1973)



See also Samuelsson, 1973.

The form of abbreviation used in this thesis will be PG (prostaglandin) followed by the appropriate group (E, F etc.) with the series as a subscript. For example, PGE₂, a prostaglandin of the E group and of the "two" series.

The prostaglandins, generally, are rapidly metabolised. In particular 90% of intravenously administered PGE₂ or PGF_{2α}, at concentrations of 500 ng/ml, is metabolised to a relatively inactive form on a single passage through the lungs (Piper, Vane & Wyllie, 1970). The major sites of metabolism are shown for PGE₂ in Fig. 6, with the structures of two of the metabolites. Numerous other metabolites of PGE₂ and PGF_{2α} have been identified (Samuelsson, Granström, Green & Hamberg, 1971). One of the earliest steps in the metabolism of PGF_{2α} or PGE₂ is the oxidation of the C-15 hydroxyl by the enzyme prostaglandin dehydrogenase, to give the 15-oxo derivative. Another early step is the reduction of the C-13,14 double bond by the enzyme prostaglandin 13,14 reductase. The 13,14 dihydro form of either PGF_{2α} or PGE₂ is not a good substrate for the dehydrogenase, so the reduction of the C-13,14 double bond is likely to be secondary to the oxidation of the C-15 hydroxyl (Samuelsson et al., 1971). Subsequently beta oxidation and omega oxidation may also occur.

General references for this Appendix:

Pickles (1969)
Horton (1972a,b)

Figure 5

The interconversion of PGE_2 , $\text{PGF}_{2\alpha}$ and PGD_2 and their conversion to other prostaglandins.

References: (A) Hensby (1974 a,b,c)

(B) Jones (1970)

Jones, Cammock and Horton (1972)

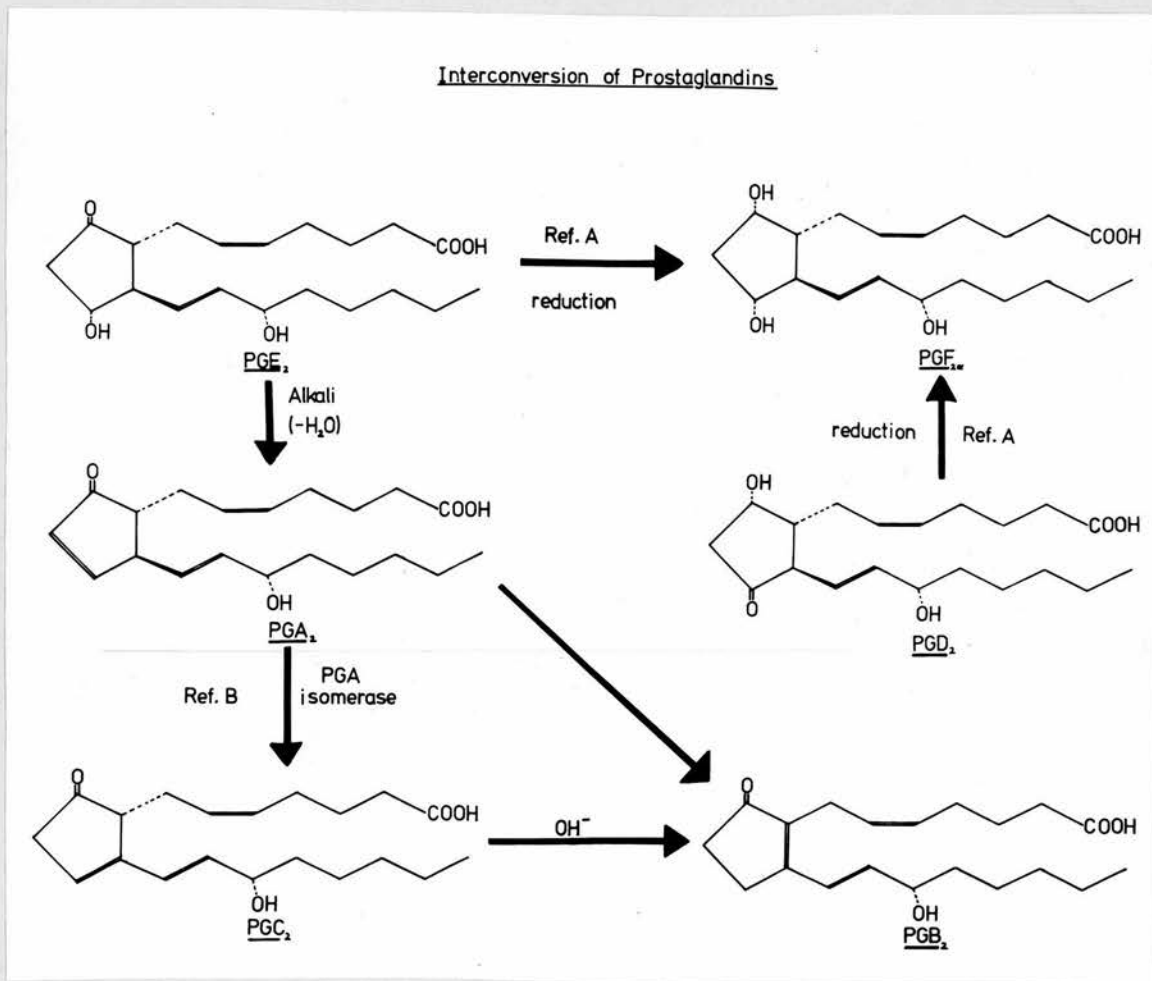
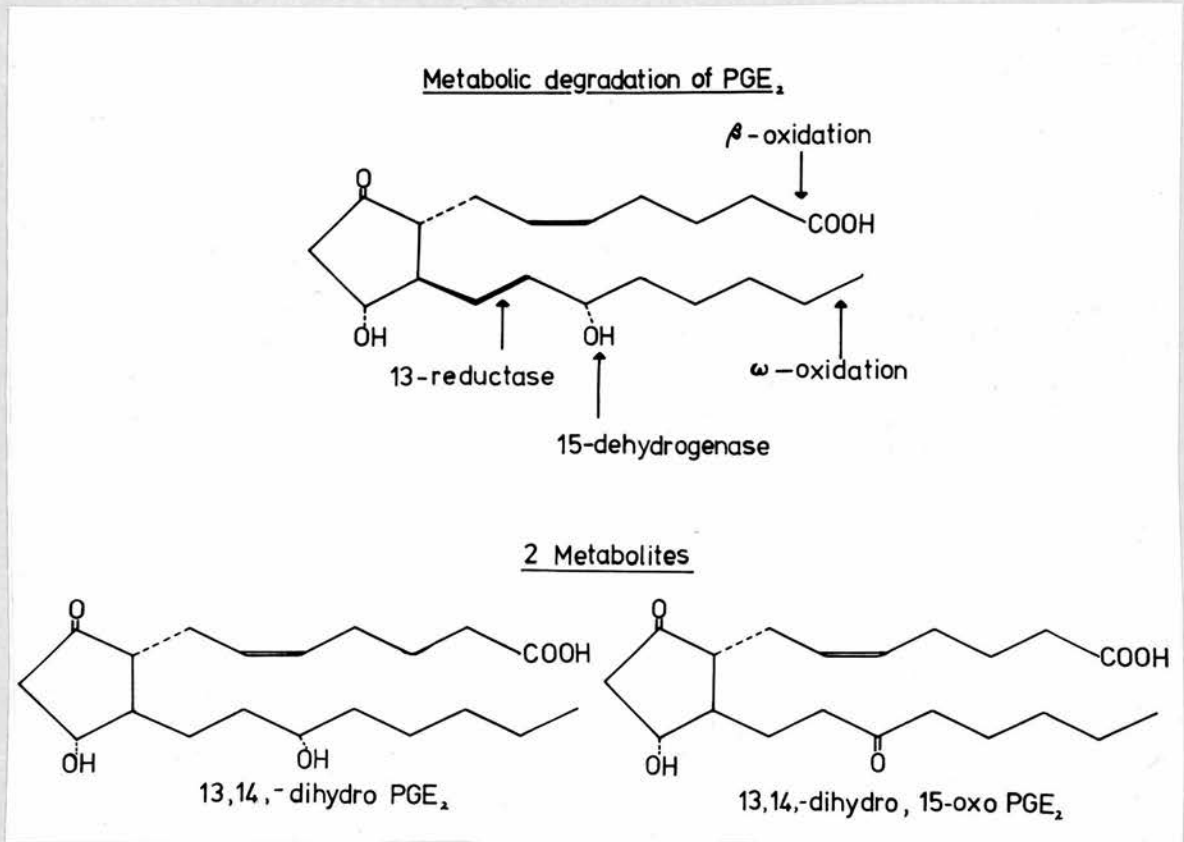


Figure 6

The metabolism of PGE₂. (See Horton, 1972 a,b).



SECTION I A

Assessment of Techniques for the extraction, separation,
biological assay and identification of prostaglandins $F_2\alpha$
and E_2 .

EXPERIMENT 1. Determination of purity of tritiated prostaglandin $F_{2\alpha}$ and E_2 (3H -PGF $_{2\alpha}$ and 3H -PGE $_2$).

Introduction

3H -PGF $_{2\alpha}$ is stable if maintained at $-20^{\circ}C$ during storage, but 3H -PGE $_2$ is notoriously unstable, even under these conditions. PGE $_2$ is always liable to dehydration and this tendency is especially marked in the radioactive form. Therefore, before any determination of efficiency of extraction, separation etc., may be made, using 3H -PGE $_2$, the purity of this tracer must first be checked. The purity of the 3H -PGF $_{2\alpha}$ was checked as a routine precaution.

Methods

The 3H -PGF $_{2\alpha}$ and 3H -PGE $_2$ were each made up to solutions of 2 μ Ci/ml in methanol. In each case 0.2 ml of the tracer solution was dropped in fine droplets onto a 50 x 200 x 0.25 mm pre-coated Silica gel T.L.C. plate, to give an origin 2 cm from the end of the plate. A micropipette with a fine screw control, attached to fine polythene tubing, was used to control the "spotting" of the samples. The final spots had a maximum diameter of 2 mm. Both plates were developed using first, solvent system F VI (90 parts redistilled ethyl acetate, 10 parts acetone, 1 part glacial acetic acid), and second, solvent system GCM (100 parts redistilled

ethyl acetate, 10 parts methanol, 1 part glacial acetic acid). In each case, for both runs, the plates were developed to give a solvent front of 16 cm.

Control plates using non-radioactive ('cold') prostaglandins and their metabolites were set up as follows: 10 μ g $\text{PGF}_{2\alpha}$, 10 μ g $\text{PGF}_{2\alpha}$, 10 μ g PGE_2 , 10 μ g 13,14 dihydro PGE_1 , 10 μ g 13,14 dihydro $\text{PGF}_{1\alpha}$, 10 μ g 13,14 dihydro, 15-oxo, PGE_2 , 10 μ g 13,14 dihydro, 15-oxo, $\text{PGF}_{2\alpha}$, 10 μ g PGB_2 , 10 μ g PGE_1 . These were set up and run under identical conditions to those used for the plates of $^3\text{H-PGF}_{2\alpha}$ and $^3\text{H-PGE}_2$.

The plates for $^3\text{H-PGF}_{2\alpha}$ and $^3\text{H-PGE}_2$ were examined using a radio T.L.C. scanner and the positions of the peaks of radioactivity were noted, relative to the origin and solvent front. Each zone of radioactivity was then scraped off the plate and extracted three times with methanol. The three fractions were combined and taken to dryness by evaporation under reduced pressure. The residue in each case was dissolved in 0.5 ml methanol, and in each case 0.1 ml of this solution was added to 10 mls of scintillant (1500 ml toluene, 900 ml 2-ethoxy-ethanol, 10.5 gm PPO (2,5-Di-phenyloxazole)) in a vial. The vials were then counted in a Nuclear Chicago scintillation counter for 4 min. each. The percentage distribution of radioactivity was calculated, from the counts per minute (c.p.m.) recorded.

The control plates were developed by spraying with a saturated solution of phosphomolybdic acid in ethanol and heating at 115°C for 15 min. The spots were ringed in pencil as they developed. Their positions relative to the origin and solvent front were recorded.

Results

The Rf values for the control and tracer plates are shown in Tables 1 and 2 together with the percentage of added radioactivity present in the different zones of the tracer plates.

Table 1

Zones	Tracer Plates			
	Rf		% added radioactivity	
	$^3\text{H-PGF}_{2\alpha}$	$^3\text{H-PGE}_2$	$^3\text{H-PGF}_{2\alpha}$	$^3\text{H-PGE}_2$
Origin	0	0	8.6	6.0
1	0.329	0.513	51.0	54.2
2	-	0.766	-	2.2
Solvent front	1	1	-	-
			Total % added radioactivity recovered	59.6
				62.4

Table 2

Control Plates		
Substance	Distance from Origin (cm)	Rf
Solvent front	15.68	1
PGF _{2β}	3.68	0.234
PGF _{2α}	5.2	0.331
PGE ₂	8.03	0.511
13,14,H ₂ -PGE ₁	10.0	0.637
13,14,H ₂ -PGF _{1α}	7.68	0.489
13,14,H ₂ ,15-oxo PGE ₂	12.06	0.768
13,14,H ₂ ,15-oxo PGF _{2α}	9.42	0.600
PGB ₂	12.04	0.767
PGE ₁	8.01	0.510

Key:- 13,14,H₂ = 13,14 dihydro
 13,14,H₂,15-oxo = 13,14 dihydro, 15-oxo.

Conclusions

The main peaks obtained for ³H-PGF_{2α} and ³H-PGE₂ clearly correspond to pure PGF_{2α} and pure PGE₂ respectively, as seen by a comparison between the Rfs recorded for the tracer and control plates. There was no detectable contamination of the ³H-PGF_{2α}. The Rf of the contaminant of the ³H-PGE₂ corresponds to that for 13,14 dihydro, 15-oxo PGE₂ or PGB₂ in the control plate. However, the contaminant represented only 3.9% of the combined radioactivity

in zones 1 and 2 and is small enough to be discounted. If significant contamination had been found a larger sample of the tracer would be run under identical conditions. Only the zone equivalent to the pure form would then be taken and extracted from the silica-gel with methanol. A final check on a small sample of this is necessary since careless handling in the interim could result again in degradation.

It is evident from the control plates that the combined F VI, GCM run is a good system for separating the prostaglandins and their metabolites although it does not distinguish between the 'one' and the 'two' series of prostaglandins.

EXPERIMENT 2. Solvent Extraction of prostaglandins.

Introduction

The main body of work to be presented will involve the determination of $\text{PGF}_{2\alpha}$ and PGE_2 in the incubates of homogenised guinea-pig uteri, thus the assessment of the extraction procedure will be carried out using boiled uterine tissue homogenates as a control system.

The procedure to be described was developed from that of Bergstrom & Samuelsson (1963), Horton & Main (1967) and Blatchley, et al., (1972) for the extraction of prostaglandin-like material.

Methods

Three female guinea-pigs were killed by stunning and incising the neck. The uteri were removed, chopped and homogenised in 25 ml Tyrode's solution, using a Polytron homogeniser, the container being kept in a bed of ice throughout. The homogenate was then boiled for 20 min. Endogenous uterine levels are low (Poyser, 1972) and with the prevention of production by the above treatment there should be no danger of endogenous content competing with added prostaglandins.

The homogenate was allowed to cool and was then divided into two equal parts. To one portion was added $0.5 \mu\text{Ci } ^3\text{H-PGF}_{2\alpha}$ in

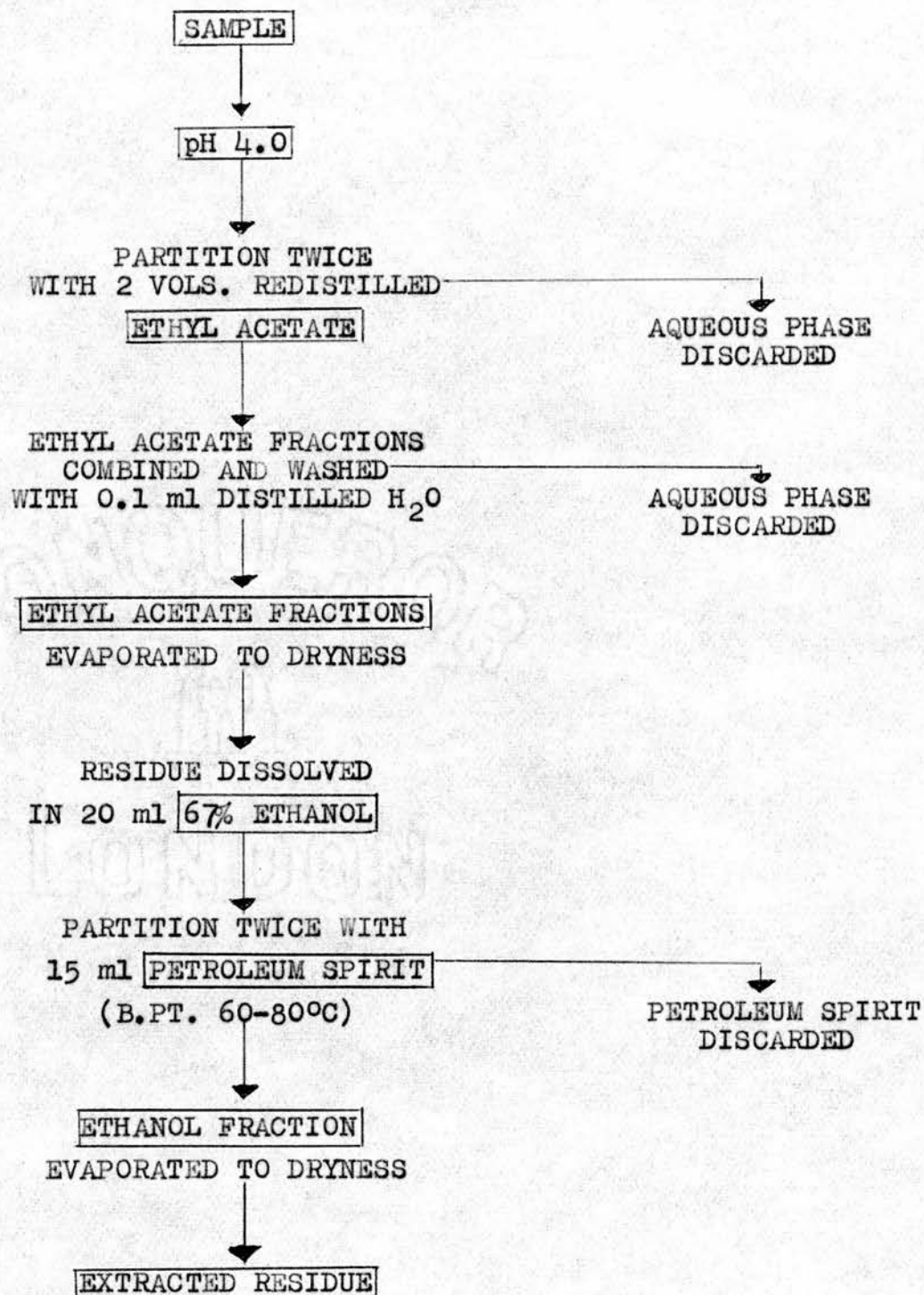
0.5 ml Tyrode's solution, and to the other was added 0.5 μCi $^3\text{H-PGE}_2$ in 0.5 ml Tyrode's solution. The purity of both tracers had been previously checked as described in Experiment 1. The subsequent treatment was identical for each portion of the homogenate.

The pH of each homogenate was adjusted to pH 4.0 with 0.1 N HCl. Each homogenate was then partitioned three times with two volumes of ethyl acetate, the ethyl acetate fractions being combined, washed with 0.1 vol. of distilled water and then evaporated to dryness, under reduced pressure. Each residue was dissolved in 20 mls of 67% ethanol, washed twice with 20 ml petroleum spirit (60-80°C B.Pt.) and then evaporated to dryness under reduced pressure. The drying was completed by placing in a dessicator, under reduced pressure, for 15 mins. This extraction procedure is summarised in Fig. 7.

Each residue was dissolved in 0.5 ml MeOH. A Scintillation fluid was made up as for Experiment 1, and 10 mls dispensed to each of 9 vials. To the first vial (Blank) was added 0.1 ml MeOH. To the next two vials was added 0.5 μCi $^3\text{H-PGF}_{2\alpha}$ in 0.1 ml MeOH ($^3\text{H-PGF}_{2\alpha}$ Controls) and similarly to another two was added 0.5 μCi $^3\text{H-PGE}_2$ in 0.1 ml MeOH ($^3\text{H-PGE}_2$ Controls). Finally, 0.1 ml of the $^3\text{H-PGF}_{2\alpha}$ extracted residue was added to each of two vials and similarly 0.1 ml of the $^3\text{H-PGE}_2$ extracted homogenate residue was added to each of two vials. Each vial was then

Figure 7

The solvent extraction of prostaglandins.



counted for 10 minutes in a Nuclear Chicago Liquid Scintillation counter. The total c.p.m. was calculated for each extracted homogenate residue and from this the percentage of radioactivity added initially and subsequently retrieved was calculated. This procedure was repeated from time to time as a routine check. The % recovery quoted is the mean of twelve determinations for each of $^3\text{H-PGF}_{2\alpha}$ and $^3\text{H-PGE}_2$.

Result

Mean % Recovery (\pm S.E.M.) after solvent extraction:-

$^3\text{H-PGF}_{2\alpha}$	=	94%	\pm	3%
$^3\text{H-PGE}_2$	=	92%	\pm	5%

Conclusions

This is clearly an efficient extraction technique. However, the residues remaining were still quite heavily pigmented. Such contamination could well prevent clear spectra being obtained in the later step of gas-chromatography mass-spectrometry and it may prove necessary to insert an extra step to obtain a cleaner extract.

EXPERIMENT 3. Separation of prostaglandins by Silicic acid column chromatography.

Introduction

This method for separating the different prostaglandins was first described by Samuelsson (1963) and recently in the adapted form by Downie, Poyser & Wunderlich (1974).

T.L.C. of prostaglandins using F VI followed by G.C.M. solvent systems gives good separation of the prostaglandins and their metabolites, as described in Experiment 1. However, to carry out further analysis the relevant zones must be scraped off and re-extracted with methanol, a time-consuming and comparatively inefficient process, especially for the recovery of nanogram quantities of prostaglandins.

The silicic acid column chromatography method to be described is to be assessed in terms of efficiency of recovery and ease of operation.

Methods

Glass columns 15 cm long, 1.2 cm i.d., with a glass scinter at their base, were used. A layer of clean sand $\frac{1}{8}$ " thick was made over the scinter. For each column 4.2 gm Sigma SIL R, 100-300 mesh, reagent grade, sieved, silicic acid was made into a slurry with approximately 5 ml of Fraction I (See Table 3).

This slurry was carefully poured into the column and allowed to settle. The column was kept moist with Fraction I until use.

0.5 μCi $^3\text{H-PGF}_{2\alpha}$ and 0.5 μCi $^3\text{H-PGE}_2$ in methanol were each taken to dryness by evaporation under reduced pressure and the drying completed in a vacuum dessicator. The dried residue was in each case dissolved in 0.5 ml of Fraction I (Table 3). The columns were allowed to become almost dry at the top and the tracer samples were then carefully applied to the top of the column, to form a thin band. Washings of the residues (total 0.2 ml in Fraction I) were also added to the column.

The columns were then developed by increasing concentrations of redistilled ethyl acetate in redistilled toluene with the addition of a small percentage of methanol as shown in the following Table 3.

Table 3

Solvent composition and volumes of fluid applied to the silicic acid columns.				
Fraction	Volume (ml)	Ethyl acetate (%)	Toluene (%)	Methanol (%)
I	40	29.9	70	0.1
II	40	39.8	60	0.2
III	130	64.4	35	0.6
IV	20	64.4	35	0.6
V	150	79.1	20	0.9
VI	40	99.1	-	0.9
VII	30	-	-	100.0

The columns were run under slight vacuum to give a flow rate of between 11 and 13 drops per 20 secs.

Each fraction from each column was then taken to dryness by evaporation under reduced pressure and the residue dissolved in 0.5 ml methanol. In each case 0.2 ml of the dissolved residue was transferred to 10 mls scintillation fluid (formula as in Experiment 1) in a vial. Control vials containing 0.5 μCi $^3\text{H-PGF}_{2\alpha}$ and 0.5 μCi $^3\text{H-PGE}_2$ respectively were also set up. All the vials were then counted for 10 min. each.

The c.p.m. recorded for each fraction was adjusted for volume to give total c.p.m. in that fraction. This figure was then expressed as a percentage of the total c.p.m. added initially, to give a percentage recovery.

Results

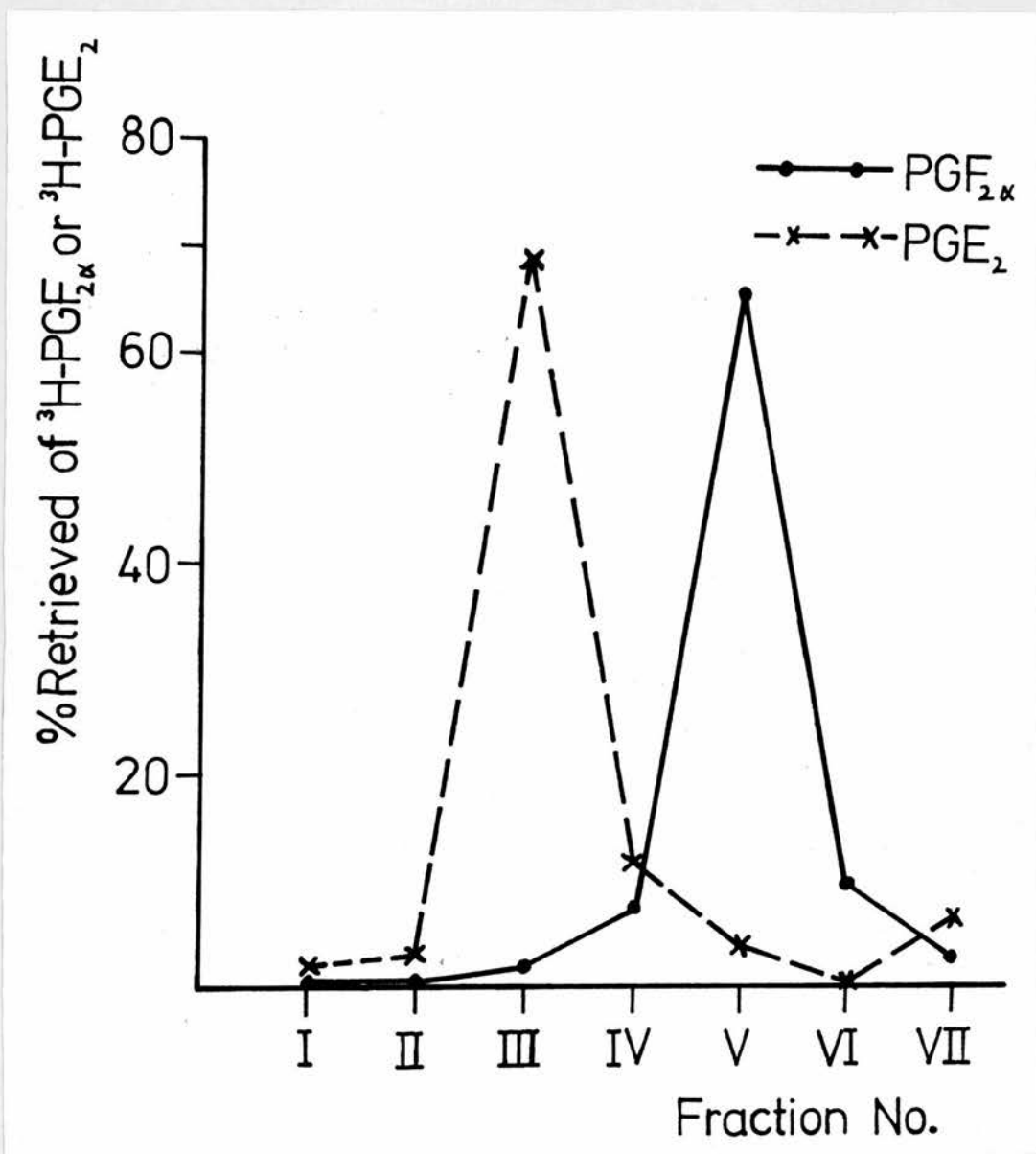
The percentage of initially added radioactivity retrieved in each fraction is shown in the following Table:-

Table 4

Fraction	% added radioactivity retrieved	
	$^3\text{H-PGF}_{2\alpha}$	$^3\text{H-PGE}_2$
I	<1	2
II	<1	3
III	2	69
IV	7	12
V	66	4
VI	10	<1
VII	3	7
Total recovery (%)	90	88

Figure 8

The separation of prostaglandins by Silicic acid column chromatography.



These figures are plotted graphically in Figure 8.

Conclusions

The percentage recovery of both $\text{PGF}_{2\alpha}$ and PGE_2 with this method is better than that found for T.L.C. It can be seen from Figure 8 that this technique gives good separation of $\text{PGF}_{2\alpha}$ and PGE_2 so that Fraction III may be assayed in terms of PGE_2 and Fraction V in terms of $\text{PGF}_{2\alpha}$. Although the columns take a day to run they require relatively little supervision once they are set up. Silicic acid column chromatography appears, therefore, to be a better technique than T.L.C. when investigating $\text{PGF}_{2\alpha}$ and PGE_2 . However, this conclusion would not necessarily apply to other prostaglandins, such as PGA_2 , which would be eluted very early in the run with silicic acid column chromatography.

EXPERIMENT 4. Biological Assay of prostaglandins on the rat fundal strip.

Introduction

This preparation is described by Vane (1957). The few small adaptations will be described, but otherwise the description will be comparatively restricted as this is such a standard preparation.

Assay Method

The rat fundus was removed as described by Vane (1957) but only two cuts were made to give a thicker strip than that described by Vane. The strip was suspended in a 5 ml organ bath in oxygenated Tyrode's solution at 37°C. Contractions of the tissue were recorded isometrically by a Grass force-displacement transducer FT03C connected via a balancing circuit to a servoscribe recorder. The sensitivity of the recorder was adjusted so that a 20 gm weight suspended from the transducer just gave a full scale deflection. A dose cycle of 5 mins. was used with drug contact time of 45 secs. and washout time of 45 secs.

Control responses of approximately $\frac{2}{3}$ full scale deflection were usually obtained to a dose of 15 ng $\text{PGF}_{2\alpha}$, or of 8 ng PGE_2 in Tyrode's solution, added to the bath. If this scale of response was not obtained the preparation was discarded and a

new one set up.

A rough dose-response curve was obtained for each of $\text{PGF}_{2\alpha}$ and PGE_2 and the sample (made up in Tyrode's solution) to be assayed was quantified as far as possible by a bracketing assay. Doses of standards or samples added to the bath were always 0.1 - 0.25 ml in volume.

EXPERIMENT 5a. Identification of prostaglandins by gas-chromatography mass-spectrometry following solvent extraction and silicic acid column chromatography.

Introduction

This method has been described by Thompson, Los & Horton, (1970). As in Experiment 2 the assessment of this method was carried out using a boiled uterine homogenate as a control system.

Methods

A boiled uterine homogenate was prepared as described in Experiment 2. When cool the homogenate was split into two parts, 2 μ g of PGF_{2 α} being added to one part (Homogenate A) and 2 μ g of PGE₂ to the other part (Homogenate B).

Each homogenate was subjected to solvent extraction and silicic acid column chromatography as described in Experiments 2 and 3.

Derivative formation is necessary before proceeding further, a process differing for PGF_{2 α} and PGE₂.

Fraction V of homogenate A (PGF_{2 α} type material), after the column chromatography, was evaporated to dryness under vacuum, and the residue dissolved in 0.2 ml methanol and transferred to a 0.5 ml stoppered tube. The flask that had contained Fraction V

was rinsed with a further 0.2 ml of methanol and the washings added to the stoppered tube. This tube was then placed in a warm ($\wedge 40^{\circ}\text{C}$) water bath and the methanol evaporated off using a jet of air. The residue was then methylated by reacting it for 30 mins. with a freshly prepared solution of diazomethane in diethylether-methanol (9:1). The excess solution was then evaporated off with a jet of air. The trimethyl-silyl ether was then formed by adding 25 μl bis-(trimethyl silyl)-trifluoroacetamide (BSTFA) to the methyl ester. This reaction takes three hours to proceed to completion.

The methyl ester/trimethyl silyl ether (MeTMS) of 500 ng $\text{PGF}_{2\alpha}$ in methanol alone was prepared in an identical manner. (Control $\text{PGF}_{2\alpha}$).

Fraction III of homogenate B (PGE_2 type material) after column chromatography, was evaporated to dryness under vacuum. The residue was dissolved in 2 ml 1N-KOH in methanol and left for 1 hr. at room temperature to convert PGE_2 to PGB_2 . The PGB_2 formed was extracted with methanol and converted to the MeTMS by a method identical to that used for $\text{PGF}_{2\alpha}$.

1 μg PGE_2 in methanol alone was also converted to PGB_2 and the MeTMS formed in an identical manner. (Control PGE_2).

These procedures resulted in four samples to be analysed, Homogenate A, Control $\text{PGF}_{2\alpha}$, Homogenate B and Control PGB_2 formed

from Control PGE₂.

10 μ l of each sample was injected on to the gas-chromatographic column without the removal of the BSTFA.

Analysis was performed on a LKB 900 gas-chromatograph mass-spectrometer. The column was 1.5 m x 1.5 mm.i.d., packed with 1 $\frac{1}{2}$ % OV1 on Suprasorb AW, 100-200 mesh, pretreated with dimethyl chlorosilane in carbon tetrachloride. The column temperature was 191°C. The carrier gas was helium which flowed at 20 ml/min. The mass spectra were recorded at an electron voltage of 27.5.

Results

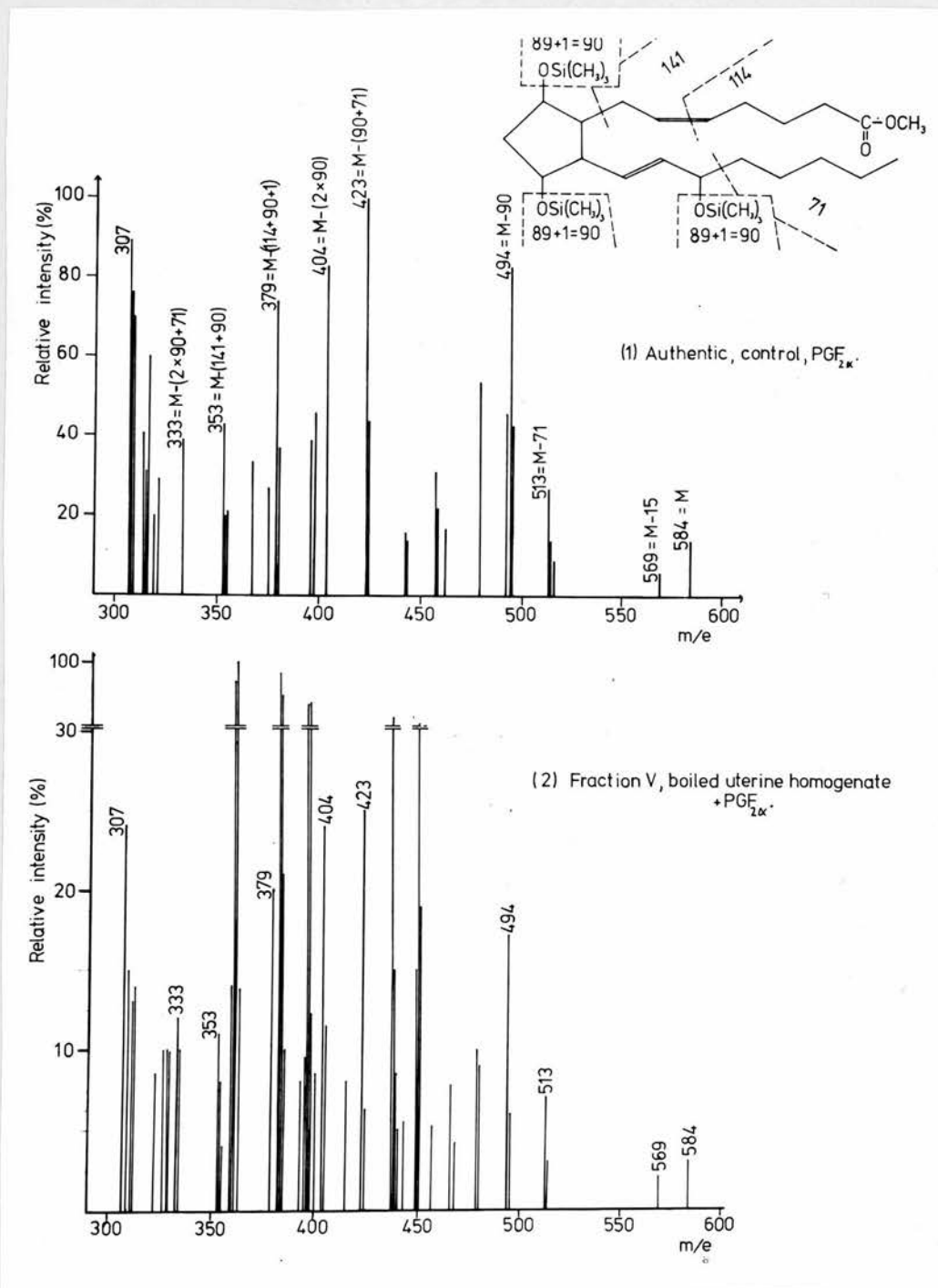
The Me/TMS derivative of control PGF_{2 α} on gas chromatography had a retention time of 8.7 min. The trace of the mass spectrum taken at this time contained the m/e peaks typical of PGF_{2 α} . Figure 9 shows the ten peaks on which positive identification was made. These peaks occur at m/e values of 307, 333, 353, 379, 404, 423, 494, 513, 569 and 584. Peaks below 300 are usually hidden in background "noise" and cannot be used for identification.

The Me/TMS derivative of Fraction V of Homogenate A (PGF_{2 α} added initially) after silicic acid column chromatography, gave a peak at 8.6 min. on gas chromatography. The trace of the mass spectrum taken at this time contained the ten m/e peaks above 300 necessary for the positive identification of PGF_{2 α} . (See Fig. 9).

Figure 9

Mass spectra of Me/TMS derivatives of

- (1) Authentic (control) $\text{PGF}_{2\alpha}$
- (2) Fraction V after solvent extraction and silicic acid column chromatography of boiled uterine homogenate A to which authentic $\text{PGF}_{2\alpha}$ had been added initially.



The Me/TMS derivative of PGB₂, formed from the control PGE₂, on gas chromatography had a retention time of 10.4 min. The mass spectrum taken at this time showed three significant peaks at m/e 321, 349 and 420 in a ratio of 4:1:1 respectively.

The Me/TMS derivative of PGB₂ formed from Fraction III of Homogenate B (PGE₂ added initially) on gas chromatography, contained too many contaminants to give a definitive peak. A mass spectrum taken at 10.4 min. (the retention time for the control sample) contained many large contaminating peaks, masking any prostaglandin peaks which may have been present.

Conclusions

The procedure for the solvent extraction and separation allows successful identification of PGF_{2α} in uterine homogenates by gas-chromatography mass-spectrometry.

However, the extracts of uterine homogenate containing PGE₂ were not clean enough to allow gas-chromatographic mass-spectrometric identification of the Me/TMS of the PGB₂ derivative. The method for the formation first of PGB₂ and then the Me/TMS derivative was shown to be successful with the control samples. Therefore, the lack of success with the uterine homogenate is almost certainly a function of insufficient extraction. The experiment with PGE₂ will, therefore, be repeated with the insertion of an additional extraction step.

EXPERIMENT 5b. Repetition of Experiment 5 for control PGE₂ and boiled uterine homogenate containing PGE₂.

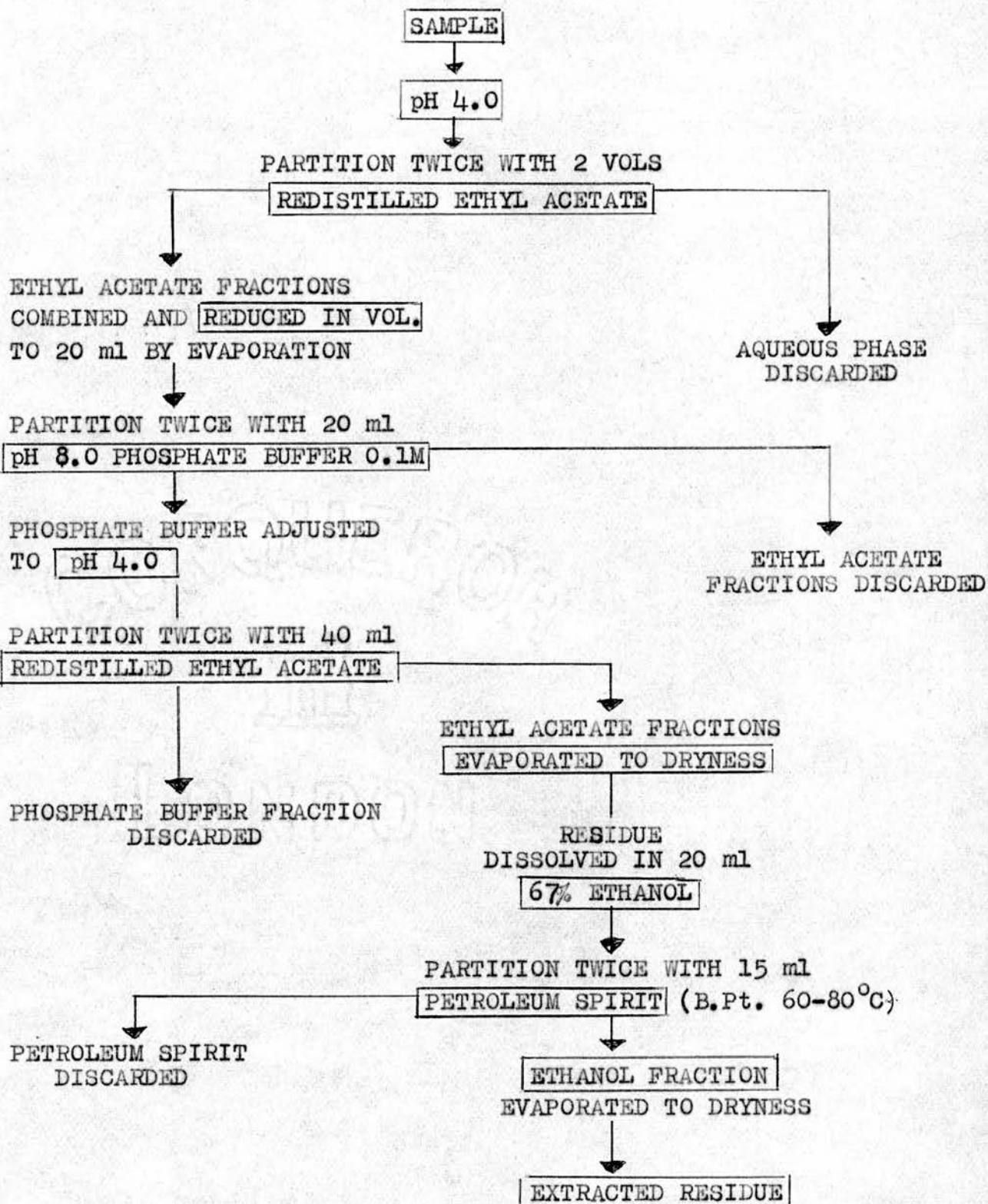
Methods

The method was exactly as before except that an additional extraction step was introduced for the uterine homogenate. The new method was as follows: The pH of the homogenate was adjusted to pH 4.0 with 0.1N HCl. It was partitioned three times with two volumes of redistilled ethyl acetate, the ethyl acetate fractions being combined and washed with 0.1 vol. of distilled water. Instead of taking the combined ethyl acetate fractions to dryness at this point, as described for Experiment 2, they were reduced in volume to about 20 mls by evaporation under reduced pressure. This 20 ml residue was then partitioned twice with 20 ml 0.1 M pH 8.0 phosphate buffer. The 40 ml of buffer was then adjusted to pH 4.0 with 0.1 N HCl and partitioned twice with 40 ml redistilled ethyl acetate. The ethyl acetate fractions were then combined and taken to dryness by evaporation under reduced pressure. The residue was dissolved in 20 ml 67% ethanol, washed twice with 20 ml petroleum spirit (60-80°C B.Pt) and then evaporated to dryness under reduced pressure. The drying was completed in a vacuum dessicator. The complete extraction process is shown in Figure 10 .

The methods for the uterine homogenate and control samples

Figure 10

Extraction of prostaglandins by solvents and phosphate buffer.



from here on were identical to those for Experiment 5a for the PGE₂ homogenates and control samples.

Results

The Me/TMS derivative of PGB₂, formed from the control PGE₂, on gas-chromatography had a retention time of 11.1 min. The mass spectrum taken at this time showed three significant peaks at m/e 321, 349 and 420 in a ratio of 4:1:1 respectively.

The Me/TMS derivative of PGB₂, formed from fraction III of the uterine homogenate to which PGE₂ was added initially, on gas chromatography still showed much contamination but a small peak occurred at 11.0 min. The mass spectrum taken at this time contained many contaminating peaks but there were also peaks at m/e 321, 349 and 420 in a ratio of 4:1:1 respectively, necessary for the positive identification of PGB₂.

Conclusions

The introduction of the extra extraction step allowed the positive identification of Me/TMS derivative of PGB₂ formed from PGE₂ added to the boiled uterine homogenate, although there was still a large degree of contamination. The PGB₂ formed from the uterine homogenate Fraction III could only have come from the added PGE₂ since endogenous PGB₂ would have been eluted in an earlier fraction in silicic acid column chromatography.

SECTION I B

RADIOIMMUNOASSAY (R.I.A.) OF PROSTAGLANDINS

An RIA system for prostaglandins was developed in these laboratories to obtain greater sensitivity than is possible using biological assay and gas-chromatography mass-spectrometry. Positive identification is lost to some degree since it is impossible to check cross-reactivity for every known substance, but the far greater speed and ease in handling large numbers of samples with RIA offsets this disadvantage. Where possible samples will be cross-checked by gas-chromatography mass-spectrometry.

The developmental work in the raising and assessment of antisera was carried out by Miss L.K. Henderson and Mrs. L. Simon of this Department.

EXPERIMENT 1. The analysis of antisera to Prostaglandin $F_{2\alpha}$ using double-antibody and solid-phased antibody techniques.

Introduction

The antisera to $PGF_{2\alpha}$ to be analysed were raised in our own laboratories in rabbits immunised with $PGF_{2\alpha}$ -Bovine Serum Albumin ($PGF_{2\alpha}$ -BSA) conjugate. The method of conjugate formation, injection regimen, and preparation of the serum are described in Dighe, Emslie, Henderson, Rutherford and Simon (1975).

We have used two methods for the precipitation of antibody-bound prostaglandins, the double-antibody and solid-phased antibody techniques. These were chosen for their ease, efficiency and reproducibility. Dextran-coated charcoal, another commonly quoted technique, was avoided because of reports suggesting that stripping of the prostaglandin from the bound fraction occurred with this method, (see Hunter, 1973). The basic double-antibody and solid-phased antibody techniques are described in Dighe et al. (1975) but methods have since changed extensively and are, therefore, described in full here.

Methods

(a) Dilution Curves - determination of the titre of the anti-sera.

(i) Double-antibody method.

A diluent was made up of 0.05 M tris hydrochloride buffer (adjusted to pH 8.0 with 1N HCl), sodium azide (0.1 gm/L) and

gelatine (0.1%).

$^3\text{H-PGF}_{2\alpha}$ (35.6 ng/ μCi) was stored in methanol in siliconised vials at -20°C . Just before use the methanol was evaporated off under nitrogen and the residue was dissolved in diluent to give a solution of 3 ng/ml (150 pg/50 μl).

Donkey anti-rabbit serum (DARS) was stored at -20°C . A stock dilution of 3 ml serum to 10 ml diluent (3/10 DARS) was maintained at 4°C for no longer than four weeks.

Normal Rabbit Serum (NRS) was obtained from non-immunised rabbits by the same method as that used for serum collection from immunised rabbits. A stock dilution of 1 ml serum to 53 ml diluent (1/53 NRS) was prepared and maintained at 4°C for a maximum of four weeks. The methods for determining the concentrations of DARS and NRS needed for an RIA are fully described by Hunter (1973).

The scintillant (No. 1) was made up of : Naphthalene, 75 gm; PPO (2,5,Di-phenyl oxazole), 7 gm; DMPOP (1,4,-Di-(2-(4 methyl-5-phenyl oxazolyl)-benzene), 0.6 gm; 2-ethoxy-ethanol, 600 ml; toluene, 1 litre. 13 ml of this scintillant will absorb 0.6 ml of an aqueous solution. Counting efficiency is then approximately 35%.

0.1 ml serum from each immunised rabbit was diluted

1 in 100 with diluent. 2-fold dilutions (1 ml each) were made to give a series ranging from 1 in 1,000 to 1 in 256,000. 50 μ l of tracer solution ($^3\text{H-PGF}_{2\alpha}$ 3 ng/ml) was added to each dilution. The solutions were then mixed on a whirlimix and incubated for one hour at room temperature. After incubation 50 μ l of the 1/53 NRS was added to each tube. The solutions were then mixed and allowed to equilibrate for 10 mins. 50 μ l of the 3/10 DARS was then added to each tube. All the solutions were then mixed and incubated at 4°C for 16 hrs. After incubation the solutions were centrifuged at 4°C, 1,200 g for 45 min., then 0.6 ml of the supernatant was removed from each tube and added to a vial containing 13 ml scintillant. The vials were each counted for 10 mins. in a Nuclear Chicago Liquid Scintillation counter, using the external standards channels/ratio method. Each dilution curve also included 4 blanks and 4 counting standards. The blanks contained 1 ml diluent, 50 μ l $^3\text{H-PGF}_{2\alpha}$ 3 ng/ml, 50 μ l 1/53 NRS and 50 μ l 3/10 DARS. The counting standards contained 1 ml diluent and 50 μ l $^3\text{H-PGF}_{2\alpha}$ 3 ng/ml. Counts from the liquid scintillation counter were recorded on a paper tape which was fed into a PDP8 computer programmed to calculate disintegrations per minute (d.p.m.), counting efficiency and percentage binding of the tracer by the antiserum. The percentage of the tracer bound by the

antiserum was calculated from the following equation:

$$\text{Percentage bound} = 100 - \frac{(\text{d.p.m.} \times 100)}{\text{average counting standard d.p.m.}}$$

If the blanks showed binding (non-specific) greater than 10% the experiment was repeated.

A dilution curve (percentage binding of the tracer by the antiserum against dilution of antiserum) was drawn for each antiserum and the 'titre' was read as that dilution which bound 60% of the $^3\text{H-PGF}_{2\alpha}$ in the absence of non-radioactive standard prostaglandin.

The assistance of Miss L.K. Henderson in carrying out the first dilution curves, and later guiding me, is gratefully acknowledged.

(ii) Dilution Curves - Solid-phased antibody method.

The diluent was the same as that used for the double-antibody method. $^3\text{H-PGF}_{2\alpha}$ (2.2 ng/ μCi) was treated as described for the double-antibody method and diluted to give a solution of 0.6 ng/ml in diluent (30 pg/50 μl).

An antiserum-cellulose complex as used by Wide (1969) and prepared according to the method of Bolton, Dighe and Hunter (1975) was used in the solid-phase R.I.A. A stock solution of cellulose-complexed antiserum was made up at a 1 in 100 dilution in diluent (containing for this 20 g/L

Sodium azide), and maintained at 4°C.

The scintillant (No. 2) for the solid-phase RIA was made up of toluene 1.5 L, 2-ethoxy-ethanol 900 ml, PPO 10.5 gm. 13 mls of this scintillant will absorb 0.6 ml aqueous solution giving a counting efficiency of just under 30%.

A series of 2-fold dilutions in 1 ml of diluent, ranging from 1 in 2000 to 1 in 1024×10^3 , were prepared in duplicate from the stock solution (1 in 100) of the PGF_{2α} antiserum-cellulose complex. The stock solution was stirred continuously, with a magnetic stirrer, while aliquots were removed from it for the preparation of the dilutions. 50 μl of ³H-PGF_{2α} 0.6 ng/ml (i.e. 30 pg) was added to each dilution of the antiserum complex. The solutions were mixed and then shaken in a mechanical shaker for 2 hr. at room temperature. After incubation the samples were centrifuged at 4°C, 1200 g for 15 min. 0.6 ml of the supernatant was removed from each tube and added to vials containing 13 mls scintillant. The vials were each counted for 4 mins. and the percentage of ³H-PGF_{2α} bound by the antiserum was calculated as described for the double-antibody method. A dilution curve was plotted and the 'titre' of the antiserum was determined.

Each dilution curve also included 4 counting standards containing 1 ml diluent and 50 μl ³H-PGF_{2α} 0.6 ng/ml (i.e. 30 pg).

Shaking was used here to keep the antibody suspended. Sucrose will be used in the other aspects of this technique, but was avoided here to make the process of serial dilution easier.

(b) Standard curves

(i) Double-antibody method

A 1/5 dilution, of the stock 1/100 dilution of Rabbit No. 6, 3rd boost, antiserum was made in diluent. This was the dilution that was equivalent to the titre when added as 50 μ l to the total reaction volume of 1.2 ml.

A series of seven 'standard' solutions of $\text{PGF}_{2\alpha}$ were prepared, i.e.: 150 pg/ml, 450 pg/ml, 1.05 ng/ml, 2.25 ng/ml, 4.65 ng/ml, 9.45 ng/ml and 19.01 ng/ml. Tubes each containing 1 ml of the standard solutions were set up in duplicate. In addition zero standards, blanks and counting standards were set up in quadruplicate, each containing 1 ml of diluent. 50 μ l of $^3\text{H-PGF}_{2\alpha}$ 3ng/ml (i.e. 150 pg) was added to each tube and the solutions were mixed and equilibrated for 10 mins. 50 μ l of the 1/500 dilution of the antiserum was added to every tube except the blanks and counting standard and, after mixing, the solutions were incubated at room temperature for 1 hour. After this incubation the procedure of addition of NRS and DARS etc. was identical to that for the dilution

curves except that NRS and DARS were not added to the counting standards.

A standard curve was plotted as percentage binding of the $^3\text{H-PGF}_{2\alpha}$ against weight of non-radioactive ('cold') $\text{PGF}_{2\alpha}$. The sensitivity of the assay was defined as the amount of 'cold' $\text{PGF}_{2\alpha}$ giving a 10% fall in binding, measured from the zero standard.

(ii) Solid-phased antibody method

The total reaction volume for this method is 0.55 ml. Two diluents are required for this assay system. The first is the same as for the double-antibody method and is referred to as 'diluent'. The second is also the same except for the addition of sucrose to give a 20% solution. This diluent is referred to as 'sucrose diluent'.

The cellulose-complexed antiserum of Rabbit No. 6, 3rd boost, was used at a dilution of 1/2,200 in sucrose diluent, that is a 1/22 dilution of the 1/100 stock solution.

The specific activity of the $^3\text{H-PGF}_{2\alpha}$ was 2.21 ng/ μCi , and it was supplied in methanol. 10.9 ng of this was reconstituted in 18.2 ml of diluent to give a solution of 0.6 ng/ml (30 pg/50 μl). The higher specific activity of this $^3\text{H-PGF}_{2\alpha}$ compared with that used for the double-antibody method allowed the sensitivity of the assay to be increased

and decreased the counting time necessary from 10 mins. to 4 mins.

A series of ten standard solutions were prepared in diluent to give the following concentrations: 20 pg/ml, 40 pg/ml, 60 pg/ml, 100 pg/ml, 140 pg/ml, 200 pg/ml, 300 pg/ml, 600 pg/ml, 1.2 ng/ml and 2.4 ng/ml. Tubes each containing 0.25 ml of the standard solutions were set up in triplicate. In addition zero standards and counting standards were set up in quadruplicate, each containing 0.25 ml diluent. Blanks were also set up in quadruplicate each containing 0.25 ml of a solution of 50 ng/ml $\text{PGF}_{2\alpha}$ in diluent. The blanks contain enough 'cold' $\text{PGF}_{2\alpha}$ to saturate the anti-serum so that any other binding of the $^3\text{H-PGF}_{2\alpha}$ observed for these tubes must be non-specific.

50 μl (30 pg) of the $^3\text{H-PGF}_{2\alpha}$ 0.6 ng/ml was added to every tube and all were then mixed.

0.25 ml of the 1/2,200 dilution of cellulose-complexed antisera in sucrose diluent was then added to every tube except the counting standards. The solution of antiserum was continuously mixed by magnetic stirrer throughout the sampling from it. 0.25 ml of sucrose diluent was added to each of the counting standards. All the solutions were then mixed and incubated for 3 hrs. at room temperature.

The tubes were then centrifuged at 2,200 r.p.m. 1181.7 G for 15 mins. and the supernatant poured off into vials, each containing 13 mls scintillant. The vials were thoroughly shaken to ensure adequate dissolution throughout the scintillant, since the sucrose diluent tends to form a separate layer otherwise.

The vials were each then counted for 4 mins. The counts were recorded on a paper tape and this was fed into a PDP8 I computer programmed to calculate the mean % $^3\text{H-PGF}_{2\alpha}$ bound by the standards and to apply the logistic curve fit formula of Parker and Waud (1971) to these values. It then supplies the observed list of values and the calculated list of values. If there is less than 5% variation between the two then the standard curve is satisfactory for experimental use. Where experimental samples are included, as in a full assay, the PDP8 computer is also programmed to calculate from the standards the amount of $\text{PGF}_{2\alpha}$ in the samples. The program was written in 'focal' by Dr. C.J. Thompson of this department.

The solid-phased RIA technique although more costly is more sensitive and faster than the double-antibody technique and has superseded the double-antibody technique for day to day use for samples expected to contain very low amounts of $\text{PGF}_{2\alpha}$.

The extensive work carried out by Mrs. L. Simon to characterise this assay is gratefully acknowledged.

- (c) Specificity Studies - determination of the degree of cross-reactivity of the antisera.
- (i) Double-antibody and solid-phase (sucrose) methods.

A standard curve for the prostaglandin to which the antiserum was raised was set up. In parallel, standard curves for different prostaglandins and their metabolites were prepared with an initial concentration of 0.05 ng/ml. Four-fold dilutions were made from this. All dilutions were set up in duplicate.

The percentage cross-reactivity of the $\text{PGF}_{2\alpha}$ antiserum with other prostaglandins or metabolites was calculated at the point of the 50% fall in binding, from the zero standard. The following calculation was then applied:

$$\% \text{ cross-reactivity of } \text{PGF}_{2\alpha} \text{ antiserum with "x"} = \frac{\text{Concentration of } \text{PGF}_{2\alpha} \text{ at 50\% fall in binding from zero standard}}{\text{Concentration of "x" at 50\% fall in binding from zero standard}} \times 100$$

where x = a prostaglandin other than $\text{PGF}_{2\alpha}$ or a metabolite. The % cross-reactivity quoted finally is the mean of three results.

Results

Antisera to $\text{PGF}_{2\alpha}$ were raised in rabbits immunised with $\text{PGF}_{2\alpha}$ -BSA conjugate.

The titre of the antiserum from Rabbit No. 6, 3rd boost, (double-antibody method) was 1/11, 200 in 1 ml volume with 150 pg of $^3\text{H-PGF}_{2\alpha}$. The dilution curve is shown in Fig. 11.

The titre of the antiserum from Rabbit No. 6, 3rd boost (solid-phase method) was 1/8,800 in 1 ml volume with 30 pg $^3\text{H-PGF}_{2\alpha}$. The dilution curve is shown in Fig. 11.

A standard curve for the antiserum from Rabbit No. 6, 3rd boost, double-antibody method with 150 pg $^3\text{H-PGF}_{2\alpha}$ is shown in Fig. 12. The sensitivity of this assay was 0.13 ng $\text{PGF}_{2\alpha}$. The mean % binding for the zero standards was 64%.

The mean standard curve obtained over six assays for the antiserum from Rabbit No. 6, 3rd boost, solid-phase method with 30 pg $^3\text{H-PGF}_{2\alpha}$ is shown in Fig. 12. The sensitivity of these assays was 0.076 ng $\text{PGF}_{2\alpha}$. The mean % binding for the zero standards was 70%.

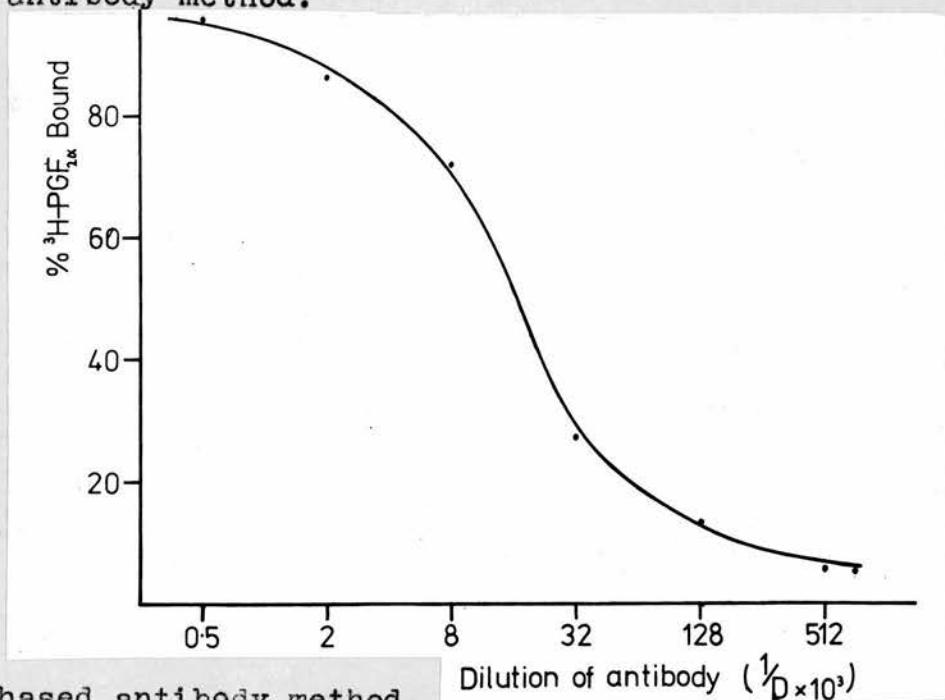
The results of the specificity studies for the antiserum from Rabbit No. 6, 3rd boost, double-antibody method, are shown in Table 5.

The results of the specificity studies for the antiserum from Rabbit No. 6, 3rd boost, solid-phase method are shown in Table 5 and a few are shown in Fig. 13.

Figure 11.

Dilution curves for $\text{PGF}_{2\alpha}$ antibodies from Rabbit No. 6,
3rd boost.

(a) Double-antibody method.



(b) Solid-phased antibody method.

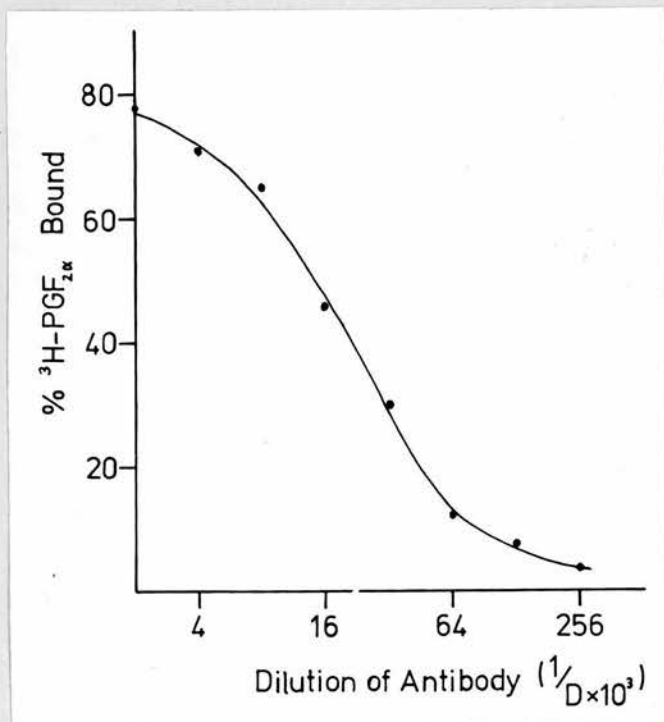
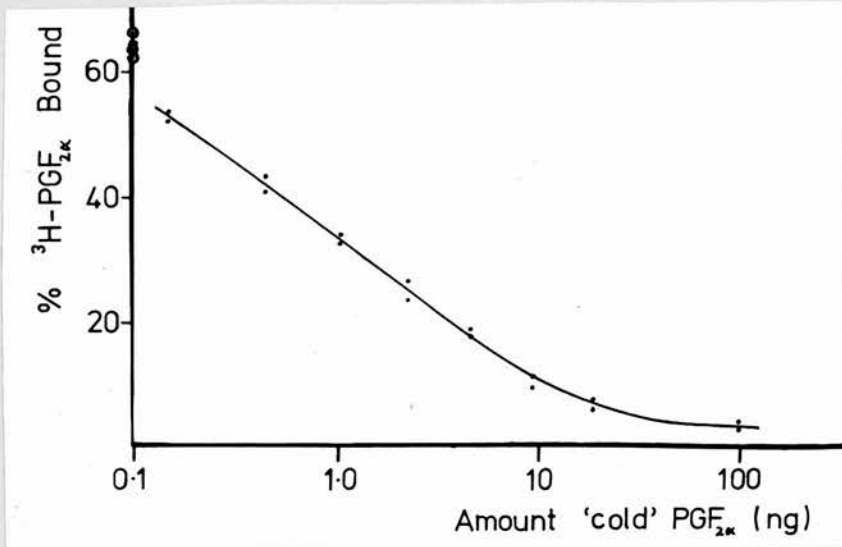


Figure 12.

R.I.A. Standard Curve to $\text{PGF}_{2\alpha}$ for antibodies from Rabbit No. 6, 3rd boost.

(a) Double-antibody method



(b) Solid-phased antibody method. (Each point is the mean of six assays, \pm S.E.M.)

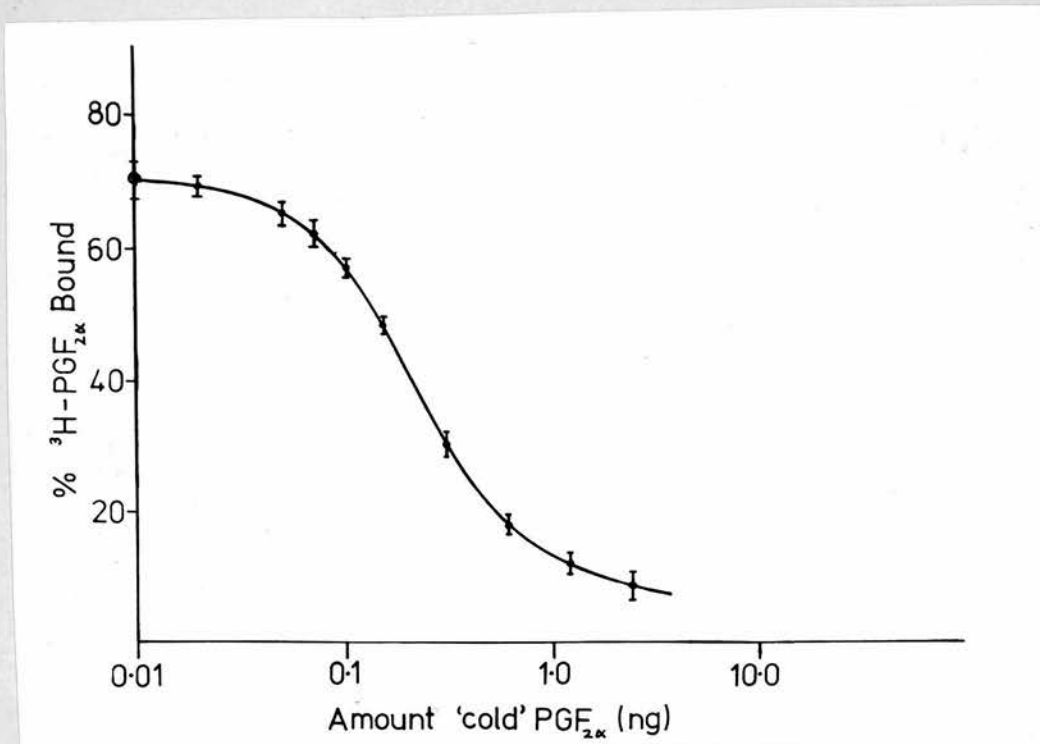


Figure 13.

Specificity studies for Rabbit No. 6, 3rd boost, solid-phased antibody method.
The cross-reactivity of the PGF_{2α} antiserum with PGB₂, PGE₂, PGE₁ and PGD₂.

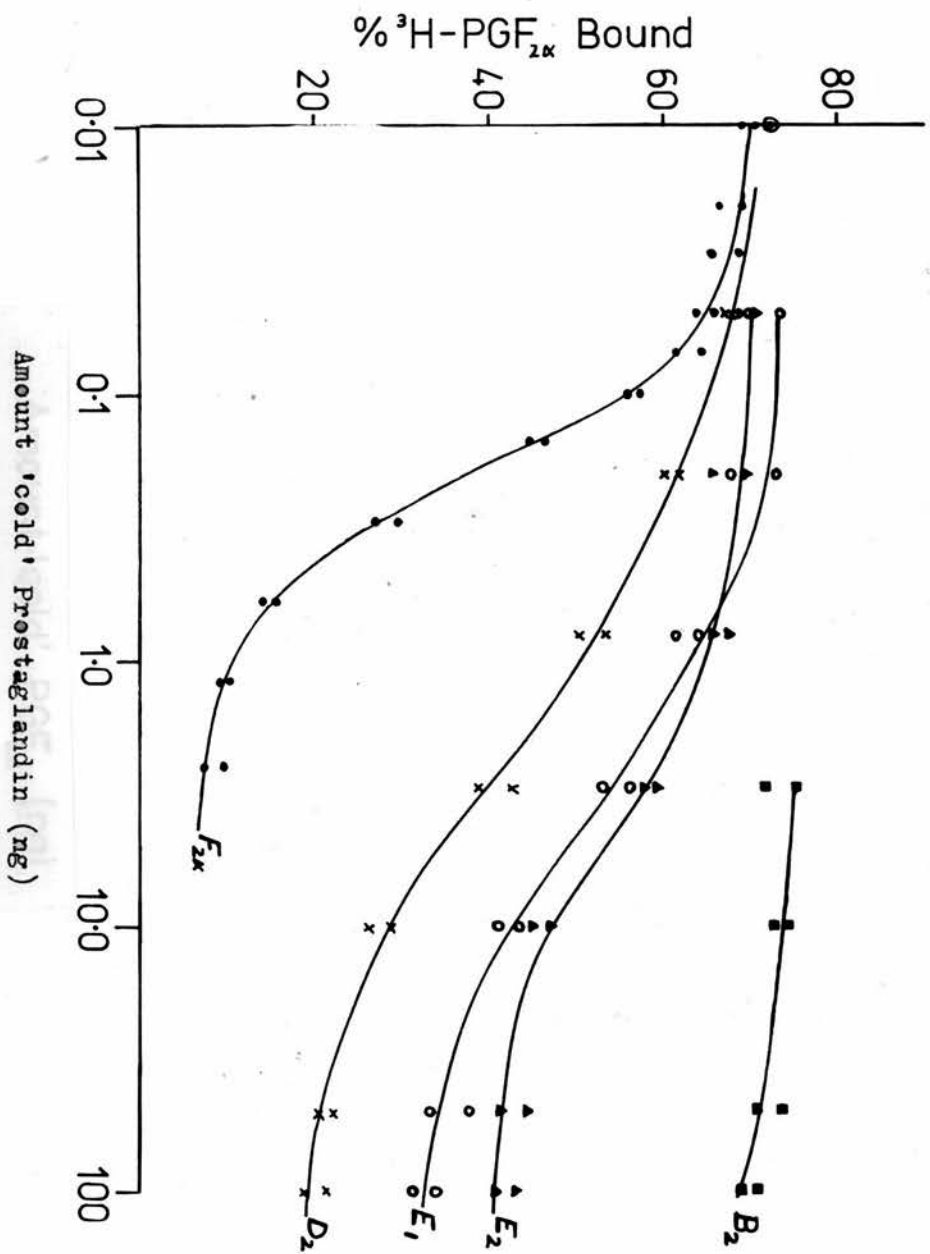


Table 5.

SPECIFICITY STUDIES		
R.I.A. Method	Double-antibody	Solid-phase
Antisera raised to	BSA-PGF _{2α}	BSA-PGF _{2α}
Rabbit No.	6 (3rd boost-)	6 (3rd boost)
Tracer Concentration	150 pg	30 pg
Final dilution of Antiserum in 1 ml	1/11,200	1/8,800
"0" Standard % binding	69%	70%
Standard Concentration for 10% fall	0.13 ng	0.074 ng
% Cross Reactivity with:-		
PGE ₂	0.7%	0.7%
PGE ₁	0.5%	0.87%
PGA ₂	0.003%	<0.116%
PGB ₂	0.1%	<0.07%
PGF _{2β}	0.3%	2.79%
PGF _{1α}	73.0%	112.0%
15-oxo-PGF _{2α}	0.2%	0.3%
PGD ₂	-	43.0%

Comments

Prostaglandins of the F_{α} series cannot be distinguished in these assay systems because of their high cross-reactivity and superimposable curves. However, past studies (Poyser, 1971) indicate that the guinea-pig uterus does not produce detectable amounts of $PGF_{1\alpha}$. Where the amount of PGF_{α} present in a sample permits it cross-checks for identity will be made by gas-chromatography mass- spectrometry.

None of the other compounds tested for cross-reactivity show parallel curves and provided parallelism of the sample is checked in the assay their presence can be discounted.

At the time of development of the double-antibody system the possible significance of PGD_2 had not been realised. However, in the solid-phase system PGD_2 has been shown to be non-parallel so its presence in any assay sample would become evident as a lack of parallelism for that sample.

The mean standard curve shown in Fig. 12 clearly indicates how very reproducible these T.I.A. systems are. Provided that blanks, internal quality controls (known amounts of prostaglandins in the test medium) and parallelism checks are included in every assay such systems are ideal for routine use.

Experiment 2. Determination of the efficiency of solvent extraction of $\text{PGF}_{2\alpha}$ from small volumes of plasma, and the effect of solvent residues on the RIA standard curve to $\text{PGF}_{2\alpha}$.

Introduction

RIA is designed to allow the assay of many samples at one time. A method of solvent extraction was developed, therefore, to allow as many samples as possible to be handled at any time to complement the RIA system. The effect of the solvent residues on the RIA standard curve to $\text{PGF}_{2\alpha}$ was also checked since any alteration of the standard curve would reduce the accuracy of the assay.

Methods

(a) Efficiency of solvent extraction from plasma

Ten 100 μl aliquots of female, non-pregnant, guinea-pig peripheral plasma were dispensed into test-tubes and to each of these was added 20 μl of a 5 $\mu\text{Ci/ml}$ solution of $^3\text{H-PGF}_{2\alpha}$ in 0.9% NaCl. 13 mls of Scintillant 2, made up as described for the solid-phased antibody RIA, was then dispensed into each of fourteen vials. One vial of scintillant alone was used to monitor the 'background' level of counts. Three vials formed counting standards, each containing 20 μl of the 5 $\mu\text{Ci/ml}$ solution of $^3\text{H-PGF}_{2\alpha}$ in 0.9% NaCl.

The plasma samples, containing $^3\text{H-PGF}_{2\alpha}$, were then extracted

as follows: 50 μ l of pH 4.0 Citrate buffer was added to each tube and the contents of each tube were then mixed for 10 secs. on a 'Whirlimix'. 2.0 mls of redistilled ethyl acetate was then added to each tube and all the tubes were shaken for 5 min. on a vortex shaker. The plasma was then frozen and the ethyl acetate decanted and retained. The plasma was thawed and extracted again with a further 2.0 mls of redistilled ethyl acetate. The two ethyl acetate fractions were combined and the ethyl acetate was blown off with air at 45°C. This extraction procedure is summarised in Figure 14. The residue in each tube was dissolved in 50 μ l of 0.9% NaCl. A 20 μ l sample was taken from each tube and added to a vial containing 13 mls of Scintillant 2, previously dispensed. All the vials were shaken and each was counted for 10 min. in a Nuclear Chicago Scintillation counter.

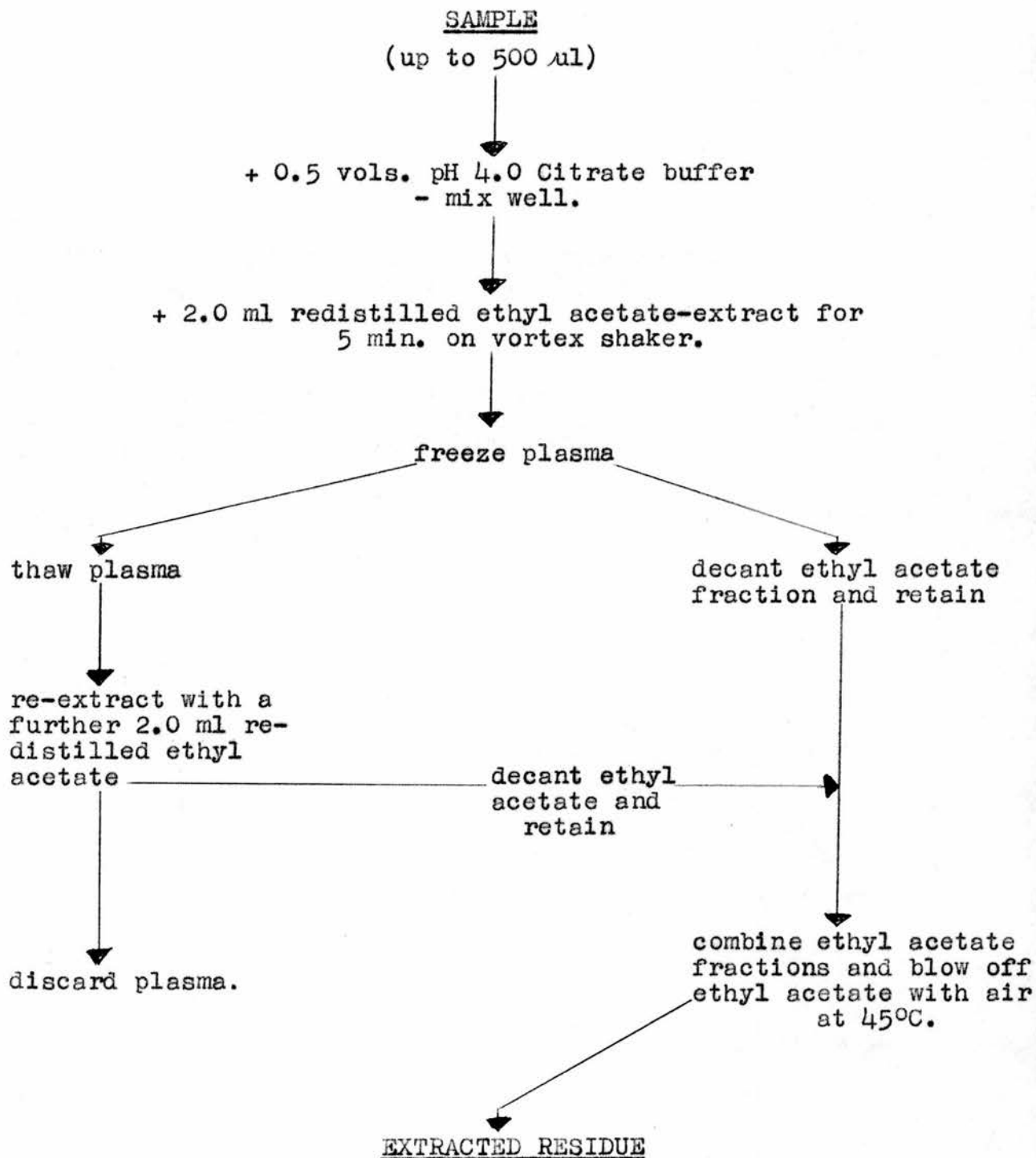
The c.p.m. recorded for the extracted samples was adjusted for volume counted, compared to the mean c.p.m. of the counting standards and expressed as a percentage of this to give a percentage recovery of $^3\text{H-PGF}_{2\alpha}$ from the plasma.

(b) The effect of solvent residues on the RIA standard curve to $\text{PGF}_{2\alpha}$.

A solvent extraction, as just described, was carried out in thirty-nine 100 μ l aliquots of Diluent 1, made up as described

Figure 14

The extraction of PGF_{2α} from small volumes of plasma



for the solid-phased antibody RIA. No radioactive $\text{PGF}_{2\alpha}$ was added. A solid-phased antibody RIA standard curve to $\text{PGF}_{2\alpha}$ was then set up, in triplicate, in these tubes along with a control standard curve, also in triplicate, in clean tubes. The mean standard curves that were obtained were then compared.

Results

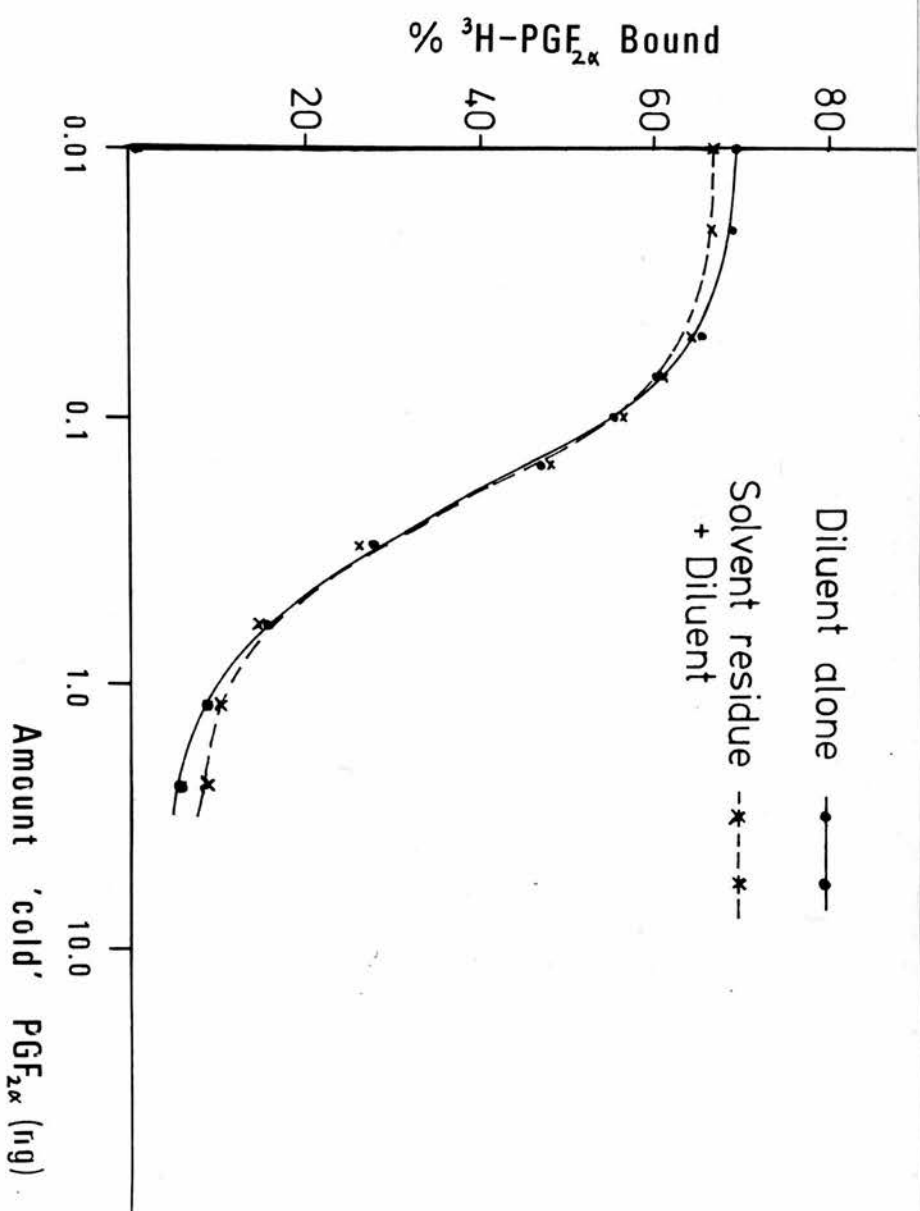
- (a) Mean % recovery of $^3\text{H-PGF}_{2\alpha} \pm \text{S.E.M.} = 97 \pm 2\%$.
- (b) The control RIA standard curve to $\text{PGF}_{2\alpha}$ and the RIA standard curve to $\text{PGF}_{2\alpha}$ in the presence of solvent residues are shown in Figure 15. There is some flattening of the top and bottom of the standard curve in the presence of the solvent residues but the central, working, area of the curve is unaffected.

Comment

This test-tube method of extraction of $\text{PGF}_{2\alpha}$ from small volumes of plasma is highly efficient, without detrimental effect to the RIA standard curve for $\text{PGF}_{2\alpha}$ and should allow the extraction of up to 200 sample aliquots at any one time.

Figure 15.

An RIA standard curve to $\text{PGF}_{2\alpha}$ alone and in the presence of solvent residues.



Experiment 3. The effect of Tyrode's solution on the RIA standard curve to $\text{PGE}_{2\alpha}$.

Introduction

Much of the work in this thesis is concerned with invitro studies on uterine homogenates in Tyrode's solution. The aim of this experiment was to determine whether the supernatant of these homogenates could enter an RIA system without prior extraction, and for this to be possible the presence of Tyrode's solution must not disrupt the RIA standard curve to $\text{PGF}_{2\alpha}$.

Methods

A set of standard solutions of $\text{PGF}_{2\alpha}$ at the concentrations described for the solid-phased antibody RIA, was made up in a solution composed of Diluent 1 and Tyrode's solution in a 40:60 volume for volume ratio. This is equivalent to 150 μl sample of uterine homogenate supernatant made up to the working volume of 250 μl (see Experiment 1) with Diluent 1. A solid-phased antibody standard curve was then set up in triplicate with these standard solutions, together with a control standard curve, also in triplicate, in Diluent 1 alone. The mean standard curves that were obtained were then compared.

Results

The control standard curve to $\text{PGF}_{2\alpha}$ in Diluent 1 alone, and



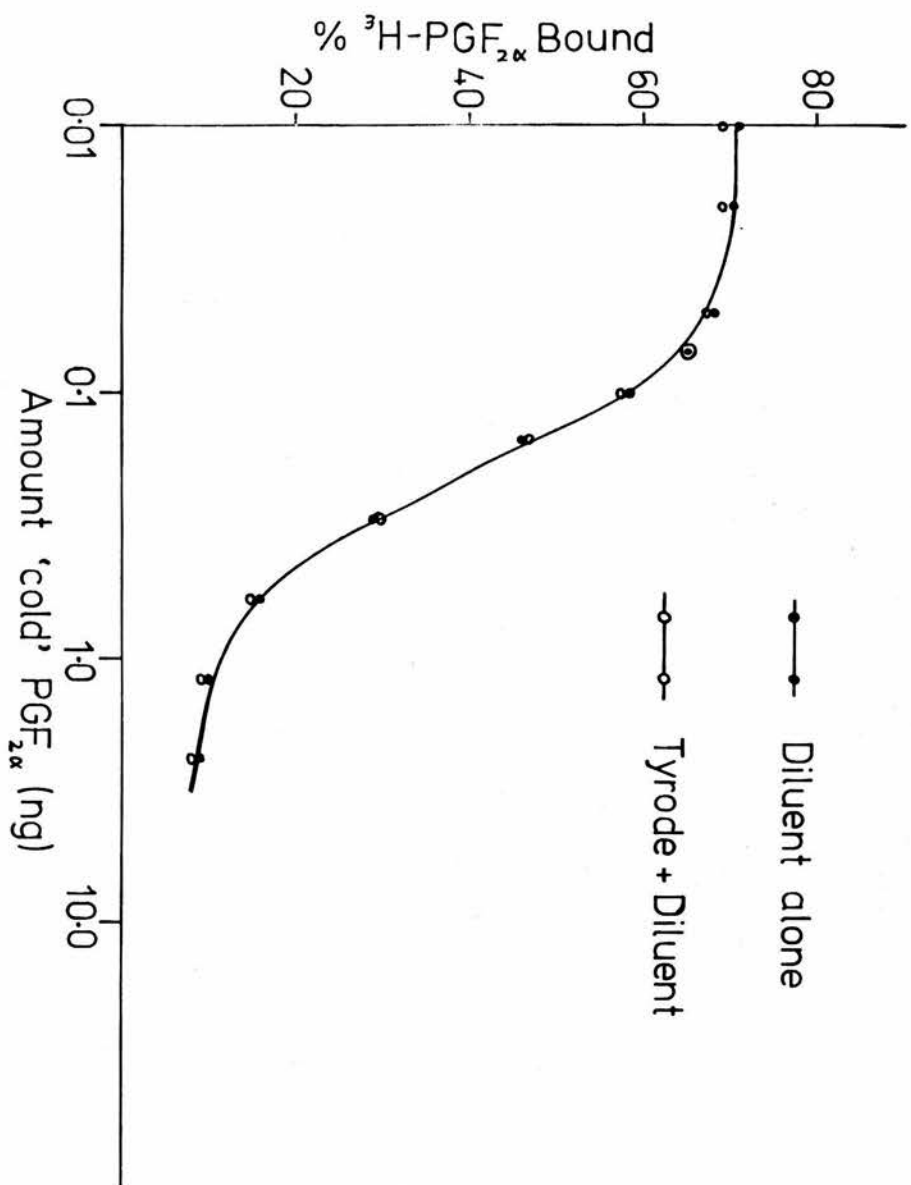
the standard curve to $\text{PGF}_{2\alpha}$ in Diluent 1/Tyrode's solution are shown in Figure 16. The two standard curves are superimposable, indicating that the presence of Tyrode's solution has no effect on the RIA standard curve to $\text{PGF}_{2\alpha}$.

Comment.

These results indicate that it is acceptable for the supernatant of uterine homogenates in Tyrode's solution to enter an RIA system without prior extraction, especially since in practice the volume samples from the supernatant is likely to be less than 150 μl . It must be borne in mind that in the absence of solvent extraction there is a greater chance of other interfering substances being present so that parallelism checks become even more important than usual. Within this framework, however, it should be possible to avoid the extraction step for uterine homogenates in Tyrode's solution, thus increasing the speed and ease of handling of these samples.

Figure 16.

An RIA standard curve to $\text{PGF}_{2\alpha}$ (solid-phased antibody method) in Diluent 1 alone and in Diluent 1/Tyrode's Solution.



Experiment 3b). Validation of Radio immunoassay for PGF_{2a} in guinea-pig uterine homogenates and plasma

For every sample: A random assay was first carried out to determine suitable aliquot volumes. Three aliquot volumes were then chosen and assayed in duplicate. Results were only accepted if values obtained for the different sample aliquots were found to lie parallel to the standard curve.

In every assay: The appropriate quality controls were included (see below) and the assay was discarded if reasonable values were not obtained for these. Some standards (from the same solutions used for the standard curve) were scattered throughout the assay and checked against the values found for the standard curve.

Quality Controls

(a) Uterine homogenates

(1) Boiled uterine homogenate. The uteri were removed from two day 7 non-pregnant guinea-pigs and were then chopped and quickly homogenised in 50 ml ice-cold Tyrode's solution. This homogenate was boiled for 15 mins and divided into two equal parts. Cold PGF_{2a} was added to one part to give a concentration of 6 ng/ml, while the other part acted as blank. Both parts were then incubated at 37°C for 90 min with continuous shaking and oxygenation, this being the standard incubation for homogenate studies. At the end of the incubation the homogenates were stored at -20°C until assayed.

Three aliquot volumes (100 µl, 200 µl and 500 µl) of the supernatant of the homogenate blank and three aliquot volumes (20 µl, 50 µl and 100 µl) of the supernatant of the homogenate containing 6 ng PGF_{2a}/ml were each extracted in quadruplicate by the method used for test samples, and the PGF_{2a} content assayed. An identical set of aliquot volumes from each of the supernatant s was set up and assayed without extraction.

Mean values \pm S.E.M. for $\text{PGF}_{2\alpha}$ in boiled uterine homogenate obtained in 3 double-antibody radioimmunoassays.

Blank			Sample containing $\text{PGF}_{2\alpha}$ 6 ng/ml		
Volume assayed	$\text{PGF}_{2\alpha}$ ng/ml		Volume assayed	$\text{PGF}_{2\alpha}$ ng/ml	
	Extracted	Not extracted		Extracted	Not extracted
100 μl	1.4 \pm 0.6	1.6 \pm 0.6	20 μl	6.9 \pm 0.6	7.8 \pm 0.8
200 μl	1.1 \pm 0.8	1.9 \pm 0.7	50 μl	6.9 \pm 0.8	7.2 \pm 0.7
500 μl	1.1 \pm 0.7	1.5 \pm 0.7	100 μl	6.6 \pm 0.8	7.3 \pm 0.8

There was no significant difference between the values obtained for the extracted and unextracted samples. Therefore after these preliminary assays no further extracted quality controls were set up and test samples were assayed without solvent extraction. In subsequent assays three aliquot volumes (100 μl , 200 μl and 500 μl) of the blank sample and three aliquot volumes (20 μl , 50 μl and 100 μl) of the sample containing $\text{PGF}_{2\alpha}$ 6 ng/ml were set up in quadruplicate and assayed without extraction. One set of duplicates was placed in the middle of the assay and the other set was placed at the end.

Mean values \pm S.E.M. for $\text{PGF}_{2\alpha}$ in boiled uterine homogenate.

Type and (Number) of Assays	Blank		Sample containing $\text{PGF}_{2\alpha}$ 6 ng/ml	
	Volume assayed	$\text{PGF}_{2\alpha}$ ng/ml	Volume assayed	$\text{PGF}_{2\alpha}$ ng/ml
Double-antibody RIA (3)	100 μl	1.4 \pm 0.5	20 μl	7.6 \pm 0.9
	200 μl	1.3 \pm 0.6	50 μl	7.5 \pm 0.7
	500 μl	1.3 \pm 0.7	100 μl	7.2 \pm 0.8
Solid-phase RIA (12)	100 μl	1.1 \pm 0.16	20 μl	7.5 \pm 0.26
	200 μl	1.01 \pm 0.12	50 μl	7.1 \pm 0.32
	500 μl	1.05 \pm 0.1	100 μl	7.15 \pm 0.30

(2) Uterine homogenates from guinea-pigs on day 7 of the oestrous cycle.

Two guinea-pigs were killed on day 7 of the oestrous cycle and their uteri removed, chopped and homogenised together in 50 ml Tynode's solution. The homogenate was divided into two halves. One half acted as control and $\text{PGF}_{2\alpha}$ was added to the other half to give a final concentration of added $\text{PGF}_{2\alpha}$ of 6 ng/ml. The homogenates were incubated as described for the boiled uterine homogenates and the incubates stored at -20°C until assayed.

Three aliquot volumes (10 μl , 20 μl and 50 μl) of the supernatant of the control homogenate and three aliquot volumes (5 μl , 10 μl and 20 μl) of the supernatant of the homogenate containing added $\text{PGF}_{2\alpha}$ were each dispensed in quadruplicate. One set of duplicates was included in the middle of the assay, the other was placed at the end.

Mean values \pm S.E.M. for $\text{PGF}_{2\alpha}$ in the supernatant of uterine homogenate from day 7 non-pregnant guinea-pigs, obtained over 12 solid phased antibody radioimmunoassays.

Control homogenate		Control homogenate + $\text{PGF}_{2\alpha}$ 6 ng/ml	
Aliquot volume	PGF_{α} ng/ml	Aliquot volume	PGF_{α} ng/ml
10 μl	12.6 \pm 0.6	5 μl	19.2 \pm 0.3
20 μl	12.2 \pm 0.45	10 μl	18.9 \pm 0.5
50 μl	12.8 \pm 0.3	20 μl	18.6 \pm 0.8

These quality controls using uteri from day 7 non-pregnant guinea-pigs directly, without boiling, represent the experimental situation more accurately than the boiled uteri homogenates, especially with regard to the aliquot volumes entering the assay. Later assays included these controls only, in place of the boiled uterine homogenate controls.

- (b) Plasma quality controls for utero-ovarian venous plasma samples.

A pool of male guinea-pig peripheral plasma (blood samples obtained by cardiac puncture in conscious animals) was maintained containing $\text{PGF}_{2\alpha}$ at a concentration of 6 ng/ml. Three aliquot volumes (20 μl , 50 μl and 100 μl) were extracted in quadruplicate at the same time and by the same method as the test samples. One set of duplicates was placed in the middle of the assay, the other at the end of the assay.

Plasma quality control containing 6 ng $\text{PGF}_{2\alpha}$ /ml: Mean values, \pm S.E.M., obtained over 4 solid-phase radioimmunoassays.

Volume assayed	$\text{PGF}_{2\alpha}$ ng/ml
20 μl	6.6 \pm 0.6
50 μl	6.54 \pm 0.16
100 μl	6.38 \pm 0.44

In all these quality controls known amounts of $\text{PGF}_{2\alpha}$ added to control samples of uterine homogenate or plasma can be accurately detected, thus validating these radioimmunoassays.

SECTION II

Production of prostaglandins by the early pregnant guinea-pig uterus in vitro.

Introduction

The transfer of day 11-12 implanted conceptuses to the spleen of non-pregnant guinea-pigs results in extension of the oestrous cycle from the normal 16-18 days to about 25 days. This suggests that the conceptus secretes a systemically active anti-luteolytic or luteotrophic factor which prevents luteal regression occurring at day 15 (Bland & Donovan, 1969).

As discussed in the introduction this action, by negating the lytic effect of the uterus, appears to be more anti-luteolytic than luteotrophic. An inhibition of the production or action of PGF_{2 α} would be a means of achieving this end. It is the aim of the experiments to be described to compare the amounts of prostaglandins produced by uteri from guinea-pigs during early gestation and when non-pregnant.

EXPERIMENT 1. A comparison of the amount of prostaglandins produced by day 15 non-pregnant and pregnant guinea-pig uteri in vitro, considered in relation to corpus luteum size.

Methods

Vaginal smears were taken daily from virgin female guinea-pigs by flushing the vagina with a small amount of 0.9% NaCl solution. Day 1 of the oestrous cycle was taken as the day preceding the post-ovulatory leucocytic influx when maximum vaginal cornification occurred. Two full oestrous cycles of normal length were observed before mating. The females were each placed with a male from day 14 of their oestrous cycles until successful mating occurred. Day 1 of pregnancy was taken as the day when spermatozoa were observed in the vaginal smear. This always corresponded to what would have been day 1 of the next oestrous cycle.

Five non-pregnant guinea-pigs and seven bilaterally pregnant guinea-pigs were used. The guinea-pigs were killed on the appropriate day by stunning and incising the neck. The ovaries were removed and immediately placed in 10% formol-saline. The uteri were removed and opened longitudinally. The pregnant uteri were carefully freed of any placental or embryonic tissue & these tissues were discarded. The uteri were then weighed individually.

In every case the uterine tissue was homogenised in 10 ml Tyrode's Solution using a Polytron homogeniser. The homogeniser blades were washed three times with 5 ml Tyrode's solution and the washings added to the homogenate, giving a final volume of 25 ml. The time-interval between removal of the uterus and completion of homogenisation was never greater than 15 min. The homogenate was oxygenated and incubated for 90 min. at 37°C with continuous shaking.

Prostaglandins were extracted by solvents, separated by silicic acid column chromatography, assayed biologically on rat fundal strip and identified by combined gas-chromatography mass spectrometry as described in Section I A. The amounts of prostaglandin produced are expressed as ng prostaglandin/100 mg uterine tissue.

The ovaries, after fixation in 10% formol-saline, were sectioned serially at 10 μ m, mounted and stained with haematoxylin and eosin. The stained sections were examined microscopically for corpora lutea. The sizes of the corpora lutea are expressed as the product of three diameters. The method used was a modification of that of Rowlands (1956). Two maximum diameters were measured at right angles to one another, using a direct-reading vernier scale mounted on the microscope. The third diameter was calculated from the number of 10 μ m sections in which the corpus luteum appeared.

All results were subjected to statistical analysis by Student's t-test.

Results

The results are shown in Fig. 17.

Day 15 non-pregnant guinea-pigs: The mean amounts (\pm standard error) of prostaglandins produced by the uteri in vitro were 113 ± 8 ng $\text{PGF}_{2\alpha}$ /100 mg tissue and 13 ± 1 ng PGE_2 /100 mg tissue. The corpora lutea varied in number between left and right ovaries and between animals, but all had regressed structurally. The mean size was 0.18 ± 0.12 mm³.

Day 15 bilaterally pregnant guinea-pigs: The mean amounts of prostaglandins produced by the uteri in vitro were 13 ± 3 ng $\text{PGF}_{2\alpha}$ /100 mg tissue and 9 ± 1 ng PGE_2 /100 mg tissue. The corpora lutea were structurally maintained in both ovaries in all cases. The mean size was 3.3 ± 0.3 mm³.

The amount of $\text{PGF}_{2\alpha}$ produced by the day 15 bilaterally pregnant uteri in vitro was significantly less ($P < 0.001$) than that from the day 15 non-pregnant uteri in vitro. There was no significant difference between the results obtained for PGE_2 production.

The mean size of the corpora lutea from the day 15 bilaterally pregnant guinea-pigs was significantly greater, ($P < 0.001$) than the mean size of those from day 15 non-pregnant

guinea-pigs.

Gas-chromatographic mass-spectrometric examination: Gas chromatographic-mass spectrometric identification was successfully made individually for the $\text{PGF}_{2\alpha}$ from the non-pregnant uteri. The Fractions III of the samples were combined for similar analysis for PGE_2 and this was successfully identified also.

The Fractions III and V from the pregnant uterine homogenates contained too little $\text{PGF}_{2\alpha}$ and PGE_2 to attempt individual identification, but in each case positive identification was made after pooling the samples.

Conclusions

The results obtained here for the in vitro production of $\text{PGF}_{2\alpha}$ by day 15 non-pregnant uteri agree closely with those reported by Poyser (1972).

The day 15 bilaterally pregnant uteri, however, produce much less $\text{PGF}_{2\alpha}$ than the non-pregnant uteri although there is no difference between the amounts of PGE_2 produced by the pregnant or non-pregnant uteri. This suggests that the conceptus is indeed exerting an anti-luteolytic action by reducing the ability of the uterus to produce prostaglandins. The structurally regressed corpora lutea of the non-pregnant animals and the structurally maintained corpora lutea of the pregnant animals is in agreement with the picture for prostaglandin production in each case.

EXPERIMENT 2. The production of prostaglandins by day 25 bilaterally pregnant guinea-pig uteri in vitro considered in relation to corpora lutea sizes.

Methods

Observation of oestrous cycles and mating procedure was as described for Experiment 1, except that in these animals day 1 of pregnancy was equivalent to what would have been day 2 of the next oestrous cycle. Five bilaterally pregnant guinea-pigs were used. The methods employed for the ovaries and uteri were exactly as described for the pregnant animals in Experiment 1.

Results

The results are shown in Fig. 17.

The mean amounts (\pm standard error) of prostaglandins produced by the day 25 bilaterally pregnant uteri in vitro were 60 ± 6 ng $\text{PGF}_{2\alpha}$ /100 mg tissue and 13 ± 1 ng PGE_2 /100 mg tissue.

The corpora lutea were structurally maintained in both ovaries in every case. The mean size was 4.7 ± 0.5 mm³.

The amount of $\text{PGF}_{2\alpha}$ produced by these uteri in vitro was significantly greater ($P < 0.001$) than that produced by day 15 bilaterally pregnant uteri but significantly less ($P < 0.001$) than that produced by day 15 non-pregnant uteri. There was no significant difference between the amount of PGE_2 produced by

the uteri of day 15 and day 25 bilaterally pregnant animals or between the amount of PGE_2 produced by these uteri and the day 15 non-pregnant uteri.

The corpora lutea of the day 25 bilaterally pregnant guinea-pigs were slightly, but significantly ($P < 0.01 > 0.001$) larger than the corpora lutea of the day 15 bilaterally pregnant uteri.

Gas-chromatographic mass-spectrometric examination: All the uterine homogenate samples were in each case too contaminated to allow identification of prostaglandins by gas-chromatography mass-spectrometry.

Conclusions

The uteri of day 25 bilaterally pregnant guinea-pigs have a greater ability to produce $\text{PGF}_{2\alpha}$ than the uteri of day 15 bilaterally pregnant guinea-pigs. This indicates that the anti-luteolytic action of the conceptus may be lessening by this time.

EXPERIMENT 3. The Production of prostaglandins by day 15 unilaterally pregnant guinea-pig uteri - a comparison between the pregnant and non-pregnant horns and with corpora lutea sizes.

Methods

Observation of oestrous cycles and mating procedure was exactly as described for Experiment 1. Four guinea-pigs became unilaterally pregnant naturally, without surgical intervention.

Treatment of ovaries and uteri was as described for Experiment 1, except that the non-pregnant and pregnant uterine horns were treated separately throughout.

Results

The results for uterine production of $\text{PGF}_{2\alpha}$ and corpus luteum size are shown in Fig. 18.

Prostaglandin production by the uteri.

Non-pregnant horns: the mean amounts (\pm standard error) of prostaglandins produced were 45 ± 3 ng $\text{PGF}_{2\alpha}$ /100 mg tissue and 10 ± 1 ng PGE_2 /100 mg tissue.

Pregnant horns: the mean amounts (\pm standard error) of prostaglandins produced were 22 ± 2 ng $\text{PGF}_{2\alpha}$ /100 mg tissue and 11 ± 1 ng PGE_2 /100 mg tissue.

The amount of $\text{PGF}_{2\alpha}$ produced by the non-pregnant horns of unilaterally pregnant animals was significantly greater than that produced by the pregnant horns ($P < 0.01 > 0.001$). However, the

amount of $\text{PGF}_{2\alpha}$ produced by the non-pregnant horns was significantly less ($P < 0.001$) than the amount of $\text{PGF}_{2\alpha}$ produced by day 15 non-pregnant uteri.

There was no significant difference between the amount of $\text{PGF}_{2\alpha}$ produced by the pregnant horns of unilaterally pregnant animals and the amount of $\text{PGF}_{2\alpha}$ produced by the uteri of day 15 bilaterally pregnant animals. There was no significant difference between any of the results so far obtained for PGE_2 . Corpora lutea.

In ovaries adjacent to non-pregnant horns, ovulation occurred in only two out of the four animals. Where ovulation had occurred the corpora lutea appeared structurally maintained. The mean size was $2.0 \pm 0.2 \text{ mm}^3$.

In ovaries adjacent to the pregnant horns, corpora lutea were structurally maintained. The mean size was $4.3 \pm 0.2 \text{ mm}^3$. There was no significant difference between the sizes of corpora lutea taken from those animals which had ovulated bilaterally (mean size $4.49 \pm 0.44 \text{ mm}^3$) and those taken from animals which had ovulated unilaterally ($4.2 \pm 0.19 \text{ mm}^3$). The mean size of the corpora lutea adjacent to the pregnant horns was slightly but significantly ($P < 0.01 > 0.001$) greater than the mean size of corpora lutea from day 15 bilaterally pregnant animals.

No test of significance could be carried out for the corpora

lutea sizes in ovaries adjacent to the non-pregnant horns because there were too few corpora lutea in these ovaries. However, the corpora lutea appeared smaller than those from ovaries adjacent to pregnant horns.

Gas chromatographic-mass spectrometric examination:

After pooling of the samples, $\text{PGF}_{2\alpha}$ was identified by gas chromatography-mass spectrometry in Fraction V from the homogenates of both the pregnant and non-pregnant uterine horns.

PGE_2 was identified in the pooled samples of Fraction III from the homogenates of the non-pregnant horns, but the samples for the pregnant horns were too contaminated to allow identification by gas chromatography-mass spectrometry.

Conclusions

The non-pregnant uterine horns of day 15 unilaterally pregnant animals do not show such a large reduction in their ability to produce $\text{PGF}_{2\alpha}$, when compared to day 15 non-pregnant uteri, as do the pregnant horns. This result suggests that there may be a local component to the anti-luteolytic action of the conceptus, the strongest action being seen in the pregnant horns. Alternatively, the effect on the non-pregnant horns may be due to a systemic anti-luteolytic action the difference between the two horns being merely a product of dilution. A systemic anti-luteolytic action was indicated by the anti-

luteolytic actions of splenic grafts of day 11-12 implanted conceptuses in non-pregnant guinea-pigs (Bland & Donovan, 1969).

The bilateral structural maintenance of the corpora lutea observed in the unilaterally pregnant animals is in agreement with the results of Deanesly (1967).

Figure 17.

Production of Prostaglandins by the guinea-pig uterus in vitro (ng prostaglandin/100 mg Uterine tissue) and Mean Corpus Luteum size (mm³).

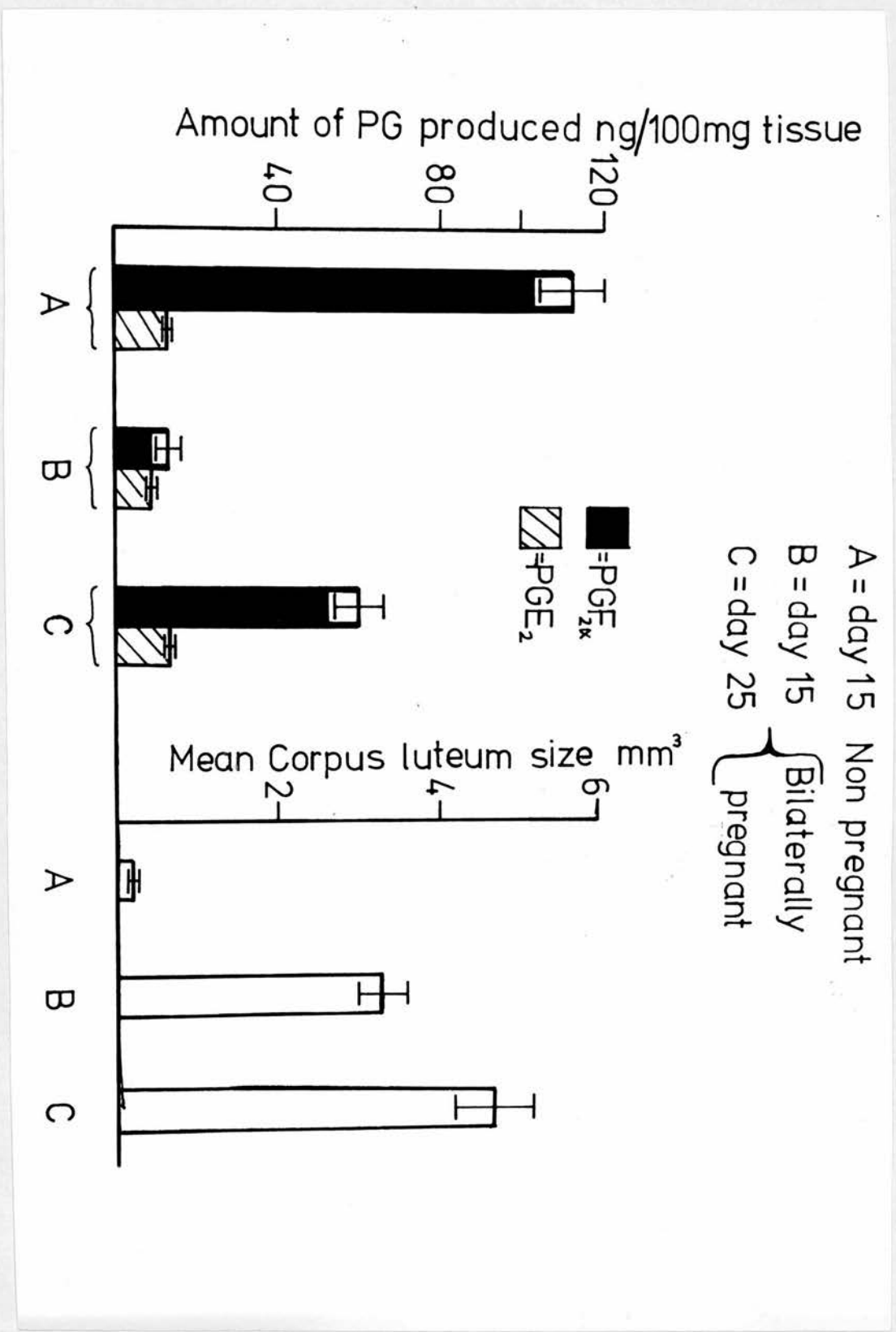
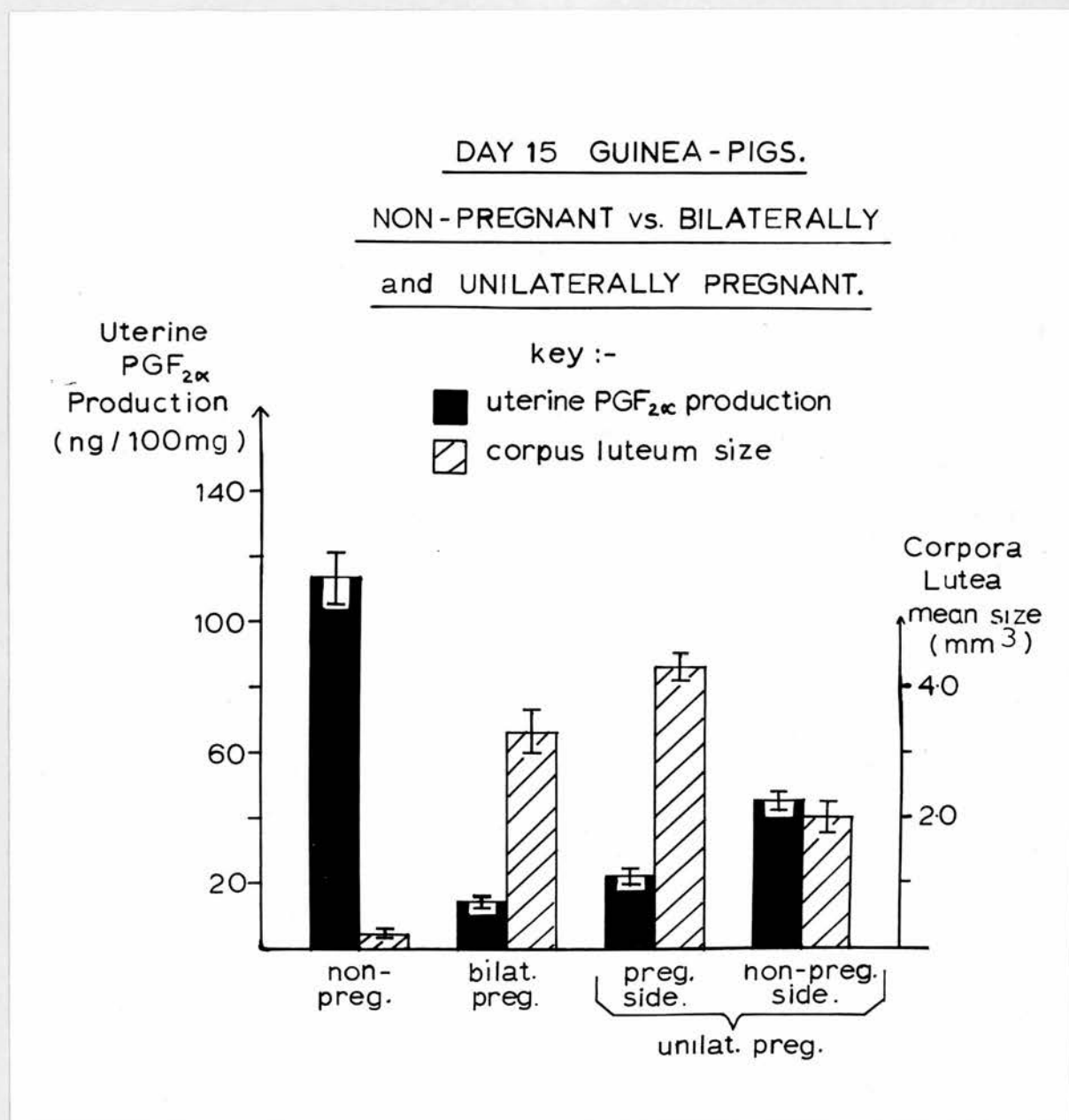


Figure 18

Production of $\text{PGF}_{2\alpha}$ by the guinea-pig uterus in vitro,
and corpus luteum size.



General Conclusions

The ability of the day 15 pregnant guinea-pig uterus to produce $\text{PGF}_{2\alpha}$ in vitro has now been shown to be very much less than that of the non-pregnant uterus on day 15 of the oestrous cycle. This result supports the hypothesis that the conceptus exerts an anti-luteolytic action around day 15 of pregnancy thus allowing luteal maintenance beyond this time, when regression would normally occur. The results from unilaterally pregnant animals suggest that there may be a local component to the anti-luteolytic action of the conceptus, the greatest effect being seen on the pregnant uterine horn. The ability of the pregnant uterus to produce $\text{PGF}_{2\alpha}$ in vitro on day 25 was greater than at day 15, indicating a lessening of anti-luteolytic activity by the conceptus. At or soon after this time in pregnancy a functional corpus luteum is no longer essential for the maintenance of pregnancy, progesterone being now adequately supplied by the placenta (Loeb and Hesselberg, 1917; Herrick, 1928; Courrier et al., 1929; Deanesly, 1963, 1972) and the lessening of anti-luteolytic activity probably reflects this change in emphasis, away from the corpus luteum. It is important, however, to notice that the ability of the day 25 pregnant guinea-pig uterus to produce $\text{PGF}_{2\alpha}$ in vitro is still very much less than that of the non-pregnant uterus on day 15 of the oestrous cycle, associated with luteal regression.

PGE_2 does not appear to be involved in the anti-luteolytic

actions of the conceptus since there was no difference in the ability to produce PGE_2 in vitro between non-pregnant and pregnant uteri.

A reduction in the ability of the uterus to produce $\text{PGF}_{2\alpha}$ in vitro is one way of explaining the low amounts of $\text{PGF}_{2\alpha}$ present in the incubates from pregnant uteri. However, increased metabolism of the $\text{PGF}_{2\alpha}$ might produce a similar result. Further experiments must be carried out to distinguish between these possibilities and to define the mechanism of the anti-luteolytic actions of the conceptus.

SECTION III

The metabolism of prostaglandins by the guinea-pig uterus (pregnant and non-pregnant) and by the guinea-pig conceptus.

Introduction

The amounts of $\text{PGF}_{2\alpha}$ in the incubate of homogenised day 15 pregnant guinea-pig uteri have been shown to be much less than those for day 15 non-pregnant uteri (Section 2). This reduction in the amount of $\text{PGF}_{2\alpha}$ present for the pregnant uteri supports the hypothesis that the conceptus has an anti-luteolytic action. This action could be achieved by an inhibition of synthesis or alternatively by an increase in the metabolism of $\text{PGF}_{2\alpha}$ by the pregnant uterus. This could result in the removal of the $\text{PGF}_{2\alpha}$ from the incubate as it was produced, and its replacement by an inactive metabolite. The conceptus was not present in the original experiments and, therefore, any metabolism would have to have been carried out by the uterus. It is possible, however, that in the intact pregnant animal the conceptus may contribute to the metabolism of prostaglandins produced by the uterus.

The aim of the experiments to be described is to determine the amount of metabolism of $\text{PGF}_{2\alpha}$ and PGE_2 carried out by the pregnant and non-pregnant guinea-pig uteri and the early guinea-pig conceptus.

Methods

Six day 15 non-pregnant and five day 15 bilaterally pregnant guinea-pigs were used. The methods used were the same as those described for Section 2, Experiment 1, with the following adaptations.

The placental and embryonic tissue (conceptuses) were retained, although kept separate & the conceptuses from a given animal were weighed together. The ovaries were not retained.

Each tissue sample type was then chopped and homogenised in Tyrode's solution as described in Section II, Experiment 1. A boiled uterine homogenate was then prepared from one day 15 non-pregnant guinea-pig as described in Section I A. Each of the homogenates was then split into two equal parts. Two flasks containing 12.5 ml Tyrode's solution alone were also set up.

0.2 μCi $^3\text{H-PGF}_{2\alpha}$ in 0.1 ml Tyrode's solution was then added to one part of each of the homogenates and to one of the Tyrode flasks. 0.2 μCi $^3\text{H-PGE}_2$ in 0.1 ml Tyrode's solution was similarly added to the other part of each of the homogenates and to the other Tyrode flask. All the samples were incubated for 90 min. at 37°C with continuous oxygenation and shaking.

Each sample was then subjected to solvent extraction in the full form (Section I A, p. 34a) and T.L.C. using first the F. VI solvent system followed by G.C.M. solvent system as described in Section I A. Control plates for $^3\text{H-PGF}_{2\alpha}$ and $^3\text{H-PGE}_2$ were also set up and run. The plates were examined for radioactive peaks on the radio T.L.C. scanner and the position of the peaks relative to the origin and solvent front was recorded. The amount of radioactivity in each peak was determined as c.p.m. (see Section I A) and expressed as a percentage of that added to the sample

prior to incubation.

Results

The mean (\pm S.E.M. where relevant) Rf of the peaks of radioactivity present after T.L.C. and the mean (\pm S.E.M. where relevant) percentage of added radioactivity recovered from each peak zone are shown in the following Table.

Table 6

SAMPLE	Incubated With			
	$^3\text{H-PGF}_{2\alpha}$		$^3\text{H-PGE}_2$	
	Peak Rf	% Radioactivity retrieved	Peak Rf	% Radioactivity retrieved
Control plates	0.330	58.1	0.512	54.4
			0.767	1.1
Tyrode alone	0.329	57.6	0.511	56.2
			0.766	1.2
Boiled uterus	0.328	57.9	0.511	58.3
			0.768	0.9
Day 15 Non-pregnant uterus	0.330	55.4	0.511	55.0
	± 0.001	± 0.5	± 0.001	± 0.7
	0.671	0.9	0.764	3.24
	± 0.001	± 0.1	± 0.001	± 0.4
Day 15 pregnant uterus	0.329	54.5	0.510	55.3
	± 0.001	± 1.2	± 0.001	± 0.3
	0.670	0.6	0.766	3.02
	± 0.001	± 0.05	± 0.001	± 0.6
Day 15 conceptus	0.330	49.1	0.512	48.5
	± 0.001	± 0.4	± 0.001	± 1.3
	0.671	5.6	0.766	7.0
	± 0.001	± 0.65	± 0.001	± 0.98

The percentage metabolism of added prostaglandin was calculated for each sample using the parent peak values on the control T.L.C. plates as zero metabolism. The results are shown in the following Table:

Table 7

Sample	Metabolism of added Prostaglandin	
	$^3\text{H-PGF}_{2\alpha}$	$^3\text{H-PGE}_2$
Tyrode alone	< 1%	< 1%
Boiled uterus	< 1%	< 1%
Day 15 Non-pregnant uterus	1.6%	5.6%
Day 15 pregnant uterus	1.1%	5.2%
Day 15 conceptus	10.2%	12.6%

Conclusions

The day 15 non-pregnant uteri and the day 15 pregnant uteri both show a very small ability to metabolise $^3\text{H-PGF}_{2\alpha}$ and $^3\text{H-PGE}_2$. There is no significant difference between the two and the extent of metabolism detected would be far too small to significantly affect the amount of prostaglandin present in the incubated day 15 non-pregnant or pregnant homogenised guinea-pig uteri.

The conceptus showed slightly greater ability to metabolise

the prostaglandins compared with the uteri. However, the level detected is not great enough to be of much significance in relation to a potential anti-luteolytic action.

It appears, therefore, that the low levels of $\text{PGF}_{2\alpha}$ found in the incubate of day 15 pregnant guinea-pig uteri compared with those for day 15 non-pregnant uteri must be the result of an inhibition, direct or indirect, of prostaglandin synthesis.

The results presented in this Section show that this preparation of incubated and homogenised uterus is a very good model for estimating the ability of the uterus to produce prostaglandins. The level of prostaglandins in the incubate is a direct measure of this ability since metabolism in this system has now been shown to be negligible.

SECTION IV

The inhibition of prostaglandin F_{2α} production by the guinea-pig uterus in vitro.

Introduction

The results presented in the last Section have led to the conclusion that the low levels of $\text{PGF}_{2\alpha}$ found in the incubate of day 15 homogenised pregnant guinea-pig uteri compared with those of day 15 non-pregnant uteri must be the result of an inhibition, direct or indirect, of prostaglandin synthesis.

A direct inhibition of $\text{PGF}_{2\alpha}$ production by the day 15 non-pregnant guinea-pig uterus in vitro has been demonstrated for Indomethacin (Poyser, 1972), an inhibitor of prostaglandin synthesis at the synthetase complex level (Vane, 1971). The conceptus may be exerting a similar action at the synthetase complex level. Alternatively the conceptus could be inhibiting the production of arachidonic acid, the precursor of $\text{PGF}_{2\alpha}$ and PGE_2 (see Fig. 4, p. 15b).

The aim of the experiments to be described is to investigate these two possibilities.

- Group II Three non-pregnant uteri alone.
- Group III Three pregnant uteri alone.
- Group IV Three groups of four conceptuses alone.
- Group V Six non-pregnant uteri each with one pregnant
uterus and one group of four conceptuses.

The excess pregnant uteri were discarded. Each sample was then pre-incubated for 2 hrs. in 10 mls. Tyrode's solution at 37°C. The samples were oxygenated and shaken continuously. After the pre-incubation 1 ml of the supernatant of each sample was removed and immediately frozen and stored at -20°C.

The conceptuses were then removed from three of the uteri in Group I to form a new group, Group I B.

The conceptuses and pregnant uteri were then removed from three of the non-pregnant uteri in Group V to form Group V B.

All samples were then homogenised (uteri and conceptuses together where appropriate) in a further 16 ml Tyrode's solution using a Polytron homogeniser. This homogenate was combined with the remaining 9 ml of pre-incubate fluid to give a final incubate volume of 25 ml. All samples were incubated for 90 min. at 37°C with continuous shaking and oxygenation. After incubation all samples were immediately frozen and stored at -20°C until assayed.

All final incubation samples and pre-incubation aliquots were assayed for PGF_{α} by R.I.A. as described in Section I B.

Results

Where non-pregnant uteri were incubated with pregnant uteri and/or conceptuses the pregnant uteri and conceptuses themselves contributed to the total amount of PGF_{α} produced by the combination of tissues. A mean figure for PGF_{α} production was obtained for pregnant uteri and for conceptuses from Group III results and Group IV results respectively. Where the non-pregnant uteri had been incubated together with either or both of these tissues the appropriate mean value was subtracted from the total value for PGF_{α} production. This gives an estimate of the contribution of the non-pregnant uterus under these circumstances. The assumption is implicit that the production of PGF_{α} by the non-pregnant uteri, if affected at all, will be reduced by the other tissues rather than vice versa.

Since a mean value is being subtracted from a figure, the individual components of which can not be measured, statistical analysis was not considered valid. However, Table 8 shows the individual results to give some indication of the degree of variation.

Table 8

GROUP (All samples day 15 of oestrous cycle of Preg- nancy)		PGF _α ng/100 mg total tissue weight			
		After Pre-Incubation only		After homogenisation & Final Incubation	
No.	Composition	Individual values	Mean	Individual values	Mean
I	Non-pregnant uteri & conceptuses present throughout	50, 27, 28	35	96, 93, 95	95
IB	Non-pregnant uteri & conceptuses present only for pre-incubation	40, 37, 50	41	95, 91, 97	94
II	Non-pregnant uteri alone	41, 60, 57	53	128, 116, 123	122
III	Pregnant uteri alone	4, 14, 15	11	32, 20, 26	26
IV	Conceptuses alone	n.d., n.d., n.d.	n.d.	4, 7, 6	6
V	Non-pregnant uteri + pregnant uteri + conceptuses present throughout	61, 35, 41	45	120, 123, 115	119
VB	Non-pregnant uteri + pregnant uteri + conceptuses present only for pre-incubation	47, 50, 54	47	100, 109, 113	107

(n.d. = not detectable)

The mean amount of $\text{PGF}_{2\alpha}$ found to be produced by day 15 uteri alone (122 ng $\text{PGF}_{2\alpha}$ /100 mg tissue) agrees well with previous findings (Poyser, 1971, Section I) as does the mean value obtained for pregnant uteri alone (26 ng $\text{PGF}_{2\alpha}$ /100 mg tissue) (see Section II). The conceptuses alone also showed a small ability to synthesise and release $\text{PGF}_{2\alpha}$, the mean amount produced being 6 ng $\text{PGF}_{2\alpha}$ /100 mg tissue. In every case where non-pregnant uteri were incubated in the presence of pregnant uteri and/or conceptuses the amount of $\text{PGF}_{2\alpha}$ produced was less than that for the non-pregnant uteri alone, even before any adjustment for the contribution to production made by the pregnant uteri and conceptuses. This result supports the assumption that the production by the non-pregnant uteri is reduced by the other tissues rather than vice versa. If this assumption did not hold a level of production at least equal to that of non-pregnant uteri alone should have been obtained.

Table 9 shows the results adjusted for the contribution of added tissues and also the percentage inhibition where relevant relative to the amount of $\text{PGF}_{2\alpha}$ produced by non-pregnant uteri alone.

Table 9

No.	GROUP Composition	PGF α ng/100 mg non-pregnant uterine tissue only		% Inhibition Relative to non- pregnant uteri alone
		After Pre-incubation	After homogenisation and final Incubation	
I	Non-pregnant uteri + conceptuses throughout	35	89	27.5
IB	Non-pregnant uteri + conceptuses present only in pre-incuba- tion	41	94	22.5
II	Non-pregnant uteri alone	53	122	0
V	Non-pregnant uteri + pregnant uteri + conceptuses present throughout	35	87	28.6
VB	Non-pregnant uteri + pregnant uteri + conceptuses present only in pre-incuba- tion	36	96	21.5
III	Pregnant uteri alone	PGF α ng/100 mg Uterine tissue		
		After Pre-incubation	After homogenisation and final Incubation	11

Conclusions

A small but consistent inhibition of PGF_α production by the day 15 non-pregnant uterus is observed in each of Groups I, IB, V and VB. At no time, however, does the observed inhibition approach that recorded for the day 15 pregnant guinea-pig uterus.

Since the results of Groups I and V and Groups IB and VB are so similar, it appears that neither the pregnant uterus nor an interaction between the pregnant uterus and conceptuses play any part in the inhibition of PGF_α production by the non-pregnant uterus. The inhibition appears to result from the conceptuses and is in each case (I and IB, V and VB) slightly greater where the conceptuses were present throughout the whole of pre-incubation and the subsequent homogenisation and final incubation.

Indomethacin (1 $\mu\text{g}/\text{ml}$) present in the homogenate of day 15 non-pregnant uteri during a 90 min. incubation inhibits $\text{PGF}_{2\alpha}$ production by 62% (Poyser, 1972). It is evident, therefore, that a significant degree of inhibition is possible in the time course of these experiments. If inhibition of PGF_α synthesis forms a major part of the mechanism of the anti-luteolytic action of the conceptus then a greater degree of inhibition would be expected than that observed in these experiments. It is also significant that the continuing presence of the conceptus is not usually necessary to achieve inhibition of PGF_α production

by the day 15 pregnant uterus, the conceptus being normally absent from the incubate as in Group III of the experiments. The results for Group I and Group V, where the conceptuses were removed after pre-incubation, agree with this picture since after homogenisation and final incubation, inhibition of PGF_{α} production was still present.

There is a tendency to bacterial growth if these uterine homogenates are maintained at 37°C for longer than 4 hrs and this prevents the extension of this work to longer incubation times to test the possibility of inhibition increasing with greater pre-incubation time.

In conclusion, some direct inhibition of uterine PGF production associated with the presence of the conceptus has been demonstrated, although this degree of inhibition does not appear to be sufficient to explain the large inhibition observed for day 15 pregnant uteri. It is possible, however, that in the intact animal there is normally a slight but continual inhibition being exerted by the conceptus. There may be some inhibitory factor secreted continuously so that on killing the animal and carrying out in vitro studies on the conceptus one sees only the effect of the amount the 'inhibitory factor' present at the time of killing.

EXPERIMENT 2. The production of PGF_{α} by the day 12 and day 15 pregnant guinea-pig uterus *in vitro* in the presence of excess arachidonic acid.

The conceptus may exert its anti-luteolytic action by inhibiting the production of arachidonic acid, the precursor of PGF_{α} and PGE_2 . If this is the case it should be possible to overcome this inhibition by the provision of excess exogenous arachidonic acid. However, the percentage conversion of arachidonic acid can be less than 0.4% (Pace-Asciak, Morawska, Cocceani & Wolfe, 1968) especially if endogenous levels are high and thus competing with the exogenous form. Therefore, for these experiments a percentage conversion of 0.1% will be assumed and the amounts added will be adjusted appropriately.

The aim of the experiment to be described is to determine whether or not the amount of PGF_{α} produced by the early pregnant guinea-pig uterus *in vitro* may be increased by the addition of exogenous arachidonic acid.

Methods

Observation of oestrous cycles and mating procedure were exactly as described before (Section II). Twelve bilaterally pregnant guinea-pigs were used. Six were used on day 12 of pregnancy and the remaining six on day 15. The animals were killed on the appropriate day, the uteri removed, slit longitudinally

inally and all placental and embryonic tissue was removed and discarded. The uteri were then each weighed and then chopped and homogenised in Tyrode's solution using a Polytron homogeniser. Three day 12 pregnant uteri and three day 15 pregnant uteri were each homogenised in 19.9 ml Tyrode's solution and 0.1 ml of ethanol was then added to each. The other three day 12 pregnant uteri and the other three day 15 pregnant uteri were each homogenised in 19 ml Tyrode's solution and to each of these was added 1 ml of a solution of arachidonic acid 200 $\mu\text{g}/\text{ml}$ in Tyrode's solution and ethanol, having the ratio Tyrode's solution:ethanol of 10:1, 1 ml of this solution of arachidonic acid with a percentage conversion of 0.1% would yield 2 μg of $\text{PGF}_{2\alpha}$, an amount far in excess of that normally produced by the early pregnant uteri.

All the homogenates were then oxygenated and incubated at 37°C with continuous shaking, for 90 min. Each sample was then immediately frozen and stored at -20°C until assayed. The PGF_{α} levels in the incubates were assayed by R.I.A. However, arachidonic acid, being a precursor of $\text{PGF}_{2\alpha}$, may cross-react with the $\text{PGF}_{2\alpha}$ -antibodies. Therefore, the following control procedures were first carried out:

- (1) The standard curve to $\text{PGF}_{2\alpha}$, in Tyrode/ethanol and in the presence of arachidonic acid, using the $\text{PGF}_{2\alpha}$ -antibodies. (Solid-phase antibody method).

One set of standards was made up in Tyrode's solution

containing 0.5% ethanol at the concentrations previously described (Section IB). Three other sets of standards were made up also in Tyrode's solution containing 0.5% ethanol, but also containing 0.5 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ arachidonic acid respectively, with standard concentrations as for the first set.

Thus four standard curves were set up with all points in duplicate, as described in Section IB, to give one control curve and three curves in the presence of 0.5 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ arachidonic acid respectively.

(2) The standard curve to $\text{PGF}_{2\alpha}$, in Tyrode alone and in the presence of arachidonic acid, using the $\text{PGF}_{2\alpha}$ -BSA antibody, following partition with petroleum spirit. (Solid-phased antibody method for the standard curve.)

The sets of standards made up as just described were used again, one control set and three sets with arachidonic acid, 0.5, 10.0 and 20.0 $\mu\text{g/ml}$. The aliquots for the four standard curves were dispensed in duplicate. Each aliquot was then partitioned with 2 ml of redistilled petroleum spirit (40-60°C B.Pt). The aqueous phase was frozen and the petroleum spirit decanted and discarded. The aqueous phase was then thawed and partitioned again with 2 ml of the redistilled petroleum spirit. After freezing the aqueous phase, the petroleum spirit was again decanted and discarded. The aqueous phase was then thawed and now entered the standard curve.

(3) Recovery of tritiated-PGF_{2 α} from Tyrode's solution containing 0.5% ethanol following double partition with petroleum spirit.

A Tyrode's/ethanol 0.5% solution was made up containing ³H-PGF_{2 α} 2 μ Ci/ml. 0.25 ml aliquots (i.e. 0.5 μ Ci) were set up in quadruplicate and subjected to a double partition with redistilled petroleum spirit (40-60°C B.Pt) by the method just described. After completion of the partitioning the aqueous phase was in each case poured into a vial containing 13 mls of scintillant (formula Section IB - Solid phase method) and each of the vials was monitored for radioactivity by counting in a Nuclear Chicago Scintillation counter for 4 mins. per vial. Four control vials were set up, to each of which had been added 0.5 μ Ci ³H-PGF_{2 α} in non-partitioned Tyrode's/ethanol 0.5% solution. The percentage of added radioactivity retrieved was calculated for each of the partitioned aliquots.

As a result of the findings of the three control procedures just described 0.25 ml aliquots from each of the uterine incubates were set up in quadruplicate and subjected to double partition with redistilled petroleum spirit by the method just described. Assay volumes were then withdrawn from the partitioned aliquots and from this point on the assay proceeded normally, using the solid-phase antibody methods described in Section IB.

Results

- (1) Standard Curve to $\text{PGF}_{2\alpha}$ in the presence of arachidonic acid.
(The Standard curves are shown in figures 19. and 20.)

The Tyrode's solution/0.5% ethanol curve was normal except for a slight flattening of the curve from the '0' standards to the 30 pg standard.

The presence of arachidonic acid caused severe disturbance of the standard curve in every case. Clearly the arachidonic acid must be removed prior to assaying the samples.

- (2) Standard Curve to $\text{PGF}_{2\alpha}$ in the presence of arachidonic acid but following double partition with redistilled petroleum spirit. (The standard curves are shown in Figure 20).

The Standard curves obtained for Tyrode's solution/0.5% ethanol alone and arachidonic acid 0.5, 10.0 and 20.0 $\mu\text{g}/\text{ml}$ became superimposable after the double petroleum spirit partitioning. There was some flattening of the curve both at the zero standard to 30 pg standard level and at the 0.8 ng to 2.4 ng standard level but this is not significant since it leaves the working area of the curve unaltered. Thus this partition with petroleum spirit appears to be an effective means of removing the arachidonic acid from samples.

- (3) Recovery of $^3\text{H-PGF}_{2\alpha}$ from Tyrode's solution/0.5% ethanol after double petroleum spirit partitioning.

% Recovery of added radioactivity = 86.6 ± 3 (\pm S.E.M.)

Figure 19

RIA Standard Curves to $\text{PGF}_{2\alpha}$ using solid-phased $\text{PGF}_{2\alpha}$ antibodies.

- Key: (1) Standards made up in Tyrode's solution.
(2) Standards in Tyrode's/0.5% ethanol solution.

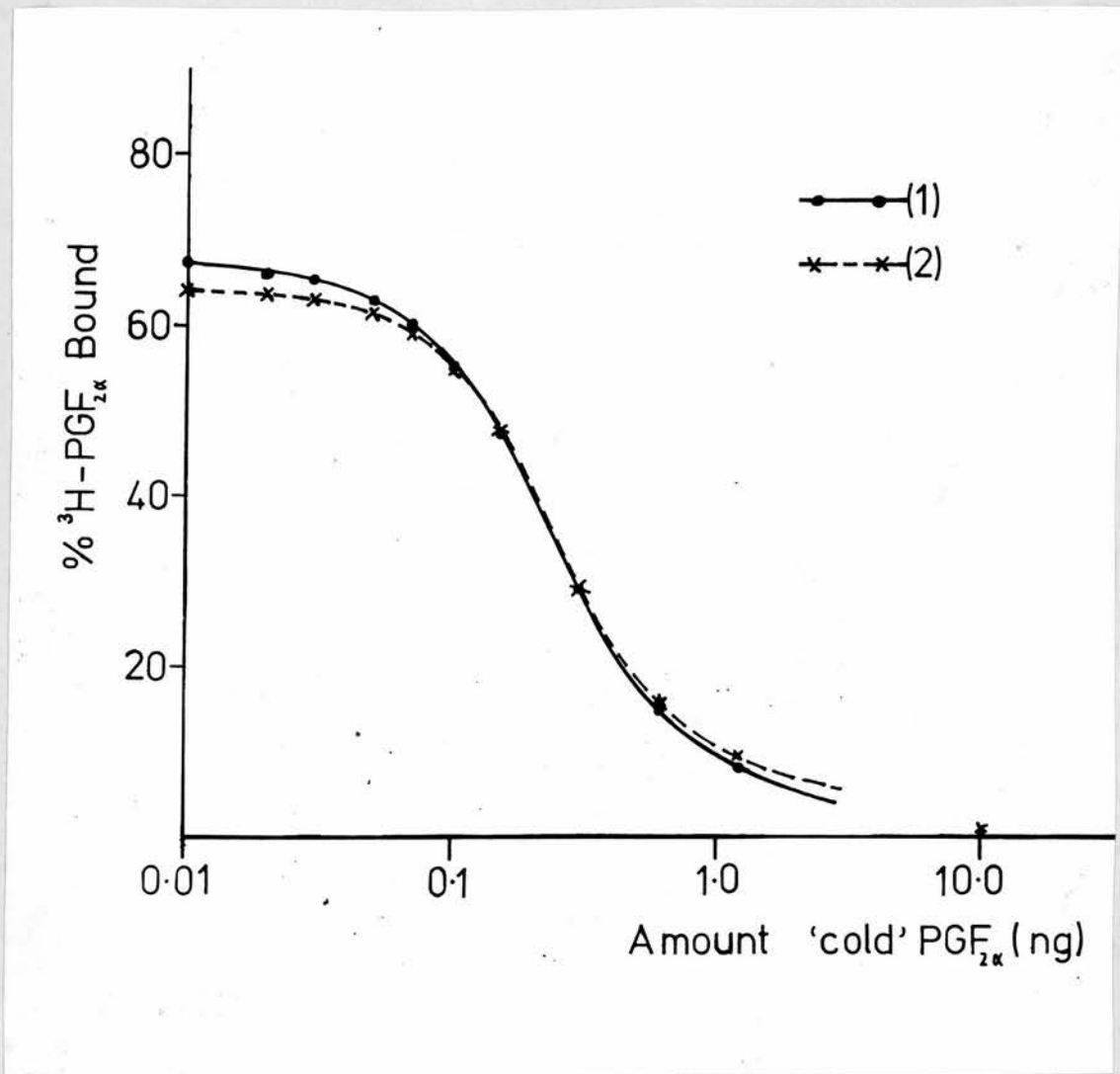
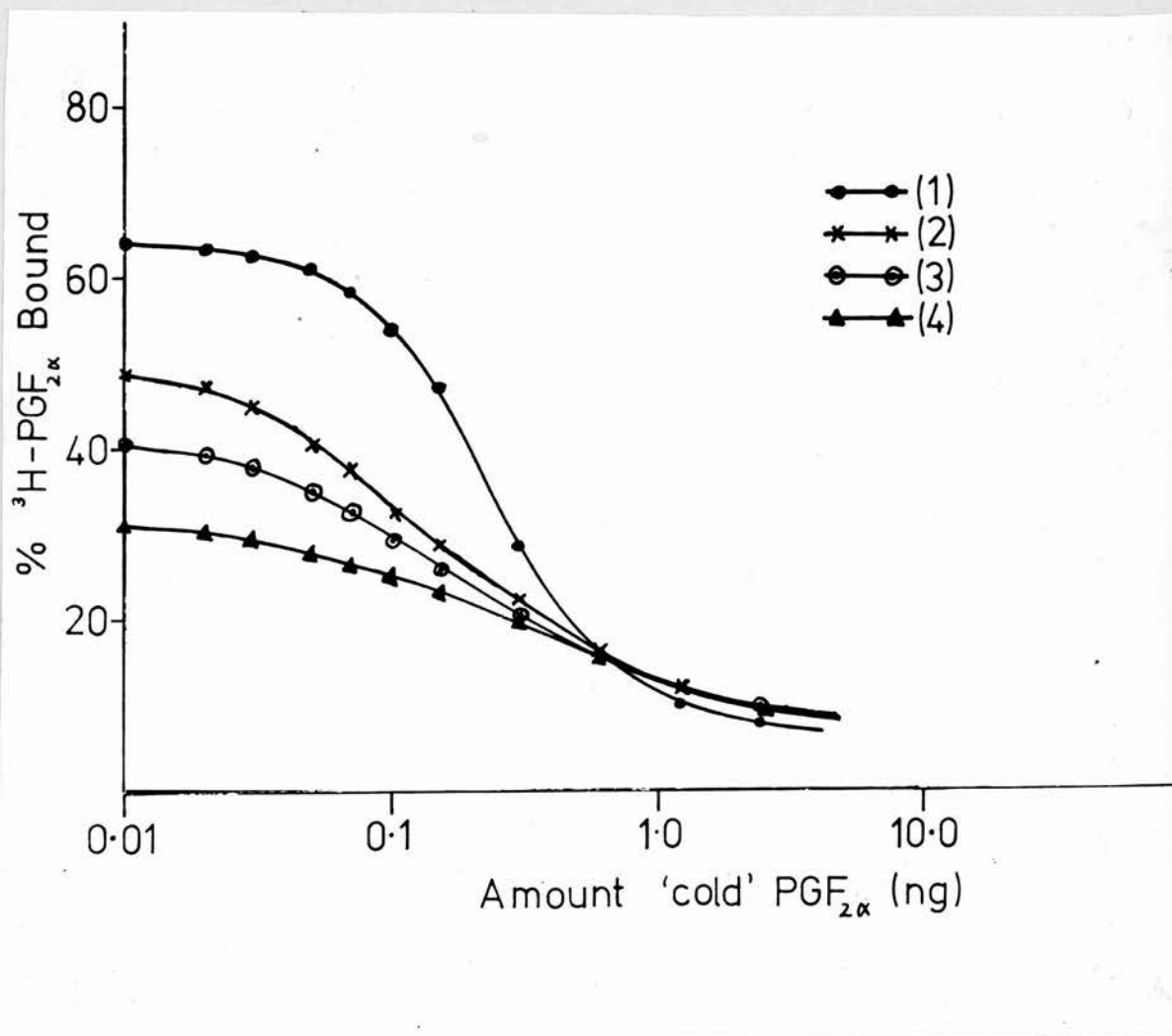


Figure 20

RIA standard curves to $\text{PGF}_{2\alpha}$ using solid-phased $\text{PGF}_{2\alpha}$ antibodies.

- Key: (1) Mean standard curve from standards in Tyrode's/0.5% ethanol solution containing 0.5, 10.0 or 20.0 $\mu\text{g}/\text{ml}$ Arachidonic acid, after double petroleum spirit partitioning in every case.
(2) Standards in Tyrode's/0.5% ethanol solution + 0.5 $\mu\text{g}/\text{ml}$ Arachidonic acid.
(3) Standards in Tyrode's/0.5% ethanol solution + 10.0 $\mu\text{g}/\text{ml}$ Arachidonic acid.
(4) Standards in Tyrode's/0.5% ethanol solution + 20.0 $\mu\text{g}/\text{ml}$ Arachidonic acid.



Although lower than for a simple ethyl acetate extraction this is sufficiently high and constant enough for routine use where arachidonic acid is present in the samples.

- (4) The in vitro production of $\text{PGF}_{2\alpha}$ by day 12 and day 15 pregnant guinea-pig uteri alone, and in the presence of exogenous arachidonic acid.

The amounts of $\text{PGF}_{2\alpha}$ produced by the homogenised and incubated uteri in vitro are shown in the following Table:

Table 10

State of Pregnancy	$\text{PGF}_{2\alpha}$ ng/100 mg Uterine tissue	
	Alone	+ Arachidonic Acid
Day 12	37.9) 47.5) 30.0) } 35.1 ± 6.2	18.7) 33.5) 42.5) } 31.5 ± 8.6
Day 15	43.4) 31.3) 32.6) } 35.8 ± 3.8	35.2) 44.3) 34.6) } 38.0 ± 2.1

There was no significant difference, by the Student's t-test, between any of the results obtained.

Conclusions

It is clear that the presence of exogenous arachidonic acid does not increase the ability of the day 12 or day 15 pregnant guinea-pig uterus to produce $\text{PGF}_{2\alpha}$ when homogenised and incubated

in vitro. It therefore appears that inhibition of the production of arachidonic acid does not constitute part of the anti-luteolytic action of the guinea-pig conceptus.

General Conclusions and Comments to Section IV.

It has been found that the presence of arachidonic acid may be successfully eliminated from uterine incubates by double partition with redistilled petroleum spirit (40-60°C B.Pt) after which the samples may enter an R.I.A. assay for $\text{PGF}\alpha$ without further treatment. The possible presence of arachidonic acid must be taken into account in any sample since it has been shown to cause severe disturbance of the standard curve to $\text{PGF}_{2\alpha}$ using the solid-phase antibody method.

The results presented in this section indicate that an inhibition of arachidonic acid production does not form part of the mechanism of the anti-luteolytic action of the conceptus. However, a slight (25-30%) direct inhibition of in vitro $\text{PGF}\alpha$ production by day 15 non-pregnant uteri was demonstrated for day 15 conceptuses. This inhibition does not account for the large reduction in the ability of pregnant uteri to produce $\text{PGF}\alpha$ in vitro when compared to non-pregnant uteri. However, this inhibition may none-the-less constitute part of the mechanism of the anti-luteolytic action of the conceptus. It is also possible that this degree of inhibition might reflect a continual, maintained,

inhibition of PGF_α production by the conceptus in the intact pregnant animal.

It would, however, be worthwhile to consider possible sites of an anti-luteolytic action further back in the sequence of steps leading to prostaglandin production. If oestrogen, in the presence of progesterone, forms the physiological stimulus to $\text{PGF}_{2\alpha}$ production and release (see Introduction) ovarian oestrogen secretion could form just such an early step on which an anti-luteolytic factor might act.

SECTION V

Corpus luteum size and levels of progesterone, prostaglandin F_{α} and oestradiol- 17_{β} in the utero-ovarian venous plasma of non-pregnant and early pregnant guinea-pigs.

Introduction

There is now much evidence to support the hypothesis that oestrogen, in the presence of progesterone, forms the physiological stimulus for increased uterine $\text{PGF}_{2\alpha}$ production and release towards the end of the oestrous cycle (see Introduction). It has only been possible to demonstrate a weak direct inhibition of uterine $\text{PGF}_{2\alpha}$ production by the conceptus (Section IV) but it is possible that the main anti-luteolytic action of the conceptus is exerted at an earlier step in the control of uterine $\text{PGF}_{2\alpha}$ synthesis and release. An inhibition of ovarian oestradiol- 17β secretion or an antagonism of the effects of the oestradiol- 17β would provide alternative mechanisms for the action of the conceptus and both of these should allow luteal maintenance beyond day 15, as is seen in pregnancy.

It is the aim of the experiments to be described to compare the utero-ovarian venous plasma levels of progesterone, oestradiol- 17β and PGF_{α} , and also luteal size, in non-pregnant and early pregnant guinea-pigs.

Methods

The oestrous cycles of virgin female guinea-pigs were recorded by daily examination of the vaginal membrane, and smears were taken by lavage with 0.9% NaCl whenever the vagina was open. The day of oestrus was taken as the day on which maximum

cornification was seen in the smear, before the post-ovulatory influx of leucocytes, and was designated day 1 of the cycle or of pregnancy. At least one cycle of normal length was completed before male and female guinea-pigs were housed together, and mating was verified by finding spermatozoa in the vaginal smear. Unilateral pregnancies were achieved by division of one oviduct between two ligatures on day 2 or 3 of pregnancy, the operation being carried out under pentobarbitone anaesthesia, 40 mg/kg i.p. (Performed by Dr. F.R. Blatchley).

Three groups of ten guinea-pigs were used. (1) Non-pregnant; (2) Unilaterally pregnant, sampled from the pregnant side; (3) Unilaterally pregnant, sampled from the non-pregnant side. Five animals from each group were used on day 12 only, the remainder on day 15 of the cycle or pregnancy.

Four animals became bilaterally pregnant by a breakdown in experimental design. Two of these were used on day 12 and two on day 15 of pregnancy.

In all animals, blood was collected from the left utero-ovarian vein by the method of Blatchley et al. (1972). (Blood collected performed by Dr. F.R. Blatchley). The volume of blood obtained (ml), duration of collection (mins.) and haematocrit were recorded to allow calculation of rate of flow of plasma as ml/min. The plasma samples were stored at -20°C until assayed. After the blood collection was completed, the presence of sterile and pregnant uterine horns was verified and if there was any doubt a^s

to whether embryo resorption was in progress the animal was discarded.

Ovaries were fixed in 10% formol saline and later serially sectioned at 10 μ m, mounted, and stained with haematoxylin and eosin. The stained sections were examined microscopically for corpora lutea. The sizes of the corpora lutea are expressed as the product of three diameters by the method of Rowlands (1956). (Histology and measurements were carried out by Dr. F.R. Blatchley).

The levels of PGF $_{\alpha}$ in the utero-ovarian venous plasma were determined by R.I.A., by the extraction and assay methods described in Section IB. Early samples were assayed by the double-antibody method, later samples by the solid-phase method. Some samples were assayed in both systems and good agreement was found between the two methods. Aliquots of 0.02 to 0.2 ml were used and a random assay was carried out first, using 0.02, 0.05 and 0.2 ml aliquots, each in duplicate. Based on the result of the random assay, three aliquot volumes were chosen to give three points lying on the steepest part of the standard curve. These were then set up in duplicate. The final result is the mean of the six values thus obtained. All samples showed parallelism with the standard curve for PGF $_{2\alpha}$.

The oestradiol levels were determined by R.I.A. using solid-phased antibody by the method of Bolton and Rutherford (In Press). Five aliquots of plasma (0.05 to 0.5 ml) were set up in duplicate. Each aliquot was made up to 0.5 ml with distilled water and

extracted with 2.5 ml of redistilled ethyl acetate, which was decanted after freezing the plasma. This extraction was repeated, the two ethyl acetate fractions combined, and the ethyl acetate blown off with air at 45°C. Then 0.5 ml of 0.05 M NaOH and 0.5 ml of redistilled CCl₄ were added to the dried extract and the oestradiol-17 β back-extracted into the NaOH with 2 min. shaking. After centrifuging at 2,300 r.p.m. at 4°C for 15 min., to break the emulsion that forms, 0.2 ml of the NaOH phase was withdrawn and the oestradiol-17 β content assayed. (Recovery of tritiated oestradiol-17 β calculated from a 0.1 ml sample of the NaOH phase was always greater than 90%).

Controls for the assay consisted of guinea-pig male and female (day 7 of oestrous cycle) peripheral plasma. Samples of these were taken from the same pool for each assay. The final result given for any sample is the mean value of all the aliquots for which the values lay on the steepest part of the standard curve. If less than four points could be used in this way, the assay was repeated with different aliquot volumes.

All samples showed parallelism with the standard curve for oestradiol-17 β . In this system oestrone is not parallel, but the cross-reactivity at 50% binding is 2.9%. The cross-reactivities at 50% binding for oestriol, oestrone-3-sulphate and oestradiol-3-glucuronide were 0.3%, 0.01% and 0.05% respectively and none of these gave curves parallel to that for oestradiol-17 β .

Progesterone was measured by R.I.A. according to the method

of Furr (1973) using an antibody raised in goats against progesterone-11 -succinyl bovine serum albumin. 20 μ l samples of 1:15 dilution of utero-ovarian vein plasma were assayed in duplicate. The limit of sensitivity of the assay was 30 ng/ml. (R.I.A. for progesterone carried out by Dr. F.R. Blatchley).

All values obtained, except those for the bilaterally pregnant animals, were subjected to statistical analysis by the Student's t-test.

Results

A standard curve for oestradiol-17 β R.I.A. is shown in Fig. 21.

Control samples:- mean oestradiol-17 β levels obtained over seven assays, \pm S.E.M.

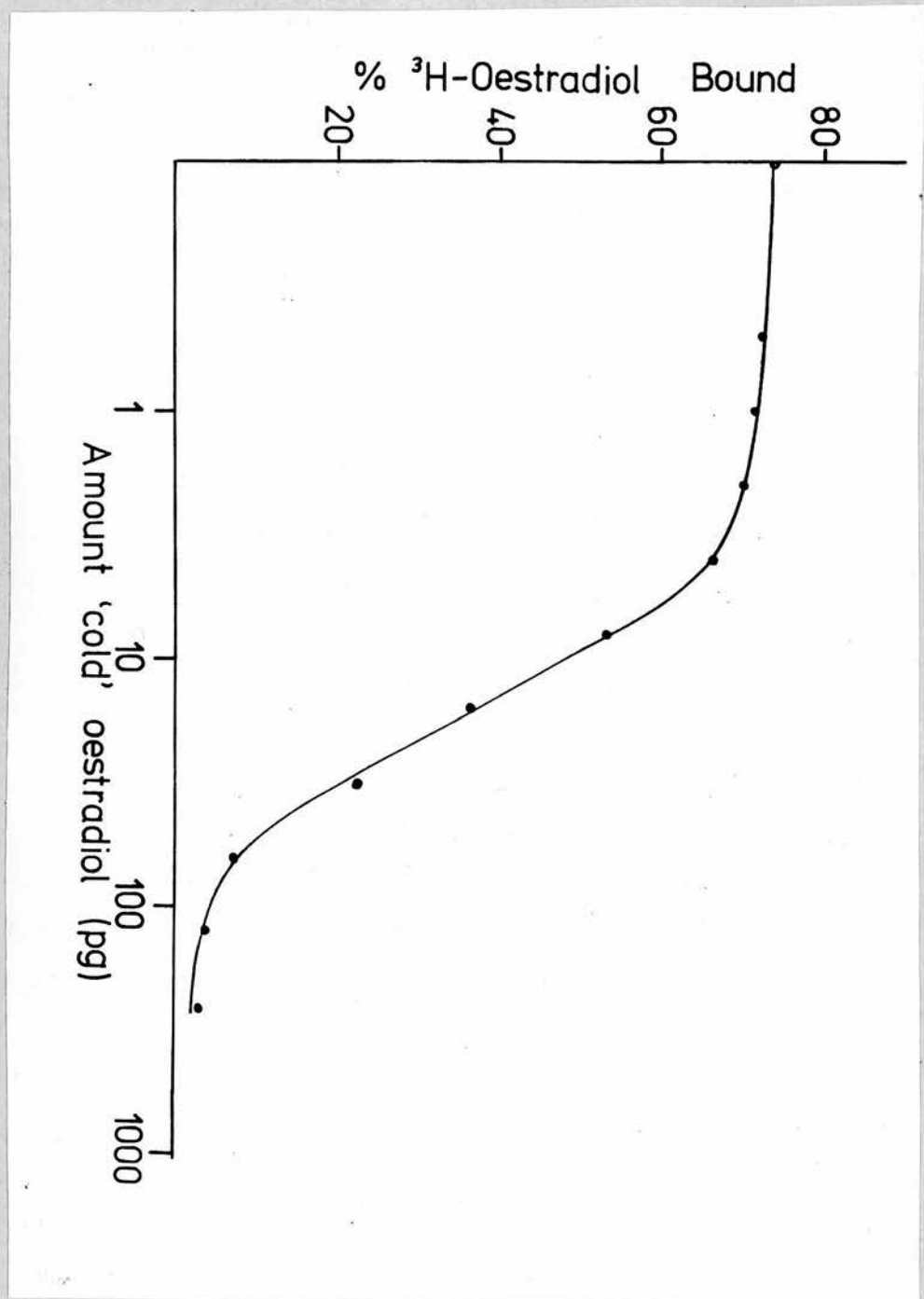
Guinea-pig peripheral plasma	}	Male	=	52 \pm 5 pg/ml
		Female (day 7 of oestrous cycle)	=	123 \pm 10 pg ml

These values show a low level of variation and any differences in utero-ovarian venous plasma levels may be assumed, therefore, to be real.

On day 12, the utero-ovarian venous plasma level of progesterone was similar in all groups of animals studied. The level in the non-pregnant guinea-pigs was 116 \pm 29.3 ng/ml (mean \pm S.E.M.) compared with 101.3 \pm 26.7 ng/ml for the non-pregnant side of unilaterally pregnant guinea-pigs and 85.5 \pm 32.1 ng/ml

Figure 21

RIA Standard Curve for Oestradiol-17 β (Solid-phase antibody method).



for the pregnant side. In the bilaterally pregnant guinea-pigs the level was 131.3 ± 7.5 ng/ml.

The PGF_α level in the utero-ovarian venous plasma of non-pregnant guinea-pigs on day 12 was 8.0 ± 1.1 ng/ml which was significantly greater ($P < 0.05$) than that recorded from the pregnant side of unilaterally pregnant animals (4.3 ± 0.2 ng/ml). The amount of PGF_α in the plasma from the non-pregnant side of unilaterally pregnant animals (5.0 ± 0.3 ng/ml) was also less than in non-pregnant guinea-pigs, but not significantly so. In the bilaterally pregnant guinea-pigs the utero-ovarian venous plasma level of PGF_α was 2.8 ± 0.3 ng/ml.

Oestradiol- 17β levels in all the groups of animals studied on day 12 showed a similar pattern to that seen for PGF_α . In non-pregnant guinea-pigs a mean value of 707 ± 88 pg/ml oestradiol- 17β was found in the utero-ovarian venous plasma, a significantly greater amount ($P < 0.01$) than that recorded for the pregnant side of unilaterally pregnant animals (279 ± 54 pg/ml). On the non-pregnant side of unilaterally pregnant animals the oestradiol- 17β level (419 ± 205 pg/ml) fell between the levels for the pregnant side and those for non-pregnant animals but was not significantly different from either. In bilaterally pregnant guinea-pigs the level was 289 ± 53 pg/ml.

By day 15, utero-ovarian venous plasma levels of progesterone in non-pregnant guinea-pigs had fallen whilst those in the pregnant

animals were maintained. In the non-pregnant guinea-pigs the level recorded, 52.5 ± 11.3 ng/ml, was significantly lower ($P < 0.01$) than that seen on day 12. It was also significantly lower than that from the pregnant side of unilaterally pregnant animals (125.3 ± 26.5 ng/ml, $P < 0.05$) and less than the level from the non-pregnant side (164.3 ± 55.0 ng/ml) on day 15 in these animals. The level at day 15 in the non-pregnant animals was also less than the level at day 15 in the bilaterally pregnant animals (103.5 ± 24.7 ng/ml).

In non-pregnant guinea-pigs the PGF_α level had increased to 15.1 ± 3.6 ng/ml by day 15. This level was significantly greater than the levels recorded from both the pregnant side (2.7 ± 0.3 ng/ml, $P < 0.01$) and the non-pregnant side (6.1 ± 1.8 ng/ml, $P < 0.05$) of unilaterally pregnant animals. On the non-pregnant side of these animals the PGF_α level was greater than that for the pregnant side, although not significantly so. In the bilaterally pregnant animals the PGF_α level was 3.1 ± 0.1 ng/ml, less than that recorded for the day 15 non-pregnant animals.

The oestradiol- 17β levels in the non-pregnant animals had increased to 1281 ± 287 pg/ml by day 15, significantly higher ($P < 0.05$) than the level for the pregnant side in unilaterally pregnant animals (461 ± 78 pg/ml). However, on the non-pregnant side, oestradiol- 17β levels (699 ± 248 pg/ml) fell between the level for the pregnant side and the level for non-pregnant animals

although not significantly different from either. In bilaterally pregnant animals, at day 15, the oestradiol-17 β level was 188 ± 36 pg/ml, which is much less than the level in the non-pregnant animals.

In the non-pregnant animals, at day 12, the mean corpus luteum size was 3.14 ± 0.3 mm³, larger than that at day 15 (2.4 ± 0.3 mm³), although the size at day 15 indicates significant structural maintenance, the smaller size suggests the onset of regression. The low progesterone levels at this time, however, indicate that at day 15 the corpora lutea were fully functionally regressed.

There was no significant difference between any of the results recorded for mean corpus luteum size in the pregnant animals. In the unilaterally pregnant animals, on the non-pregnant side the mean luteal size on day 12 was 3.62 ± 0.3 mm³, and on day 15, 3.09 ± 0.4 mm³. On the pregnant side the mean luteal size on day 12 was 3.60 ± 0.2 mm³, and on day 15, 3.46 ± 0.4 mm³. In the bilaterally pregnant animals the mean luteal size on day 12 was 2.92 ± 0.3 mm³ and on day 15, 3.38 ± 0.4 mm³.

These results considered so far are summarised in Table 11.

Table 11

Corpus Luteum Size and Utero-Ovarian Venous Plasma Levels of Progesterone, PGF α and Oestradiol-17 β in Non-Pregnant and Early Pregnant Guinea-Pigs.

	Unilaterally Pregnant								Bilaterally Pregnant	
	Non-Pregnant Control		Non-Pregnant Side		Pregnant Side		Pregnant Side		Day 12	Day 15
	Day 12	Day 15	Day 12	Day 15	Day 12	Day 15	Day 12	Day 15	Day 12	Day 15
Corpus Luteum Size (mm ³ \pm S.E.M.)	3.14 \pm 0.3	2.4 \pm 0.3	3.62 \pm 0.31	3.09 \pm 0.4	3.60 \pm 0.2	3.46 \pm 0.4	2.92 \pm 0.3	3.38 \pm 0.4		
UTERO-OVARIAN VENOUS PLASMA LEVELS (\pm S.E.M.)	8.0 \pm 1.1	15.1 \pm 3.6	5.0 \pm 0.3	6.1 \pm 1.8	4.31 \pm 0.2	2.7 \pm 0.3	2.8 \pm 0.3	3.1 \pm 0.1		
	707 \pm 88	1281 \pm 287	419 \pm 205	699 \pm 248	279 \pm 54	461 \pm 78	289 \pm 53	188 \pm 36		
	116.4 \pm 29.3	52.5 \pm 11.3	101.3 \pm 26.7	164.3 \pm 55.0	85.5 \pm 32.1	125.3 \pm 26.5	131.3 \pm 7.5	103.5 \pm 24.7		

(Corpus luteum size and Progesterone levels determined by F.R. Blatchley.)

The mean values for collection time, blood volume, plasma volume and utero-ovarian venous plasma flow and blood flow in ml/min. are shown in the following Table.

Table 12.

		UTERO-OVARIAN VENOUS BLOOD				
		Collection Time (min.)	Blood Volume (ml)	Plasma Volume (ml)	Plasma Flow (\pm S.E.M.) ml/min	Blood Flow (\pm S.E.M.) ml/min
Non-Pregnant Control	Day 12	55	12.6	6.7	0.128 \pm 0.02	0.235 \pm 0.04
	Day 15	62	12.2	6.8	0.114 \pm 0.02	0.204 \pm 0.04
Unilaterally Pregnant-Sterile Side	Day 12	58	12.3	6.8	0.114 \pm 0.01	0.204 \pm 0.02
	Day 15	59	15.8	8.5	0.146 \pm 0.01	0.267 \pm 0.02
Unilaterally Pregnant-Pregnant Side	Day 12	56	16.8	8.4	0.156 \pm 0.02	0.306 \pm 0.04
	Day 15	60	16.7	9.2	0.156 \pm 0.03	0.279 \pm 0.06
Bilaterally Pregnant	Day 12	60	14.5	12.0	0.200	0.324
	Day 15	63	15.4	8.0	0.130	0.247

There was no significant difference between any of the values obtained for plasma flow rate or for blood flow rate. However, for some individual values of PGF_α levels etc. which had appeared rather different to the rest of their group it was noticed that their values for plasma flow showed a similar trend in the opposite direction so that if the results were adjusted for flow, to give a value for output in terms of amount per minute, the variation within the group was sometimes reduced. For the sake of comparison Table 13 shows the figures of oestradiol- 17β , Progesterone and PGF_α in terms of both amount per ml plasma and output as amount, per ml, per minute.

It can be seen that the relationships for the output values between non-pregnant and pregnant sides of unilaterally pregnant animals, between Day 12 and Day 15 is general and between non-pregnant and pregnant animals are the same as those found when comparing the utero-ovarian venous plasma levels of oestradiol- 17β , progesterone and PGF_α .

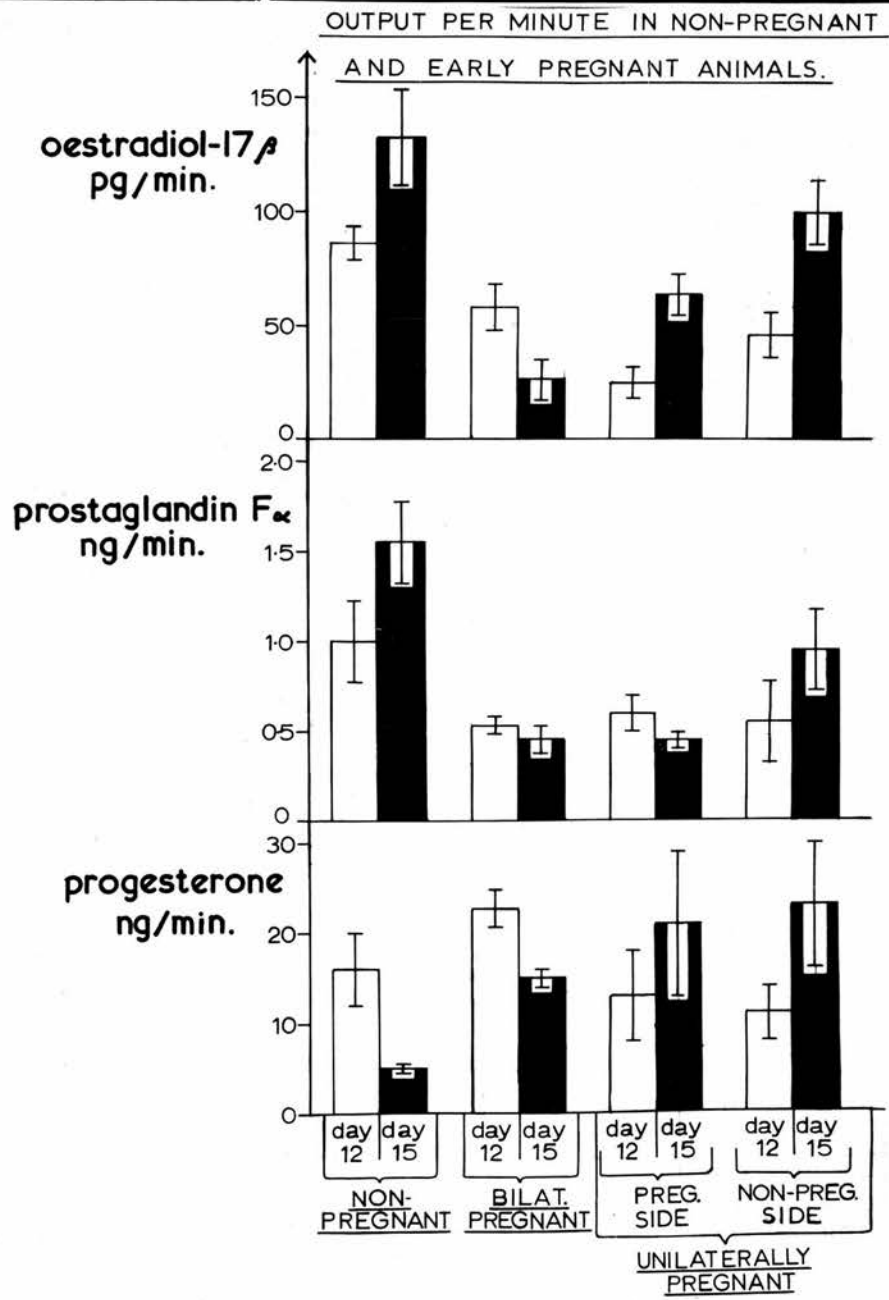
The results for output per minute of PGF_α , oestradiol- 17β and progesterone are shown in Fig. 22.

TABLE 13 - Results for Utero-ovarian venous plasma levels - adjusted for rate of flow (\pm S.E.M.).

		UTERO-OVARIAN VENOUS PLASMA							
		Oestradiol-17 β		Progesterone		PGF α			
		pg/ml	pg/min	ng/ml	ng/min	ng/ml	ng/min	Rate of flow ml/min	
NON-PREGNANT	DAY 12	A	707 \pm 88	86 \pm 8	116 \pm 29	16 \pm 4	8.0 \pm 1.1	1.0 \pm 0.2	0.128 \pm 0.02
	UNILATERALLY PREGNANT	B	419 \pm 205	46 \pm 10	101 \pm 27	11 \pm 3	5.0 \pm 0.3	0.6 \pm 0.2	0.114 \pm 0.01
C		279 \pm 54	25 \pm 7	86 \pm 32	13 \pm 5	4.3 \pm 0.2	0.7 \pm 0.1	0.156 \pm 0.02	
BILATERALLY PREGNANT	DAY 15	D	289 \pm 53	47 \pm 10	131 \pm 8	23 \pm 2	2.8 \pm 0.3	0.6 \pm 0.1	0.200
	E	1281 \pm 287	133 \pm 21	53 \pm 11	5 \pm 0.4	15.1 \pm 3.6	1.6 \pm 0.2	0.114 \pm 0.02	
NON-PREGNANT	UNILATERALLY PREGNANT	F	699 \pm 248	100 \pm 14	164 \pm 55	23 \pm 7	6.1 \pm 1.8	0.9 \pm 0.2	0.146 \pm 0.01
		G	416 \pm 78	64 \pm 9	125 \pm 27	21 \pm 8	2.7 \pm 0.3	0.4 \pm 0.04	0.156 \pm 0.01
BILATERALLY PREGNANT	COLUMN	H	188 \pm 36	26 \pm 9	104 \pm 25	15 \pm 1	3.1 \pm 0.1	0.41 \pm 0.1	0.130
		I	AVScp < 0.01 EvsGp < 0.05	AVScp < 0.001 AVSBp < 0.02 EvsFp < 0.02	AVSBp < 0.1 EvsGp < 0.05	AVSBp < 0.05 EvsFp < 0.05 EvsGp < 0.1	AVScp < 0.05 EvsFp < 0.05 EvsGp < 0.01	AVSBp < 0.1 EvsFp < 0.1 EvsGp < 0.001	
Results are not statistically significant from one another except where shown:-									

Figure 22.

GUINEA-PIG UTERO-OVARIAN VENOUS PLASMA
OESTRADIOL-17 β , PROSTAGLANDIN F α and PROGESTERONE.



Conclusions

In the non-pregnant guinea-pigs, utero-ovarian venous plasma levels of PGF_{α} increased from day 12 to day 15 of the oestrous cycle, while progesterone levels fell over the same period, indicating functional luteal regression around day 15. The pattern of these results is in agreement with previous observations (Blatchley et al., 1972, Earthy et al., 1975). However, the levels of PGF_{α} found here are much less than those reported by Blatchley et al., (1972), while falling within the range found by Earthy et al., (1975) for day 15 of the oestrous cycle. The levels reported by Blatchley et al. (1972) were determined by bio-assay while those of Earthy, et al., (1975) were assayed by R.I.A. as were the levels described here. The difference is not due to a low recovery since recovery for R.I.A. extraction (see Section IB) from plasma is always greater than 90%. Since there is good agreement between bio-assay and R.I.A. for uterine homogenates the difference is unlikely to be purely due to the different assay methods. Earthy et al. (1975) suggested that the large variation observed for the PGF_{α} levels may indicate a pulsatile release of $\text{PGF}_{2\alpha}$, similar to that observed for sheep (Thorburn, Cox, Currie, Restall & Schneider, 1972) with only some peaks of release being detected. Alternatively, the difference in PGF_{α} levels observed by different workers may be simply indicative of a large variation between individual animals. The use of acute sampling techniques

necessitates the pooling of results from different animals in circumstances where individual profiles may be more useful, although virtually impossible to obtain for small animals such as guinea-pigs.

In the pregnant guinea-pigs there was no rise in utero-ovarian venous plasma levels of $\text{PGF}\alpha$ at day 15, the levels remaining low. This clearly reflects the reduced ability of the uterus to produce $\text{PGF}\alpha$ at this time (see Sections II - IV). Progesterone secretion was maintained at a high level at day 15 in all the pregnant guinea-pigs, in contrast to the fall observed at this time in the non-pregnant animals. In the unilaterally pregnant animals progesterone levels were bilaterally maintained, indicating bilateral functional luteal maintenance. This is in agreement with the results of Deanesly (1967) and indicates that the conceptus in one horn can influence the non-gravid horn in some way. There was a large variation in the progesterone levels between individual animals. Thus when the figures were pooled the final result was frequently of little significance. This is again a drawback of the acute technique of sampling.

In the unilaterally pregnant guinea-pigs, at day 12 and at day 15, utero-ovarian venous plasma levels of $\text{PGF}\alpha$ for the pregnant side were less than those for the non-pregnant side, although both sides showed lower levels than those of the non-pregnant animals.

This is the same pattern as was observed for the in vitro production of $\text{PGF}_{2\alpha}$ by the individual uterine horns of day 15 unilaterally pregnant guinea-pigs (see Section II). The greater reduction in utero-ovarian venous plasma levels of PGF_{α} observed for the gravid side of unilaterally pregnant animals supports the suggestion, based on the results for uterine PGF_{α} production, that there is a local component to the anti-luteolytic action of the conceptus. The bilateral maintenance of progesterone secretion in unilaterally pregnant animals indicates that the slightly higher PGF_{α} levels for the non-pregnant side are not sufficient to affect luteal function and progesterone secretion for that side.

Oestradiol- 17β levels in all the animals studied showed a very similar pattern to that exhibited by PGF_{α} . Oestradiol- 17β levels in the utero-ovarian venous plasma of non-pregnant guinea-pigs increased from day 12 to day 15 of the oestrous cycle, in agreement with the observations of Joshi et al. (1973) and supporting the hypothesis that oestrogen, in the presence of progesterone, forms the physiological stimulus for the increased uterine production and release of $\text{PGF}_{2\alpha}$ towards the end of the oestrous cycle. In all the pregnant guinea-pigs, however, the utero-ovarian venous plasma levels of oestradiol- 17β remained low at day 15, in contrast to the high levels at this time in the non-pregnant animals. It appears, therefore, that at least part of

the mechanism of the anti-luteolytic action of the conceptus is to inhibit ovarian oestradiol secretion, thus removing the stimulus to $\text{PGF}_{2\alpha}$ production and release.

Ovarian oestradiol- 17β will be diluted systemically prior to acting on the uterus and it is to be expected that after this dilution the blood levels to each uterine horn will be approximately equal. However, as already described, the utero-ovarian venous plasma PGF_{α} levels and the in vitro uterine $\text{PGF}_{2\alpha}$ production are greater for the non-pregnant side than the pregnant side in unilaterally pregnant guinea-pigs. If the 'oestrogen-stimulus' is equal for both horns there must be another factor to be considered. It has been shown that the conceptus can cause approximately 30% inhibition of in vitro $\text{PGF}_{2\alpha}$ production by the day 15 non-pregnant uterus (see Section IV) and it is possible that such a direct action may explain the differences in production and release of PGF_{α} by the non-pregnant and pregnant uterine horns in unilaterally pregnant animals.

In this context, it is interesting to observe that the local component of the action of the conceptus appears to extend also to ovarian oestradiol- 17β secretion. On both days 12 and 15 of pregnancy the utero-ovarian venous plasma levels of oestradiol- 17β were lower on the pregnant side than the non-pregnant side in unilaterally pregnant animals, although the levels for both sides

were lower than for the non-pregnant animals. It seems that to some degree there is a local relationship between a uterine horn of a gravid uterus and its ipsilateral ovary similar to that between a uterine horn and its ipsilateral ovary in the non-pregnant animal (see Introduction). It is possible that variations in blood flow between non-pregnant and pregnant sides of unilaterally pregnant animals might cause the apparent utero-ovarian venous plasma levels to differ between sides. However, it has been shown that there is no significant difference between the plasma flow rate for any of the groups, and a similar calculation for blood flow gave the same result. When the utero-ovarian venous plasma levels were adjusted for flow rate to give a value for output per minute the relationships observed for the plasma levels still held. These may, therefore, be assumed to represent genuine differences in production and release or secretion.

The results for $\text{PGF}\alpha$ and oestradiol- 17β levels in the utero-ovarian venous plasma also indicate that the effect of the conceptus is quite marked even by day 12 of pregnancy since in all cases $\text{PGF}\alpha$ and oestradiol levels were lower on day 12 of pregnancy than on day 12 of the oestrous cycle.

The results for the progesterone levels and corpora lutea sizes in the day 15 non-pregnant animals suggest that structural and functional luteal regression constitute two separate events.

Thus the corpora lutea of the day 15 non-pregnant animals appear substantially structurally maintained, yet the progesterone levels are low indicating that functionally they are regressed. It is evident from this that measuring luteal size alone does not provide an adequate assessment of luteal function and for this progesterone levels must be determined.

Several significant points have resulted from this Section, and may be summarised as follows:

- (1) Utero-ovarian venous plasma levels of progesterone were low in day 15 non-pregnant animals associated with high levels of PGF_{α} and oestradiol- 17β . This is in agreement with published results.
- (2) Utero-ovarian venous plasma levels of progesterone were high in day 12 and day 15 pregnant animals associated with low levels of PGF_{α} and oestradiol- 17β .
- (3) An inhibition of ovarian oestradiol- 17β secretion appears to be at least part of the mechanism of the anti-luteolytic action of the conceptus.
- (4) Results from unilaterally pregnant animals indicate a local component to the action of the conceptus for both PGF_{α} and oestradiol- 17β levels. This agrees with the results for in vitro uterine PGF_{α} production for unilaterally pregnant animals. Progesterone secretion in these animals is, however, bilaterally maintained.

- (5) The anti-luteolytic actions of the conceptus are evident by day 12 of pregnancy for $\text{PGF}\alpha$ and oestradiol 17β levels.
- (6) There was no significant difference between plasma flow or blood flow rate for any of the groups. The relationships observed, therefore, indicate genuine differences in production and release or secretion.
- (7) Luteal size alone is not an adequate measure of luteal function. Knowledge of progesterone levels is essential.

SECTION VI

The effect of exogenous oestrogen on the course of pregnancy and the production of PGF_α by the pregnant guinea-pig uterus in vitro.

Introduction

Utero-ovarian venous plasma levels of oestradiol-17 β are low on day 15 of pregnancy, in contrast to the high levels at this time in the non-pregnant animal (Section V). Since oestrogen, in the presence of progesterone, appears to be the physiological stimulus to PGF_{2 α} production by and release from the uterus (see main Introduction and Section V) the administration of exogenous oestrogen to the pregnant animal might be expected to stimulate uterine PGF_{2 α} production and possibly thereby terminate the pregnancy.

The aim of the experiments in this Section is to determine whether the anti-luteolytic action of the conceptus may be overcome by the administration of exogenous oestrogen. In vitro uterine PGF_{2 α} production and cardiac plasma progesterone levels will be determined for normal and oestrogen-treated pregnant guinea-pigs.

EXPERIMENT 1. The effect of exogenous oestrogen administered from day 10 to day 15 of pregnancy - systemic progesterone levels during the treatment and the production of $\text{PGF}\alpha$ by the uterus in vitro on day 15.

Methods

The observation of oestrous cycles and the mating procedure were as described before (Section II). Six bilaterally pregnant guinea-pigs were used. These were divided into two groups:-

Group I. (3 animals) 0.5 ml of a 20 $\mu\text{g}/\text{ml}$ solution of oestradiol benzoate (10 μg) in arachis oil was administered daily by s.c. injection on days 10 to 15 of pregnancy, inclusive. ('Test' animals).

Group II. (3 animals) 0.5 ml of arachis oil was administered by daily s.c. injection on days 10 to 15 of pregnancy, inclusive. ('Control' animals).

The treatment of the two groups was identical in all other respects. 2.0 ml of blood was obtained from every animal by cardiac puncture, being withdrawn into a syringe containing 50 i.u. heparin in 0.05 ml sterile saline, on Monday, Wednesday and Friday of each week during two control cycles and up to and including day 15 of pregnancy. The blood was immediately centrifuged for 10 mins. at 4°C , 2,200 r.p.m., and the plasma

stored at -20°C until use.

All animals were killed, by stunning and incising the neck, on day 15 of pregnancy, and their body weight recorded. The ovaries were removed and immediately placed in 10% formol saline. The uteri were removed and incised, the condition of the placentae and embryos being noted and these tissues being then removed and discarded. The uteri were then chopped, homogenised and incubated exactly as described in Section II. The incubates were subsequently stored at -20°C until use.

The ovaries were serially sectioned at $10\ \mu\text{m}$, mounted, stained with haematoxylin and eosin, and examined microscopically for corpora lutea. Luteal size was recorded as described in Section II.

Progesterone levels in the cardiac plasma were assayed by R.I.A. by the method of Dighe and Hunter (1974) but using a double-antibody rather than solid-phased antibody method. The petroleum spirit (B.pt $40-60^{\circ}\text{C}$) was purified only by redistillation prior to use for extraction of progesterone from the plasma. Recovery using this form of the solvent was always greater than 90%. (The assay of progesterone was carried out by Dr. N.L. Poyser, of this Department).

The levels of PGF_{α} in the uterine incubates were determined

by solid-phase R.I.A. as previously described. The amounts of $\text{PGF}\alpha$ produced by the uteri are expressed as ng/100 mg uterine tissue.

Results

The results are shown in Tables 14, 15 and 16.

In all animals treated with exogenous oestrogen there was evidence of failure of pregnancy seen as placental detachment and lack of vascularity. The placentae (maternal and foetal) were very white suggesting a low blood flow rate in contrast to the body of the uterus which was a healthy pink colour. The two animals showing the greatest placental detachment also showed increased $\text{PGF}\alpha$ production by their uteri in vitro, compared to that of the controls. However, only one of these (R7) showed any sign of luteal regression in the form of falling cardiac plasma progesterone levels. None of the oestrogen treated animals showed structural luteal regression when compared with the control animals.

CONTROL CYCLES						
Systemic Progesterone Levels ng/ml Plasma						
Day of Oestrous Cycle	Test Animals			Control Animals		
	G7	78	R7	W8	B8	P7
10		4.6		1.9	3.1	3.6
11	4.2			1.2		
12		4.6				
13	0.74			0.15	2.5	3.0
14						
15		0.31			0.15	0.25
16	0.22			0.12		
1		1.6			0.14	0.19
2	0.17			0.16		
3		1.2	0.50		0.35	0.56
4	1.8		0.77	2.6		
5					3.9	2.5
6		5.0				
7	2.6		2.5	2.6	4.0	
8		5.4				
9	6.7		2.8	2.2		3.6
10		7.6			2.4	
11	6.8		2.2	2.5		4.6
12					3.5	
13		0.68				
14	0.29		0.30	0.19	0.28	0.43
15		1.0				
16	0.13		0.37	0.15		0.13
1	0.23	0.31	0.26		0.14	0.22
2				0.19		
3					0.19	
4	1.5	1.3	2.0			
5				2.5	1.6	2.3
6	0.12	4.8	3.8			
7				2.3		
8	2.8	3.8	5.0		3.1	3.0
9				1.6		
10					3.1	
11	4.3	3.8	3.5			3.6
12				0.42	3.7	
13	0.5	3.0	0.69			1.7
14				0.24		
15	0.18	0.19	0.2		0.22	0.27
16					0.13	

'EXPERIMENTAL' CYCLES						
Systemic Progesterone Levels ng/ml Plasma						
Day of Pregnancy	Test Animals 10 µg Oestradiol Benzoate S.C. Days 10P - 15P			Control Animals 0.5 ml Arachis Oil S.C. - Days 10P - 15P		
	G7	78	R7	W8	B8	P7
1P	0.24	0.19	0.2	0.22		0.39
2P					0.24	
3P	2.0	2.2	0.36			0.75
4P				2.7		
5P	4.0	3.8	4.2		3.0	2.5
6P				3.3		
7P			4.2		3.8	
8P	4.3	4.5		4.4		2.6
9P					3.1	
10P	4.4	4.2	4.6			4.5
11P				4.0		
12P	6.5	7.1	5.2		5.2	3.4
13P				6.8		
14P			4.7		2.2	
15P	5.8	7.0	0.9	9.7	3.8	6.6

Conclusions

All the oestrogen treated animals exhibited signs of the initiation of termination of pregnancy although these were not reflected in cardiac plasma progesterone levels. It appears, therefore, that the dose of oestrogen, or the duration of administration may be on the edge of a threshold for producing termination of pregnancy. It would be worthwhile, therefore, to determine the effects of an increased duration of administration.

EXPERIMENT 2. The effect of exogenous oestrogen administered from day 10 to day 21 of pregnancy - systemic progesterone levels during the treatment and the production of $\text{PGF}\alpha$ by uterus in vitro on day 21.

Introduction

The results of Experiment 1 suggest that it would be worthwhile to extend the duration of oestrogen administration beyond day 15 of pregnancy. A functional corpus luteum is essential for the maintenance of pregnancy until day 20-25 of pregnancy (Loeb & Hesselberg, 1917; Courrier et al., 1929) after which placental progesterone secretion is sufficient to maintain pregnancy (Heap & Deanesly, 1966; Illingworth & Deanesly, 1972; Heap & Illingworth, 1973). Day 21 of pregnancy was chosen, therefore, for the limit of the extension of duration of oestrogen administration since beyond this time placental progesterone would be expected to maintain pregnancy even in the presence of functional luteal regression.

Methods

The observation of oestrous cycles and mating procedure were as described before (Section II). Six bilaterally pregnant guinea-pigs were used. These were divided into two groups:-

Group I. (4 animals). Treated with 10 μg oestradiol benzoate in arachis oil (0.5 ml) daily, administered by

s.c. injection, on days 10 to 21 of pregnancy, inclusive. ('Test' animals).

Group II. (2 animals). Treated with 0.5 ml arachis oil daily, administered by sc. injection, on days 10 to 21 of pregnancy, inclusive. ('Control' animals).

All other treatment of the two groups was exactly as that described for experiment 1 except that cardiac blood samples were taken during one, rather than two, control cycles prior to mating in some animals.

Also the animals were not killed until day 21 of pregnancy, the procedures that were then carried out were identical to those described for day 15 of pregnancy in experiment 1.

Results

The results are shown in Tables 17, 18 & 19 and Figures 23, 24 & 25.

All the animals treated with exogenous oestrogen were aborting on day 21 of pregnancy. In three out of four of these animals (P22, B1 and G22) the cervix had dilated and the conceptuses had entered the vagina, the uterine horns containing mainly placental remnants. In these animals the corpora lutea were structurally regressed on day 21 of pregnancy and cardiac plasma progesterone levels had fallen by day 12 - 13 of pregnancy indicating functional luteal regression within 2 - 3 days of the onset of the oestrogen treatment. This fall in cardiac plasma progesterone in the

oestrogen treated animals occurred slightly earlier than that normally seen in association with functional luteal regression in the oestrous cycle when the levels show a slight fall on day 13, the main fall occurring around days 14-15. (Blatchley et al., 1972; Earthy et al., 1975). The mean in vitro PGF_{α} production by the uteri of the animals treated with exogenous oestrogen (145 ng PGF_{α} /100 mg tissue) was significantly greater ($P < 0.01$) than that of the control animals on day 21 of pregnancy (67 ng PGF_{α} /100 mg tissue).

In one of the animals treated with oestrogen (Y1) the placentae were detached on day 21 of pregnancy and the conceptuses were presenting at the cervix although the cervix was not dilated. In this animal, although in vitro uterine PGF_{α} production was high (126 ng PGF_{α} /100 mg uterine tissue) on day 21 of pregnancy the corpora lutea appeared structurally maintained (size = 4.0 mm^3) at this time and cardiac plasma progesterone levels had increased to give values higher than those observed in the control animals for days 18 - 21 of pregnancy. This large increase suggests a compensatory production of progesterone, possibly by the placenta, in addition to the corpus luteum.

The body weight of the animals used in experiment 2 was noticeably less than that for the animals used in experiment 1. Slight opening of the vaginal membrane was observed on day 21 of pregnancy for P22, B1 and G22, but not for any of the others.

Table 17.

		CONTROL CYCLES					
		Systemic Progesterone Levels ng/ml Plasma					
		Control Animals		Test Animals			
Day of Oestrous Cycle		B22	W22	P22	B1	G22	Y1
Oestrous →	16					0.11	
	1				0.16	1.15	0.38
	2						
	3				0.23		0.7
	4						
	15				1.9	2.55	
	6						4.8
	7					2.43	
	8				2.8		5.0
	9						
	10				3.8	2.9	2.6
	11						
	12				2.4	2.3	
	13						0.55
	14	0.22				0.7	
	15				0.17		0.24
Oestrus →	16		0.06		0.08		
	1	0.21		0.19		0.09	0.19
	2		0.36		0.09		
	3					0.35	
	4		1.78	1.3			1.1
	5	2.62			2.9	1.12	
	6			2.4			3.8
	7		2.03		1.7		
	8	3.35		3.65		4.05	5.1
	9		4.20		2.1		
	10					3.8	
	11	3.46	3.86	3.5			5.2
	12				2.1	2.85	
	13	2.36		1.7			3.9
	14		0.38		0.22		
	15	0.15		0.14		0.31	0.3
Oestrus →	16		0.09		0.1		0.18
		← MATED →					

Day of Pregnancy	EXPERIMENTAL CYCLES					
	Systemic Progesterone Levels ng/ml Plasma					
	Control Animals Arachis Oil 0.5 ml Daily days 10-21 of pregnancy		Test Animals Oestradiol Benzoate 10 ug s.c. daily days 10-21 of pregnancy			
	B22	W22	P22	Bl	G22	Y1
1P					0.08	
2P	0.18	0.06	0.22			0.28
3P				0.56	0.45	
4P	2.1					2.8
5P		2.7	2.65	3.5		
6P	2.8				2.7	
7P		2.5	2.3	3.3		4.1
8P					3.2	
9P	3.9	3.6	2.6			3.8
10P				2.7	2.9	
11P	3.7					2.7
12P		3.4	1.0	0.49		
13P	3.6				0.2	
14P		5.2	0.4	0.15		4.6
15P					0.12	
16P	5.9	5.3	0.17			3.9
17P				0.21	0.26	
18P	6.95					14.7
19P		9.6	0.21	0.18		
20P	14.9			0.15	0.09	
21P	18.6	16.7	0.15	0.12	0.21	31.0

Figure 23.

EFFECT OF EXOGENOUS OESTROGEN ON
CARDIAC PLASMA PROGESTERONE LEVELS IN
EARLY PREGNANT GUINEA-PIGS.

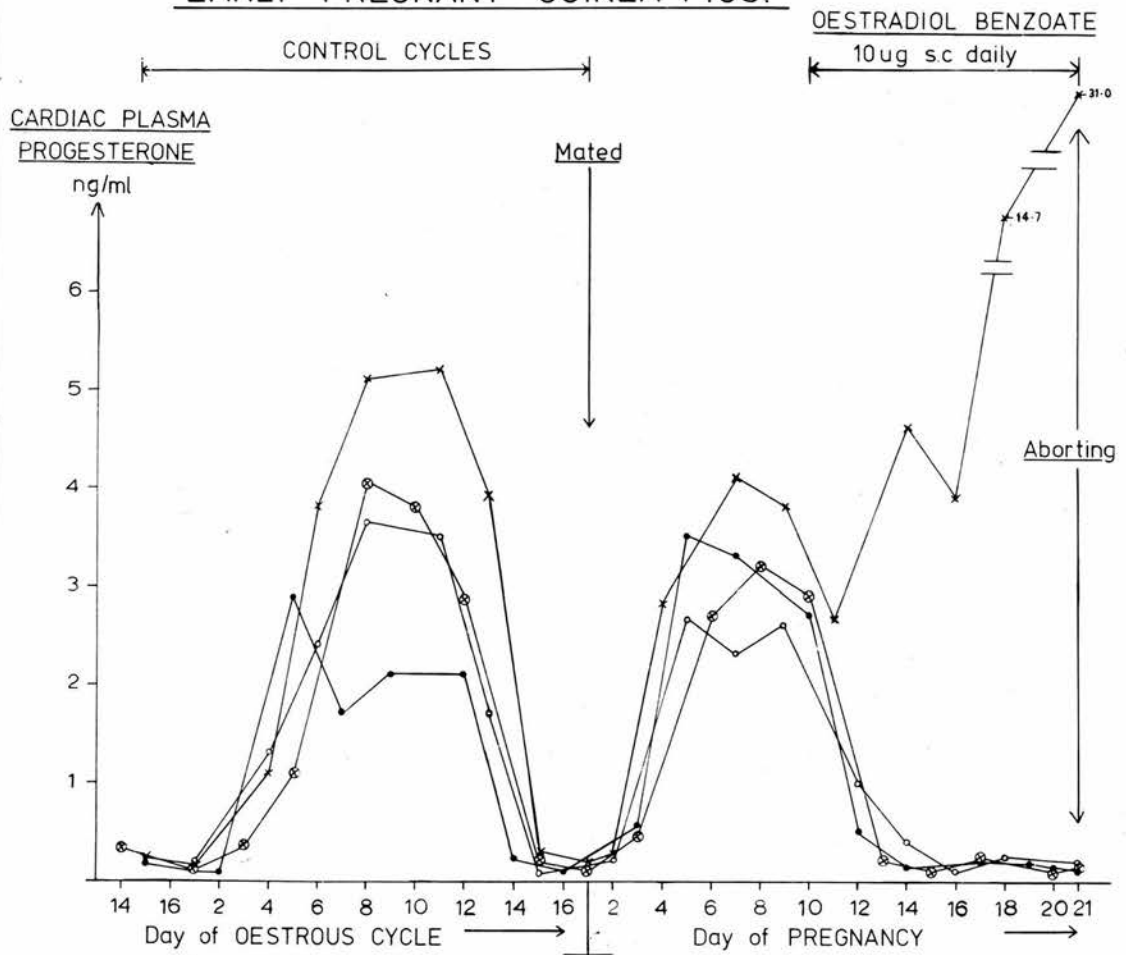


Figure 24.

EFFECT OF ARACHIS OIL ON
CARDIAC PLASMA PROGESTERONE LEVELS IN
EARLY PREGNANT GUINEA-PIGS.

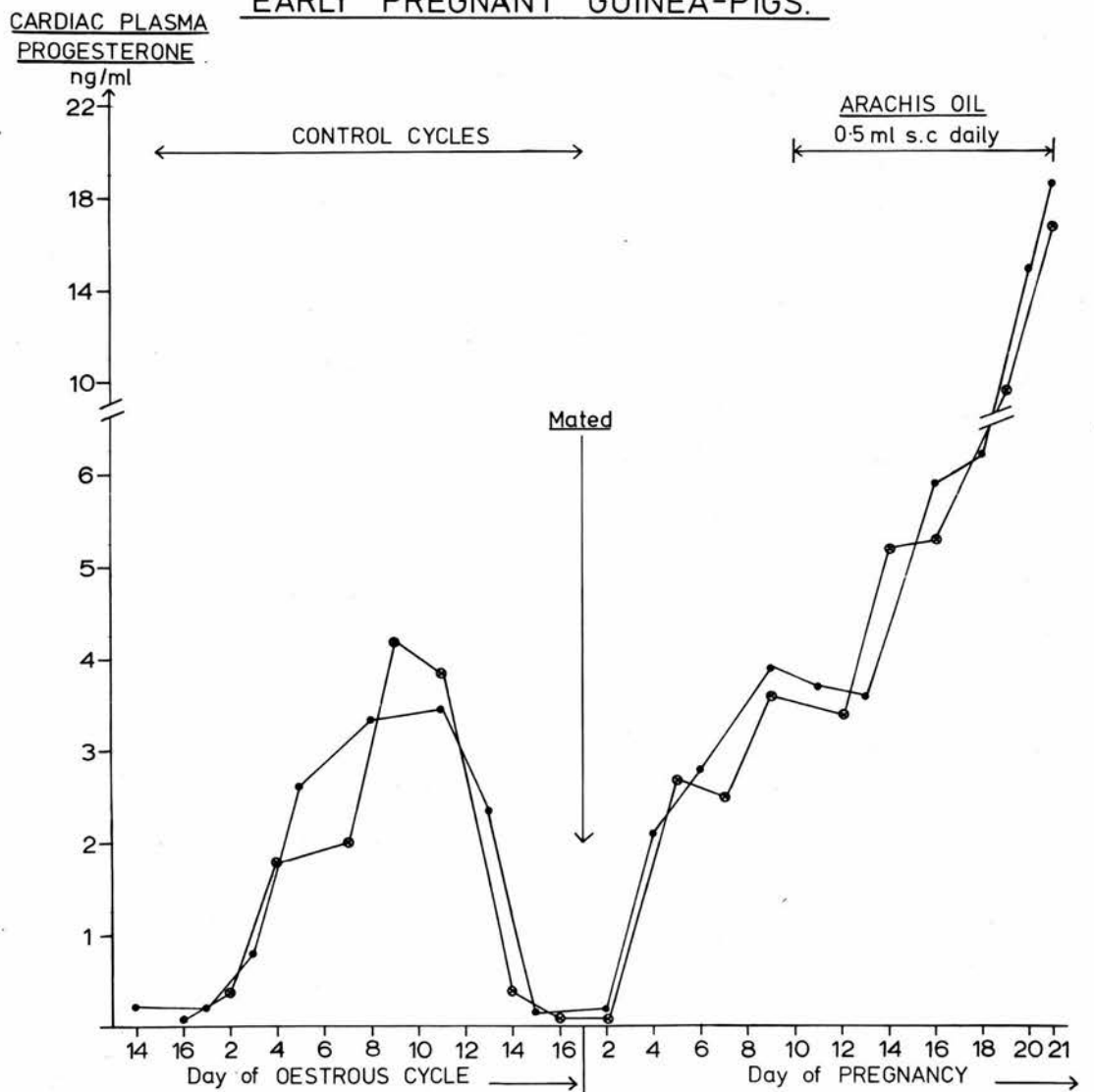
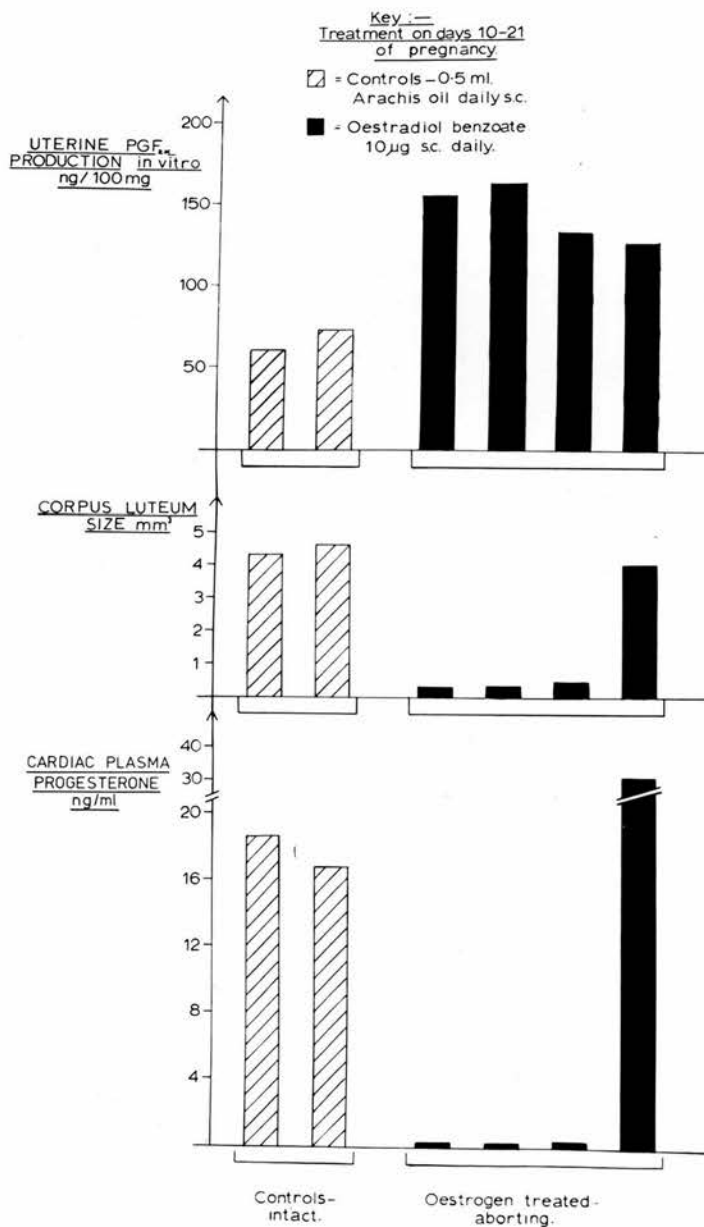


Figure 25.

STUDIES ON DAY 21 OF PREGNANCY
Effect of Exogenous Oestrogen on Uterine $PGF_{2\alpha}$
Production in vitro, Corpus Luteum Size and
Cardiac Plasma Progesterone Levels.



Conclusions

It is evident from these results that the administration of exogenous oestrogen to pregnant guinea-pigs can overcome the anti-luteolytic action of the conceptus thus causing termination of pregnancy.

In experiment 2 functional luteal regression was seen in three out of the four oestrogen treated animals by day 12 - 13 of pregnancy although this was not seen in experiment 1. Since the animals in experiment 2 were of much less body weight than those for experiment 1 it would appear that the oestrogen treatment used in experiment 1 was close to the threshold of effectiveness with respect to dose level rather than duration of administration. It can be seen from these results that, once the threshold is reached, the effect of the oestrogen takes only 2 - 3 days to become evident in the falling cardiac plasma progesterone levels.

The actual cause of abortion in these animals cannot be identified from these results. The placentae appeared very pale and this reduced vascularity might have been due to the increased uterine $\text{PGF}\alpha$ production, stimulated by the oestrogen. This loss of vascularity might be a primary cause of abortion with functional luteal regression as a secondary cause or the two may be complementary. It is interesting that the placental detachment observed in these experiments appears similar to that

described by Deanesly (1972) in pregnancy termination in ovariectomised animals. She was able to overcome this with exogenous progesterone which suggests that functional luteal regression might be the primary cause of abortion in the experiments just described.

It is interesting that although the progesterone levels had been low for seven days, by day 21 of pregnancy in animals P22, B1 and G22 no signs of oestrus had been observed in this time although there was slight opening of the vaginal membrane on day 21. This was probably associated with the actual onset of abortion. It is surprising that the pregnancy did survive for this duration in the presence of such low progesterone levels. The picture of persistently low progesterone levels from day 12 - 13 of pregnancy with a lack of return to oestrus contrasts to that seen if the conceptuses are surgically removed on days 12 - 13 of pregnancy following which oestrus recurs at the expected time of about day 16 (see main Introduction). This indicates that recurrence of oestrus requires the complete loss of the conceptuses from the uterus.

In one oestrogen treated animal in experiment 2 (Y1) the corpora lutea were structurally maintained and cardiac plasma progesterone levels were high yet there was placental detachment and increased in vitro uterine PGF_{α} production. The high

progesterone levels may indicate a compensatory mechanism attempting to overcome the placental detachment, which here appears to be due to the high uterine PGE₂ production. It would have been interesting to follow the cardiac plasma progesterone levels for a further time in this animal. Determination of uterine and utero-ovarian venous plasma levels of progesterone would have given information as to the source of the progesterone.

The results of the experiments described here show that the anti-luteolytic actions of the conceptus can be overcome by the administration of exogenous oestrogen although in one animal this treatment appeared to cause an increase in progesterone levels. The absence of effect on cardiac plasma progesterone levels in experiment 1 indicates that the dose of oestradiol benzoate used was on the threshold for effectiveness.

SECTION VII

Production of PGF_{2 α} , in vitro, by the uteri of spontaneously aborting guinea-pigs.

Introduction

The previous section has shown that termination of pregnancy by administration of exogenous oestrogen is associated with an increase in the amount of $\text{PGF}_{2\alpha}$ produced by the uteri in vitro compared to that for control animals at a similar stage of pregnancy. Spontaneous abortion may result from either genetic or placental deficiency and it is possible that prostaglandins may be involved in luteal regression at this time, the expulsion (where resorption does not occur) of the conceptus or placental disruption.

The animals considered in this section were observed to be aborting spontaneously and it was the aim of these experiments to compare in vitro uterine $\text{PGF}_{2\alpha}$ production and corpus luteum size in these animals to that of animals at a similar stage of intact pregnancies.

Methods

Observation of oestrous cycles and mating procedure were as described before (Section II). Five bilaterally pregnant guinea-pigs at various stages of pregnancy, observed to be aborting spontaneously from the appearance of blood in the vagina, were used. For each aborting animal a control bilaterally pregnant guinea-pig was used at the same stage of pregnancy (i.e. a total of five control animals).

The animals were killed by stunning and incising the neck on the

day that abortion was first noted or on the appropriate day for the control animals.

In all animals, the ovaries were removed, immediately placed in 10% formol saline and treated in an identical manner to that described in Section II, mean luteal size being determined in each case.

The uteri were in every case removed and freed of any placental and embryonic tissue, these tissues being discarded after noting their appearance. The uteri were then weighed, chopped, homogenised and incubated in Tyrode's solution exactly as described in Section II. After incubation the homogenates were stored at -20°C until use. The levels of PGE_{α} in the incubates were determined by solid-phase R.I.A., as previously described (Section IB). The incubates were then subjected to the full solvent extraction procedure for prostaglandins (Section IA) and identification of the PGE_{α} as $\text{PGF}_{2\alpha}$ was made by combined gas chromatography-mass spectrometry (GC-MS) where possible.

In three of the aborting animals and in three control animals a 2.5 ml cardiac blood sample was collected prior to killing, by the methods described in Section VI. After centrifuging, the plasma was stored at -20°C until use. The progesterone levels in the plasma were determined by R.I.A. as described in Section VI. The assays were carried out by Miss A. Pelais, of this Department.

Results

The results are summarised in Table 20. and fig. In all the aborting animals in vitro uterine $\text{PGF}_{2\alpha}$ production was much greater than that for intact pregnant animals, at an equivalent stage of pregnancy. Successful GC-MS identification of $\text{PGF}_{2\alpha}$ was made in four of the five aborting animals, but one sample was too heavily contaminated to allow this. Successful identification of $\text{PGF}_{2\alpha}$ was similarly made for two of the intact pregnant animals, but the amounts present were low and contamination prevented identification in the other samples.

The results for mean corpus luteum size were not consistent. Complete structural regression was only seen in one aborting animal, at day 25 of pregnancy, where the conceptuses had passed beyond the cervix into the vagina. However, cardiac plasma progesterone levels, where measured, in the aborting animals were always lower than in the intact animal at an equivalent stage of pregnancy. These low levels indicate functional luteal regression in these animals.

In the aborting animals decreased corpus luteum size and cardiaceplasma progesterone levels were always associated with increased uterine in vitro $\text{PGF}_{2\alpha}$ production, when compared with the intact pregnant animals. However, in any pairs of aborting and control animals there was no correlation between the extent of

Table 20.

Condition of pregnancy, corpus luteum size, cardiac plasma progesterone and in vitro uterine PGF α production in aborting and intact pregnant guinea pigs.

	DAY OF PREGNANCY											
	15		16		23		25		27			
	Aborting	Intact	Aborting	Intact	Aborting	Intact	Aborting	Intact	Aborting	Intact		
Cervix dilated	Slightly	No	Slightly	No	No	No	Yes	No	No	No		
Position of embryos	uterus	uterus	uterus	uterus	uterus	uterus	vagina	uterus	uterus	uterus		
Placenta detached	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No		
Mean corpus luteum size (mm 2)	2.0	3.1	1.8	3.6	3.6	4.2	0.9	4.6	3.8	4.9		
Cardiac Plasma progesterone (ng/ml)	-	-	2.12	5.9	6.68	52.0	0.09	45.0	-	-		
<u>In vitro</u> Uterine PGF α production	ng PGF α /100 mg tissue	GC-MS identification as PGF 2α	168	29	186	32	147	63	155	72	114	48

the decrease in corpus luteum size and cardiac plasma progesterone levels with the extent of the increase in in vitro uterine $\text{PGF}_{2\alpha}$ production, in the aborting animal compared to its respective control animal.

Conclusions

The results presented in this Section have shown an association between an increase in the ability of the uterus to produce $\text{PGF}_{2\alpha}$ in vitro and the occurrence of abortion in guinea-pigs at several stages of early pregnancy. A decrease in luteal size and a fall in cardiac plasma progesterone level were also observed in the aborting animals. However, no causal relationship can be established between these observations and the phenomenon of abortion.

It is interesting that complete structural luteal regression was only observed in the aborting animal where the cervix was fully dilated and the embryos had passed from the uterus to the vagina. Functional luteal regression, but not full structural luteal regression, was present in the other aborting animals, where cardiac plasma progesterone levels were determined. This indicates that structural and functional luteal regression can be distinct events in the pregnant animal, a conclusion already reached, based on the results of Sections V and VI, for the non-pregnant animal. The possible connection between cervical

dilation and entry of the embryo into the vagina with structural luteal regression is worthy of further investigation.

The measurement of utero-ovarian venous plasma levels of $\text{PGF}_{2\alpha}$ and measurement of luteal progesterone content in addition to luteal size and cardiac plasma progesterone levels in aborting guinea-pigs would allow better evaluation of possible cause and effect relationships. The uterine in vitro production of $\text{PGF}_{2\alpha}$ measures only the ability of the uterus to produce $\text{PGF}_{2\alpha}$ and an equivalent production and release of $\text{PGF}_{2\alpha}$ cannot be assumed for the uterus in vitro. Utero-ovarian venous plasma levels of $\text{PGF}_{2\alpha}$ would, therefore, clarify this position. Similar information for oestradiol- 17β levels would also be relevant.

It is interesting that these abortions occurred at the 'risk' times. Thus abortions occurred on days 15 and 16 pregnancy when uterine production and release of $\text{PGF}_{2\alpha}$ would normally increase in the non-pregnant animal and also on days 23, 25 and 27, the time of transfer from luteal to placental maintenance of pregnancy. Uterine in vitro production of $\text{PGF}_{2\alpha}$ in pregnant animals normally increases around this time (see results for control animals, Section II) although the amount produced is still much less than that for a day 15 non-pregnant guinea-pig uterus (Section II).

It may be concluded, therefore, that the ability of the

uterus to produce $\text{PGF}_{2\alpha}$ is greater in aborting guinea-pigs than in intact animals at an equivalent stage of early pregnancy. There is evidence of functional and some structural luteal regression in the aborting animals. However, no definitive role can be established for the increased uterine $\text{PGF}_{2\alpha}$ production in vitro in connection either with luteal regression, placental detachment or extrusion of the embryo in the aborting animals. The conceptuses in the aborting animals at day 15 and 16 of pregnancy would probably in fact have been resorbed rather than expelled via the vagina. Measurement of the various additional parameters considered earlier might clarify the role of uterine production and release of $\text{PGF}_{2\alpha}$ in abortions occurring at an early stage of pregnancy.

SECTION VIII

Studies on unilaterally pregnant guinea-pigs with the non-pregnant uterine horn transected and separated from the pregnant uterine horn.

Introduction

The anti-luteolytic actions of the conceptus appear to have both local and systemic components.

The local action is suggested by the greater reduction in utero-ovarian venous plasma levels of oestradiol-17 β and PGE α on the pregnant side, compared to the non-pregnant side in unilaterally pregnant animals. Similarly a greater reduction in the ability to synthesise PGE $_{2\alpha}$ in vitro is seen for the pregnant uterine horn compared to the non-pregnant horn in unilaterally pregnant animals. The slight direct inhibition of PGE α synthesis by the conceptus, seen in Section would also form part of the local component of the anti-luteolytic action of the conceptus.

However, the bilateral luteal maintenance observed in unilaterally pregnant guinea-pigs is evidence for the systemic anti-luteolytic actions of the conceptus.

The relative significance of the two components of the anti-luteolytic action of the conceptus requires further investigation. The aim of the experiments to be presented in this section is to prevent the local action of the conceptus on the non-pregnant uterine horn by transection and special separation of that horn from the pregnant horn. The in vitro uterine PGE α production and luteal function will be compared for each side in these animals and compared to that for control animals.

Experiment 1. Observations on the course of pregnancy in guinea-pigs with unilaterally transected uteri: determination of uterine in vitro PGF_α production, cardiac plasma progesterone levels and luteal size on days 7 and 15 of the oestrous cycle and day 15 of pregnancy.

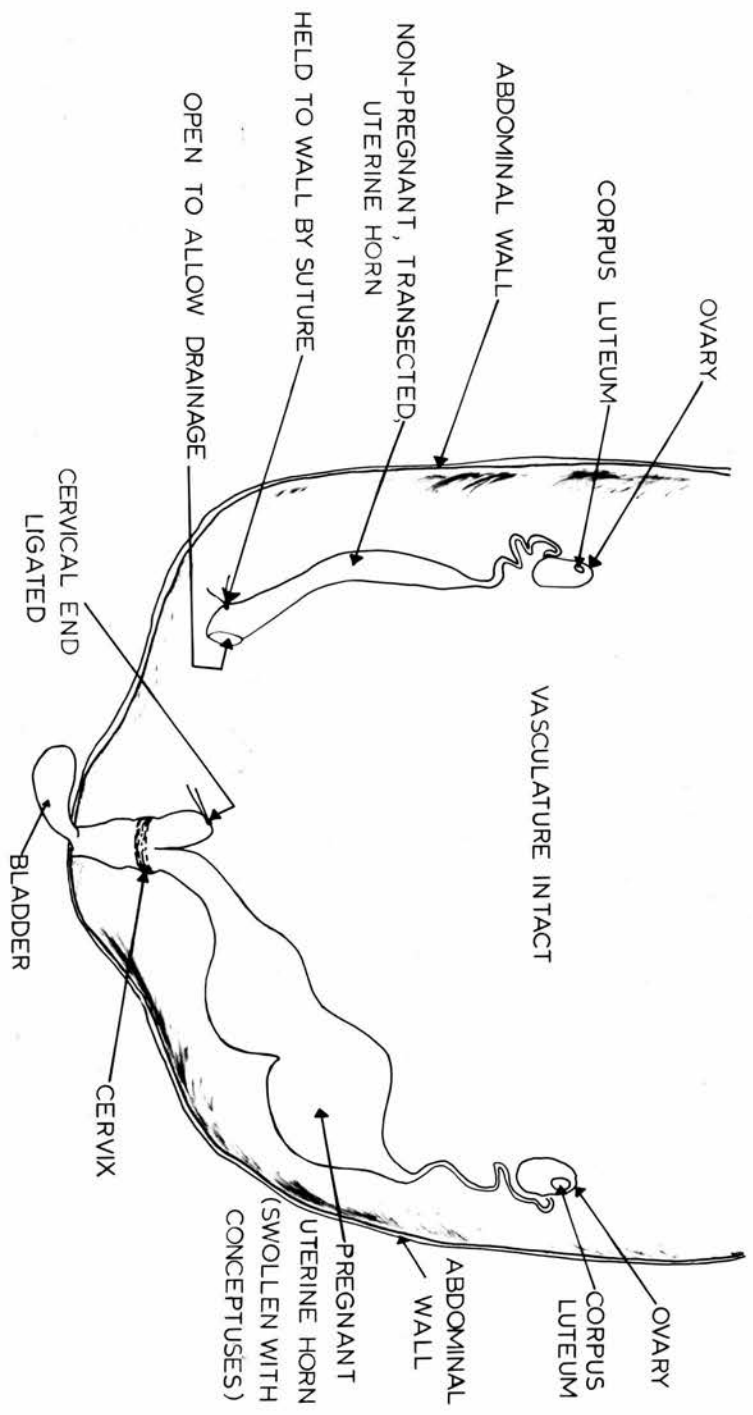
Methods

In all guinea-pigs at least two preliminary control oestrous cycles were observed, as described before (Section II). Ten guinea-pigs were mated by the method previously described (Section II) to form the pregnant-controls. Five of these animals became bilaterally pregnant, the other five became unilateral pregnancies. All ten pregnant-control guinea-pigs were used on day 15 of pregnancy.

Eight guinea-pigs were used as non-pregnant controls, five being used on day 15, and three on day 7, of the oestrous cycle.

In thirteen non-pregnant guinea-pigs unilateral uterine transection was performed under pentobarbitone anaesthesia (30 mg/Kg i.p.). In each case the right uterine horn was ligated at the cervical junction and transected just superior to the ligature (see Fig. 26a). The transected horn was carefully drawn over to the abdominal wall on the right side and secured by silk suture, the transection incision being left open to allow drainage of any uterine fluid. Care was taken throughout to avoid damage to any of the vasculature. After two normal

Diagram to show Uterine Transection



post-operative cycles had been observed five of these animals were mated using the method previously described (Section II). These animals were used on day 15 of pregnancy. The remaining eight formed non-pregnant controls. Five of these were used on day 15, and three on day 7 of the oestrous cycle.

In all the animals used on day 15 of the oestrous cycle or pregnancy a 2.5 ml cardiac blood sample was taken just prior to killing. The method used was that described before (Section VI). The blood was centrifuged immediately and the plasma stored at -20°C until use.

In every case the animals were killed on the appropriate day, by stunning and incising the neck. The uteri were removed and freed of any placental or embryonic tissue where necessary, these tissues being discarded. In every animal the two uterine horns (transected and pregnant, pregnant and non-pregnant or left and right) were treated separately throughout. Thus each uterine horn was weighed, chopped and homogenised in Tyrode's solution, exactly as described in Section II. The homogenates were each then incubated for 90 mins, at 37°C , with continuous oxygenation and shaking. The incubates were then stored at -20°C until use.

In all animals used on day 15 of pregnancy the ovaries were also removed and immediately placed in 10% formal saline. The ovaries were then serially sectioned and stained, and corpus luteum size was determined, by the methods described in Section II.

Ovaries from the other animals were discarded.

The progesterone levels in the cardiac plasma samples were determined by RIA, by the method described in Section VI. The assays were kindly carried out by Miss A. Pelanis of this Department.

The PGF_{α} levels in the uterine incubates were determined by solid-phase RIA, as described in Section IB. The amount of PGF_{α} produced in vitro was expressed as ng/100 mg uterine tissue.

All the results were subjected to statistical analysis by the Student's t-test.

Results

The results are shown in Tables 21 and 22 and Fig. 26b.

In each of the animals with transected uteri the transected uterine horn was still open at the cervical end, allowing successful drainage of uterine fluid. There was no distension of the transected horn, but in each case there was slight thickening of the uterine wall and shortening of the horn in comparison to the non-pregnant uterus. In the day 15 unilaterally pregnant animals with transected uteri, the non-pregnant (transected), uterine horn showed decidualisation similar to that of the pregnant horn, but slightly greater than that of the non-pregnant uterine horn of control, intact, day 15 unilaterally pregnant animals. The ovaries of all animals appeared macroscopically normal.

On day 15 of pregnancy, in the unilaterally pregnant guinea-pigs with transected uteri, the mean amount of PGF_{α} produced in vitro

Table 21

Uterine production of PGF α in vitro, corpus luteum size and cardiac plasma progesterone levels in pregnant guinea-pigs with intact or transected uteri.

	Day of Pregnancy							
	15							
	Control Bilaterally Pregnant		Control Unilaterally Pregnant		Non-Pregnant		Uterus Transected Intact Horn Pregnant	
Uterine Horn	Left	Right	Pregnant	Non-Pregnant	Intact Pregnant	Transected Non-Pregnant		
Mean amount of PGF α produced by uterine horn <u>in vitro</u> ng PGF α /100 mg tissue \pm S.E.M.	23 \pm 4	21 \pm 3	28 \pm 3	48 \pm 2	25 \pm 3	72 \pm		
Mean Corpus Luteum size in adjacent ovary - mm $\bar{5}$ \pm S.E.M.	3.1 \pm 0.5	3.3 \pm 0.5	3.8 \pm 0.6	2.8 \pm 0.6	4.0 \pm 0.4	1.87 \pm 0.3		
Mean Cardiac Plasma Progesterone level ng/ml \pm S.E.M.	6.7 \pm 1.7		5.8 \pm 0.6		2.53 \pm 0.5			

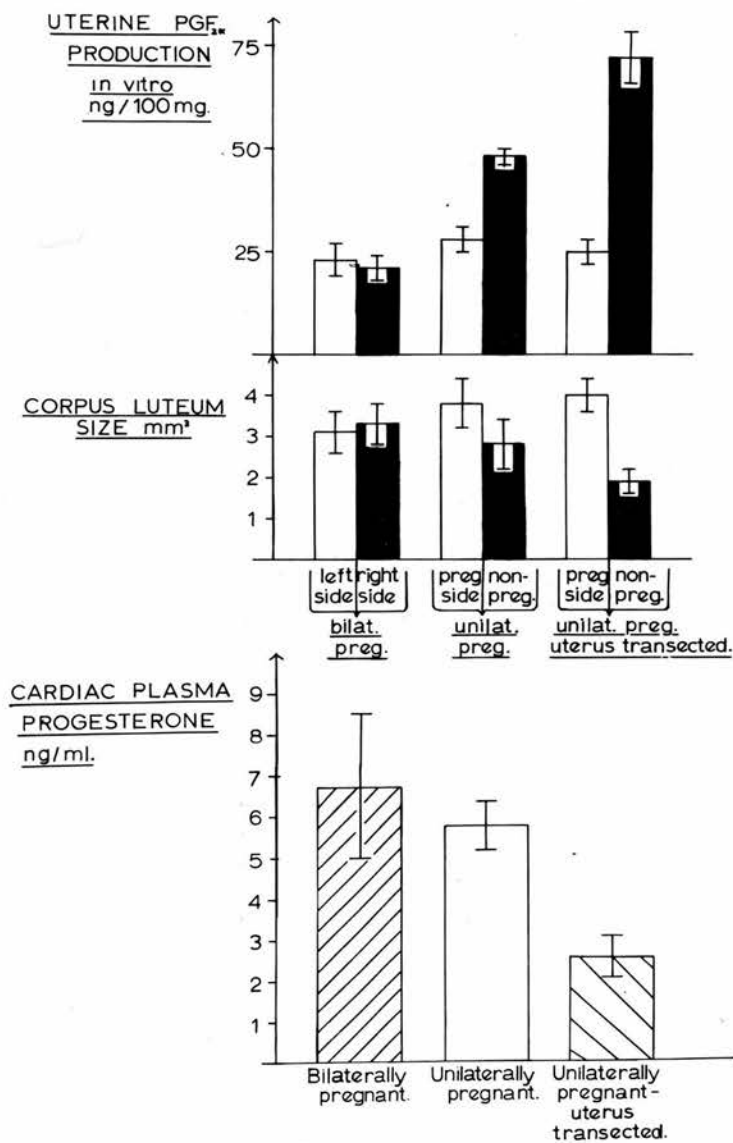
Table 22

Uterine production of PGF_X in vitro and cardiac plasma progesterone levels in non-pregnant guinea-pigs with intact or transected uteri.

		Day of Oestrous Cycle.							
		7				15.			
Uterine Horn	Control		Uterus Transected		Control		Uterus Transected		
	Left	Right	Intact (Left)	Transected (Right)	Left	Right	Intact (Left)	Transected (Right)	
Mean amount of PGF _X produced by uterine horn <u>in vitro</u> ng PGF _X /100 mg tissue \pm S.E.M.	46 \pm 4	42 \pm 4	44 \pm 2	47 \pm 6	118 \pm 3	112 \pm 5	116 \pm 4	119 \pm 5	
Mean cardiac plasma progesterone level ng/ml \pm S.E.M.	—		—		0.03 \pm 0.24		0.31 \pm 0.2		

Figure 26b

DAY 15 PREGNANT GUINEA-PIGS.
The Effect of Uterine Transection on $PGF_{2\alpha}$
Production by the Uterus in vitro, Corpus
Luteum Size and Cardiac Plasma
Progesterone Levels.



by the transected (non-pregnant) uterine horn (72 ng/100 mg tissue) was significantly greater ($P < 0.001$) than that produced by the intact (pregnant) uterine horn (25 ng/100 mg tissue). In the control intact, unilaterally pregnant guinea-pigs at day 15 the mean amount of PGF_{α} produced by the non-pregnant uterine horns in vitro was 48 ng/100 mg tissue, significantly greater ($P < 0.001$) than that produced by the pregnant uterine horns in vitro (28 ng/100 mg tissue), but significantly less ($P < 0.01$) than that produced in vitro at day 15 by the transected, non-pregnant, uterine horns in the unilaterally pregnant guinea-pigs with transected uteri.

There was no significant difference between the mean amounts of PGF_{α} produced in vitro by the right and left uterine horns of the bilaterally pregnant animals or of any of the non-pregnant animals. Nor was there any significant difference between the mean amounts of PGF_{α} produced in vitro by intact and transected uteri on either day 7 or day 15 of the oestrous cycle.

In the unilaterally pregnant guinea-pigs with transected uteri the mean size of the corpora lutea in the ovaries adjacent to the transected, non-pregnant, uterine horns on day 15 of pregnancy was 1.87 mm^3 , significantly smaller ($P < 0.01$) than that for the corpora lutea in the ovaries adjacent to the intact, pregnant, uterine horns (4.0 mm^3).

There was no significant difference between the mean sizes of

corpora lutea in the right and left ovaries of day 15 bilaterally pregnant animals. However, in day 15 intact, unilaterally pregnant guinea-pigs, the mean size of the corpora lutea in the ovaries adjacent to the non-pregnant uterine horns was 2.8 mm^3 , smaller than the corpora lutea in the ovaries adjacent to the pregnant uterine horns (3.8 mm^3), but not significantly so.

The mean cardiac plasma progesterone level in the day 15 unilaterally pregnant guinea-pigs with transected uteri was 2.5 ng/ml , significantly less ($P < 0.02$) than that for day 15 intact, unilaterally pregnant animals (5.8 ng/ml) but significantly greater ($P < 0.01$) than the level for either day 15 intact, non-pregnant animals (0.03 ng/ml) or day 15 non-pregnant animals with transected uteri (0.3 ng/ml). There was no significant difference between the mean cardiac plasma progesterone level in day 15 bilaterally pregnant animals (6.7 ng/ml) and that for day 15, intact, unilaterally pregnant animals (5.8 ng/ml).

Conclusions

Corpora lutea in intact, day 15, unilaterally pregnant guinea-pigs are normally bilaterally maintained (Deanesly, 1967). The results presented here agree with this observation and the mean cardiac plasma progesterone level in these animals has also been found to show no significant difference from that for day 15 bilaterally pregnant animals. Thus in day 15 intact

unilaterally pregnant and bilaterally pregnant guinea-pigs the systemic anti-luteolytic actions of the conceptus are seen in the bilateral structural luteal maintenance and high circulating levels of progesterone, the presence of PBG presumably aiding the latter (Illingworth, et al., 1973).

In contrast, however, in the day 15 pregnant guinea-pigs with transected uteri, the corpora lutea in the ovary adjacent to the non-pregnant, transected, uterine horn were significantly smaller than those in the ovary adjacent to the pregnant uterine horn. Also in those animals with transected uteri, the mean cardiac plasma progesterone level was significantly less than that for intact day 15 unilaterally pregnant guinea-pigs. It is evident, therefore, that interrupting the continuity between the pregnant and non-pregnant uterine horns, by transection of the non-pregnant horn, interferes with the anti-luteolytic actions of the conceptus.

The results presented here also show that loss of continuity between pregnant and non-pregnant uterine horns is associated with an increase in ability to produce PGF_{α} in vitro for the transected, non-pregnant horn. The ability to produce PGF_{α} in vitro remained very low for the pregnant uterine horns of these animals. In intact, unilaterally pregnant guinea-pigs the non-pregnant uterine horn normally shows a greater ability to produce PGF_{α} in vitro than does the pregnant horn (see also Section II), this observation leading initially to the suggestion of a local component to the anti-

luteolytic actions of the conceptus. However, the amount of PGF_{α} produced in vitro by the transected, non-pregnant uterine horns of unilaterally pregnant animals is significantly greater than for those from intact animals, although still significantly less than the amount of PGF_{α} produced in vitro by the uteri of non-pregnant guinea-pigs on day 15 of the oestrous cycle. The surgical procedures involved in uterine transection do not themselves lead to increased production of PGF_{α} in vitro by the operated uterine horns. This is clearly demonstrated by the low production of PGF_{α} in vitro observed for both operated and intact uterine horns from guinea-pigs on day 7 of the oestrous cycle. There was no significant difference between the amount of PGF_{α} produced in vitro by the intact uteri and that produced by the uteri with one horn transected for non-pregnant guinea-pigs at this time. Both uterine horns, from animals with one horn transected, showed the normal cyclical, increase in the amount of PGF_{α} produced in vitro on day 15 of the oestrous cycle.

It may be concluded, therefore, that interruption of the continuity between non-pregnant and pregnant uterine horns of unilaterally pregnant guinea-pigs, by transection of the non-pregnant horn, results in an increase in the ability of the non-pregnant horn to produce PGF_{α} in vitro, on day 15 of pregnancy. This again indicates interference with a local component to the anti-luteolytic actions of the conceptus. The ability to produce

PGF α in vitro of the non-pregnant, transected, uterine horn of unilaterally pregnant animals does not, however, return to the level observed for the non-pregnant uterus on day 15 of the oestrous cycle, suggesting that there is also a significant systemic component to the anti-luteolytic actions of the conceptus.

The reduced luteal size and increased uterine production of PGF α in vitro for the non-pregnant side in unilaterally pregnant animals with transected uteri, together with their reduced circulating levels of progesterone may indicate the pending demise of the conceptuses in these animals. Further studies are necessary to determine whether the smaller luteal size represents the onset of regression or merely a slower growth rate. The reduced circulating level of progesterone may indicate a reduced luteal progesterone contribution, a reduced circulating level of PBG or both; both conditions being potentially fatal for the conceptus.

The results of this experiment clearly indicate a strong local component to the anti-luteolytic actions of the conceptus in addition to a significant systemic component. The importance of the local component for the survival of the conceptus remains to be determined.

Experiment 2. Observations on the course of pregnancy in guinea-pigs with unilaterally transected uteri. Determination of uterine in vitro PGF_{α} production, corpus luteum size and cardiac plasma progesterone levels on day 20 of pregnancy.

Introduction

It has been shown in experiment 1 that the corpora lutea in the ovary adjacent to the transected, non-pregnant uterine horn of unilaterally pregnant guinea-pigs are smaller than those in the ovary adjacent to the pregnant horn on day 15 of pregnancy. It is the aim of this experiment to determine the status of the corpora lutea in the ovary of the non-pregnant transected side at a later stage of pregnancy and to compare them with the corpora lutea of the ovary on the gravid side. This should indicate whether the corpora lutea in the ovary adjacent to the transected non-pregnant horn on day 15 of pregnancy, in unilaterally pregnant animals, are starting to regress at this stage or merely growing more slowly than those in the ovary adjacent to the pregnant uterine horn.

Methods

Observation of oestrous cycles, uterine transection methods and mating procedure were exactly as described for experiment 1. Three intact spontaneously unilaterally pregnant guinea-pigs and three unilaterally pregnant guinea-pigs with a transected, non-

pregnant uterine horn were used.

All animals were used on day 20 of pregnancy. Blood sampling by cardiac puncture immediately prior to killing, determination of plasma progesterone levels, treatment of uteri and determination of in vitro production of PGE_α , treatment of ovaries and determination of luteal sizes were all exactly as described for experiment 1.

The results were subjected to statistical analysis by the Student's t-test.

Results

The results are shown in Table 23 .

The appearance of both intact and transected unilaterally pregnant uteri on day 20 of pregnancy was very similar to that observed on day 15 of pregnancy in Experiment 1. The ovaries in all animals appeared macroscopically normal.

In the control, intact, unilaterally pregnant animals there was no significant difference between the amount of PGE_α produced in vitro by the non-pregnant and pregnant uterine horns, or between the size of corpora lutea in the ovaries on the non-pregnant and pregnant sides.

In the unilaterally pregnant animals with transected uteri the transected, non-pregnant uterine horn produced significantly ($P < 0.05$) more PGE_α in vitro than the intact, pregnant uterine horn. Also in these animals both the transected, non-pregnant, uterine

Table 23

Day 20 of Pregnancy - Uterine PGF α production in vitro, corpus luteum size and cardiac plasma progesterone levels, on day 20 of pregnancy, in guinea-pigs with intact or transected uteri.

	CONTROL		UTERUS TRANSECTED			Results of Student's t-test
	UNILATERALLY PREGNANT		INTACT HORN PREGNANT			
Uterine Horn	Pregnant	Non-Pregnant	Intact Pregnant	Transected Non-Pregnant		
Mean amount of PGF α produced by uterine horn in vitro (ng/100 mg tissue \pm S.E.M.)	61 \pm 2 A	62 \pm 2 B	73 \pm 1 C	83 \pm 3 D	I	CVSDP < 0.05 > 0.02 AVSCP < 0.01 > 0.001 BVSDP < 0.01 > 0.001
Mean Corpus luteum size in adjacent ovary (mm $\bar{5}$ \pm S.E.M.)	4.8 \pm 0.3 W	4.3 \pm 0.2 X	4.1 \pm 0.2 Y	3.1 \pm 0.2 Z	II	YVSZP < 0.01 > 0.001 XVSZP < 0.01 > 0.001
Mean Cardiac plasma progesterone level (ng/ml \pm S.E.M.)	29.6 \pm 1.7 E		19.0 \pm 1.2 F		III	EVSF P < 0.01 > 0.001

horns produced significantly ($P < 0.01$) more $\text{PGF}\alpha$ in vitro than their counterparts in the control, unilaterally pregnant animals. The corpora lutea in the ovaries adjacent to the transected, non-pregnant, uterine horns were significantly ($P < 0.01$) smaller than those in the ovaries adjacent to the pregnant horns of the same animals and those in the ovaries adjacent to the non-pregnant uterine horns of the control, intact, unilaterally pregnant animals. None of the corpora lutea in the ovaries of any of the animals with or without transected uteri showed any involution or other signs indicative of structural regression. The mean cardiac plasma progesterone level in the unilaterally pregnant animals with transected uteri was significantly less ($P < 0.01$) than that for the control unilaterally pregnant animals.

Conclusions

In the day 20 unilaterally pregnant guinea-pigs with transected uteri, the corpora lutea in the ovary adjacent to the transected, non-pregnant uterine horn appear to be structurally maintained although significantly smaller than those in the ovary adjacent to the pregnant uterine horn in the same animals, and significantly smaller than those in the ovaries adjacent to the non-pregnant uterine horns of the control, intact, unilaterally pregnant animals. The mean size of the corpora lutea in the ovaries adjacent to the transected, non-pregnant uterine horns on day 20 of pregnancy is,

however, slightly larger than that recorded for day 15 of pregnancy in experiment 1. It would appear, therefore, that the corpora lutea observed in the ovaries adjacent to the non-pregnant, transected, uterine horns on day 15 of pregnancy, which were smaller than those in the ovaries adjacent to the pregnant horns, were not starting to regress, but were growing more slowly than those adjacent to the pregnant uterine horn, and that this is still the case on day 20 of pregnancy.

In the control, intact, unilaterally pregnant guinea-pigs corpora lutea on both sides are still structurally maintained on day 20 of pregnancy, in agreement with the observations of Deanesly (1967), and the mean luteal size on both sides is greater than that found for day 15 of pregnancy in experiment 1. The amount of $\text{PGF}\alpha$ produced by both uterine horns of these animals, in vitro, is greater than that for day 15 of pregnancy, in agreement with the results of Section II, although still much less than that associated with luteal regression as seen for non-pregnant uteri on day 15 of the oestrous cycle (see experiment 1 and Section II). The mean cardiac plasma progesterone level of these animals, on day 20 of pregnancy, is greatly increased compared to that found for control, unilaterally pregnant guinea-pigs on day 15 of pregnancy in experiment 1.

In the unilaterally pregnant guinea-pigs with transected uteri the mean cardiac plasma progesterone level was significantly lower

on day 20 of pregnancy than that found for the control, intact, animals at the same time. However, the level found for those animals with transected uteri is still within the range previously found for intact, bilaterally pregnant animals at this stage of pregnancy (Section VI) and, therefore, probably does not represent an immediate threat to the well being of the conceptus. However, in the unilaterally pregnant animals with transected uteri both uterine horns produced significantly more PGF_{α} in vitro than did the uterine horns of the control animals on day 20 of pregnancy, and although the transected non-pregnant horns did produce slightly more PGF_{α} in vitro than the pregnant horns in the same animals the difference was not as marked as it was on day 15 of pregnancy. At that time increased PGF_{α} production in vitro was observed only for the non-pregnant transected uterine horns. It would appear, therefore, that interruption of the continuity between pregnant and non-pregnant uterine horns by transection has, by day 20 of pregnancy, resulted in a sufficient reduction in the inhibition of events leading to PGF_{α} production to allow stimulation of uterine PGF_{α} production to occur also on the pregnant side. Increased ovarian oestradiol secretion on the non-pregnant, transected side could also act on the pregnant uterine horn to cause this result. These results would suggest, therefore, that there is a local component to the anti-luteolytic actions of the conceptus acting at the level of ovarian oestrogen secretion. This was suggested by previous results also (Section V).

Determination of oestradiol levels in the utero-ovarian venous plasma of these animals would clarify this situation. The amount of PGF_{α} produced in vitro by both uterine horns of the unilaterally pregnant animals with transected uteri, although greater than that for the control animals, is still less than that associated with luteal regression indicating that there is still quite effective anti-luteolytic activity present. Day 20 is, however, approaching the time when the corpus luteum is no longer necessary for the maintenance of pregnancy (see Introduction) and consequently the necessity for anti-luteolytic activity is reducing at this time.

Thus although uterine transection in unilaterally pregnant guinea-pigs has quite clearly interfered with local components of the anti-luteolytic actions of the conceptus, none of the effects would appear serious enough by day 20 to be likely to cause termination of the pregnancy.



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Experiment 3. Observations on the course of pregnancy in guinea-pigs with unilaterally transected uteri: Determination of uterine PGF_{α} production in vitro, cardiac plasma progesterone levels and luteal size in animals which aborted.

Introduction

The results of the last two experiments showed that uterine transection did interfere with local components to the anti-luteolytic actions of the conceptus, but it did not appear that the reduced rate of luteal growth or reduced cardiac plasma progesterone levels were serious enough to be fatal for the conceptus.

The aim of this experiment was to determine whether or not pregnancy will continue to term in guinea-pigs with unilaterally transected uteri. If abortion occurred uterine PGF_{α} production in vitro, cardiac plasma progesterone levels and corpus luteum size were determined.

Methods

The observation of oestrous cycles and method of uterine transection were exactly as described for experiment 1. After the uterine transection four control oestrous cycles were observed prior to mating. Mating procedure was as described for experiment 1. After mating the animals were examined daily for opening of the vaginal membrane and were kept until term or the occurrence of

abortion. The onset of parturition or abortion was taken as the day that the vaginal membrane opened and blood appeared in the vagina.

Five guinea-pigs pregnant in one uterine horn, the other having been transected were used. All animals were used on the first day of parturition or abortion. Blood sampling by cardiac puncture immediately prior to killing, determination of plasma progesterone levels, treatment of uteri and determination of in vitro production of PGF_{α} , treatment of ovaries and determination of luteal sizes were all exactly as described for Experiment 1.

Where fluid had accumulated in the transected, non-pregnant uterine horn the volume was measured, progesterone levels were determined (by Miss A. Pelanis) by R.I.A. as described in Section VI and PGF_{α} levels were determined by solid-phase R.I.A. as described in Section IB.

Results

The results are shown in Table 24.

Pregnancy did not continue to term in any of the five guinea-pigs. Four animals aborted between days 20-23 of pregnancy, but in the fifth, pregnancy, survived until day 36. In every animal the cervical end of the transected, non-pregnant, uterine horn had healed over and fluid had accumulated causing extreme distension of the horn. The volume of fluid varied between animals, as did the PGF_{α} and progesterone levels in the fluid.

STUDIES ON DAY OF DETECTION OF ABORTION

Animal No. Stage of Pregnancy and State of Cervix	Cardiac Plasma Progesterone ng/ml	Intact, Pregnant Side		Transsected, Non-Pregnant Side				
		Uterine PGF _α Production in vitro ng PGF _α / 100mg tissue	Mean Corpus Luteum size mm ²	Uterine PGF _α Production in vitro ng PGF _α / 100mg tissue	Mean Corpus Luteum size mm ²	Uterine Fluid in Transsected Horn		
						Vol (ml)	PGF _α ng/ml	Progesterone ng/ml
1 Day 20 Cervix dilated	0.08	129	0.69	196	0.5	3.8	15.1	1.8
2 Day 21 Cervix dilated	0.09	120	0.47	149	0.33	4.1	11.2	1.7
3 Day 21 Cervix not dilated	0.36	124	1.13	167	0.98	2.9	9.5	2.1
4 Day 23 Cervix dilated	0.13	107	0.68	132	0.62	3.2	2.6	1.4
5 Day 36 Cervix dilated	0.16	164	0.32	226	0.30	3.7	3.5	1.7

Cardiac plasma progesterone levels were low in every animal associated in every case with small, structurally regressing corpora lutea. However, in one animal (No. 3) the mean corpus luteum size and cardiac plasma progesterone level was slightly higher than for the others. In this animal although there was blood in the vagina and the placentae were detached, the cervix was not dilated and the conceptuses were still in the uterus.

Uterine in vitro PGF_{α} production was high for both uterine horns in every animal, but in every case the amounts produced by the transected, non-pregnant uterine horn were greater than those produced by the intact, pregnant uterine horn.

Conclusions

Abortion in these animals was in every case associated with low cardiac plasma progesterone levels, structural luteal regression and high uterine in vitro PGF_{α} production. This is a very similar picture to that observed for spontaneous abortion in Section VII. Termination of these pregnancies can not be directly blamed on the loss of continuity between the non-pregnant and pregnant uterine horns by the transection because the accumulation of uterine fluid in the transected horns of these animals is likely to have been a contributory factor. The volume of fluid which accumulated was far greater than was ever observed by Ginther (1969a, 1971) in non-pregnant guinea-pigs where one uterine horn was ligated. The picture more closely resembles that seen in sheep with an auto-transplanted ovary where a

similar accumulation of uterine fluid was observed (Harrison, Heap, Horton and Poyser, 1972). Harrison et al., considered the accumulation of uterine fluid to be a consequence of the prolonged progesterone regime of these animals resulting from persistently maintained corpora lutea. Thus, with the closure of the transected uterine horn in these guinea-pigs preventing drainage the accumulation of large volumes of fluid might well be the result of the progesterone regime of pregnancy. Mechanical distension of the guinea-pig uterus has previously been shown to stimulate $\text{PGF}_{2\alpha}$ production by the uterus in vitro, (Poyser, Horton, Thompson and Los, 1970, 1971) and it seems likely that the very high amounts of PGF_{α} produced in vitro by the transected uterine horns are due to the distension by the accumulated uterine fluid. Although a high amount of PGF_{α} produced by the transected uterine horn was probably released in the uterine vein it should not have been able to directly affect the corpora lutea of the contralateral ovary, adjacent to the pregnant uterine horn, since 90% of $\text{PGF}_{2\alpha}$ is removed from the blood on one passage through the lungs (Piper, et al., 1970). However, the involvement of some action interfering with the maintenance of pregnancy resulting from the accumulation of uterine fluid in the transected horn rather than due to the loss of continuity between pregnant and non-pregnant uterine horns is supported by the results of Eckstein and McKeown (1955), where numbers of corpora lutea were compared for each ovary in unilaterally pregnant guinea-pigs on day

65 of pregnancy with one uterine horn transected in a very similar manner to that used in these experiments. Eckstein and McKeown (1955) observed only slight distension by uterine fluid in the transected horn and the pregnancies were successfully maintained at least until day 65 of pregnancy, a time very close to term.

It is possible that the animals used in this experiment were left for too many control cycles before use, allowing the cervical end of the transected horn to heal and close resulting in fluid accumulation during pregnancy. This problem could be overcome by the introduction of an artificial drainage channel for the transected horn, under which conditions the experiment would be worthy of repetition.

General Conclusions and Discussion to Section VIII

The results presented in this section strongly suggest that there is a significant local component to the anti-luteolytic actions of the conceptus which is disrupted if the continuity between pregnant and non-pregnant uterine horns of unilaterally pregnant guinea-pigs is interrupted by transection of the non-pregnant uterine horn.

There has previously been some controversy as to the functional state of the corpora lutea in the ovary adjacent to the non-pregnant uterine horn of unilaterally pregnant guinea-pigs, although there is now much evidence indicating that they are structurally and functionally maintained (Deanesly, 1967 & Sections II, V and this

Section). However, in attempts to investigate this both Deanesly (1967) and Oxenreider and Day (1967) used experiments involving transection of the non-pregnant uterine horn. Oxenreider and Day (1967) performed an identical transection procedure to that described in this Section. However, when animals were examined on day 25 of pregnancy the ovaries on the non-pregnant side were found to be without corpora lutea and it was impossible to determine whether or not ovulations had originally occurred in these ovaries. Deanesly (1967) is not completely clear as to her site of transection except to say that one uterine horn was severed or ligatured "near the middle", but she found no difference between corpora lutea on the left and right sides when examining the animals at day 56 of pregnancy. It is possible that by this stage of pregnancy any differences between the sides with regard to the corpora lutea have evened out since this is well beyond the time when an anti-luteolytic action by the conceptus is necessary. Alternatively, Deanesly (1967) may have left too small a portion of the uterine horn free of the local component of the anti-luteolytic actions of the conceptus for the loss of this component to become evident. It is clear, however, that the pregnancies in her animals had successfully survived to day 56 of pregnancy, in agreement with the results of Eckstein and McKeown (1955) and in contrast to the results of Experiment 3 of this Section. It would appear, therefore, that interruption of the continuity between the pregnant and non-pregnant uterine horns in unilaterally pregnant animals does not normally

put the entire pregnancy at risk. The abortions seen in experiment 3 were, therefore, almost certainly due to some factor or factors resulting from the distension of the non-pregnant uterine horn by accumulation of uterine fluid.

The results of these experiments indicate, therefore, that although there is a significant local component to the anti-luteolytic actions of the conceptus there is also a strong systemic component normally capable of maintaining the early pregnancy by maintaining luteal function, if the local component is in some way prevented from exerting its action.

SECTION IX

Oestrous cycle length in non-pregnant guinea-pigs treated with guinea-pig embryonic homogenate.

Introduction

An anti-luteolytic role for the conceptus, similar to that suggested for the guinea-pig, has also been proposed for the sheep (see main Introduction). In support of this it has been shown that homogenates of 14 days old sheep embryos will prolong the functional life-span of the corpus luteum if infused into the uterus of non-pregnant ewes from day 12 of the oestrous cycle (Rowson & Moor, 1967). A single infusion on day 10 caused only a slight extension of the luteal life-span while intra-muscular (i.m.) injections had no effect at all. Thus in sheep the anti-luteolytic action of the conceptus appears to need a continuing, local, type of action to achieve its full effect.

The experiments presented in this Section form a set of pilot experiments designed to determine whether the anti-luteolytic actions proposed for the guinea-pig conceptus may be demonstrated in a manner similar to that just described for the sheep.

Experiment 1. Oestrous cycle length in non-pregnant guinea-pigs after daily intra-peritoneal (i.p.) injections of guinea-pig embryonic tissue homogenate.

Methods

(a) Preparation of embryonic tissue homogenate (also referred to as foetal homogenate).

20 donor female guinea-pigs of known past breeding potential and of known mating date were provided from a breeding centre. A day was selected on which all were between 12 and 14 days pregnant. On this day all were killed by stunning and incising the neck, the abdomen was then incised and the uteri and their contents quickly removed under sterile conditions, placed in a sterile container and kept moist with sterile saline. All conceptuses, (embryos and their membranes, but not the maternal decidua at the point of attachment) were removed from the uteri using sterile technique and stored in sterile containers, each containing 6 conceptuses, at -20°C until use.

On the day of use the necessary number of conceptuses was thawed and then homogenised under sterile conditions, in 0.7 ml sterile saline per conceptus. A Polytron homogeniser was used, the blades of which had been sterilised with Hibitane (10% in ethanol) and thoroughly washed with sterile saline. 1.5 ml homogenate was then approximately equivalent to one conceptus, (0.7 ml saline + 1 conceptus of average volume of 0.8 ml). Homogenates of conceptuses were kept in sterile containers at 4°C

for no longer than 10 hours prior to use.

(b) General methods

Three virgin, female, guinea-pigs were observed for three control oestrous cycles prior to use. Observation of oestrous cycles was as described in Section II. During the last control cycle and throughout all subsequent cycles a 2.0 ml blood sample was collected on Monday, Wednesday and Friday of each week by cardiac puncture as previously described in Section VI. After collection the blood was immediately centrifuged and the plasma stored at -20°C until use. The cardiac plasma progesterone levels were determined by R.I.A. by the method described in Section V. (Assays were carried out by Miss A. Pelanis).

All animals were treated as follows. Starting on day 7 of the first oestrous cycle after the control cycles, 1.5 ml of sterile saline was injected i.p. five times daily at four-hourly intervals, starting at 9.00 a.m. each day. Injections were ceased when oestrus recurred. Two more control cycles were then recorded before any further treatment. After the completion of these cycles, injections of embryonic homogenate were started on day 7 of the next oestrous cycle. 1.5 ml of homogenate was injected i.p. five times daily at four-hourly intervals, the first injection of the day being at 9.00 a.m. The injections were continued until oestrus recurred. Oestrous cycle length and cardiac plasma progesterone levels were recorded for one further oestrous cycle after the cessation of this treatment.

Results

The results are shown in Table 25.

Oestrous cycle length and cardiac plasma progesterone levels did not differ between control cycles and cycles during which the guinea-pigs were treated with either saline or embryonic homogenate.

Comment

Treatment of non-pregnant guinea-pigs with i.p. injections of embryonic homogenate had no demonstrable effect on oestrous cycle length or cardiac plasma progesterone levels. Similarly in sheep i.m. injections of sheep embryonic homogenate were without effect on luteal life-span. It is possible that, as for the sheep, the guinea-pig embryonic homogenate needs to be administered locally (i.e. to the uterus) before any anti-luteolytic actions of the conceptus can be demonstrated.

Table 25

The effect of foetal homogenate administered i.p. to non-pregnant guinea-pigs.

(a) Oestrous Cycle Length (days)

Animal No.	Treatment				
	Control	Saline i.p.	Control	Foetal Homogenate i.p.	Control
1	16,17,17	16	17,17	16	16
2	16,16,16	16	17,16	17	17
3	16,17,17	17	16,17	17	16

(b) Mean Cardiac Plasma Progesterone Levels

Day of Oestrous Cycle	Treatment				
	Control	Saline i.p.	Control	Foetal Homogenate i.p.	Control
1	0.08	0.08	0.09	0.07	0.09
2	0.1	0.1	0.4	0.22	0.26
3	1.2	1.4	1.7	1.3	1.3
4	1.9	2.6	2.5	2.3	2.8
5	3.6	3.1	2.9	3.7	3.1
6	3.9	3.6	3.6	4.1	3.9
7	3.7	3.6	3.4	4.0	3.7
8	4.0	4.1	3.9	4.3	4.2
9	4.6	4.6	4.9	4.7	4.7
10	4.9	4.8	5.1	5.0	4.9
11	4.4	4.1	4.1	4.6	4.0
12	2.9	2.7	3.0	2.7	2.2
13	2.0	2.1	2.1	1.9	1.9
14	1.1	1.4	1.1	1.1	1.3
15	0.4	0.5	0.4	0.6	0.6
16	0.1	0.1	0.1	0.08	0.1
17	0.06	0.09	0.07	0.07	0.08

Experiment 2. Oestrous cycle length in non-pregnant guinea-pigs treated with daily intra-uterine injections of guinea-pig embryonic tissue homogenate.

Methods

The guinea-pig embryonic tissue homogenate was prepared in exactly the same way as for experiment 1.

Two control oestrous cycles were observed, by the methods previously described, in three virgin female guinea-pigs. An indwelling intra-uterine catheter was then inserted into the uterine lumen of one uterine horn of the uterus in each animal. The catheter used was adapted from that used for chronic cannulation of blood vessels in rats (Dow & McQueen, 1972).

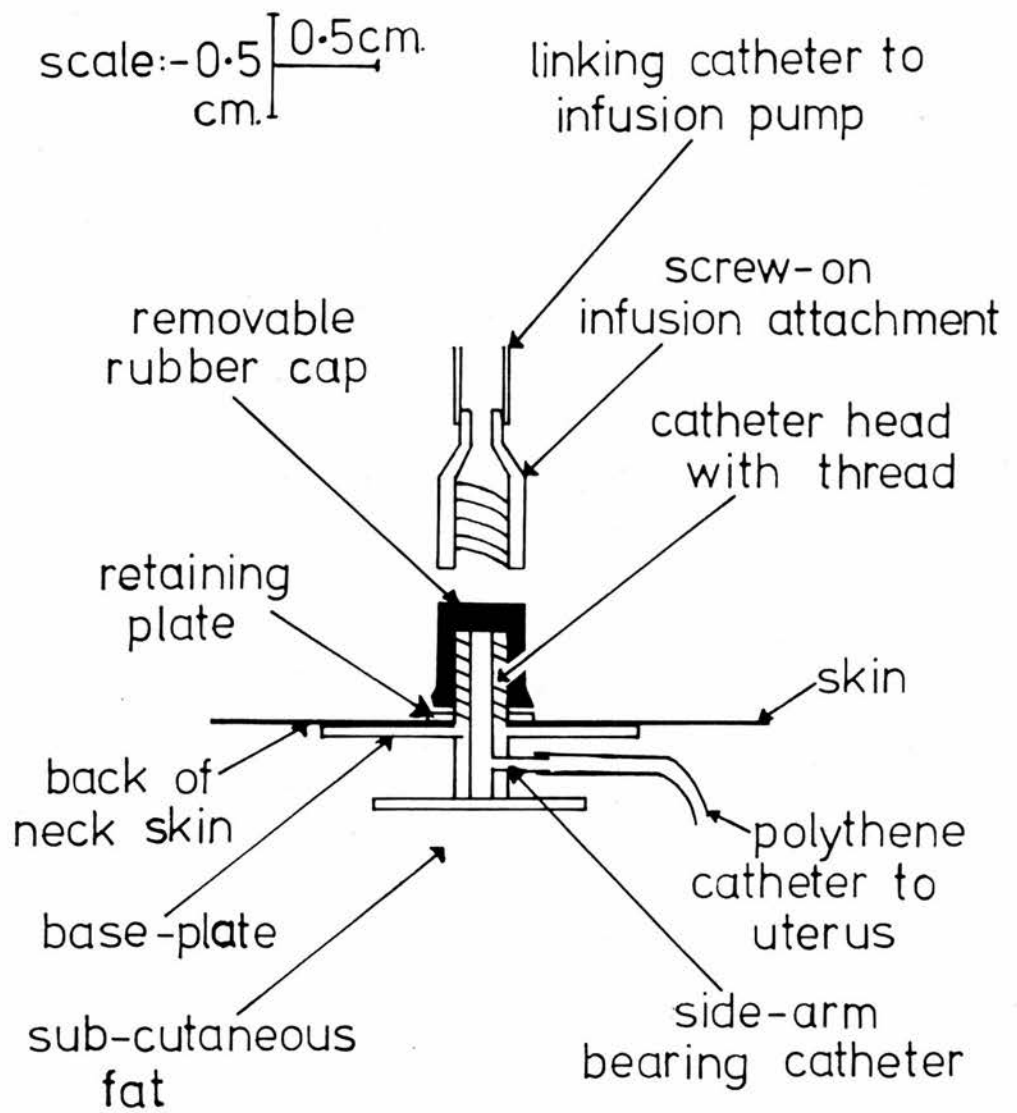
Method of uterine catheterisation: The animals were anaesthetised with Nembutal 30 mg/kg i.p. They were then shaved all over and their skin thoroughly washed with Hibitane (10% in ethanol). Sterile technique was used subsequently throughout. A small incision (\sim 12 mm) was made on the right-hand side of the back of the neck and the skin across the back of the neck was freed of connective tissue. A small hole was then punched in the skin at a point in the centre of the back of the neck. The catheter head was then passed through the incision and under the skin until the threaded screw for external catheter attachment protruded through the punched hole in the skin. The retaining

plate was then screwed on from the outside to hold the catheter head against the neck skin. The catheter tubing was then passed under the back skin to the lower front abdomen and an incision was made to expose the uterus. The catheter tubing tip, which had a small retaining cuff, was then passed into the superior tip of the left uterine horn, through a small incision, and secured by fine silk purse string suture. The peritoneum and skin incisions were then also closed by suture. The outer screw of the catheter head was closed by a small rubber cap and the catheter was flushed with sterile saline. The dimensions and structure of the catheter used in these animals are shown in Fig. 27.

General Method

Following the uterine catheterisation two control oestrous cycles were observed, as described before, prior to any further treatment. Starting on day 7 of the next oestrous cycle 0.5 ml sterile saline was injected into the uterus via the catheter every hour for 12 hours daily, the first injection being at 9.00 a.m. every morning. In this way a total of 6 mls was administered daily. Cardiac blood samples were obtained during the saline treatment and cardiac plasma progesterone levels were determined exactly as described for experiment 1. Oestrous cycle length was recorded throughout this experiment. Two further control cycles were then recorded for each animal. An injection regimen

Figure 27 The dimensions and structure of the uterine catheter.



for embryonic homogenate exactly the same as that just described for sterile saline was started on day 7 of the next oestrous cycle and continued until the recurrence of oestrus. In this way homogenate equivalent to 4 embryos and their membranes was administered daily. Cardiac blood samples were obtained and cardiac plasma progesterone levels were determined, as previously described during the administration of the homogenate. Following recurrence of oestrus a further two oestrous cycles were recorded.

Results

The results are shown in Tables 26a and b.

Following each intra-uterine injection of embryonic homogenate there was extensive extrusion of the homogenate from the vagina. This was observed to a lesser degree also for the saline injections.

Comments

Neither the injections of embryonic homogenate, nor the saline injections had any effect at all on oestrous cycle length or cardiac plasma progesterone levels as compared with the control cycles. This result differs to that obtained in the sheep by Rowson & Moor (1967), where a single intra-uterine injection of embryonic homogenate to non-pregnant ewes on day 10 of the oestrous cycle caused some elongation of the oestrous cycle. The lack of effect of the intra-uterine injections of embryonic homogenate in

Table 26 (a)

the effect of intra-uterine injections of foetal homogenate
in non-pregnant guinea-pigs.

Oestrous Cycle Length (Days)

Animal No.	Control	Uterine Cathetn.	Control	Intra-Uterine Saline	Control	Intra-Uterine Foetal Homogenate.	Control
1	16,18,16	↑	16,16	17	16,17	17	16
2	17,17,15		16,17	16	15,16	16	16
3	16,16,16		16,16	17	17,16	17	17

Terminology note :- the terms 'foetal homogenate' and 'embryonic tissue homogenate' are synonymous in the context of this experiment.

Table 26 (b) The effect of intra-uterine injections of foetal homogenate in non-pregnant guinea-pigs.

Day of Oestrous Cycle	Mean Cardiac Plasma Progesterone Levels (ng/ml)					
	Control	Uterine Catheter	Control	Intra-Uterine Saline	Control	Intra-Uterine Foetal Homogenate
1	0.1		0.09	0.1	0.09	0.1
2	0.1		0.15	0.3	0.25	0.3
3	1.6		1.3	1.8	1.7	1.4
4	1.9		1.9	2.1	2.0	2.0
5	2.7		2.9	2.9	2.8	2.6
6	3.8		3.6	3.9	3.6	3.8
7	3.9		3.5	4.1	3.6	3.7
8	4.2		3.5	3.9	3.9	4.0
9	4.3		4.0	4.1	4.2	4.1
10	4.7		4.9	4.3	4.8	4.4
11	4.6		4.8	4.3	4.1	4.5
12	3.1		3.0	3.3	4.0	3.9
13	2.4		2.1	2.5	2.9	2.3
14	1.0		1.3	1.6	1.1	1.4
15	0.4		0.6	1.0	0.8	0.8
16	0.2		0.1	0.3	0.2	0.1
17	0.09		0.09	0.1	0.09	0.1
18	0.1		0.09	0.09	0.08	0.06

Terminology note :- the terms 'foetal homogenate' and 'embryonic tissue homogenate' are synonymous in the context of this experiment.

the guinea-pig may be the result of too much of the homogenate being lost from the vagina at the time of injection or it may be that the luteolytic influence of the uterus in the guinea-pig is harder to overcome than that of the sheep, requiring more continuous contact between the uterus and embryonic homogenate. Alternatively, the volume of homogenate injected into the uterus may have been large enough to cause distension and possibly thereby directly stimulate production and release of $\text{PGF}_{2\alpha}$ (Poyser, et al., 1970, 1971). Such an action would negate any anti-luteolytic actions of the embryonic homogenate so that the net result might be seen as no effect at all on oestrous cycle length or cardiac plasma progesterone levels.

The problems of volume of homogenate administered and duration of contact with the uterus could both be overcome by the use of continuous, slow, intra-uterine infusion of the embryonic homogenate. Inclusion of a loose ligature around the cervix of these animals should serve both to reduce vaginal loss of homogenate and to encourage the homogenate to pass into the contralateral uterine horn, even though contact between the two horns is very slight above the level of the cervix.

Experiment 3. Oestrous cycle length in non-pregnant guinea-pigs given a continuous intra-uterine infusion of guinea-pig embryonic tissue homogenate.

Methods

The guinea-pig embryonic tissue homogenate was prepared exactly as for experiment 1.

Two control oestrous cycles were observed by methods previously described (Section II), in two virgin, female, guinea-pigs. An indwelling intra-uterine catheter was then inserted into the uterine lumen of the left uterine horn of the uterus in each animal by the method described in experiment 2. In these animals a loose ligature was placed around the cervix. Following the uterine catheterisation two control oestrous cycles were observed prior to any further treatment.

Infusion of sterile saline was started on day 7 of the next oestrous cycle. Using a stepping motor with an infusion pump, the saline was infused into the uterus at the rate of 0.15 ml/hour. A polythene tubing linkage system was used between the infusion pump and the catheter head. During the infusion the animals were housed alone, on a grid base, in an area of 10 ins. x 8 ins. and fed and watered normally, except that any hay that was used was sterilised. The infusion was continued until the recurrence of

oestrus. The links between the infusion pump and the catheter head on the animals were frequently checked for kinking. It was found that any further restriction of movement for the guinea-pigs, while reducing the problems with kinking of linking catheters, resulted in distress for the animals and they were, therefore, always kept in an area which allowed them to turn round and lie down freely. During the saline infusion, cardiac blood samples were collected and cardiac plasma progesterone levels were determined as described before (experiment 1).

Following the saline infusion two control oestrous cycles were observed prior to any further treatment. Infusion of guinea-pig embryonic homogenate was started on day 7 of the next oestrous cycle. The homogenate was infused into the uterus at a rate of 0.15 ml/hr, that is homogenate equivalent to approximately 2.5 embryos and their membranes was administered during each 24 hours. Infusion of homogenate was continued until the recurrence of oestrus. During the infusion cardiac blood samples were collected and cardiac plasma progesterone levels were determined as described before (experiment 1).

Following the homogenate infusion, oestrous cycle length and cardiac plasma progesterone levels were observed as previously described until at least two further control cycles had been recorded.

Results

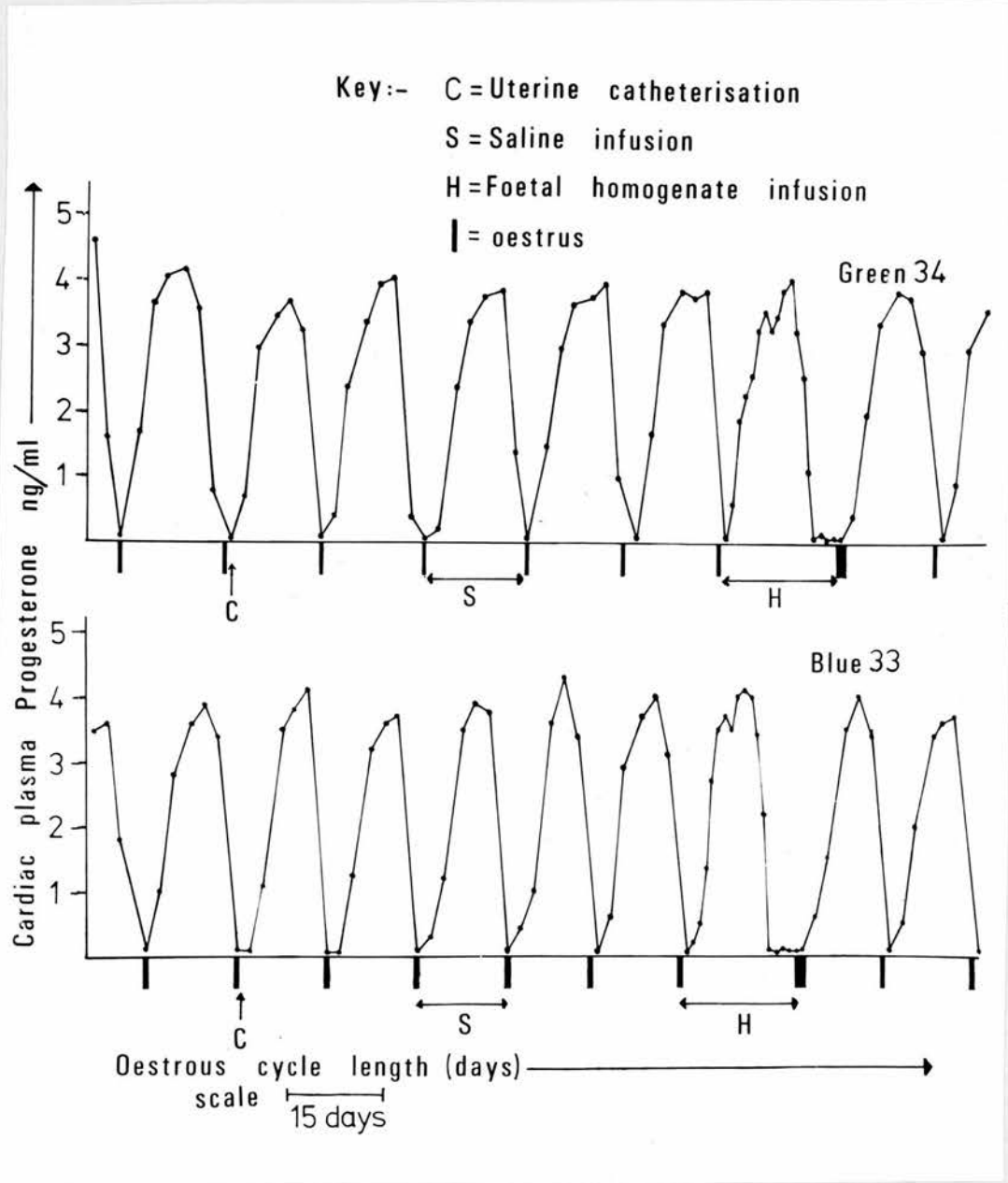
The results are shown in Fig. 28.

The saline infusion had no effect on either oestrous cycle length or luteal life-span as judged by cardiac plasma progesterone levels. In contrast the infusion of embryonic homogenate caused elongation of the oestrous cycle and oestrus, although marked by opening of the vaginal membrane, was not associated with the marked proliferation of cornified epithelial cells in the vaginal smear that normally appears for 12-24 hours during oestrus. Instead there were 48 hours in which the vaginal smear was almost free of cells although those which were present were predominantly cornified epithelial cells with some leucocytes always present, in contrast to the normal absence of leucocytes at oestrus. Luteal life-span, however, was unaffected by the infusion of embryonic homogenate, cardiac plasma progesterone levels starting to fall from day 12 of the oestrous cycle and falling to the low levels associated with functional luteal regression by day 15.

Comments

The effect of the intra-uterine infusion of embryonic homogenate in the non-pregnant guinea-pig is remarkably similar to the effect of exogenous progesterone in the non-pregnant guinea-pig (Deanesly, 1968) and sheep (Ginther 1969b), both treatments causing elongation of the oestrous cycle without

Figure 28 Oestrous cycle length and cardiac plasma progesterone levels in guinea-pigs receiving intra-uterine infusions of saline or guinea-pig foetal homogenate.



affecting luteal life-span. The increase in oestrous cycle length was not due to some physical effect of the intra-uterine infusion since a similar infusion of saline was without effect. The extended oestrous cycles must be, therefore, the result of the action, direct or indirect, of some substance present in the embryonic homogenate. This result contrasts sharply to the results of similar experiments by Rowson & Moor (1967) in the non-pregnant ewe. In their experiments luteal life-span was extended in association with elongation of the oestrous cycle in response to intra-uterine infusions of embryonic homogenate.

General Conclusions to Section IX

In these pilot experiments it has not been possible to demonstrate an anti-luteolytic action for the guinea-pig conceptus under experimental conditions in which such an action has been successfully demonstrated for the sheep conceptus (Rowson & Moor, 1967). The number of guinea-pigs used in these experiments was necessarily low because of the high cost of obtaining the conceptuses for embryonic homogenate. None-the-less, since each animal acted as its own control the results obtained are valid

although they would benefit from repetition in further animals.

There are four main possibilities that must be considered.

(1) It is possible that the guinea-pig anti-luteolytic factor is less stable than that of the sheep and the mode of treatment of the homogenate may be causing its destruction. Fresh embryonic homogenate that has never been subjected to freezing could be tried to test this, but some factor must have been present in the frozen and thawed homogenate to produce the extended oestrous cycles observed in experiment 3.

(2) The uncatheterised uterine horn and its adjacent ovary may be over-riding any anti-luteolytic actions of the embryonic homogenate on the catheterised uterine horn. This would seem unlikely if, as suggested, the anti-luteolytic factor is systemically born and acts to inhibit ovarian oestradiol- 17β secretion, since it would be expected to act on both ovaries and to inhibit PGF_{α} synthesis by both uterine horns. This could, however, be tested by repeating experiment 3 in hemi-hysterectomised and ipsilaterally ovariectomised guinea-pigs.

(3) The anti-luteolytic actions of the guinea-pig conceptus may be more complex than those of the sheep conceptus. Secretion of an anti-luteolytic factor by the guinea-pig conceptus may require a continuing foeto-maternal interaction which can not be mimicked

by an infusion of embryonic homogenate. In non-pregnant guinea-pigs splenic grafts of 11-12 day implanted conceptuses from donor pregnant uteri cause elongation of the oestrous cycle and extension of luteal life-span, while ectopic pregnancies where there has never been any foeto-maternal interaction in the uterus are not associated with any extension of oestrous cycle length or luteal life-span (Loeb, 1914, 1915; Bland & Donovan, 1965, 1969). These results suggest that while an initial foeto-maternal interaction at uterine level is essential, this can be interrupted and the conceptus, having had that initial interaction, will still show anti-luteolytic activity.

(4) Since it is hard to draw any conclusions from what is not happening, the most useful approach would be to investigate further events associated with the elongated oestrous cycles observed in experiment 3. Determination of utero-ovarian venous plasma levels of oestradiol- 17β , progesterone and $\text{PGE}\alpha$ could be made in an acute study on day 15 of the oestrous cycle in guinea-pigs that had received intra-uterine infusions of embryonic homogenate. Knowledge of these levels would indicate more clearly whether any anti-luteolytic actions were being produced by the embryonic homogenate. It would be interesting also to determine PBG levels in these animals. Some stimulation of PBG production could be responsible for the progesterone-like actions of the intra-uterine infusions of embryonic homogenate.

GENERAL DISCUSSION

General Discussion

The day 15 pregnant guinea-pig uterus has now been shown to biosynthesise $\text{PGF}_{2\alpha}$ in vitro in much smaller amounts than the day 15 non-pregnant guinea-pig uterus, day 15 being about the normal time of the onset of luteal regression in the non-pregnant animal. The low amounts of $\text{PGF}_{2\alpha}$ produced in vitro by the day 15 pregnant guinea-pig uterus were shown to reflect a true reduction in biosynthetic capacity since it was found that these uteri were not able to metabolise either $\text{PGF}_{2\alpha}$ or PGE_2 to any significant extent. Utero-ovarian venous plasma levels of PGF_α were found to rise from day 12 to day 15 of the oestrous cycle in non-pregnant guinea-pigs, while progesterone levels fell over the same period, in agreement with previous observations (Blatchley et al., 1972, Earthy et al., 1974). In the pregnant guinea-pigs, however, there was no rise in utero-ovarian venous plasma levels of PGF_α at day 15, the levels remaining low, reflecting the reduced ability of the uterus to produce $\text{PGF}_{2\alpha}$ at this time. Progesterone levels remained high at day 15 in these animals indicating functional maintenance of the corpora lutea. Since $\text{PGF}_{2\alpha}$ appears to be the uterine luteolysin in the guinea-pig (see Introduction) these results support the hypothesis that the guinea-pig conceptus secretes an anti-luteolytic factor in early pregnancy which acts to reduce the amount of $\text{PGF}_{2\alpha}$ produced by the uterus and released into the uterine veins, thus allowing maintenance of the corpus luteum and

secretion of progesterone up to and beyond day 15.

In sheep the uterine luteolysin also appears to be $\text{PGF}_{2\alpha}$ (McCracken et al., 1972 a,b; Goding et al., 1972; Goding 1973, 1974). In this species acute sampling techniques have shown that $\text{PGF}_{2\alpha}$ is detectable in uterine venous plasma around the time of luteal regression, but not earlier in the cycle (Bland, Horton & Poyser, 1971). However, chronic techniques for sampling from the utero-ovarian vein have since been developed and the frequent sampling now possible has shown that $\text{PGF}_{2\alpha}$ is in fact released in peaks of normally less than 6 hrs. duration, the frequency and intensity of which increase from day 12 to day 16 of the oestrous cycle, while progesterone levels are falling over this period (Thorburn, Cox, Currie, Restall & Schneider, 1972, 1973; Barcikowski, Carlson, Wilson & McCracken, 1974). In the pregnant sheep, although peaks of $\text{PGF}_{2\alpha}$ release into utero-ovarian or uterine venous plasma are still found around days 12 and 13 (Thorburn et al., 1973; Pexton, Weems & Inskeep, 1975), these peaks are absent from day 14 onwards (Thorburn et al., 1973; Barcikowski et al., 1974) and progesterone levels are maintained. These results present a similar picture to that obtained for the guinea-pig and support the early suggestion of an anti-luteolytic role for the sheep conceptus made by Rowson & Moor (1967 - see Introduction). In contrast, however, Wilson,

Butcher & Inskoop (1972 a,b) using an acute technique have reported higher $\text{PGF}_{2\alpha}$ levels in utero-ovarian venous plasma and endometrium in pregnant sheep at day 13 than in non-pregnant sheep. It is difficult to reconcile this result with those previously described and a more extensive study into the biosynthetic capacity for $\text{PGF}_{2\alpha}$ of the non-pregnant and early pregnant sheep endometrium is merited. / Since peaks of $\text{PGF}_{2\alpha}$ release in utero-ovarian venous plasma are still present around days 12 and 13 (Thorburn et al., 1973; Pexton et al., 1975) it is possible that the anti-luteolytic actions of the conceptus are not evident until after this time in the ewe. Wilson et al., (1972 a,b) may then have sampled during a peak of production and release of $\text{PGF}_{2\alpha}$ in their pregnant ewes, and between peaks in their non-pregnant ewes. This possibility is supported by the fact that the utero-ovarian venous plasma level of PGF_2 quoted by these workers for their non-pregnant ewes is only equivalent to the base-line values (between peaks) found by Thorburn et al., (1972) at a similar time in the oestrous cycle. The absence of an anti-luteolytic action for the conceptus before day 13 is further suggested by the results of Moor & Rowson (1966 a,b) demonstrating that the corpus luteum is not influenced by the conceptus during the first 12 days after oestrus. A close relationship between the conceptus and ovary then develops between the 12th and 13th day after oestrus. (Moor, Rowson,

Hay & Caldwell, 1969).

The timing of events in the guinea-pig contrasts to those in the sheep since the anti-luteolytic actions of the conceptus are evident by day 12 of pregnancy in the guinea-pig. At this time both the ability of the uterus to produce $\text{PGF}_{2\alpha}$ in vitro and utero-ovarian venous plasma levels of PGF_{α} are lower in the pregnant animal, than in the non-pregnant animal. There is, however, no difference in the in vitro biosynthetic capacity for PGE_2 between non-pregnant and early pregnant guinea-pig uteri. Synthesis of PGE_2 is in each case so low as to be of a background, residual nature and there is no evidence of redirection of synthesis towards PGE_2 in pregnancy, which could have been a possible mechanism to achieve reduced $\text{PGF}_{2\alpha}$ production. The reduction in uterine in vitro $\text{PGF}_{2\alpha}$ production in pregnancy appears, therefore, to be the product of inhibition at an earlier stage in the control of $\text{PGF}_{2\alpha}$ production and release. Little consideration has been given to the prostaglandin PGD_2 although it has been shown to be present in the tissues of many species (Nugteren & Hazelhof, 1973). The potential biological significance of PGD_2 was not apparent until much of the work of this thesis had been completed. However, in the earlier work PGD_2 , if present, would have been eluted in fraction I during silicic acid column chromatography and no significant biological activity was ever associated with these fractions from incubated uterine homogenates (see Section IA). For subsequent samples assayed in solid-phase

R.I.A. the $\text{PGF}_{2\alpha}$ -BSA antibodies used showed a 40% cross-reactivity at the 50% binding point for PGD_2 , with PGD_2 giving a non-parallel standard curve. Had significant amounts of PGD_2 been present in either the uterine homogenates or the utero-ovarian venous plasma samples, the samples should have shown a marked lack of parallelism with the $\text{PGF}_{2\alpha}$ standard curve. However, all samples showed good parallelism and care was always taken to check this since it provides valuable evidence of identity when using R.I.A. It appears highly unlikely, therefore, that PGD_2 is produced by and released from the non-pregnant or early pregnant guinea-pig uterus.

The common precursor of $\text{PGF}_{2\alpha}$, PGE_2 and PGD_2 is arachidonic acid, the availability of which could be a limiting factor to the synthesis of any of these prostaglandins. However, this did not prove to be the case for $\text{PGF}_{2\alpha}$ production by the pregnant guinea-pig uterus nor could more than a slight direct inhibition of $\text{PGF}_{2\alpha}$ production be demonstrated for the conceptus acting on the non-pregnant guinea-pig uterus in vitro. Such a direct inhibition could be interpreted as an inhibition at the synthetase complex level, controlling the synthesis of the $\text{PGF}_{2\alpha}$ from the arachidonate. However, the *in vitro* system that was used for this investigation was hampered by the crudity of both the uterine and embryonic preparations. It is quite possible, for instance, that in the presence of the chopped or homogenised conceptuses the mobility of co-factors or free arachidonate may differ to that for control

(uterus alone) preparations. It should be possible to develop a basic uterine synthetase extract from day 15 non-pregnant guinea-pit uteri, using which conversion of labelled arachidonate into $\text{PGF}_{2\alpha}$ and PGE_2 could be monitored. The effect of various extracts of homogenised conceptuses could then be determined in this system. Until the possibilities of such a system have been investigated some direct inhibition of the synthetase complex by an anti-luteolytic factor can not be ruled out.

In the non-pregnant guinea-pig the physiological stimulus to increased uterine production and release of $\text{PGF}_{2\alpha}$ towards the end of the oestrous cycle appears to be ovarian oestradiol- 17β , after progesterone priming (see Introduction). Ovarian venous plasma levels of oestradiol- 17β rise sharply around days 14-15 of the oestrous cycle (Joshi et al., 1973) and a similar picture has now been obtained for utero-ovarian venous plasma. It is possible that the developing follicles constitute the source of this oestradiol- 17β . It has now been shown that in early pregnant guinea-pigs utero-ovarian venous plasma levels of oestradiol- 17β remain low on day 15 in contrast to the sharp rise observed at this time in non-pregnant animals. An inhibition of ovarian oestradiol- 17β secretion would appear, therefore, to form at least part of the mechanism of the anti-luteolytic actions of the guinea-pig conceptus. It is significant that, like the reduction in PGF_{α} levels, utero-ovarian venous plasma levels of oestradiol- 17β were reduced even by day 12 of pregnancy, compared with the levels found on day 12 of

the oestrous cycle. This provides further evidence that the guinea-pig conceptus is exerting an anti-luteolytic action by day 12 of pregnancy.

In sheep, the physiological stimulus to uterine $\text{PGF}_{2\alpha}$ production and release towards the end of the oestrous cycle also appears to be oestradiol- 17β (McCracken, Baird, Carlson, Goding & Barcikowski, 1973; Barcikowski et al., 1974). In this species also utero-ovarian venous plasma levels of oestradiol fail to rise in early pregnancy around days 14-16 (Cox, Thorburn, Currie & Restall, 1974) in contrast to the rise that occurs around this time in non-pregnant ewes (Cox, Mattner & Thorburn, 1971). Thus in the ewe, also, at least part of the mechanism of the anti-luteolytic actions of the conceptus appears to be an inhibition of ovarian oestradiol- 17β secretion.

In the ewe, a further characteristic of the anti-luteolytic actions of the conceptus is that they appear to be species specific, since intra-uterine infusions of homogenate of pig conceptuses to non-pregnant ewes were without effect on luteal function (Rowson & Moor, 1967). The pig, at first sight, appears to present a similar picture to that seen in the guinea-pig and sheep for events associated with the oestrous cycle and early pregnancy. Hysterectomy extends the luteal life-span (Spics et al., 1960) and $\text{PGF}_{2\alpha}$ has been shown to be luteolytic in this species (Diehl & Day, 1974; Kraeling, Barb & Davis, 1975). Towards the end of the oestrous cycle when peripheral plasma

levels start to fall the peripheral plasma levels of total unconjugated oestrogens rise, reaching their maximum level 1-2 days prior to oestrus (Henricks, Guthrie & Handlin, 1972) and at the same time as the oestrogen levels rise, the peaks of PGF in the utero-ovarian venous plasma increase in frequency and intensity (Gleeson & Thorburn, 1973). In contrast, in early pregnancy progesterone levels are maintained and the pre-oestrus peak of oestrogen is absent (Guthrie, Henricks & Handlin, 1972; Robertson & King, 1974). Infusion of a saline extract of freeze-dried porcine embryonic membranes into the uterus of non-pregnant gilts did not extend the life-span of the corpora lutea, but increased pregnancy rates were achieved for unilaterally pregnant gilts by giving infusions of saline extracts of the complete conceptus into the sterile uterine horn (Longenecker & Day, 1972). So far then the picture is very similar to that seen in the sheep and guinea-pig. However, although in the pig the ovaries are essential for the maintenance of pregnancy through most of gestation (du Mesnil du Buisson & Danzier, 1957) progesterone has an opposite effect to that seen in sheep and guinea-pigs. Perry (1954) considers that the greater proportion of embryonic death in large litters of swine may be traceable to the larger numbers of corpora lutea, with the consequent excess of progesterone. Supporting this is the observation that progesterone treatment of early pregnant gilts causes luteal regression, (Sammelwitz & Nalbandov, 1958; Spies, Zimmerman, Self & Casida, 1959). In contrast oestrogen has no detrimental effect on

luteal function causing instead luteal maintenance in gilts (Chakraborty, England & Stormshak, 1972). Denamur (1968) suggests that luteal maintenance in the oestradiol-treated gilt might be due to the ability of the oestrogen to suppress the effect of a uterine luteolysin. It is possible also that oestrogen is involved in an anti-luteolytic action for the conceptus since the pig blastocyst shows a considerable ability to incorporate labelled androstenedione into oestrogens by day 14-16 of pregnancy (Perry & Heap, 1973). Given the apparent difference between the physiological roles of oestrogen in the pig and the sheep it is not really surprising that intra-uterine infusions of pig conceptuses had no effect on luteal life-span in non-pregnant sheep. It would be a better test of species specificity to determine the effect of sheep conceptus in the non-pregnant guinea-pig since the basic relationships between PGF_α , luteal life-span and the ovarian steroid hormones appear similar in these two species.

In the guinea-pig, in contrast to the pig, it has now been shown that administration of exogenous oestradiol benzoate to early pregnant animals will cause abortion, presumably by overcoming the anti-luteolytic actions of the conceptus. This abortive action of oestradiol was associated with a noticeably bloodless appearance of the placenta and high uterine in vitro $\text{PGF}_{2\alpha}$ production. In the non-pregnant animal oestrogen is well known for its ability to cause uterine hyperaemia, such an effect

being seen in guinea-pigs (Markee, 1932), rats (Spaziani & Suddick, 1967; McKercher, Van Orden III, Bhatnager & Burke, 1973), sheep (Huckabee, Crenshae, Curet, Mann & Barron, 1970; Anderson & Hackshaw, 1974) and rabbits (Kaiser, 1947). In the pregnant rabbit, however, Abdul-Karim & Bruce (1972) noticed that while causing hyperaemia of the myometrium oestrogen caused a large decrease in placental blood flow, a picture similar to that observed for the aborting guinea-pigs that had received oestrogen treatment. Large amounts of PGE (172 ng/ml) are present in the uterine venous plasma of early pregnant rabbits (Venuto, O'Dorisio, Stein & Ferris, 1975). Indomethacin treatment reduces this level to 23 ng/ml PGE and also causes a severe reduction in total utero-placental blood flow (Venuto et al., 1975). In the rat, indomethacin also blocks oestrogen-induced uterine hyperaemia with a concomitant, parallel block of uterine PGE and PGF synthesis in vitro (Ryan, Clark, Van Orden, Farley, Edvinsson, Sjoberg, Van Orden III & Brody, 1974) while in the dog PGE is known to be a potent uterine vasodilator (Clark, Ryan & Brody, 1973). There appears, therefore, to be a strong association between PGE, PGF, oestrogen and uterine or utero-placental haemodynamics. It is tempting to speculate that the oestrogen-induced hyperaemia of the non-pregnant uterus or early pregnant myometrium is associated with PGE and that the oestrogen-induced reduction in placental blood flow in early pregnancy is associated with PGF, possibly reflecting

different synthetic capacities for these tissues. However, in the sheep at parturition there is a large rise in oestradiol levels and also in uterine secretion of $\text{PGF}_{2\alpha}$ (Challis, Harrison, Heap, Horton & Poyser, 1972) and this is associated with a marked rise in absolute uterine blood flow, (Bedford Challis, Harrison & Heap, 1972). A further investigation of the actions of oestrogen on blood flow in the non-pregnant uterus or early pregnant uterus and placenta with the measurement of PGE and PGF production in each case would be worthwhile. Determination of PGE levels or production has been limited by the sensitivity of available assay methods. Attempts to raise antibodies to PGE_2 -BSA resulted usually in a mixed population of PGE_2 and PGB_2 antibodies and sometimes to a population of PGB_2 antibodies.

Initial separative steps have associated procedural losses that are crucial at a nanogram level and attempts to convert PGE_2 to PGB_2 and assay using PGB_2 antibodies, showed this conversion to be unreliable at the nanogram level. However, the recent development of highly specific antibodies to PGE_2 (Dray, Charbonnel & Maclouf, 1975) should now permit determinations of PGE_2 to be made at the picogram level.

If, as it would appear, inhibition of ovarian oestradiol secretion is an essential step in the anti-luteolytic actions of the conceptus, how is this achieved? If the theoretical 'anti-luteolytic factor' is having a direct effect at the level of the ovary it is possible that it would be more correctly regarded as

luteotrophic, the prevention of luteolysis being a secondary effect. Early pregnancy has been shown to be independent of the maternal pituitary in the guinea-pig (Heap et al., 1967). It is possible, however, that hypophysectomy may be simply mimicking an inhibitory action occurring at pituitary level, normally brought about by the conceptus. If inhibition of ovarian oestrogen secretion is initiated at the pituitary level then luteal progesterone would be a potential candidate for such an action. Under these circumstances the anti-luteolytic actions of the conceptus, if involving a direct stimulation of luteal progesterone secretion, would be more correctly described as luteotrophic. However, administration of progesterone to non-pregnant guinea-pigs while causing extension of the oestrous cycle had no effect on luteal life-span (Deanesly, 1968, Ginther, 1969b). A similar result is seen in the sheep (Ginther, 1969b). Deanesly (1968) considered the delaying of oestrus to be the result of an inhibition of oestrogen. However, if oestrogen constitutes the physiological stimulus for increased output of $\text{PGF}_{2\alpha}$ from the uterus, and subsequent luteal regression, what are the hormonal events that lead to luteal regression in the progesterone-treated animals? This raises the whole problem of the true relationship between different hormonal events in the oestrous cycle. Do oestradiol secretion and $\text{PGF}_{2\alpha}$ production and release cause progesterone levels to fall or is progesterone withdrawal the stimulus to

oestradiol secretion and $\text{PGF}_{2\alpha}$ production and release? Studies into early pregnancy cannot proceed very much further until the true relationship between hormonal events in the oestrous cycle is understood. Much of the work relating to the oestrous cycle was carried out before the sensitive assays for progesterone, oestrogen and $\text{PGF}_{2\alpha}$ became available and would bear repetition within this new framework. It is possible that in progesterone treated animals only the peak of ovarian oestradiol- 17β output is abolished, removing the stimulus to ovulation, but leaving enough oestradiol- 17β present to initiate events leading to luteal regression. A simple measurement of utero-ovarian venous plasma levels of oestradiol- 17β , progesterone and $\text{PGF}_{2\alpha}$ in control and progesterone treated guinea-pigs and sheep would go much of the way to answering these important questions.

There are several other major drawbacks to the hypothesis of a progesterone-mediated inhibition of ovarian oestradiol- 17β secretion. In the guinea-pig it is now clear that a marked anti-luteolytic action is in evidence by day 12 of pregnancy, as seen in the reduced uterine in vitro biosynthetic capacity for $\text{PGF}_{2\alpha}$ and reduced utero-ovarian venous plasma levels of oestradiol- 17β and $\text{PGF}_{2\alpha}$ at this time. Therefore, if this hypothesis is to hold, either a progesterone concentrating mechanism and/or an increased production rate for progesterone should be evident by day 12 of pregnancy. However, maternal MCR for progesterone does not start

to fall significantly until day 15 of pregnancy (Illingworth et al., 1970) and PBG can not be detected in maternal peripheral plasma before this time (Illingworth et al., 1973). The main increase in luteal production rate of progesterone does not occur until around days 26-28 of pregnancy when it increases from the luteal phase value of $0.18 \mu\text{g}/\text{min}$ to $1.32 \mu\text{g}/\text{min}$ (Challis et al., 1971), the extra-ovarian contribution being only about $0.1 \mu\text{g}/\text{min}$ until day 28 of pregnancy (Illingworth & Deanesly, 1972). However, by day 15 of pregnancy luteal production rate of production is $0.40 \mu\text{g}/\text{min}$, approximately twice that of the luteal phase of the oestrous cycle ($0.18 \mu\text{g}/\text{min}$) (Challis et al., 1971), but the authors have not determined production rate earlier than this in pregnancy. The significance of the increase in production rate of progesterone that is present by day 15 of pregnancy is hard to evaluate. Deanesly (1968) believed that the doses of progesterone that she gave to non-pregnant guinea-pigs, which caused lengthening of the oestrous cycle, were sufficient to create blood levels equivalent to those of early pregnancy. However, this requires validation and the significance of the lutetrophic response in early pregnancy or the effects of exogenous progesterone in non-pregnant animals can not be evaluated until some quantitative relationship has been identified between the blood level or dose of progesterone and the response it produces. In the sheep the situation is very different since PBG is not found in this species,

(Illingworth et al., 1973) and the secretory activity of the corpus luteum in early pregnancy is little different to that of the luteal phase of the normal cycle (Bedford, Harrison & Heap, 1972). Also in this species the presence of the maternal pituitary is essential for the maintenance of early pregnancy (Denamur & Martinet, 1961). It is possible that part of the action of the conceptus may be to stimulate secretion of a pituitary luteotrophin, which acts at the luteal level, prevention of luteolysis being secondary to the luteotrophic response. However, pituitary LH has been ruled out as one possible candidate for this pituitary luteotrophin since the administration of LH did not result in luteal maintenance in the ewe (Cerini, Beck, Chamley, Cumming, Findlay & Goding, 1973). This is a complex field where much further investigation is required, but some useful information might result from pilot studies using gonadotrophin-antibodies in early pregnant ewes.

Luteal progesterone would be expected to act systemically, particularly if its action involves the maternal pituitary, and luteal function in both ovaries should be equally affected whether the pregnancy was unilateral or bilateral. However, a definite local component to the anti-luteolytic actions of the conceptus has now been demonstrated for the guinea-pig. In unilaterally pregnant animals uterine in vitro biosynthetic capacity for $\text{PGF}_{2\alpha}$ and utero-ovarian venous plasma levels of oestradiol- 17β and PGF_{α} are all greater on the non-pregnant side than on the pregnant side.

Furthermore, if in unilaterally pregnant animals, the continuity between pregnant and non-pregnant uterine horns is interrupted by uterine transection the uterine in vitro $\text{PGF}_{2\alpha}$ biosynthetic capacity of the non-pregnant horn increases, and the corpora lutea in the ovary on the non-pregnant, transected, side grow more slowly than those in intact unilaterally pregnant animals. In the sheep the local anti-luteolytic actions of the conceptus are even more marked and in this species uterine transection in a uni-cornuate pregnancy, bearing bilateral corpora lutea, results in complete luteal regression on the non-pregnant transected side (Moor & Rowson, 1966c). Investigation is required to determine the mechanism of luteal regression in this case, and the nature of the stimulus to increased uterine production of PGF_{α} in vitro for the non-pregnant horn in guinea-pigs with transected uteri. Utero-ovarian venous plasma sampling is required, chronically for the sheep and acutely for the guinea-pig, with analysis of the samples for oestradiol- 17β , progesterone and PGF_{α} . If ovarian oestradiol- 17β is to be the stimulus to PGF_{α} production by the non-pregnant uterine horn, one must either propose a local route for the distribution of oestradiol- 17β from the ovary to the uterus or alternatively the conceptus could be exerting a dual action, normally blocking the secretion of oestradiol but also acting as an antagonist to the actions of oestradiol. The first situation is hard to visualise, but can not be ruled out, the second seems unlikely in the guinea-pig,

since small doses of exogenous oestradiol have been shown to overcome the anti-luteolytic actions of the conceptus and cause abortion. The difficulty in explaining the significance of these oestradiol- 17β levels raises the question as to whether oestradiol- 17β is truly the physiological stimulus to $\text{PGF}\alpha$ synthesis and release. Good evidence in support of oestradiol- 17β as the physiological stimulus would be provided by the demonstration of blockade of luteal regression in animals immunised against oestradiol- 17β . Preliminary experiments in guinea-pigs passively immunised against oestradiol- 17β have not, however, resulted in any inhibition of luteal regression (N.L. Poyser, personal communication) and further investigation of this is in progress.

More emphasis is now being placed on the local component to the anti-luteolytic actions of the conceptus. As a result it becomes important to investigate further the possibility of a direct inhibitory action of the conceptus at the synthetase-complex level in the guinea-pig, using purer preparations in an in vitro system, as mentioned earlier in the discussion. If positive results could be achieved with such a system it would provide a simple assay preparation. At the moment the only available assay preparation is oestrous cycle length in non-pregnant guinea-pigs receiving intra-uterine infusions of embryonic homogenate. This system is far from ideal, one of the most serious drawbacks being

the high cost of the embryonic homogenate. An alternative approach would be that of tissue culture. Moor (1970) makes a passing observation that successful prolongation of luteal life-span was seen in non-pregnant ewes receiving intr-uterine infusions of monolayer cultures of disaggregated sheep conceptuses (referred to as Hay, Moor, Rowson & Lawson: unpublished observations). In the non-pregnant guinea-pig, large celled trophoblast from day 12 implanted conceptuses is the minimum tissue that must be present as a splenic graft if prolonged luteal life-span is to be achieved (Bland & Donovan, 1969). Human trophoblast has been successfully cultured to yield HCG and this would appear, therefore, to be a reasonable proposition for the guinea-pig and sheep. However, even where normality of the culture is carefully checked in terms of oxygen uptake and other safeguards there is some evidence for the cultured human trophoblast that the biosynthesised HCG may differ to native HCG in structure or conformation (Tojo, Mochizuki & Maruo, 1974). In ectopic pregnancy, where there is no fetomaternal interaction in the uterus, oestrous cycle length and luteal life-span are unaffected (Loeb, 1914, 1915; Bland & Donovan, 1965). In contrast day 12 conceptuses, having had a fetomaternal uterine interaction, which are then grafted to the splenic capsule of non-pregnant guinea-pigs cause extension of the oestrous cycle and luteal life-span. Thus if tissue culture is to be used for the guinea-pig to obtain an anti-luteolytic factor, initial samples of

trophoblast must be taken from conceptuses which have undergone some interaction with maternal decidua.

The phenomenon of ectopic pregnancy in the guinea-pig is intriguing. Evidently the developing embryo survives quite adequately in the presence of the very low progesterone levels associated with luteal regression. If the foetal placenta produced significant amounts of progesterone oestrous cycle length would be extended, so this can not be the case. In the usual intra-uterine pregnancy progesterone not only prevents decidual collapse (Deanesly, 1972) but also controls the uptake and utilisation of glucose (Yochim, 1971). Apart from being essential for the initiation of decidualisation, this would also appear to be of nutritive significance. The active decidual cells also contain glycogen and lipid (Finn, 1971). Thus in the intra-uterine pregnancy adequate nutrition of the embryo requires an intact decidua for which the maintenance of luteal progesterone secretion and prevention of luteolysis are essential. Where the pregnancy is outside the 'hostile' situation of the uterus, the embryo presumably derives its nutrition from diffusion of products from an adjacent vascular bed. Thus in ectopic pregnancy the whole question of an embryonic anti-luteolytic factor or luteotrophin is avoided.

In conclusion, for uterine pregnancy in guinea-pigs, evidence

has now been obtained which supports the hypothesis that the early conceptus secretes an anti-luteolytic factor which acts to reduce uterine $\text{PGF}_{2\alpha}$ production and release, thereby allowing luteal maintenance and continued progesterone secretion. An inhibition of ovarian oestradiol- 17β secretion appears to constitute at least part of the mechanism of the anti-luteolytic actions of the conceptus. There is also a significant local component to the anti-luteolytic actions of the conceptus, the mechanism of which is not clear although a direct inhibition at the synthetase complex level has not been ruled out. Events associated with the oestrous cycle and the true relationships between progesterone, oestrogen, $\text{PGF}_{2\alpha}$ and PGE_2 must be clarified before further work on early pregnancy can be truly worthwhile.

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Sources of Drugs and Chemicals

All reagents and solvents were Analar grade, supplied by BDH chemicals except for the following:-

Arachidonic acid (99% pure)	Sigma Chemical Company
Arachis oil	Hopkin and Williams
Bis-(Trimethylsilyl)- Trifluoroacetamide	Sigma Chemical Company
Oestradiol benzoate	Koch-Light Laboratories
Oestradiol-17	" " "
Pentobarbitone	Abbott Laboratories Ltd.
Progesterone	Koch-Light Laboratories
Silicic Acid	Sigma Chemical Company
Silic a Gel	Merck
Sucrose	May and Baker Ltd.
Tri(Hydroxymethyl)methylamine (Tris)	Koch-Light Laboratories

Radioactive compounds:-

^3H -Oestradiol-17 β

^3H -Progesterone

^3H -PGF $_{2\alpha}$

^3H -PGE $_2$

All supplied by the Radio-
chemical Centre, Amersham.

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