A STUDY OF THE OVARIAN RESPONSE OF NEW ZEALAND ROMNEY EWES SEQUENTIALLY SUPEROVULATED WITH PREGNANT MARE'S SERUM GONADOTROPHIN

A thesis presented in partial fulfilment of the requirements for the degree of Master of Agricultural Science in Animal Science at Massey University

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ABSTRACT

A series of experiments investigated the nature and causes of ovarian refractoriness in ewes sequentially treated with Pregnant Mare's Serum Gonadotrophin (P.M.S.G.). 70 ewes were subjected to the following treatments over 3 oestrous cycles of the 1972 breeding season:- 1. Injected with P.M.S.G. at each of 3 cycles (cycles 1,2 and 3);

2. Two injections of P.M.S.G. (at cycles 1 and 3) separated by a normal oestrous cycle;

3. Injected with P.M.S.G. at two successive cycles (cycles 2 and 3);

4. Injected at 1 cycle only (cycle 3).

These treatments were replicated at 1000 i.u. and 1500 i.u. P.M.S.G. and 9 ewes acted as an uninjected control group. The ewes were blood sampled and slaughtered at the end of these treatments and ovulation data were obtained by recovery of the reproductive tracts. The terminal ovulation rates showed that ewes were refractory to a second injection of P.M.S.G. and this condition persisted. The refractoriness was to some extent alleviated by the spacing of injections (Treatment 2 above).

Biological Inhibition Tests (using mice) analysed the plasma of the above ewes for evidence of anti-gonadotrophins. Although such factors were not detected in the blood of these ewes, the test did reveal antibody production against P.M.S.G. in the plasma of a further group of ewes which had been chronically treated with the hormone for 6 weeks. It was concluded that ovarian refractoriness, which is rapidly attained in sequentially treated ewes, is

not due to the development of serological antibodies against the exogenous gonadotrophin.

Another experiment, carried out early in the 1973 breeding season, investigated ovarian follicle development in 30 ewes which were sequentially treated with P.M.S.G. for up to 3 oestrous cycles. Ewes were laparotomised or killed on Day 10 of the oestrous cycles following treatment and measurements on follicle development were taken. A group of control ewes were observed at a similar time to the treated ewes.

Counts on ovarian surface follicles differed little between treated and control ewes, at each of the observations. However, the ovaries of slaughtered ewes were sectioned to allow estimation of total ovarian follicular populations and to make some assessment of follicular atresia. Ewes slaughtered after 1 injection of P.M.S.G. had lower numbers of normal antral follicles per ovary than did control ewes or ewes observed at similar times after 2 or 3 injections.

It was suggested that exhaustion of ovarian follicular populations may precipitate a refractory condition but that this condition persists because of an endogenous hormonal imbalance. Further work should be done to investigate this latter possibility.

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P R E F A C E

This investigation was conducted at the Sheep Production Centre, part of the Department of Sheep Husbandry, Massey University. The experimental work was carried out between April of 1972 and May of 1973 and represents original research by the author under supervision of Dr. M.F. McDonald, Reader, Sheep Husbandry Department, Massey University.

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INTRODUCTION

INTRODUCTION

"The outstanding deficiency of the New Zealand sheep is low reproductive rate and yet it is in this aspect that a great potential exists."

(Coop, 1972)

Pregnant Mare's Serum (P.M.S.) as a source of gonadotrophin is of potential value for increasing the reproductive rate of sheep. Superovulation with this hormone preparation makes rapid proliferation of a small number of animals possible. The overall efficiency of this treatment may be facilitated by ovum transfer.

Studies in New Zealand have shown Pregnant Mare's Serum Gonadotrophin (P.M.S.G.) capable of increasing litter size of the Romney ewe (Wallace, 1954; Larsen, 1971). However, the large litters produced after gonadotrophic stimulation are most susceptible to pre-natal loss than those of normal size (Cumming, 1965) and the ability of P.M.S.G. to effectively increase fecundity in the ewe is thus limited. To realise the full reproductive potential of the ewe, fertile ova from superovulated animals may be transferred to a number of recipient ewes for development. This would avoid the problem of uterine overcrowding.

With the introduction of "exotic" breeds of sheep into New Zealand, rapid multiplication of the small number imported would aid efficient dispersal of their influence throughout the country and hence, offer desirable characteristics to the national flock.

To obtain large numbers of offspring from the few parent individuals, the "exotic" ewes could be superovulated and mated to rams of the same breed. As this system would predispose to loss of whole litters in the pre-natal stages, it may be more desirable to transfer fertile ova from the superovulated "exotics" to ewes of a more common breed. This type of operation has been suggested by Clarke (1973).

If donor ewes were able to be superovulated at successive oestrous cycles, the number of ova available for transfer could be increased thus enhancing the efficiency of multiplication procedures. Although sequential superovulations would be desirable, such treatments are not feasible. With increasing numbers of P.M.S.G. treatments, ewes become progressively less responsive to the injected hormone. The reduced ovulation rates obtained with sequential gonadotrophic stimulations is referred to as "refractoriness" in response to the exogenous hormone. The development of a refractory condition of the ovary with administration of a protein hormone (e.g. P.M.S.G.), may be due to the production of serological antibodies against the hormone molecules. However, other explanations include loss of sensitivity of the ovaries, exhaustion of mature follicles, or an endogenous hormonal imbalance being augmented by large amounts of exogenous gonadotrophin.

If the factors responsible for refractoriness to P.M.S.G. were overcome, then sequential treatment of ewes could become feasible and the yield of ova over a short period of time would be potentially larger. This project was designed to elucidate causes of refractoriness in ewes sequentially treated with P.M.S.G. and methods of overcoming the condition were sought.

CHAPTER I

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

The indication, by Cole and Miller (1933), that P.M.S. augmented the manifestation of oestrus in anoestrous ewes, created a widespread interest in the hormone preparation as a source of gonadotrophin. Since this observation, P.M.S.G. has been utilised both for the induction of oestrus and ovulation in anoestrous animals and the superovulation of cyclic animals. Extensive use of P.M.S. as a laboratory source of Follicle Stimulating Hormone (F.S.H.)-like activity has also contributed to knowledge on the preparation's physiological and biochemical properties. Literature related to the use of P.M.S.G. in domestic animals is reviewed below. The present state of knowledge on the gonadotrophin's mode of action is also considered.

By virtue of their protein moeity, gonadotrophins become effective antigens when injected into heterologous species. P.M.S.G. conforms to this pattern and the review presented in this chapter embodies a consideration of antibody formation against this molecule. A resumé of information available on Biological Inhibition Tests, used to detect anti-gonadotrophins, is also pertinent to this study.

Finally, the incomplete knowledge of sheep ovarian follicular dynamics will be reviewed.

Pregnant Mare's Serum Gonadotrophin

The capacity of P.M.S.G. to exhibit F.S.H.-like activity in sheep has been reported in many studies. Efforts have been directed towards its use in:-

- Inducing out of season breeding, being used alone (Cole et al., 1945; Robinson, 1950) or in conjection with progestagens (Dutt, 1953; Robinson, 1954; McDonald, 1961; Gordon, 1958b; 1963b; 1972; Roberts and Edgar, 1966):

- Augmenting fecundity during the breeding season (Robinson, 1951; Wallace, 1954; Gordon, 1958a; 1963a; Cumming and McDonald, 1967; Newton et al., 1971; Tempest and Boaz, 1970; Bindon et al., 1971; Hunt et al., 1971):

Endocrinological studies, utilising its F.S.H.-like properties (Short et al., 1963; McCracken et al., 1969; Hunt et al., 1971):

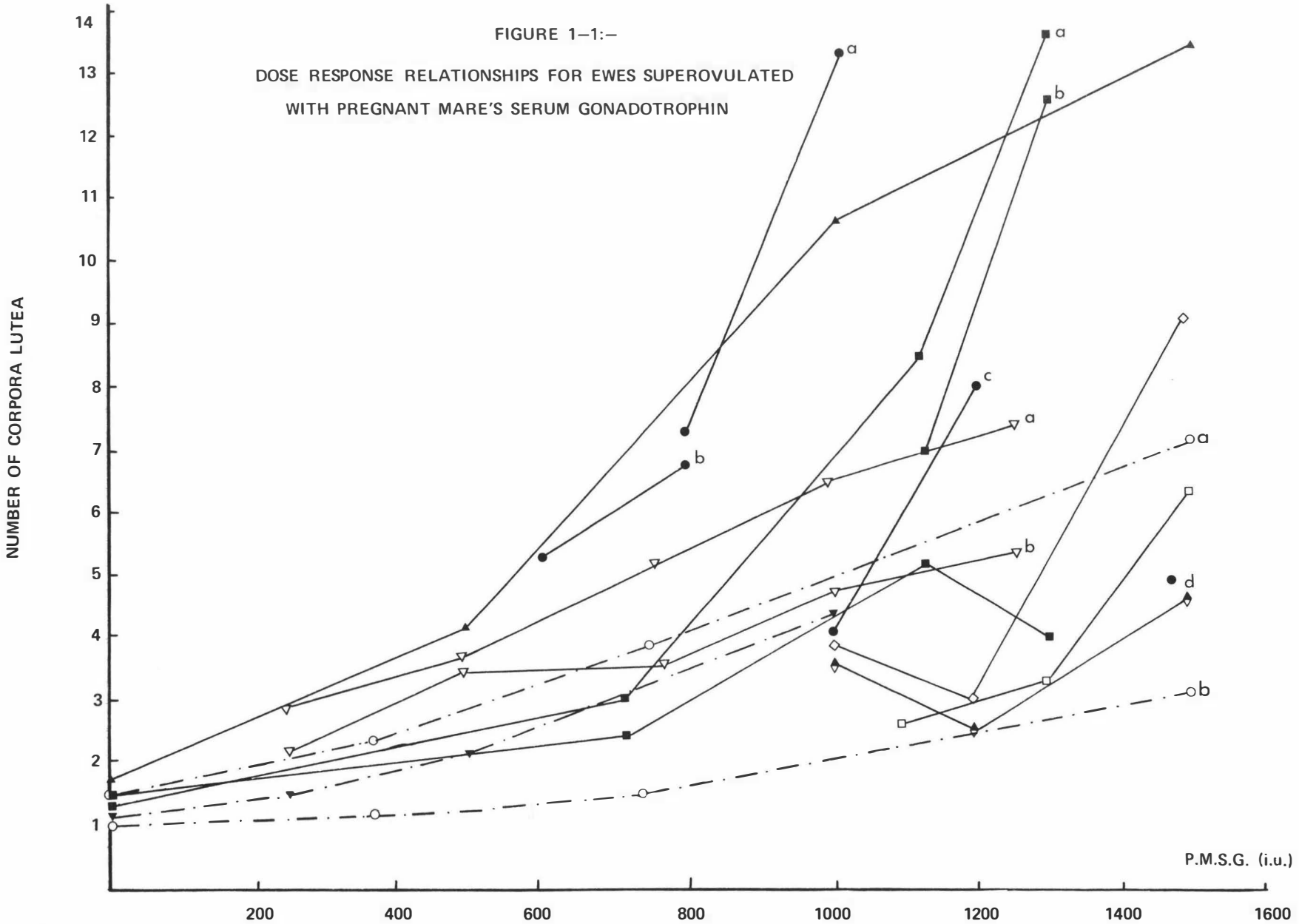
- Production of ova for transplantation to foster 'recipients' following fertilization in the 'donor' (Averill and Rowson, 1959: Larsen, 1971) and for studies on the in vitro fertilization and culture of ova (Moor and Cragle, 1971).

Notwithstanding the value of P.M.S. as a source of experimental gonadotrophin, the hormone also has practical value as a superovulatory hormone (Gordon, 1963a; 1972; Findlay and Vaughn, 1964; McDonald, 1966; Robinson, 1967c). Gordon (1969) has pointed out the practical difficulties in the use of the hormone at the farm level.

Superovulatory responses to P.M.S.G. in sheep have been recorded by a number of authors. Results of some of these studies are shown in Fig. 1-1 which plots ovulation response (number of Corpora Lutea) with increasing doses of P.M.S.G.

FIGURE 1–1:–

DOSE RESPONSE RELATIONSHIPS FOR EWES SUPEROVULATED WITH PREGNANT MARE'S SERUM GONADOTROPHIN



Although a definite trend is seen with increasing dose rates of P.M.S.G., most workers (Robinson, 1951; Wallace, 1954; Averill, 1958; Larsen, 1971) report marked variation of ovarian response in individual animals. This effect is greater at higher doses. Response becomes progressively less with increasing doses (Averill, 1958) and falls off above some critical value (Robinson, 1951). The incidence of cystic and luteinised follicles increases with dose level (Robinson, 1951 and Larsen, 1971).

Hammond et al. (1942) and Moore and Shelton (1964) obtained a clear relationship between dose level and number of antral follicles when injecting sheep with Horse Anterior Pituitary extracts (H.A.P.). These workers could not achieve linearity of response with P.M.S.G. Wallace (1954), Holst (1969) and Bindon et al. (1971) reported a relationship between P.M.S.G. dose level and the number of corpora lutea in sheep ovaries (see Fig. 1-1). Because of the large variance amongst animals on each dose level, Robinson (1951) was able to plot a linear curve within the limits of the standard deviations of the mean responses to increasing dose. Lamond (1964b) derived a linear increase in log-ovulation rate with log-dose of P.M.S.G. The inclusion of follicle counts with numbers of corpora lutea as a measure of ovarian response, produced larger standard errors associated with the mean response than when ovulation rate was considered alone (Larsen, 1971). Holst (1969) has shown significant dose effects of P.M.S.G. in inducing increases in the number of 'persistent' follicles and the number of corpora lutea plus 'persistent' follicles.

To counteract differences in response recorded by individual workers in different environments, Averill (1958) suggested the use of regression analysis, when drawing comparisons. The slope of the response line would then offer a common denominator for all trials.

Recently, Hunt et al. (1971) have noted a better ovarian response (number of corpora lutea) when sheep F.S.H. was used in preference to P.M.S.G. for superovulating sheep.

Factors Affecting Response to Pregnant Mare's Serum Gonadotrophin

1. Breed

Some of the discrepancies between results obtained by different authors (see Fig. 1-1) in ewe response to P.M.S.G. may be resolved when breed differences, or differences in inherent fecundity, are taken into account. Robinson (1951) used breeds of a higher natural fecundity (natural ovulation rate of 1.4-1.9) than did Wallace (1954) (1.13-1.17) and produced a steeper curve than the latter.

McDonald and Ch'ang (1966) found differences in ovulation rates between normal ewes of the same strain when they were exposed to different environments. Using P.M.S.G., Braden et al. (1960) showed differential responses between ewes of the same breed which were obtained from different sources. Wallace (1954) has described a similar effect when treating Romney ewes of different strains.

The finding that breeds of an inherently higher fecundity will respond better to superovulation is borne out by the work of Bradford et al. (1971) yet Bindon et al. (1970) have found that ewes of a higher natural ovulation rate may need higher doses of P.M.S.G. to

effect maximal superovulation, than ewes of comparatively lower fecundity.

2. Liveweight and Nutrition

Hammond (1952) postulated that high plane feeding of ewes produced more follicles with an antrum than did low plane feeding. The influence of liveweight on ovarian activity has subsequently been verified (Coop, 1966; Allison, 1968). However, Wallace (1954) found increased nutrition, resulting in elevated liveweight (flushing), to have little effect on ewe ovulatory response to P.M.S.G. The increase in ovulation rate, effected by the hormone, was noted on all planes of nutrition but was larger in ewes on a sub-maintenance diet than for flushed ewes (Allen and Lamming, 1961). In agreement with these latter authors, Lamond (1963) noted that administration of P.M.S.G. may compensate for nutrient deficiencies on low planes of nutrition. Lamond also presented tentative evidence that, for ewes suffering from undernutrition, responses to gonadotrophin may be more closely aligned to bodyweight.

Recent work (Newton et al., 1970; Bradford et al., 1971) indicates little evidence of a relationship between liveweight of the ewe and the ovulatory response to P.M.S.G. Tait (1971) showed no evidence of an interaction between response to P.M.S.G. and nutritional levels when considering number of lambs born per ewe. Allison (1973) and Eastwood (1973) reported that ewes fed on a high plane of nutrition (higher liveweight) showed a better ovarian response to P.M.S.G. than those on a low plane of nutrition (lower liveweight) but the differences were statistically not significant in both cases.

The fasting of heifers after a low plane of nutrition will cause a decrease in ovulation response to P.M.S.G. but will not do so after a high plane of nutrition. Fasting also leads to a greater variation in the heifer's response to the hormone (Lamond, 1970).

Breed and inherent fecundity thus seem more likely to cause differences in response to P.M.S.G. than differences in liveweight.

3. Season of Administration

Seasonal differences in the incidence of multiple ovulations in ewes have been noted by Radford (1959). McDonald and Ch'ang (1966) reported a similar variation during the breeding season of Romney ewes which showed negligible changes in liveweight over the period of observation. These latter authors noted the natural ovulation rate of ewes to rise between the first and third oestrous cycles of the breeding season.

Braden et al. (1960) detected better superovulatory responses to P.M.S.G. when treating ewes early in the breeding season in preference to treatment late in anoestrous. However, with non-superovulatory doses, P.M.S.G. may induce similar ovarian responses in anoestrous and cyclic ewes (Lamond, 1962). With Suffolk ewes, Averill (1958) noted a slightly (non-significant) greater ovulatory response to P.M.S.G. treatment in the breeding season, compared with progesterone-P.M.S.G. treatment in the non-breeding season. A small difference between ewes superovulated in different breeding seasons was also evident and may have been due to the time of the season in which the treatment was imposed.

Variations within a season in ewe sensitivity to oestrogen have been recorded by Raeside and McDonald (1959), Reardon and Robinson (1961) and Gibson and Robinson (1971). Liveweight and

nutritional status affect this response although light and temperature play a dominant part (Gibson and Robinson, 1971).

4. Age of Ewe

Robinson (1951), Averill (1958) and Gordon (1963a) have all reported that there is no differential response in ewes of different ages when the animals are superovulated with P.M.S.G.

5. Day of Injection

Administration of P.M.S.G. between days 12 and 15 of the oestrous cycle will usually effect similar ovulation rates in ewes (Robinson, 1951; Wallace, 1954; Bindon et al., 1960; Cumming, 1965; Bindon et al., 1971; Larsen, 1971).

There is an interaction between P.M.S.G. and progestagens which affects the ovarian response when the two hormones are used concurrently (Lamond, 1964a;b). Type of progestagen pretreatment may influence time after treatment at which P.M.S.G. should be injected, for optimal ovulation results from the gonadotrophin, (Lamond 1964b; Roberts and Edgar, 1966). Hulet and Foote (1967 and 1969) noted that P.M.S.G. treatment on the day of termination of progestagen synchronisation, yielded lower ovulation rates than if the same ewes were injected again during the following oestrous cycle.

6. Nature of P.M.S.G.

Whole serum appears to offer a better response in ewes (Gordon, 1958a) and in cows (Rowson, 1951 and Brock and Rowson, 1952) than freeze-dried (whole serum) powders. The latter are in turn better than purified powders. This fact may account for some of the

differences in response that are seen in Fig. 1-1. For example, Robinson (1951) used whole serum and obtained considerably larger responses than Wallace (1954) who used a dried preparation.

Mode of action of Pregnant Mare's Serum Gonadotrophin

Lamond (1964a) stated that:

> "Knowledge about the way whereby gonadotrophins act on the ovary is meagre".

However, with recent advances in assay techniques it is now easier to monitor hormonal fluctuations in vivo (see Pant et al., 1972). Study of the endocrinology of reproduction is also aided by the use of ovarian transplants (McCracken et al., 1969) which enable blood flowing to and from the gland to be monitored in the conscious animal.

There are suggestions that the effect of P.M.S.G. is mediated indirectly, through the pituitary. For example, Rennels and O'Steen (1967) exhibited a significant decrease in pituitary gonadotrophin (L.H. and F.S.H.) of rats treated with this hormone. The direct response of the ovary to P.M.S.G. is displayed by the administration of the hormone to hypophysectomised mice and rats (Lamond and Emmens 1959; Flux and Li, 1965). However, ovulation will not occur in the absence of the hypothalamus (Quinn and Zarrow, 1964; 1965), suggesting that neuro-endocrine pathways are able to mediate and enhance ovarian response to the exogenous gonadotrophin. Stimulated follicles will become luteinised (Cole, 1969), but it is thought that P.M.S.G. must elicit a

release of pituitary L.H. before ovulation may occur (Quinn and Zarrow, 1964; De La Lastra, 1972). Lamond and Emmens (1959) have shown that responses of mice given P.M.S.G. are lessened by hypophysectomy and suggest that endogenous L.H. is the limiting factor in mouse response. Administration of H.C.G. to such animals will enhance the effect of P.M.S.G. (Lamond and Bindon, 1966). Apart from the role played by endogenous L.H., in ovulatory response to P.M.S.G., the exogenous hormone may itself contribute to the luteinising of follicles and ovulation of the same (Williams, 1945a;b). A preparation of P.M.S.G. used by Lamond (1960) was estimated to have approximately one third the L.H. potency of H.C.G., as a source of L.H. and Schmidt-Emendoff et al. (1962) found a preparation of the hormone to have an F.S.H./L.H. ratio of 2.10-like.

To what extent the L.H. moeity in P.M.S.G. preparations may contribute to the L.H. required to ovulate stimulated follicles in the ewe is not known. Bindon et al. (1971) and Allison (1973) have suggested the use of hypophysectomised sheep to clarify this point.

Pigon et al. (1960) discovered a decrease in pituitary L.H. content when ewes were given P.M.S.G. The importance of the pre-ovulatory surge in L.H. is well documented (see Pant et al., 1972). Hence the reliance of superovulation on an adequate supply of endogenous L.H. is easy to imagine. In fact, differences in L.H. contents of circulating blood or pituitaries of ewes may be primary cause of different responses among ewes given P.M.S.G. (Bindon et al., 1971).

In the ewe, P.M.S.G. will cause follicle growth in the anoestrous period (Robinson, 1954) and effective superovulation during the breeding season (Larsen, 1971 and many others). Using ovarian transplants, Baird et al. (1968) found P.M.S.G. to cause release of oestradiol-17-Beta from the ovary with a delay of 2+ days from the time of infusion. The delay before a rise in L.H., effected by the same hormone, is 41-46 hours (Cumming et al., 1971). Although L.H. infusions will increase the steroid output of the autotransplanted ovary (McCracken et al., 1969), which could in turn elicit the release of endogenous L.H. (Short, 1972), the above suggests that P.M.S.G. may itself stimulate the direct release of endogenous L.H. from the pituitary. This could occur before a rise in circulating oestrogens and hence be a direct effect of the gonadotrophin on the pituitary. This argument is based on the fact that P.M.S.G. will produce a rise in circulating levels of L.H. before a rise in oestrogens.

An ovulating dose of L.H. extracts may (Braden et al., 1960; Hunt et al., 1971) or may not (Robinson, 1951) augment a more efficient superovulatory response to P.M.S.G. in ewes.

Greep (1973) in reviewing the state of knowledge about gonadotrophins indicates that the primary site of action of F.S.H. is on the granulsoa cells of growing follicles. Action of such hormones is thought to be mediated by cyclic A.M.P.* (Greep, 1973).

* 3'-5' - Adenosine Monophosphate

Chemical Characteristics of Pregnant Mare's Serum Gonadotrophin

Information on the chemistry of P.M.S.G. has been reviewed by Geshwind (1963) and Papkoff (1969).

P.M.S.G. is a molecule consisting of peptide chains, carbohydrate and sialic acid (Gospodarowicz; 1972). Estimates of molecular weight have ranged from 28,000 (Papkoff, 1969) to 68,000 (Morris, 1964). Gospodarowicz (1972) decided that, with a molecular weight of 53,000, being reduced and alkylated to 23,000, P.M.S.G. is an oligomeric molecule composed of two sub-units.

Immunology of Pregnant Mare's Serum Gonadotrophin

Soon after the discovery of P.M.S.G., by Cole and Hart (1930), Selye *et al.* (1934) detected the formation of antibodies being produced against the exogenous gonadotrophin. With this realisation a large interest was taken in the field of anti-hormones. Reviews on the early work have been prepared by Collip *et al.* (1940), Thompson (1941), Leathem (1949), Rowlands and Parkes (1965) and Wright (1965).

Thompson (1941) stated that the early literature seemed:-

> "Almost hopelessly confused by the number of hormones, the variance of their sources, the wide variety of test animals experimented upon and the number of different approaches used in the study of the problem".

Rowlands and Parkes (1965) decided that:-

"Responses of animals to prolonged injections of anterior pituitary extracts of heterozoic origin is that the hypertrophy produced initially by the target organ is not maintained indefinitely. Ultimately a state of physiological hypophysectomy is reached. In these circumstances the ovaries have lost their capacity to respond to injected gonadotrophin".

But refractoriness of the ovaries was not thought due to insensitivity of the organ by other workers. Follicular exhaustion of the ovary was excluded as a possibility because animals incapable of further response to one gonadotrophin were sensitive to preparations from other species (Hafez et al., 1964), and refractoriness was not seen to develop when homologous extracts were injected (in fact pro-gonadotrophic responses were reported (Katzman et al., 1947).

Initially antibodies to hormones were called "anti-hormones" yet it was not known whether anti-hormones and antibodies to hormones were the same (Geshwind, 1963). In fact poor correlations were observed between anti-hormonal activity and any chemical methods used to quantitate the extent of the reaction e.g. Van Den Ende (1941). Some authors supported the view that the more pure a hormone preparation was, the less likely it was to be antigenic (Rowlands and Parkes, 1965).

P.M.S.G. is regarded as a good antigen when injected into heterozoic species (Rowlands and Parkes, 1965). This may be

attributed to its high content of peptide sub-units and its long half life in the blood stream (Parlow, 1961). For the latter reason a single injection was found to be as effective as multiple injections in causing increased ovarian weight in immature mice (Connell, 1965). The rate of loss from the blood stream appears to be independent of dose (Lamond, 1959).

Preparations of P.M.S.G. are regarded as being very impure (see Flux and Li, 1965). Hence the administration of the hormone may enhance the formation of a number of antibodies not related to the gonadotrophin molecules (Van Den Ende, 1941, Flux and Li, 1965). However, fewer antigen-antibody complexes are formed with more pure preparations.

Components of preparations other than the hormone fraction may also be potent antigens (Van Den Ende, 1941). The fact that impurities may be more strongly antigenic than the hormone itself has been displayed by Segal et al. (1960) and Segal et al. (1962).

Antibodies to P.M.S.G. include a fraction reacting with horse serum proteins. These are partly absorbed by normal mare's serum without any loss of anti-P.M.S.G. potency (Flux and Li, 1965). Injecting normal mare's serum into ewes for two months will induce antibody production of a lower titre than a similar treatment with P.M.S.G., demonstrating that the hormone fraction has specific antibodies associated with it (Cole et al., 1957).

Ewe Ovarian Weight Response to Pregnant Mare's Serum Gonadotrophin

Robinson (1950, 1951) and Allen and Lamming (1961) measured ovarian weight after stimulating ewes with P.M.S.G. and found linear relationships between ovarian weight and dose level of the hormone. A straight line is also obtained by the regression of ovarian weight on the number of corpora lutea present (Robinson, 1951).

Hutchinson and Robertson (1966), observing unstimulated ewes, have found a significant relationship between total follicular volume and ovarian weight in ovaries devoid of corpora lutea.

Assay of Gonadotrophins and Anti-gonadotrophins

The value of using immature mice to assay gonadotrophins has been indicated by Brown (1955). Green (1955) states that the immature mouse ovary secretes hormone within 12 hours of an injection of Equine Pituitary Gonadotrophin. The degree of response may then be quantitated by measuring uterine or ovarian weights.

Uterine and ovarian responses in the mouse have been recorded with H.C.G. (Lamond and Bindon, 1966), with F.S.H. (Brown, 1955, Igarashi and McCann, 1964 and Bell, 1969) and with P.M.S.G. (Green, 1955; 1956; Suhiro *et al.* 1955; Sasamoto, 1972; Christiansen and Eleftheriou, 1972).

Hypophysectomised mice may be used for the specific assay of F.S.H., measuring its augmentation of the response by H.C.G. (Lamond and Emmens, 1957; Uteroi and Meyer, 1967; Lamond and Bindon, 1966; Bindon and Lamond, 1966). Lamond and Bindon (1966)

state that the uterine weight is a more sensitive indicator of F.S.H. potency than ovarian weight, even though the ovary is the primary target of the hormone. Intraperitoneal injection increases the sensitivity of the assay of P.M.S.G. in immature mice, as compared with subcutaneous injection (Sasamoto, 1972). Lin and Bailey (1965), Zarrow et al. (1972) and Bell (1969) have adequately demonstrated that the response varies significantly between strains of mice.

Cole and Erway (1941) developed a 48 hour assay test for Equine Pituitary Gonadotrophin with results expressed in International Units. The test was based on measuring the ovarian response in 25-day-old rats. Cole et al. (1957) modified this assay to measure the inhibition of response to injected P.M.S.G., by antiserum against the hormone. Results are reported by indicating the % inhibition of the response. The same method was used by Pigon et al. (1960) to measure anti-P.M.S.G. titres in chronically treated ewes and by Jainudeen et al. (1966) for a similar study with cattle sequentially treated with the hormone. Nakahari et al. (1964) employed immature mice to detect antibodies against P.M.S.G. which were produced in cattle. Johnson (1962) used both rats and mice for the detection of anti-gonadotrophins.

Since Ouchterlony (1949) developed the technique of investigating antibody-antigen systems by means of double diffusion through an agar gel, the method has been widely used (see Rees-Midgely 1969).

In particular, Segal et al. (1960) and Segal et al. (1962) have used the technique to diagnose the immunology of gonadotrophins. Flux and Li (1965) have investigated the cross reactions amongst gonadotrophins by this means and compared the results with those from biological inhibition tests, performed in hypophysectomised rats. Cross reactions and impurities in the hormone-antisera complexes complicate analysis by this method although these drawbacks may be overcome by the use of electrophoresis, chromatography and the removal of unwanted antibodies by absorption with specific antigens (Flux and Li, 1965 and Rees-Midgely, 1969). The presence of non-precipitating antibodies must always be considered when interpreting results from this method (Rees-Midgely, 1969) and there are some indications that antigen-antibody complexes involving P.M.S.G. may be of this kind (Johnson, 1962).

Refractoriness of the ovary in ewes sequentially treated with Pregnant Mare's Serum Gonadotrophin

Refractoriness due to "anti-substances" was noted by Parkes (1942). When rabbits were stimulated with Horse Anterior Pituitary Extracts (H.A.P.) the initial hypertrophy of the ovaries was followed by a decrease in the number of follicles developing with subsequent treatments. Adams (1953) noted a similar effect.

A summary of the literature pertaining to the refractory condition is given in Table 1-1.

TABLE 1-1

Literature reporting refractory conditions of the ovary after Pregnant Mare's Serum Gonadotrophin treatment

Species	Authors
Bovine	Dzuik et al. (1948); Willett et al. (1953); Cole et al. (1957); Hafez et al. (1964); Nakahari et al. (1964); Jainudeen et al. (1966); Laster et al. (1971).
Ovine	Pigon et al. (1960); Mulet and Foote (1967, 1969); Larsen (1971).
Murine	Fowler and Edwards (1957); Edwards and Fowler (1960); Lin and Bailey (1965); Land and McLaren (1967).
Rodentia	Johnson (1962); Greenwald (1963).
Largomorpha	Parkes (1942); Adams (1953).

Fowler and Edwards (1957) produced a refractory condition in mice by injecting P.M.S.G. and H.C.G. Edwards and Fowler (1960) decided that, since four successive ovulations could result from four series of gonadotrophic stimulation, follicular exhaustion was not likely to be responsible for refractoriness. Lin and Bailey (1965) substantiated this claim by producing one to ten superovulations in mice on similar treatments.

Land and McLaren (1967) have postulated anti-P.M.S.G. to be responsible for refractoriness developing in mice subjected to successive superovulations.

Dzuik et al. (1948) detected no consistent reduction in superovulation when cows were repeatedly stimulated with gonadotrophins, the interval between treatments varying from 49 to 359 days. However Willett et al. (1953) obtained a refractoriness by treating cows with a variety of gonadotrophins. Hafez et al. (1964) and Laster et al. (1971) realised lower ovarian responses to retreatment of cows with P.M.S.G. Cole et al. (1957) and Jainudeen et al. (1966) demonstrated anti-gonadotrophic activity in cows repeatedly stimulated with P.M.S.G., the former using precipitation tests and biological inhibition tests and the latter using biological inhibition tests alone.

Cole et al. (1957) and Nakahari et al. (1964) decided that antibodies to P.M.S.G. would only be produced in cattle if the animals were treated with supra-physiological levels of the hormone.

An animal does not normally develop antibodies to endogenous gonadotrophins. However, in some cases, when a protein hormone of a different species is introduced, the antibody formed against it also reacts with the endogenous hormones. Hence, rats immunized with ovine L.H. developed an antibody which neutralised endogenous L.H. (Wakabayashi and Tamaoki, 1966). Lin and Bailey (1965 and Witschi and Johnson (1960) have also hinted that repeated injections of P.M.S.G. may inhibit endogenous hypophyseal control.

Jainudeen et al. (1966) decided that normal follicle development continued under the influence of endogenous hormones while antibodies to injected P.M.S.G. rendered exogenous influence incapable of stimulating the ovaries of cows.

Greenwald (1963) showed that anti-P.M.S.G. would not interfere with the endogenous control of cyclic behaviour in hampsters. To reinforce this argument, Land and McLaren (1967) found that even though the response of mice to Human Chorionic Gonadotrophin (H.C.G.) ceased after repeated injections, normal cycles resumed later, in spite of continued treatment. These latter authors thus concluded that the effect was that of an immunity to the injected hormone, not a refractoriness of the ovary. However they did not seek any evidence of antibodies being present.

Antisera to one particular hormone may also inhibit the action of other exogenous hormones (see Lunfeld and Eshkol, 1969). The basis of this cross-reactivity may lie in the similarity in the antigenic sites on the hormones (Geshwind, 1963). Similarities in taxonomic relationship between animals may also enhance cross-reactivity (Katzman et al., 1947 and Geshwind, 1963).

P.M.S.G. is sufficiently similar to Equine L.H. to result in confluent patterns in agar gel diffusion tests (Desjardins and Hafs, 1965). These authors also saw Ovine L.H. to be neutralised (biologically) by antisera to Equine L.H. They also saw that antisera to P.M.S.G. and Ovine L.H. would not inhibit the action of H.C.G. in hypophysectomised rats. Ely and Chen (1967) also showed antiserum to Ovine L.H. to inhibit P.M.S.G. action.

In considering the above results it is important to note that because an antibody is agglutinating, precipitating or neutralising, it does not necessarily follow that these processes parallel one another (Geshwind, 1963).

Pigon et al. (1960) subjected ewes to chronic treatment with P.M.S.G. and developed a potent antiserum. The treatment resulted in a lowered pituitary content of L.H. which may be one of the factors responsible for the failure of such ewes to ovulate.

Hulet and Foote (1967) demonstrated a fall off in ovulation rate when ewes were superovulated at six successive oestrous cycles. Investigating the phenomenon more fully, Hulet and Foote (1969) showed the development of a definite refractory condition yet did not seek evidence of antibody production. Information on the dissipation of the condition with time was revealed. Larsen (1971) found a similar effect in New Zealand Romney and Border-Leicester-Romney cross ewes when the ewes were treated over three successive cycles; a high incidence of cystic and luteinised follicles resulted from the treatments.

Conflicting reports on the effects of dose level of administered hormone on the refractory condition make conclusions difficult. Parkes (1942) showed that a geometric rise in dosage every few days failed to overcome the increasing insensitivity of the ovary. Willett et al. (1953) and Jainudeen et al. (1966) decided that increases in dose of P.M.S.G. would partially overcome the condition. This was not verified with similar treatment of sheep (Larsen 1971). Pigon et al. (1960) reported that 200 I.U. elicited a more rapid manufacture of antibodies in sheep than 500 I.U. when P.M.S.G. was given at chronic levels. Higher doses were seen to be more effective for antibody production in cows (Cole et al., 1957) and rats (Johnson, 1952).

Antibodies to P.M.S.G. have been noted in the blood streams of treated animals some considerable time after sequential treatments with the hormone (Nakahari et al., 1964; Jainudeen et al., 1966) and have been postulated to be present up to a year from treatment (Willett et al., 1953; Hulet and Foote, 1969). However Pigon et al. (1960) stated the effect to be dissipated over three weeks subsequent to the treatment.

A second series of gonadotrophic injections, some time after the first, will effect an upsurge in antibody titre (Cole et al., 1957; Pigon et al., 1960; Nakahari et al., 1964). This is typical of an immune-like response (Nossal, 1971).

Follicular growth and atresia in the ewe ovary

Growth of ovarian follicles in the ewe has been studied by Grant (1934), McKenzie and Terrill (1937), Kammalade et al. (1952), Santalucito et al. (1960), Robertson and Hutchinson (1962). Hutchinson and Robertson (1966) Smeaton and Robertson (1971). Holst et al. (1972) and Brand and De Jong (1973). Follicular dynamics has been investigated in the mouse (Peters and Levy, 1966; Petersen, 1970), the pig (Robinson and Malbandov, 1951; Parlow et al., 1964) and the cow (Rajakoski, 1960; Erickson 1966; Choudary et al., 1968). Folliculogenesis in a variety of mammals has been reviewed by Mauleon (1969), follicular atresia by Ingram (1961).

The preovulatory surge in follicular growth has been recognised for a considerable time (Grant 1934). McKenzie and Terrill (1937) stated that the number of small follicles (1-2 mm.) in the ewe ovary

was quite variable and some individuals had consistently more than others. "Great variation" in the number of ovarian follicles was also observed by Brand and De Jong (1973) in 33 normal Texel ewes. The latter authors found a pair of ovaries to contain 32.2% (Mean= 84.2,S.E.=38.1) normal and 67.8% (Mean=177.1,S.E.=103.4) atretic follicles with diameters greater than 2 mm. However, of the normal tertiary follicles, 82.7% had a diameter of less than 1 mm. Choudary et al.(1968) found a mean relative proportion of 23.9% normal and 76.3% atretic follicles present per pair of ovaries in cyclic heifers of unspecified breed.

Kammalade et al. (1952) and Santaucito et al. (1960) stated that follicle growth tended to be continuous throughout the cycle. Robertson and Hutchinson (1960) and Hutchinson and Robertson (1966) upheld this concept and considered that follicles destined to ovulate at the ensuing oestrus begin to enlarge 4-5 days after the onset of the previous oestrus and then pause until a pre-ovulatory surge in growth. However Smeaton and Robertson (1971) point out that these conclusions were deduced from observations on the ovaries of different animals killed at various stages of the cycle. This latter study traced the pattern of follicular growth by injecting Indian ink into the follicles and thus monitored subsequent development of follicles previously observed. This method relied on the assumption that the follicles continued to grow normally after the administration of ink. The study suggested that there may be three or more waves of follicle growth during one cycle. Follicles enlarging at the beginning of the cycle may become atretic and not ovulate. This concept of follicular

growth is consistent with the fact that one may show three peaks of oestradiol-17-beta secretion from the ewe ovary on days 3-4, days 6-9 and days 11-15 after oestrus (Mattner and Braden, 1972). Holst et al. (1972) show these peaks to be concomitant with an increase in follicular volume of the ovary. Brand and De Jong (1973) have recently found only two growth waves of follicles in sheep ovaries. The first was detected from days 1-10 and the second from day 6 of one cycle to day 1 of the next.

Peters and Levy (1966) and Petersen (1970) have traced follicular growth in the mouse ovary by using radio-active thymidine.

Brand and De Jong (1973) suggest that the growth pattern of enlarging follicles is influenced by the levels of circulating progesterone. The end of the first wave of growth, detected in their study, was at the time one would expect progesterone levels to have reached maximum levels in the blood and enlargement of preovulatory follicles on day 16 was at a time of expected low levels of plasma progesterone.

There is a sharp increase in the size of the corpus luteum of the ewe between 36 hours after ovulation and 5 days after onset of oestrus (Hutchinson and Robertson, 1966). Luteal regression begins about day 10 (Grant, 1934; Santalucito et al., 1960; Hutchinson and Robertson, 1966). Rapid degeneration occurs between this time and the onset of the next oestrus (Hutchinson and Robertson, 1966).

PURPOSE AND SCOPE OF THE INVESTIGATION

The production of antibodies to P.M.S.G. has been demonstrated in sheep. The fall in ovulatory response of ewes treated sequentially with this hormone has been postulated due to the development of such anti-P.M.S.G. factors.

The first part of this project considered the development of a refractory condition in response of ewes treated with P.M.S.G. over three oestrous cycles. Plasma from these ewes, and that of ewes chronically treated with the gonadotrophin, were tested by a Biological Inhibition Test, using immature mice. The hypothesis that ovarian refractoriness in P.M.S.G.-treated ewes was due to anti-gonadotrophic agents could thus be tested.

On the basis of these investigations, it was considered necessary to elucidate other possible casual factors in the development of a refractory state of the ovaries in sequentially treated ewes. Hence in a further experiment, the possibility that the supply of normal mature (antral) Graafian follicles was depleted by repeated treatment, was examined.

CHAPTER II

MATERIALS AND METHODS

Chapter II

MATERIALS AND METHODS

Sheep and their Management

Ewes used in this study were 'cast for age' (five or more years) Romneys with no records of previous lambing performance. Sheep for Experiment 1 were purchased in March, 1972 from a mixed line of ewes offered for auction at a local sale. The animals for Experiments 2 (8 sheep) and 3 (40 sheep) were 'cast for age' from various flocks on the Massey University farms. The latter ewes were consequently derived from an environment similar to that in which the experiments were conducted.

All ewes were rotationally grazed on Ryegrass-White Clover pastures with yard and surgical facilities nearby. Sheep of Experiment 1 were offered pasture adequate to effect only a slight loss of body weight over the duration of the trial. The district suffered drought conditions during January-February, 1973 and sheep of Experiment 3 were subjected to feed restrictions at this time. The drought ended on March 5th, 1973 and good autumn pastures were available for the experimental period. Liveweight changes of ewes over the duration of Experiments 1 and 3 are shown in Fig.2-1 and Fig.2-2 respectively.

Ewes were identified by serially numbered ear tags and corresponding numbers branded on their shoulders. Vasectomised rams ('teasers'), fitted with Sire Sine mating harness, were run

FIGURE 2–1:– EWE LIVEWEIGHTS AT THE BEGINNING AND END OF EXPERIMENT 1

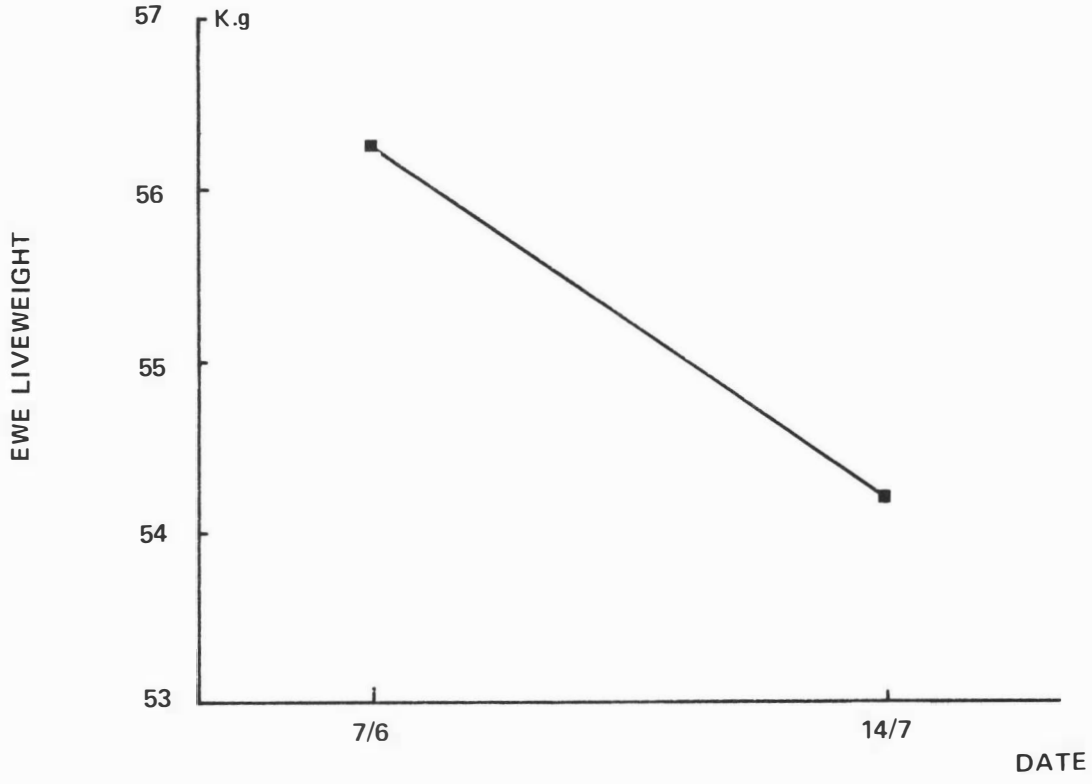
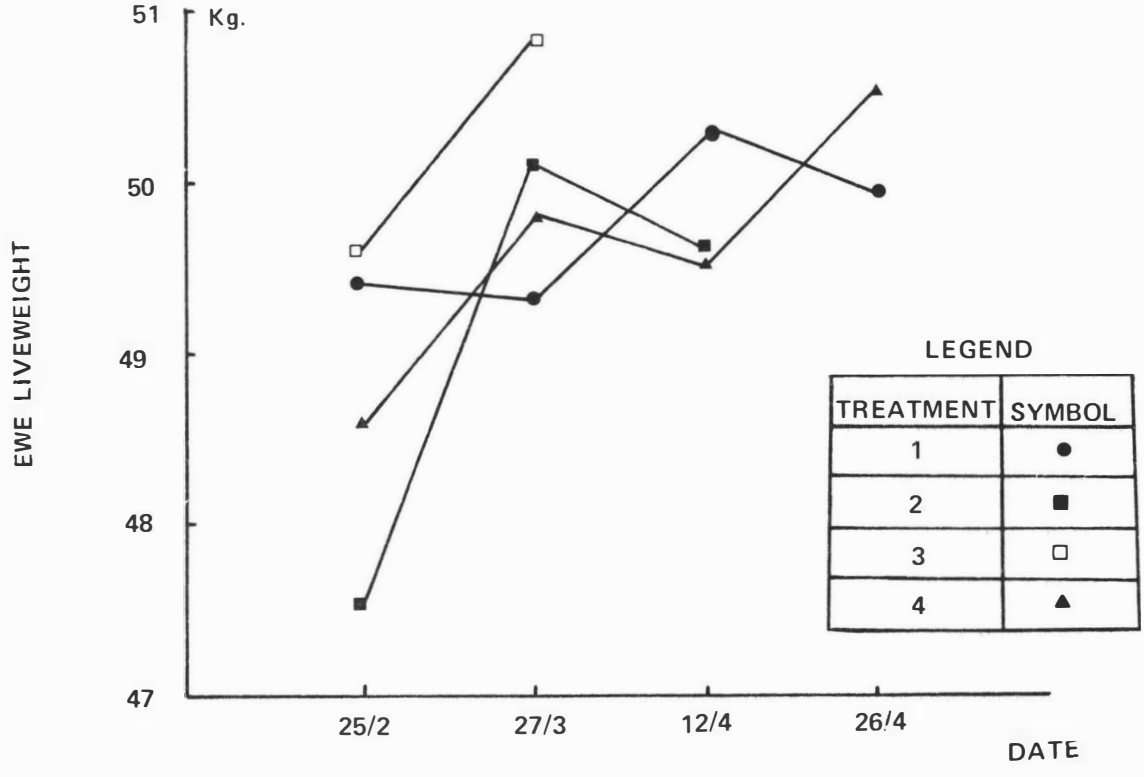


FIGURE 2–2:– EWE LIVEWEIGHT CHANGES DURING EXPERIMENT 3



with the ewes during all experimental treatments except when the latter had recently been laparotomised or mated. When laparotomies were performed the operated ewes were run without a ram for 3 days to prevent 'rape' services while they recovered. The ram to ewe ratio was never less than 1/40 and was usually greater.

Observations to record oestrus in ewes were made at 08.00–09.00 hr. and 17.30–18.30 hr. Marked ewes were withdrawn from the flock to increase the efficiency of oestrus detection in the remaining ewes. The sheep were yarded to inspect the rump regions for crayon markings that signified service by the teaser rams.

Prior to laparotomy, ewes were yarded and starved overnight. This diminished the risk of loss associated with anaesthesia and surgery. After operation, ewes were placed in pens to recover and then allowed to move back to pasture in their own time.

Ewes of Experiment 1 were subjected to progestagen treatment in the middle of the breeding season and had therefore experienced a number of oestrous cycles prior to the treatment. Experiment 2 was carried out in the non-breeding season and ewes were not treated with progestagens. Synchronisation of oestrous cycles of ewes in Experiment 3 was initiated before the 1973 breeding season began. In 1973 ewes on the same farm as that used for these experiments, were first seen to display oestrus on 18 February. This was 2 days after progestagen sponges had been inserted intravaginally into sheep of Experiment 3.

Mice and their Husbandry

Mice were employed in a test for anti-gonadotrophins (Experiment 2). They were descended from the inbred NOS strain derived from a stock of albino mice of unknown origin and have been previously described by MacKenzie (1972). This strain is known to be characteristically oestrogen sensitive (Anon, 1965). Only immature females, weighing between 7-10 grams, and being approximately 25 days of age, were used.

The mice were collected over a period of 4 months (December, 1972 to March, 1973) by weaning litters that had reached the desired age or weight. Both these factors were taken into account in order to obtain mice of a uniform size i.e. stage of maturity. Mice from larger litters took longer to reach the desired weight than those of smaller litters.

Plastic mouse boxes, with a flooring of saw dust, were used. Pelleted food and water were provided ad libitum. The mice were kept in the same room and held at ambient temperatures.

Experimental Design

The investigation involved three experiments:-

Experiment 1: The development of an ovarian refractoriness in ewes subjected to various sequences of P.M.S.G. injections over three oestrous cycles.

Experiment 2: Biological Inhibition Test for anti-gonadotrophins.

Experiment 3: Ovarian follicular development in ewes super-ovulated up to three times with P.M.S.G.

Flow diagrams outline the design of Experiments 1 and 2 in Fig.2-3 and Fig.2-4. Design of Experiment 3 is set out in Fig.2-6.

Experimental Procedure

Experiment 1

Eighty-two animals were available for treatment. Initially, the oestrous cycles of all ewes were synchronised by progestagen administration. This technique aided the conduct of the experiment as all ewes could be treated within chronological limits, thus eliminating a temporal bias from the treatments. Furthermore, synchronisation facilitated simplification of treatment, in that time of injection, blood sampling and slaughter of experimental units could be carried out with efficient utilization of labour and resources.

Synchronisation was effected by the insertion of intravaginal pessaries (polyurethane sponges) impregnated with 40 mg. 6α-methyl - 17α- acetoxyprogesterone (M.A.P.).* The hormone was diffused into sponges in 3 ml. of 95% ethanol.

Sponges were inserted on 24-5-72 and withdrawn on 7-6-72.

All ewes in the experiment were weighed (straight off pasture) on the completion of progestagen treatment and again just prior to slaughter (Fig.2-1).

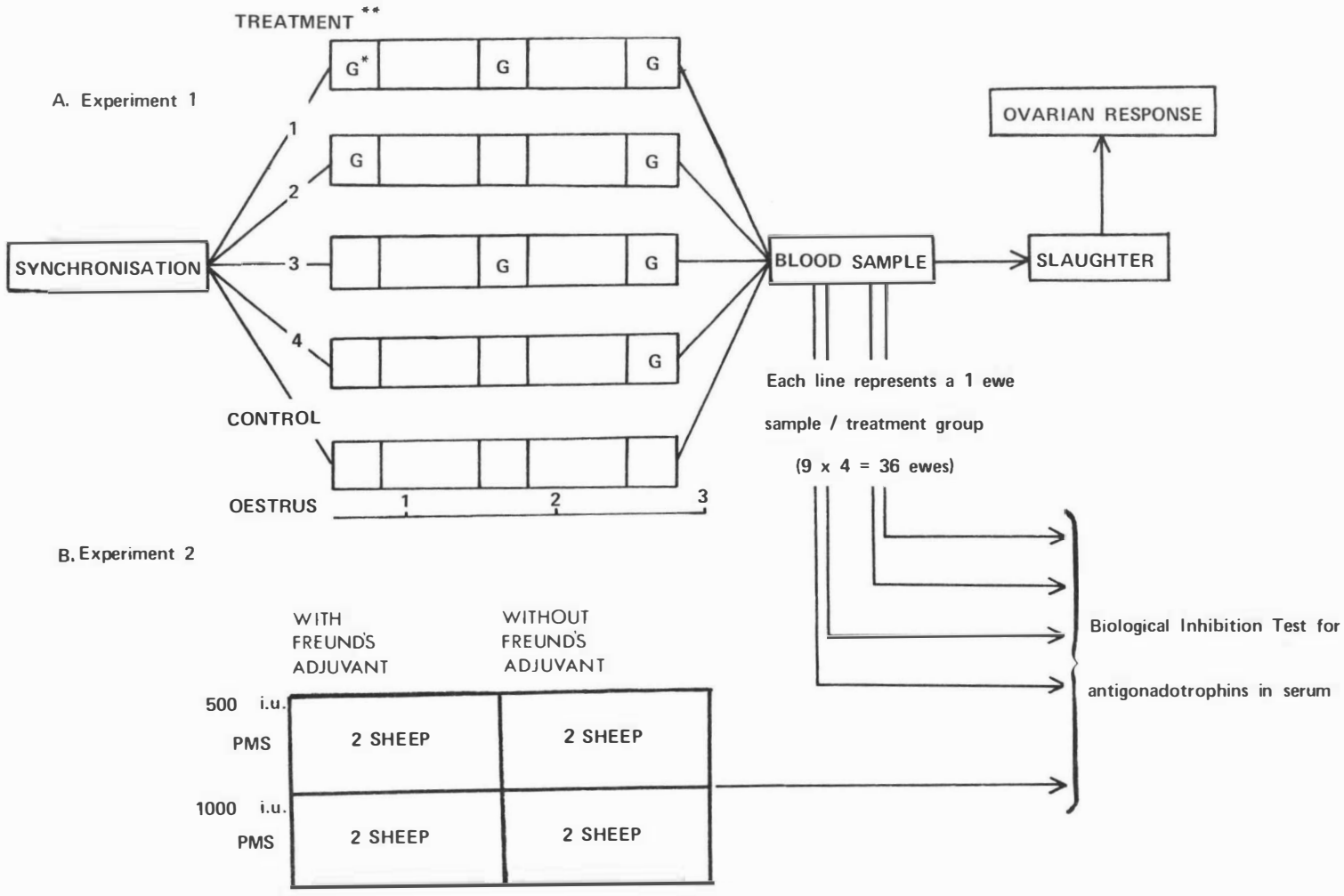
The sheep were allocated to treatment groups by consulting a table of random numbers. P.M.S.G.** was injected subcutaneously at dose levels of either 1000 i.u. or 1500 i.u. Ewes on each dose level were subjected to the following sequences of injections (see Fig. 2-3):-

* Upjohn Company

** Paines and Byrne Ltd.

FIGURE 2–3:–

EXPERIMENTAL DESIGN : EXPERIMENT ONE AND EXPERIMENT TWO

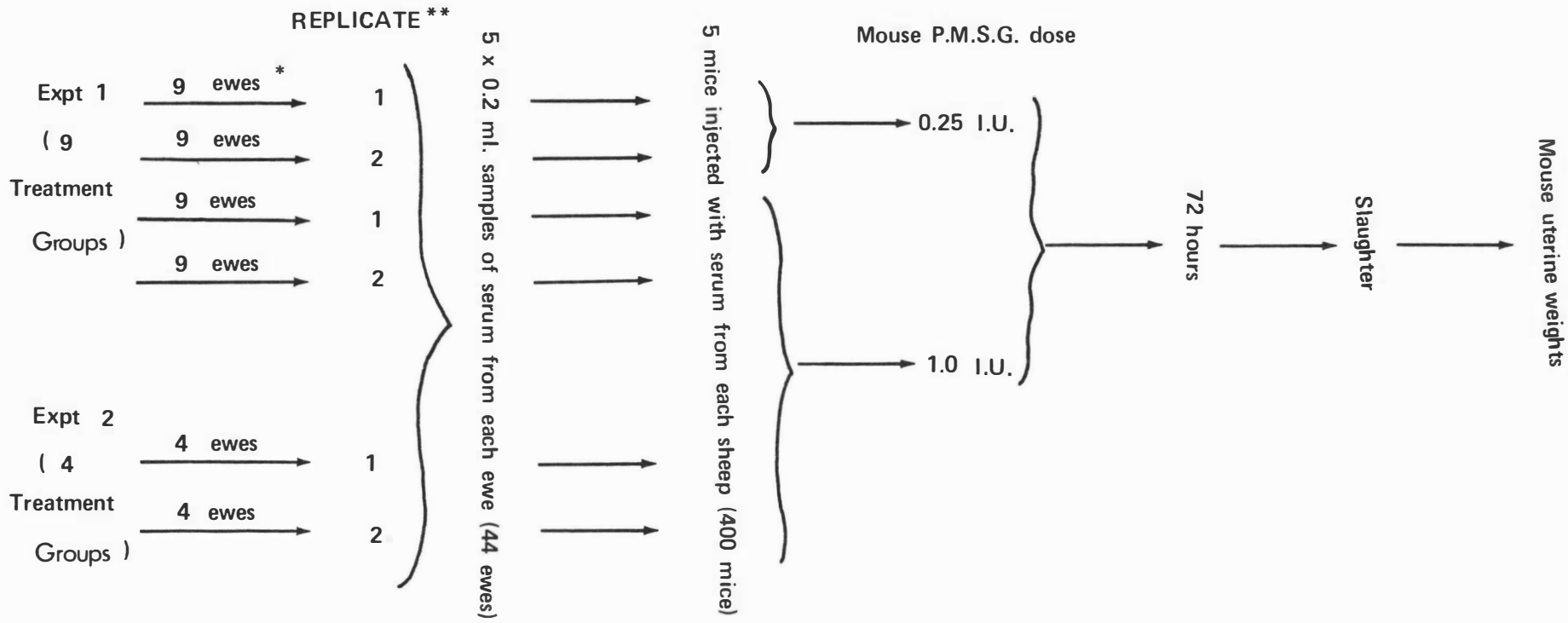


* G = Gonadotrophin

** Treatments 1–4 replicated at dose levels of 1000 i.u. and 1500 i.u. P.M.S.G.

FIGURE 2–4:–

DESIGN OF EXPERIMENT TWO



* Each line represents one ewe / treatment

** 2 replicates (1 ewe / replicate) for each ewe treatment group

Treatment 1: Injected at each of three oestrous cycles (cycles 1,2 and 3).

Treatment 2: Two injections (at cycles 1 and 3) separated by a normal oestrous cycle.

Treatment 3: Injected at two successive oestrous cycles (cycles 2 and 3).

Treatment 4: Injected at one oestrous cycle only (cycle 3).

The experiment was thus of 4 x 2 factorial design. Nine uninjected, 'synchronised' ewes constituted a reference group, enabling comparative responses of the treatment to be guaged. Further reference to the experimental groups will be made using the following abbreviations:-

L1, L2, L3, L4 refer to animals given 1000 i.u. P.M.S.G. in sequences outlined in treatments 1 to 4 above.

H1, H2, H3, H4 refer to animals given 1500 i.u. P.M.S.G. in sequences outlined in treatments 1 to 4 above.

Either 9 or 10 sheep were designated to each of these groups at the beginning of the experiment but 1 death from undetermined cause (Group H1) and 2 accidental pregnancies (Group H3) caused three sheep to be excluded from the results. One ovary of a ewe in Group L3 was lost as reproductive tracts were recovered from animals being slaughtered at the local freezing works and this sheep was excluded from analysis of ovarian response data.

Sequences of injection of P.M.S.G. as described above were designed to quantitate the ovulation rate of sequentially treated

ewes (Treatments 1 and 3) compared with ewes given 1 injection (Treatment 4) or control ewes (uninjected). Treatment 2 was included to investigate the terminal ovarian response of ewes given staggered injections. Two dose levels of P.M.S.G. were given to see whether the development of a refractory condition is dependent of the amount of gonadotrophin administered.

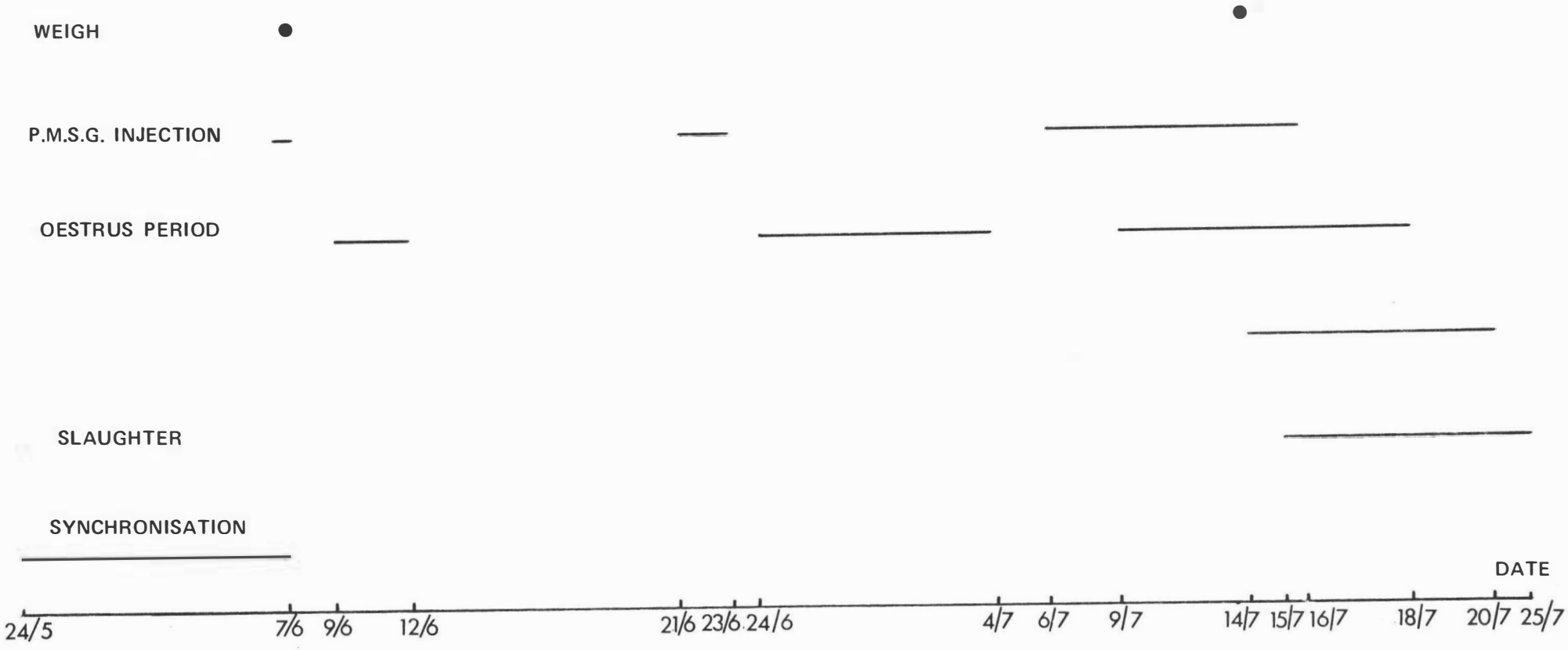
The first injections were made on the day of sponge withdrawal and subsequent injections were made on Day 12 of the oestrous cycle (Day 0 taken as the day of onset of oestrus). Because of variation in oestrous cycle lengths of individual ewes, the initial synchronisation became less defined as the experiment proceeded. Ewes were injected on Day 12 of their individual cycles regardless of this.

Five days after the onset of the third oestrus from sponge withdrawal, the ewes were blood sampled. Blood samples were taken from the jugular vein with 10 ml. Vacutainers* (heparin coated glass tubes which enable sampling direct from the vein, by means of a two way needle). The blood was centrifuged and the collected plasma was deep-frozen for analysis in Experiment 2.

Animals were slaughtered 6 days after the onset of oestrus following their final treatment with P.M.S.G. Those ewes not showing oestrus were slaughtered on a date derived thus: oestrous cycles lengths of all ewes on a similar treatment were averaged and the ewes having silent oestrus were slaughtered 6 days after this period of time had elapsed since their last oestrus.

*Biolab.

FIGURE 2–5:–
CALENDAR OF EVENTS FOR EXPERIMENT ONE



Reproductive tracts were recovered from the slaughtered ewes and data on ovarian response (ovarian weight, number of ovulations, number of follicles greater than 3 mm. and incidence of cystic of inactive ovaries) were recorded.

A calendar of events of this experiment is given in Fig. 2-5.

Experiment 2

Mouse tests for anti-gonadotrophins in the plasma of ewes from Experiment 1 were carried out between December 1972 and February 1973.

Anti-gonadotrophins in the plasma of animals chronically treated with P.M.S.G. were also detected by this method. Eight 'cast for age' ewes were injected subcutaneously with either 500 i.u. or 1000 i.u. P.M.S.G. Half the ewes were given their first injection in Freund's adjuvant (a water in oil emulsion of the hormone with heat killed mycobacteria added; Rees-Midgely, 1969). Sheep were injected on Monday and Thursday of each week, for 6 weeks (13-11-72 to 22-12-72). Plasma was collected on the Friday of the final week of treatment.

The Biological Inhibition Tests used were similar to the rat tests used by Cole *et al.* (1957), Pigon *et al.* (1960), Flux *et al.* (1965), and Jainudeen *et al.* (1966) to detect anti-gonadotrophins. This test relies on the inhibition of immature mouse uterine weight response to injected gonadotrophins by the introduction of antibodies to that gonadotrophin.

Determination of a suitable dosage of P.M.S.G. for stimulation of uterine weight in immature mice was carried out by means of a pilot test over a range of dose levels (0.2 i.u. to 1.0 i.u. P.M.S.G.).

Four sheep from each treatment group in Experiment 1 were randomly selected to donate plasma samples for the tests (9 groups, 36 ewes). Plasma of 2 sheep per group was tested in mice injected with 1 i.u. P.M.S.G. and the plasma of the other 2 sheep of that group was tested in mice injected with 0.25 i.u. P.M.S.G. The hormone was given to the mice as a single subcutaneous dose of the gonadotrophin dissolved in 0.2 ml. of 0.15% saline. 0.25 ml. samples of plasma from each ewe tested were injected (intra-peritoneally) into the test mice. Five plasma samples from each ewe were injected into mice given 1 i.u. and five into mice injected with 0.25 i.u. P.M.S.G. This sampling procedure is outlined in Fig. 2-4.

Mice were allotted to treatment groups by random numbers. They were weighed and earmarked before being placed in boxes. Each box housed only one treatment group of 5 mice. After being injected with P.M.S.G. and plasma, the mice were left for 72 hours. After re-weighing they were killed by cervical dislocation and the uteri were removed. These organs were dissected free of fat and any adhering tissue and then weighed on a torsion balance. Weighing of the animals before and after treatment allowed the detection of any adverse effects of injection e.g. plasma samples may have become contaminated during storage.

Samples of those sheep chronically treated (i.e. given twice weekly injections of P.M.S.G.) were tested only in mice injected with 1 i.u. of gonadotrophin. Procedure was the same as above except that all sheep's samples were tested. Since there were only two sheep on each treatment, a sample from each sheep permitted 2 replicate tests per ewe treatment group, to be carried out.

Experiment 3

Forty sheep were treated at the beginning of the 1973 breeding season. Intravaginal pessaries of similar preparation to those used in Experiment 1, were inserted on 16-2-73 and withdrawn on 28-2-73. Because ewes had not experienced recent overt oestrus prior to the progestagen treatment, a 10 mg. injection of progesterone dissolved in 1 ml. of peanut oil, was injected intramuscularly 2 days before sponge withdrawal. It was hoped such an injection would provide a more effective progesterone priming of the ewe's capability to display overt oestrus than has previously been noted in ewes treated early in the breeding season (see Cumming, 1965 and Larsen, 1971). On withdrawal of the pessaries some of the ewes were suspected to have experienced silent oestrus. Laparotomy of two such ewes confirmed that they had ovulated.

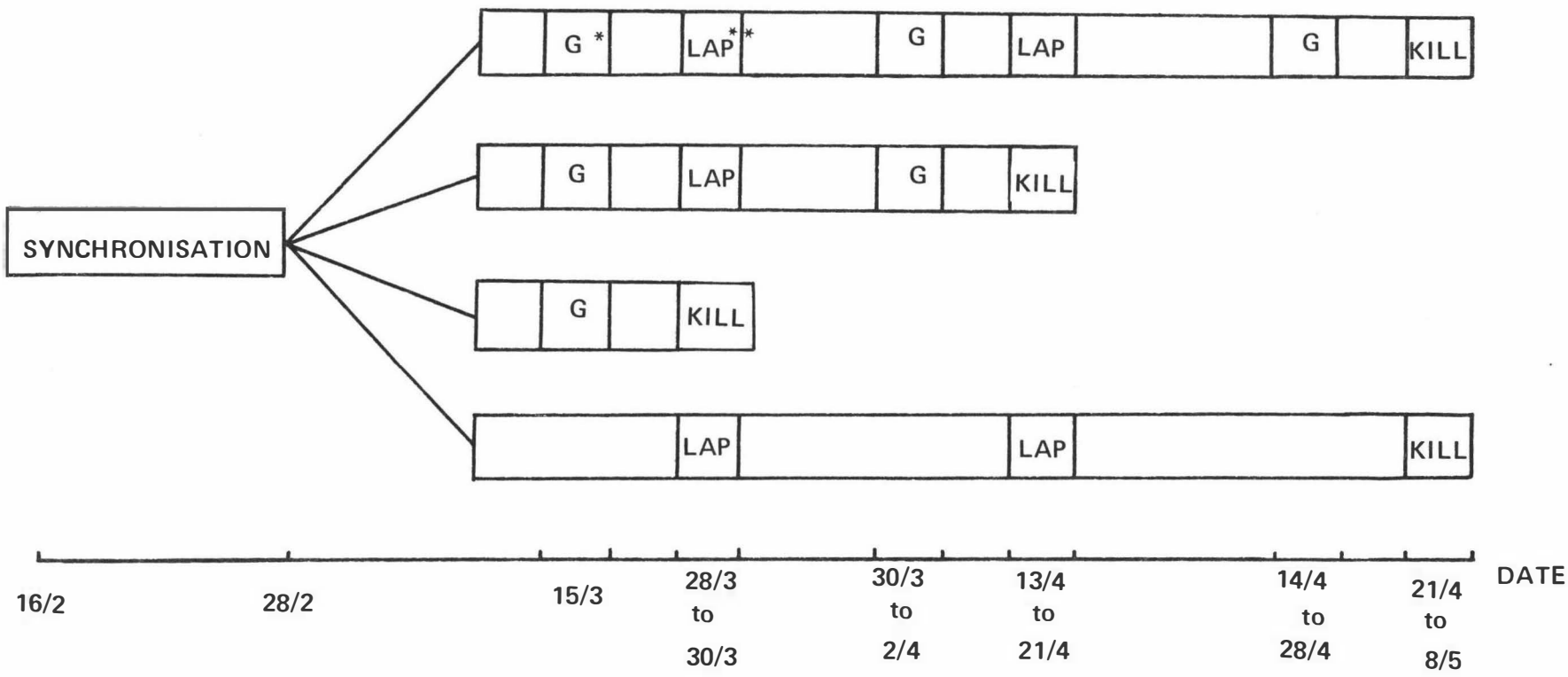
P.M.S.G. was injected on Day 12 of the cycle following sponge withdrawal. Subsequent injections were made on either Day 12 or 13 of the cycle.

The experimental design is shown in Fig. 2-6. Ewes were designated to 4 groups of 10 animals by random numbers. Group numbers were later rendered unequal by 1 death and 1 suspected pregnancy. Ewes were either uninjected (control) or injected once, twice or thrice (Fig. 2-6) over three oestrous cycles.

Day 10 of the oestrous cycle was chosen as a reference point to monitor ovarian activity. Observation at this point allowed measurement of ovulation rate at the previous oestrus as well as

FIGURE 2–6:–

DESIGN OF EXPERIMENT THREE



*G = Gonadotrophin

** Lap = Laporotomy

enabling assessment of follicles likely to be stimulated by a following injection of gonadotrophin. Treated ewes were either laparotomised or slaughtered on Day 10 (10 ewes being slaughtered after the first 2 superovulations and the third treatment group and control being slaughtered after the final superovulation).

Laparotomy was performed under light anaesthesia ("Nembutal"*, on a laparotomy cradle. A ventral incision, slightly off the mid-line, allowed exposure of the reproductive tract. Counts of corpora lutea and follicles were made. Follicle sizes were established by the use of sterile slide callipers (see Fig. 2-7) and were always made by the same person. The follicles were assigned to three groups; 1-3 mm. (small), 3-5 mm. (medium) greater than 5 mm. (large).

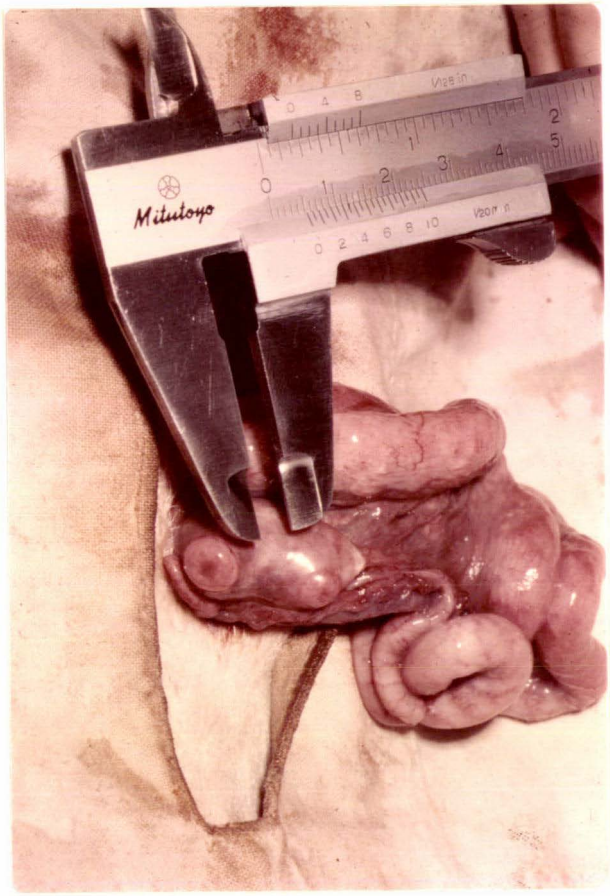
Ovaries recovered from the slaughtered animals were immediately scored as for laparotomised animals and fixed in 10% buffered formalin. After fixation (this time was variable depending on the size of the ovaries) the ovaries were placed in 50% alcohol for 24 hours and then stored in 70% alcohol. To assess the total ovarian follicular population all ovaries were sectioned into 2 mm. slices. These ovarian slices were surrounded by a thin layer of paraffin wax to contain the slices in correct sequence. Such "plates" of material were immersed in trays of 70% alcohol. Fig. 2-8 shows ovaries set in wax before being put in alcohol.

Counts of all antral follicles were made on all ovarian slices. Addition of the number of follicles in all slices from one ovary gave an indication of the total follicle population in that ovary. These counts were made with the aid of a large magnifying glass.

* Pentobarbitone Sodium (Abbott Laboratories).

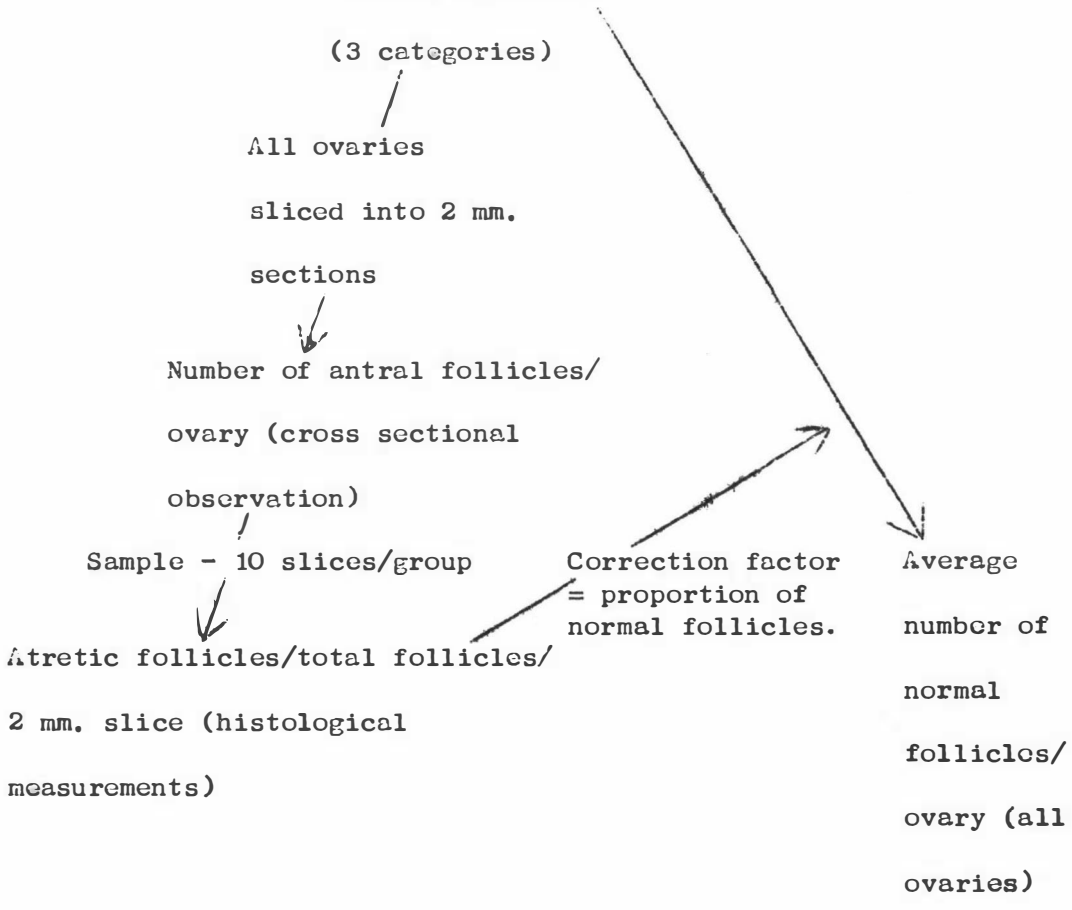
Fig. 2-7:- *In vivo* measurement of ovarian follicles with sterile slide callipers

Fig. 2-8:- 2 mm. ovarian slices semi-embedded in 70% alcohol


Mitutoyo

Ten randomly selected 2 mm. slices from ovaries of ewes in each of the 4 experimental groups (right or left ovaries) were selected for histological analysis. After processing in graded alcohols and xylene, these samples were embedded in paraffin wax and sectioned at 6μ. The sections were stained with Haematoxylin and Eosin. Antral follicles were now able to be assessed for any signs of atresia or luteinisation and the percentage of the total population examined that were atretic, was computed. Gross measurements were corrected with respect to this figure which was derived for each separate group.

The assessment of follicular population may be summarised thus:-



This procedure was not designed to measure every follicle in every ovary. However an indication of the follicular population in ovaries of animals on each treatment is attained and some idea of the degree of atresia of these follicles derived. The limitations of this method will be discussed in Chapter Seven.

Analysis of Date

Analyses of discrete data utilised Chi^2 or the G statistic (Sokal and Rolf 1969).

Ovarian response data from Experiment 1 were from groups of unequal size. However, a two way analysis of variance for unequal sub-classes was available on a computer programme*, and the experiment was analysed as for a 2x2 factorial design. The general linear equation for the model analysing ovarian response was:-

$$Y_{ijk} = u + t_i + d_j + (td)_{ij} + e_{ijk}$$

where

u = the overall mean when equal frequencies exist in each subclass

t_i = effect of treatment ($i = 1,2,3,$ or 4)

d_j = effect of dose ($j = 1$ or 2)

$(td)_{ij}$ = individual interaction effects expressed as deviations from mean u.

e_{ijk} = error peculiar to each y_{ijk}

*Courtesy of Prof. R.E. Munford.

Bartlett's test for homogeneity of variances was carried out on discrete ovarian response data as a test of normality of the data distributions (Snedecor and Cochran, 1967). On the basis of these tests all discrete data were transformed to $\sqrt{x+1}$ before any tests assuming normality were carried out.

Comparisons of treatment means were made following analyses which yielded statistically significant results. Comparisons were made using the Student-Newman-Keuls test (Sokal and Rolf 1969).

Mouse uterine weights (Experiment 2) were transformed to logarithms and analysed by covariance. Significant treatment effects were further analysed by the Student-Newman-Keuls test.

CHAPTER III

SYNCHRONISATION AND OESTROUS PHENOMENA

Chapter III

SYNCHRONISATION AND OESTROUS PHENOMENA

Experiments 1 and 3 yielded data on progestagen synchronisation of oestrous cycles and oestrous phenomena of subsequent cycles. Effects of repeated P.M.S.G. treatment of ewes were apparent as well as information on the efficiency of synchronisation by intravaginal progestagen administration.

Factors Affecting the Onset of Oestrus following Progestagen Sponge Withdrawal

1. P.M.S.G. administration

Immediately following the cessation of progestagen treatment of ewes in Experiment 1, some sheep were given P.M.S.G. This did not significantly affect the time interval from sponge withdrawal to the onset of oestrus (Table 3-1). Distributions of the onset of oestrus in ewes either given, or not given P.M.S.G. are shown in Fig. 3-1.

The number of ewes showing overt oestrus immediately following sponge withdrawal was not affected by the administration of P.M.S.G. (Table 3-3).

2. Injection of Progesterone.

Synchronisation of ewes given a progesterone injection 2 days before sponge withdrawal (Experiment 3) is compared with that of ewes given only M.A.P. (Tables 3-2 and 3-4 and Fig. 3-2). There is a temporal bias in this comparison as the two different treatments were carried out at different times of the year and in different

TABLE 3-1

EFFECT OF P.M.S.G. ADMINISTRATION ON THE TIME INTERVAL BETWEEN PROGESTAGEN SPONGE WITHDRAWAL AND THE ONSET OF OESTRUS

Treatment	Number of Ewes in Oestrus Days from Sponge Withdrawal				Total Number of Ewes
	Day 2	Day 3	Day 4	Day 5	
Progestagen and P.M.S.G.	16(47.0%)[A]	9(26.5%)	8(23.5%)	1(3.0%)	34
Progestagen Alone	13(37.2%)	15(42.8%)	7(20.0%)	0	35
Total	29	24	15	1	69

G = 3.264 - Not Significant

TABLE 3-2

EFFECT OF A PROGESTERONE INJECTION ON THE TIME INTERVAL BETWEEN PROGESTAGEN SPONGE WITHDRAWAL AND THE ONSET OF OESTRUS

Treatment	Number of Ewes in Oestrus Days from Sponge Withdrawal			Total Number of Ewes
	Day 2	Day 3	Day 4	
M.A.P. Alone (Expt 1)	13(37.2%)	15(42.8%)	7(20.0%)	35
M.A.P. and Progesterone (Expt 4)	20(66.7%)	9(30.0%)	1(3.3%)	30
Total	33	24	8	65

G = 7.76 - $P < 0.05$

A. () Percentage of Ewes coming into oestrus that were in oestrus on the day indicated.

TABLE 3-3

EFFECT OF P.M.S.G. ADMINISTRATION ON THE OCCURRENCE OF OESTRUS FOLLOWING PROGESTAGEN TREATMENT

Treatment	Number of Ewes Showing Overt Oestrus	Number of Ewes Showing Silent Oestrus	Total Number of Ewes	% of Ewes Showing Overt Oestrus
Progestagen and P.M.S.G.	34	3	37	91.9
Progestagen Alone	35	8	43	81.4
Total	69	11	80	

CHI^2 = 1.068 - Not Significant

TABLE 3-4

EFFECT OF A PROGESTERONE INJECTION ON THE OCCURRENCE OF OESTRUS FOLLOWING PROGESTAGEN SPONGE TREATMENT

Treatment	Number of Ewes Showing Overt Oestrus	Number of Ewes Showing Silent Oestrus	Total Number of Ewes	% of Ewes Showing Overt Oestrus
M.A.P. Alone	35	8	43	81.4
M.A.P. and Progesterone	30	10	40	75.0
Total	65	18	83	

CHI^2 = 0.193 - Not Significant

FIGURE 3–1:–

DISTRIBUTION OF ONSET OF OESTRUS FOLLOWING SPONGE WITHDRAWAL : EXPERIMENT ONE

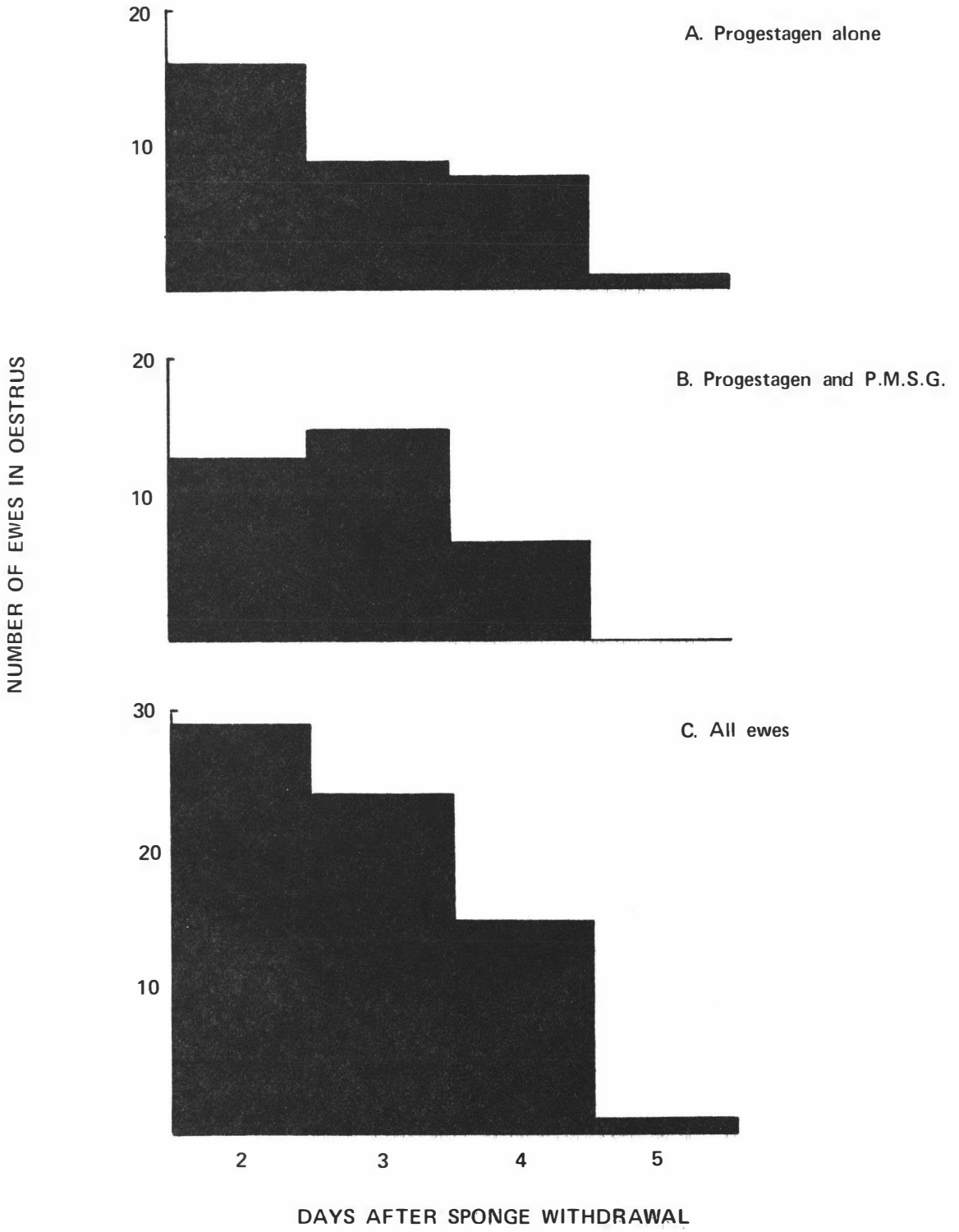
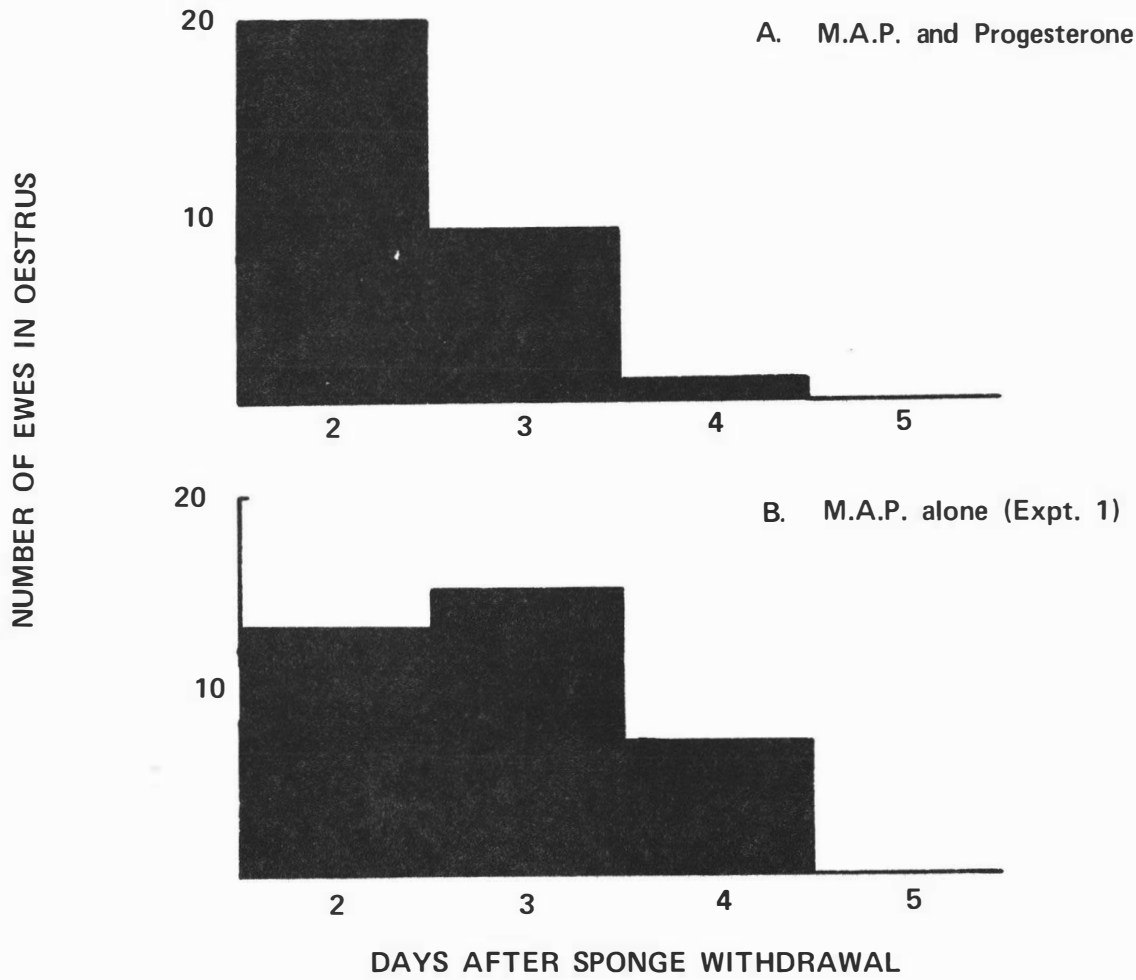


FIGURE 3–2:–

THE EFFECT OF A PROGESTERONE INJECTION ON THE ONSET OF OESTRUS OF EWES GIVEN AN INTRAVAGINAL PROGESTAGEN TREATMENT



breeding seasons. Seasonal variation in ewe response to progesterone is known to occur (Lamond 1964c). For this reason, the comparison is not strictly valid but is drawn because of indications that supplementation of M.A.P. treatment with progesterone may be effective in producing a more defined synchronisation.

Progesterone supplementation of M.A.P. treatment caused ewes to come into heat over a shorter time than if M.A.P. was given alone. The distributions of the onset of oestrus were significantly ($P < 0.05$) different for these two groups (Table 3-2 and Fig. 3-2). Of those ewes showing oestrus, 80.0% were in oestrus over a 2 day period with M.A.P. alone compared with 96.7% when M.A.P. was supplemented with a progesterone injection (Table 3-2).

Supplementation of progestagen sponge treatment did not significantly reduce the proportion of ewes showing silent heats (Table 3-4).

Effect of Sequential P.M.S.G. Treatment on Synchronisation of Oestrous Cycles Subsequent to Progestagen Treatment

1. Variation in the Time of Onset of Oestrus

Treatments in Experiment 1 had a significant effect on the time of onset of oestrus, one oestrous cycle after sponge withdrawal ($P < 0.005$). Treatments 1 and 2 caused a greater dispersion in the time period in which ewes came into oestrus than did Treatments 3 and 4 (Table 3-5 and Fig. 3-3). This trend was also apparent two cycles from sponge withdrawal (Table 3-6 and Fig. 3-4), but was less marked ($P < 0.01$).

TABLE 3-5

TREATMENT EFFECTS ON THE SYNCHRONISATION OF OESTRUS,

ONE OESTROUS CYCLE FROM PROGESTAGEN TREATMENT:- EXPERIMENT 1

Treatment	Dose (P.M.S.G.)	Days from the first display of Oestrus					Total Number of Ewes
		0	1	2	3	4 - 10	
1	L	3	2	2	0	3	10
	H	2	2	1	0	4	9
Total		5	4	3	0	7	19
2	L	0	1	6	1	1	9
	H	1	1	3	2	2	9
Total		1	2	9	3	3	18
3	L	0	4	3	2	0	9
	H	1	3	3	0	0	7
Total		1	7	6	2	0	16
4	L	1	1	5	2	0	9
	H	0	1	6	2	0	9
Total		1	2	11	4	0	18
Total		8	15	29	9	10	71

Test of Independence: G Statistic	D.F.	G	Significance
Treatment x Dose x Distribution	31	43.238	$P < 0.05$
Treatment x Distribution	12	35.318	$P < 0.005$
Dose x Distribution	4	0.038	Not Significant
Treatment x Dose x Distribution Interaction	12	8.848	Not Significant

TABLE 3-6

TREATMENT EFFECTS ON THE SYNCHRONISATION OF OESTRUS,

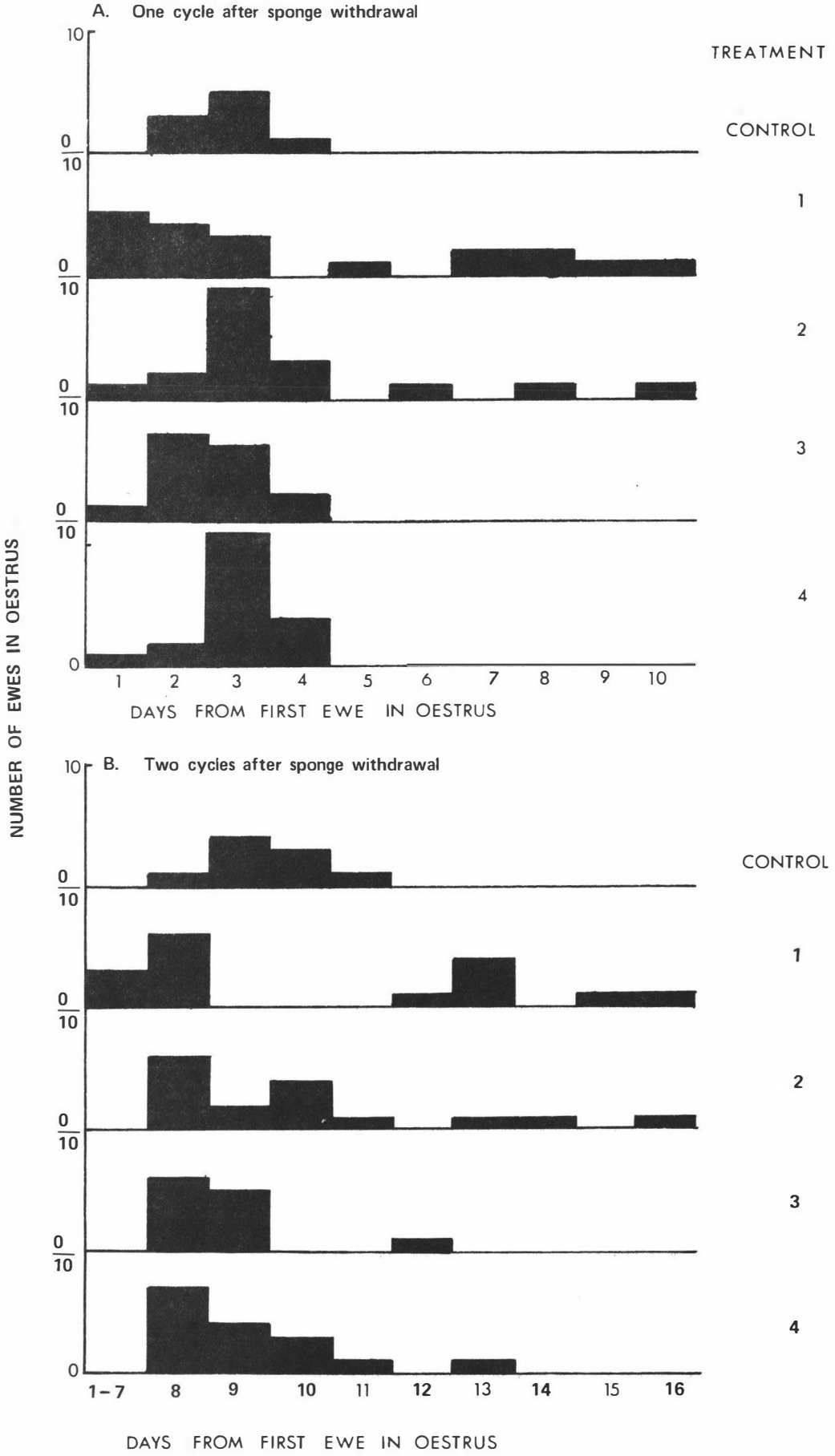
TWO OESTROUS CYCLES FROM PROGESTAGEN TREATMENT:- EXPERIMENT 1

Treatment	Dose (P.M.S.G.)	Days from the first display of Oestrus					Total Number of Ewes
		0 - 7	8	9	10	11 - 16	
1	L	3	3	0	0	2	8
	H	0	3	0	0	5	8
Total		3	6	0	0	7	16
2	L	0	2	2	2	2	8
	H	0	4	0	2	2	8
Total		0	6	2	4	4	16
3	L	0	4	2	0	1	7
	H	0	2	3	0	0	5
Total		0	6	5	0	1	12
4	L	0	2	4	2	1	9
	H	0	5	0	1	1	7
Total		0	7	4	3	1	16
Total		3	25	11	7	14	70

Test of Independence: G Statistic	D.F.	G	Significance
Treatment x Dose x Distribution Independence	31	56.526	$P < 0.01$
Treatment x Distribution	12	35.608	$P < 0.01$
Dose x Distribution	4	0.322	Not Significant
Treatment x Dose x Distribution x Interaction	12	6.346	Not Significant

FIGURE 3--3:-

EFFECT OF REPEATED GONADOTROPHIC STIMULATION ON SYNCHRONISATION OF OESTROUS CYCLES



The effect of doses of P.M.S.G. on the temporal distribution of onset of oestrus was not significant at either of the two periods.

Table 3-7 gives the results of sequentially treated, laparotomised ewes (Experiment 3). Uninjected ewes still came into heat over a 4 day period whereas treated ewes were dispersed over 8 days. Laparotomy did not appear to affect the time of onset of oestrus.

2. Manifestation of Oestrus

All ewes in Experiment 1 displayed overt oestrus at the second oestrous period from sponge withdrawal. At the final observation, more intensive P.M.S.G. treatments (Treatments 1 and 3) caused a non-significantly greater number of ewes to experience silent oestrus (Table 3-8).

Data from Experiment 4 (Table 3-9) showed that increasing numbers of sequential P.M.S.G. treatments enhanced the incidence of silent oestrus ($P < 0.05$). This effect does not appear to have been due to the number of laparotomies as control ewes which were laparotomised three times, continued to exhibit overt oestrus.

CHAPTER SUMMARY

1. Administration of P.M.S.G. immediately following sponge withdrawal had little effect on oestrous behaviour at the first subsequent oestrous period.
2. An intramuscular injection of 10 mg. progesterone, 2 days before sponge withdrawal, may cause more effective synchronisation but probably does not decrease the number of ewes showing silent oestrus. These results are not conclusive for want of appropriate control animals.
3. Oestrous cycles of ewes given progestagen via the intravaginal route may remain effectively synchronised for 3-4 cycles. This effect is disrupted by the administration of P.M.S.G.

TABLE 3-7

TREATMENT EFFECTS ON THE MANIFESTATION OF OESTRUS:- EXPERIMENT 1

Treatment	Dose (P.M.S.G.)	Number of Ewes Having Overt Oestrus	Number of Ewes Having Silent Oestrus	Total Number of Ewes
1	L	8	2	10
	H	8	1	9
Total		16	3	19
2	L	8	1	9
	H	8	1	9
Total		16	2	18
3	L	7	2	9
	H	5	2	7
Total		12	4	16
4	L	9	0	9
	H	7	2	9
Total		16	2	18
Total		60	11	71

G = 11.110 - Not Significant

TABLE 3-8

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON

MANIFESTATION OF OESTRUS

Treatment	Number of Ewes Showing Overt Oestrus	Number of Ewes Showing Silent Oestrus	Total Number of Ewes	% Ewes Showing Overt Oestrus
Injection at 1 cycle	10	0	10	100.0
Injection at 2 cycles	8	2	10	80.0
Injection at 3 cycles	6	3	9	66.7
Uninjected Control	9	0	9	100.0
Total	33	5	38	

$G = 3.128 \quad P < 0.05$

CHAPTER IV

OVARIAN RESPONSE TO P.M.S.G. IN SEQUENTIALLY TREATED EWES

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OVARIAN RESPONSE TO P.M.S.G. IN SEQUENTIALLY TREATED EWES

This chapter reports ovarian response data of ewes in Experiment 1. Some ovulation data from Experiment 3 is also included. Observations of ovaries from sheep in Experiment 1 were made at the end of the experiment and are thus 'terminal' responses of ewes to the treatments imposed. The ovaries of sheep in Experiment 3 were observed at a series of laparotomies and post-mortem.

Results are presented as transformed data and raw data are given in Appendix 1.

Transformation of Discrete Data

Bartlett's tests on ovulatory data (number of corpora lutea) revealed that variances of the 8 treatment groups were heterogeneous (Appendix 2). The most suitable transformation for data involving counts and zero values is $\sqrt{x + 1}$ (Sokal and Rolf 1969). After this transformation had been applied to the number of corpora lutea per ewe, Bartlett's test showed that heterogeneity had been reduced but variances just failed to attain homogeneity for this variable. As this transformation was considered the most likely to produce homogeneity, no other transformations were tested. In the light of the marked reduction in heterogeneity attained, all discrete data were transformed to $\sqrt{x + 1}$ for analysis of variance. For ease of computation, the transformed data were increased by a factor of 10.

FIGURE 4–1:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON THE NUMBER OF EWES OVULATING

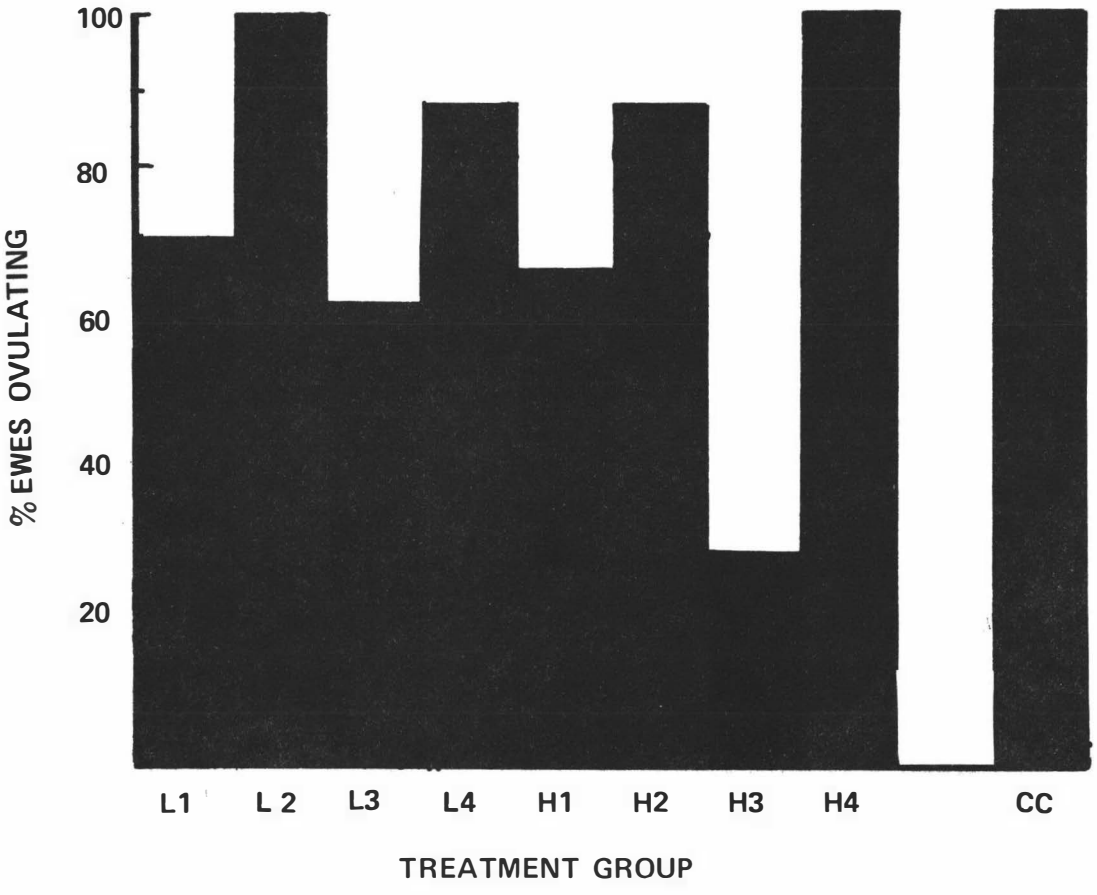


TABLE 4-1

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT OF THE NUMBER OF EWES OVULATING

A. Experiment 1

Treatment	Dose	Total Number of Ewes	Number of Ewes Ovulating
1	L	10	7
	H	9	6
Total		19	13
2	L	9	9
	H	9	8
Total		18	17
3	L	8	5
	H	7	2
Total		15	7
4	L	9	8
	H	9	9
Total		18	17
Grand Total		70	54

Test of Independence: G Statistic

	Significance
Ewes Ovulating x Treatment x Dose Independence	$P < 0.05$
Ewes Ovulating x Treatment	$P < 0.01$
Ewes Ovulating x Dose	N.S.
Ewes Ovulating x Treatment x Dose Interaction	N.S.

B. Experiment 3

Ewe Treatment	Total Number of Ewes	Number of Ewes Ovulating
1 (3 injections)	9	7
2 (2 injections)	20	18
3 (1 injection)	30	30

$G = 6.716$ $P < 0.05$

Ewes Ovulating

The presence of a corpus luteum was taken as denoting that ovulation had occurred. Table 4-1a shows that treatments of Experiment 1 significantly ($P < 0.01$) affected the numbers of ewes ovulating. Fewer ewes ovulated when given sequential treatments with P.M.S.G. (Treatments 1 and 3) than when given a single injection (Treatment 4) or staggered injections (Treatment 2). The effects of dose and dose x treatment interaction were not significant. These results are displayed in Fig. 4-1.

In Experiment 3, increasing numbers of sequential treatments with P.M.S.G. increased the proportion of ewes which were anovular ($P < 0.05$). The effect is partly masked (Table 4-1b) because fewer ewes were observed as the number of injections increased. Furthermore, the stress of laparotomy may have enhanced the anovular condition in these ewes.

Incidence of Cystic Ovaries

Treatment, dose and treatment x dose interaction had no effect on the proportion of ewes in Experiment 1 which developed cystic ovaries (Table 4-2). Ewes having cystic ovaries were only seen in Treatments 1 and 3 (sequential treatments with P.M.S.G.). The cystic condition evident in a ewe on Treatment H3 is seen in Fig.4-2.

Ovulation rate

1. Corpora Lutea : All ewes (Experiment 1)

Ovulation rate was determined by counting the number of corpora lutea per ewe. Treatments of Experiment 1 significantly affected ($P < 0.05$) the number of corpora lutea per ewe that were observed at

Fig. 4-2:- Cystic condition of the ovary (this ovary taken from a ewe on Trt. H3, Expt. 1)

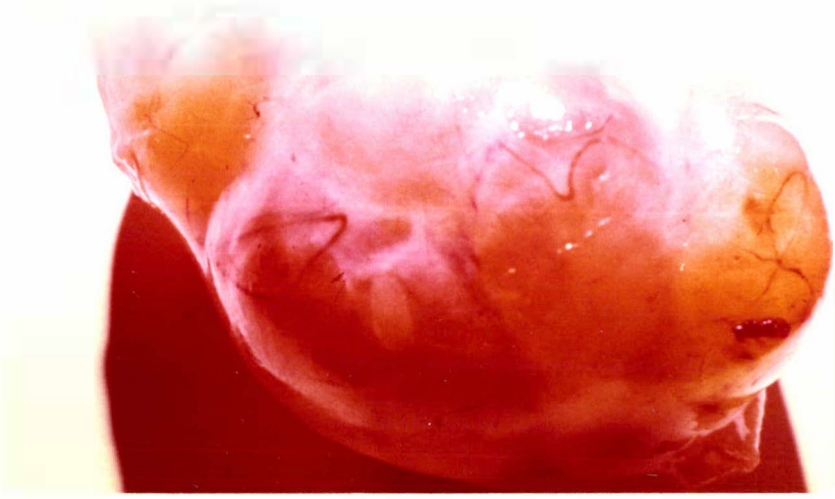


TABLE 4-2

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON THE INCIDENCE OF CYSTIC OVARIES

Treatment	Dose	Total Number of Ewes	Number of Ewes with Cystic Ovaries
1	L	10	1
	H	9	0
Total		19	1
2	L	9	1
	H	9	0
Total		18	1
3	L	8	1
	H	7	2
Total		15	3
4	L	9	0
	H	9	0
Total		18	0
Grand Total		70	5

Test of Independence: G Statistic

	Significance
Cystic Condition x Treatment x Dose Independence	N.S.
Cystic Condition x Treatment	N.S.
Cystic Condition x Dose	N.S.
Cystic Condition x Treatment x Dose Interaction	N.S.

slaughter (Table 4-3). Effects of dose level and dose x treatment interaction were not significant. Raw data is plotted in Fig. 4-3 and Fig. 4-4 presents the same data after transformation.

Comparison of treatment means, by the Student-Newman-Keuls test, is outlined in Appendix 3. Treatment 1 caused significantly lower terminal ovulation rates than Treatments 2 ($P < 0.01$) and 4 ($P < 0.01$). Treatment 4 produced significantly greater terminal ovulation rates than Treatment 3 ($P < 0.05$) (Table 4-5). Differences in all other treatment comparisons were not significant.

2. Corpora Lutea : Ewes ovulating (Experiment 1)

Consideration of the ovulation rates of those ewes ovulating (Table 4-4) revealed that treatment effects remained significant ($P < 0.05$). Treatments 2,3 and 4 caused significantly greater terminal ovulation rates per ewes ovulating than Treatment 1. ($P < 0.05$, $P < 0.01$ and $P < 0.05$ respectively) (Table 4-6). Data from these ewes are presented in Fig. 4-9 and Fig. 4-10.

3. Corpora Lutea : All ewes (Experiment 3)

Table 4-7 and Fig. 4-17 display data of ewes given 1,2 or 3 sequential injections of P.M.S.G., compared with control animals. After one stimulation, the treated ewes had significantly greater ($P < 0.001$) ovulation rates than the control animals. Ovulation rates of ewes treated 2 or 3 times with P.M.S.G. did not differ from those of control animals observed at the same time.

FIGURE 4–3:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON EWE OVULATION RATE : RAW DATA

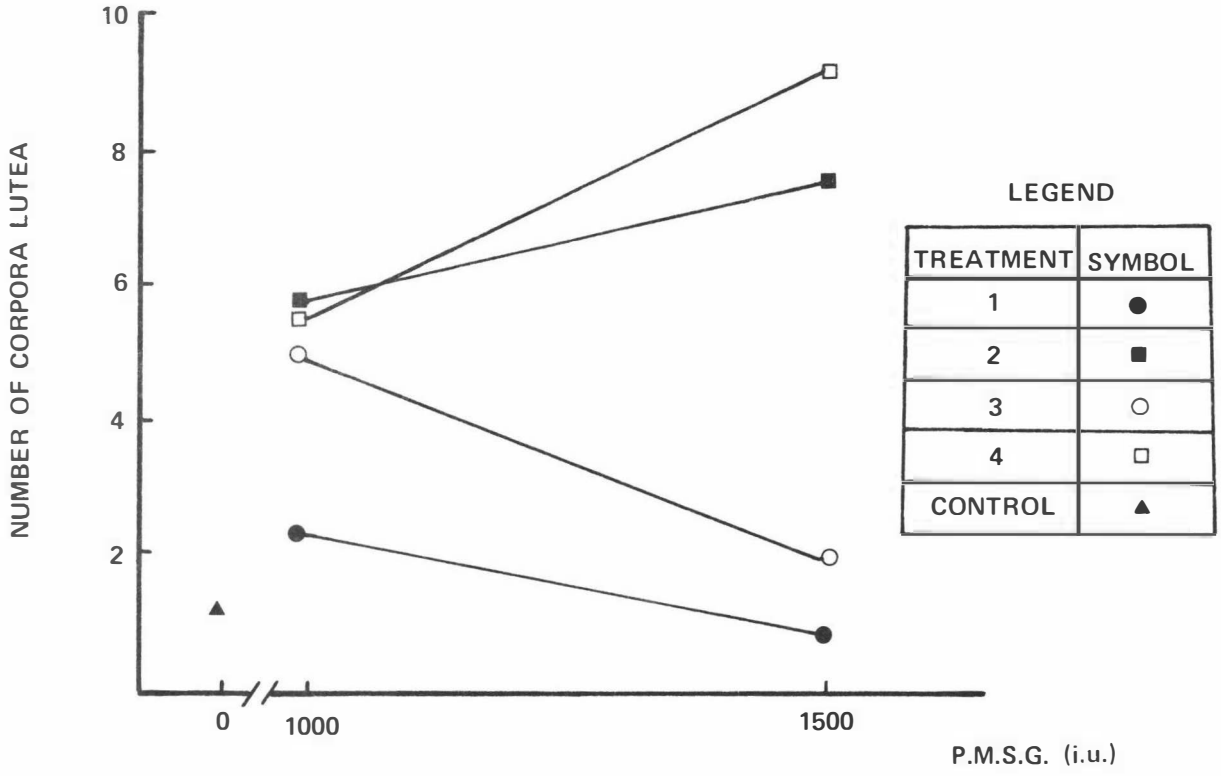


FIGURE 4–4:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON EWE OVULATION RATE : TRANSFORMED DATA

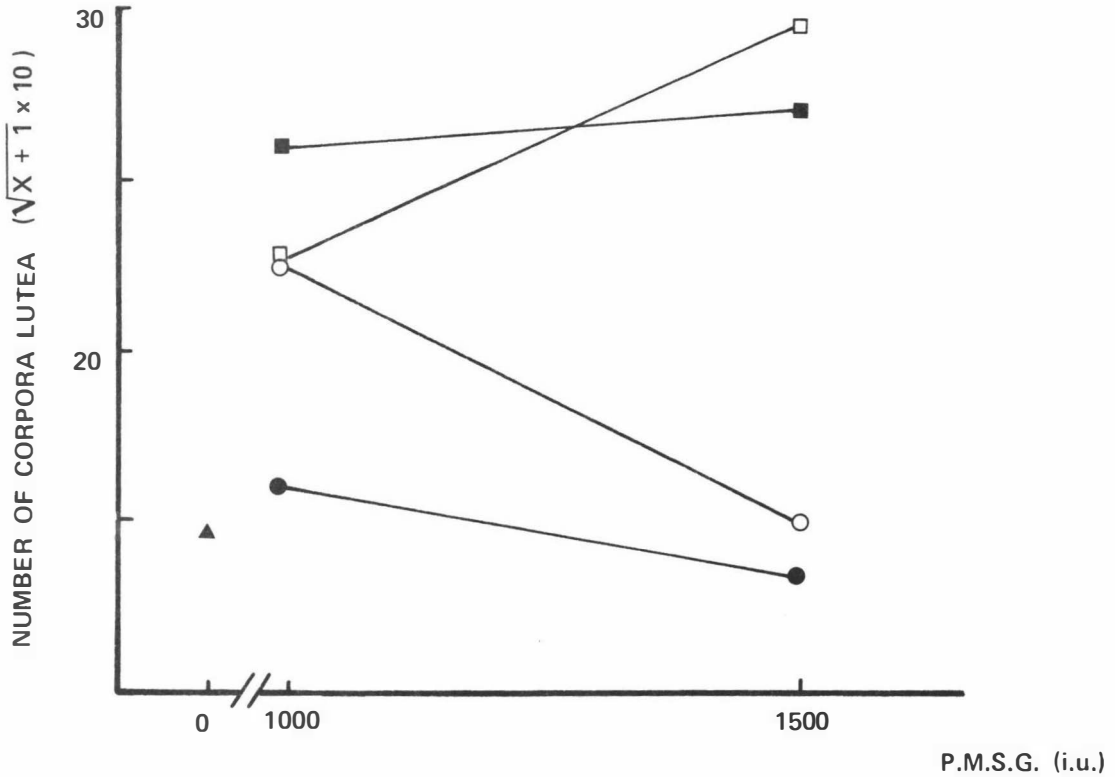


TABLE 4-3

OVARIAN RESPONSE : ALL EWES

Estimated Means and Standard Errors

Variable	Means		Standard Errors			
	DOSE		TREATMENT			
	Low	High	1	2	3	4
Corpora Lutea	21.56	21.07	14.61	26.43	13.41	25.81
(transformed counts)	±1.74	±1.79	±2.40	±2.47	±2.70	±2.47
Corpora Lutea +	26.66	31.53	22.75	28.77	33.26	31.58
Follicles > 3mm.	±1.65	±1.70	±2.28	±2.33	±1.81	±2.33
(transformed counts)						
Ovarian weight	7.35	9.63	5.87	6.69	11.09	10.31
(Gms.)	±1.17	±1.20	±1.61	±1.65	±2.56	±1.65

Analysis of Variance

Source of Variation	D.F.	Mean Squares		
		Corpora Lutea	Corpora Lutea + Follicles > 3mm.	Ovarian weight
Dose	1	4.14	410.58 **	90.04
Treatment	3	603.97 **	376.81 *	115.43
Dose x treatment	3	143.88	111.50	55.81
Residual	62	109.43	98.36	49.28

* $P < 0.05$
** $P < 0.01$

TABLE 4-4

OVARIAN RESPONSE : EWES OVULATING

Estimated Means and Standard Errors

Variable	Means and Standard Errors					
	DOSE		TREATMENT			
	Low	High	1	2	3	4
Corpora Lutea	24.18	25.73	16.89	27.45	28.98	26.69
(transformed counts)	±1.93	±2.08	±2.89	±2.52	±3.93	±2.52
Corpora Lutea +	27.37	30.85	19.97	29.98	34.23	32.32
follicles > 3mm.	±1.82	±1.95	±2.72	±2.38	±3.70	±2.38
(transformed counts)						
Ovarian weight	7.37	11.68	4.60	6.97	13.51	10.73
(Gms.)	±1.28	±1.38	±1.93	±1.68	±2.62	±1.68

Analysis of Variance

Source of Variation	D.F.	Mean Squares		
		Corpora Lutea	Corpora Lutea + Follicles 3mm.	Ovarian weight
Dose	1	4.25	200.43	200.96 *
Treatment	3	359.67 *	516.53 **	216.32 **
Treatment x dose	3	54.21	65.18	71.89
Residual	46	108.54	96.25	48.04

* $P < 0.05$

** $P < 0.01$

TABLE 4-5

COMPARISON OF MEANS[A] : OVARIAN RESPONSE OF ALL EWES (EXPERIMENT 1)

Comparison of Treatments	Difference of means		
	Corpora Lutea[B]	Corpora Lutea + Follicles >3mm.[B]	Ovarian Weight
TRT 1 vs TRT 2	11.82 **	6.02	0.82
" 1 vs " 3	3.80	10.51 *	5.21
" 1 vs " 4	11.82 **	8.83 *	4.43
" 2 vs " 3	8.02	4.48	4.38
" 2 vs " 4	0.62	2.81	3.61
" 3 vs " 4	7.39 *	1.67	0.77
Low dose vs High dose	0.48	4.86 *	2.28

TABLE 4-6

COMPARISON OF MEANS[A] : OVARIAN RESPONSE OF EWES OVULATING (EXPERIMENT 1)

Comparison of Treatments	Difference of means		
	Corpora Lutea[B]	Corpora Lutea + Follicles >3mm.[B]	Ovarian Weight
TRT 1 vs TRT 2	10.55 *	10.00	2.18
" 1 vs " 3	12.00 **	14.26 **	8.90 **
" 1 vs " 4	10.55 *	12.34 **	6.12 *
" 2 vs " 3	0.76	4.25 **	6.54 *
" 2 vs " 4	1.54	2.34	3.76
" 3 vs " 4	2.21	1.91	2.77
Low dose vs High dose	1.61	3.46	4.31 *

* $P < 0.05$

** $P < 0.01$

A - Student-Newman-Keuls Test

B - Difference in Transformed Means

TABLE 4-7

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT, COMPARED WITH UNTREATED EWES : CORPORA LUTEA (TRANSFORMED DATA)[A]

OBSERVATION	MEAN NUMBER OF CORPORA LUTEA STD. ERROR ($\sqrt{X + 1}$)		ANALYSIS OF VARIANCE	
	P.M.S.G. TREATED[B]	CONTROL[C]	MEAN SQUARES	F
1	25.90 (30)[D]	14.45 (10)	Between = 981.97	19.83***
	±1.28	±2.22	Within = 49.50	
2	16.41 (20)	13.59 (9)	Between = 50.07	2.07
	±1.09	±1.63	Within = 24.16	
3	13.57 (9)	16.44 (9)	Between = 37.13	1.93
	±1.46	±1.46	Within = 19.19	

TABLE 4-8

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT COMPARED WITH UNTREATED EWES : CORPORA LUTEA PLUS FOLLICLES GREATER THAN 1 mm. (TRANSFORMED DATA)[A]

OBSERVATION	MEAN NUMBER OF CORPORA LUTEA+ FOLLICLES 1 mm. + STD. ERROR ($\sqrt{X+1}$)		ANALYSIS OF VARIANCE	
	P.M.S.G. TREATED[B]	CONTROL[C]	MEAN SQUARES	F
1	43.38 (30)[D]	39.25 (10)	Between = 127.92	1.17
	±1.90	±3.29	Within = 108.73	
2	35.17 (20)	32.03 (9)	Between = 60.89	0.74
	±2.02	±3.02	Within = 82.26	
3	32.03 (9)	37.17 (9)	Between = 118.69	1.24
	±3.25	±3.25	Within = 95.23	

A - Experiment 3 data

B - Treated once, twice or three times respectively with P.M.S.G. before observations one, two and three.

C - The same animals laparotomised twice and then killed.

D - () Number of ewes/treatment group.

*** $P < 0.001$

Fig. 4-5:- Superovulated ovaries taken from a ewe given one injection of P.M.S.G. (Trt. H4, Expt. 1). 36 ovulations.

Fig. 4-6:- Inactive ovaries of a ewe in Treatment H1 (Expt. 1). Corpora albicans denote response to previous gonadotrophic stimulation.

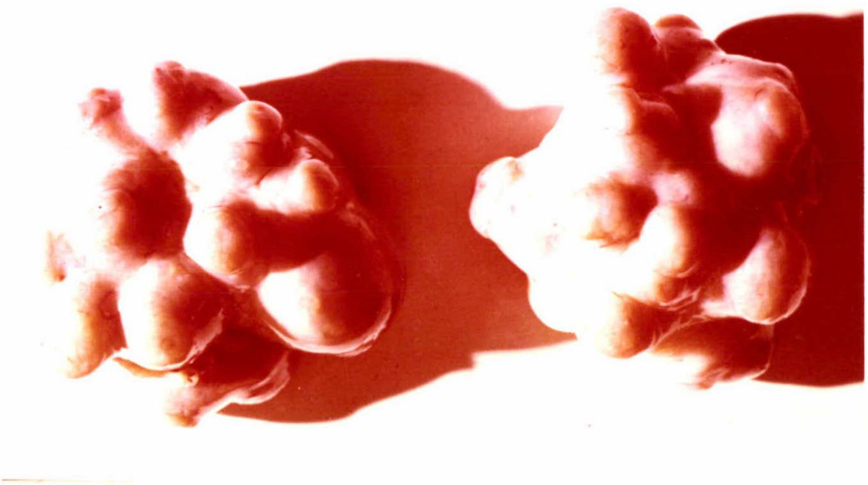
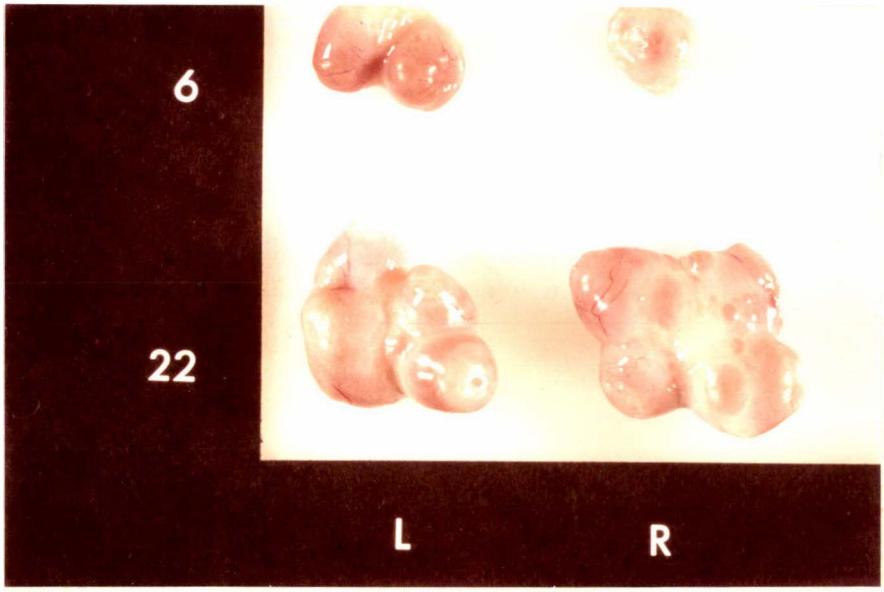
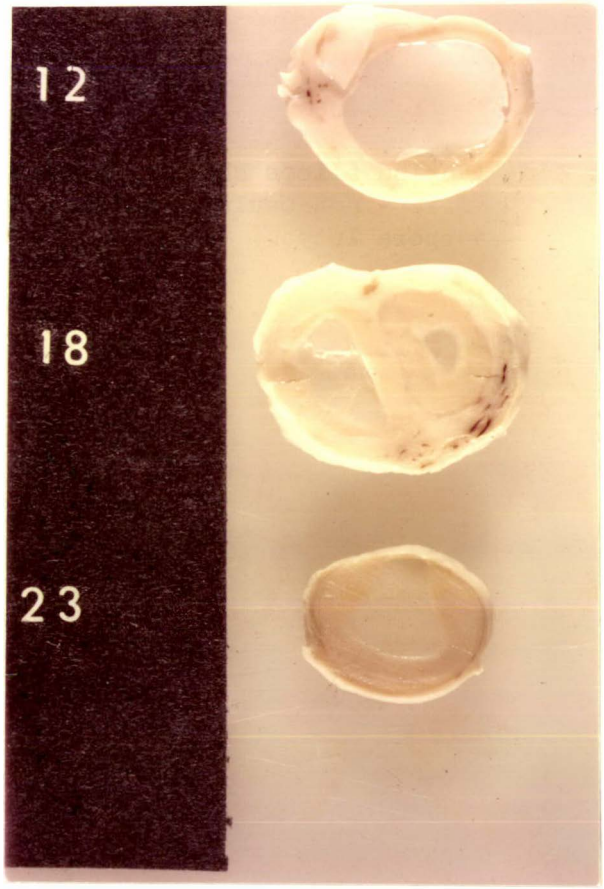


Fig. 4-7 Ovaries observed at Day 10 of the oestrous cycle. Ewe 6 - control, ewe 22 - superovulated.

Fig. 4-8 Ovarian slices showing luteinisation of follicles. No. 12 - non-ovulatory. No's 18 and 23 contain corpora lutea.


6
22
L
R


12
18
23

Examples of superovulated ovaries from ewes in Experiment 1 (taken 6 days after the onset of oestrus) are shown in Fig. 4-5 and 4-6. Ovaries of ewes in Experiment 3 (10 days after the onset of oestrus) are seen in Fig. 4-7. The cross sections seen in Fig. 4-8 are through luteinised follicles. At Day 10 of the oestrous cycle, luteinised follicles may resemble corpora lutea but the two are distinguished by the presence or absence of an ovulation point.

Corpora Lutea plus follicles

1. Corpora Lutea + follicles greater than 3 mm. : All ewes (Experiment 1)

Both treatment and dose had significant effects ($P < 0.05$ and $P < 0.01$ respectively) on this measurement (Table 4-3). Interaction between the two main effects was not significant. The number of corpora lutea + follicles > 3 mm. of ewes on Treatment 1 was significantly lower than ewes on Treatments 3 ($P < 0.05$) and 4 ($P < 0,05$). The high dose of P.M.S.G. (1500 i.u.) caused a significantly greater response ($P < 0.01$) than did the low dose (1000 i.u.)

These data are plotted in Fig. 4-11 and 4-12.

2. Corpora Lutea + follicles greater than 3 mm. : Ewes ovulating (Experiment 1)

When data from ovulating ewes only are analysed (Table 4-4), treatment and dose effects remain significant ($P < 0.05$ and $P < 0.01$, respectively). Treatments 2,3 and 4 produce significantly greater response in this measurement than Treatment 1 ($P < 0.01$) (Table 4-6 and Figs. 4-13 and 4-14).

3. Corpora Lutea + follicles greater than 1 mm. : All ewes (Experiment 3)

Ewes treated once, twice or three times with P.M.S.G. do not

FIGURE 4–9:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON OVULATION RATE OF EWES OVULATING : RAW DATA

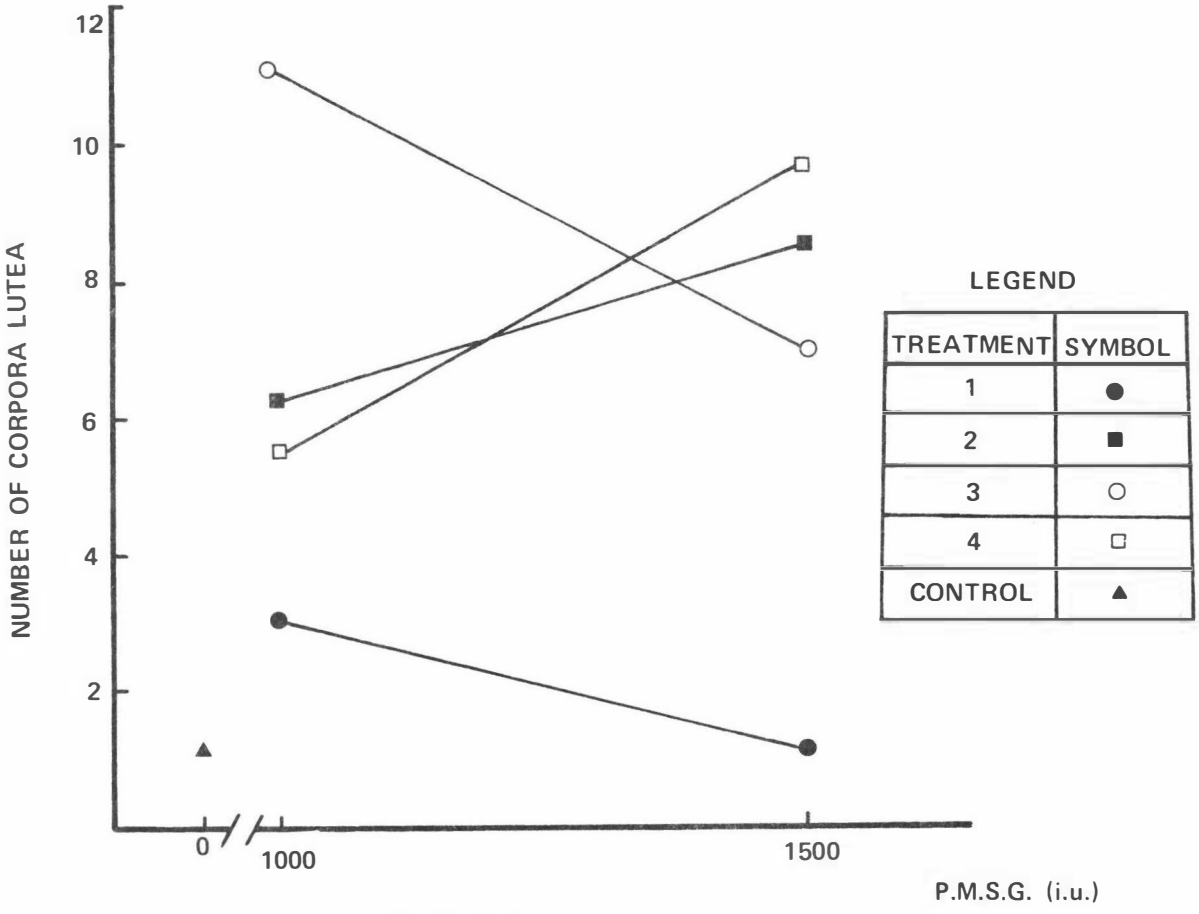


FIGURE 4–10:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON OVULATION RATE OF EWES OVULATING : TRANSFORMED DATA

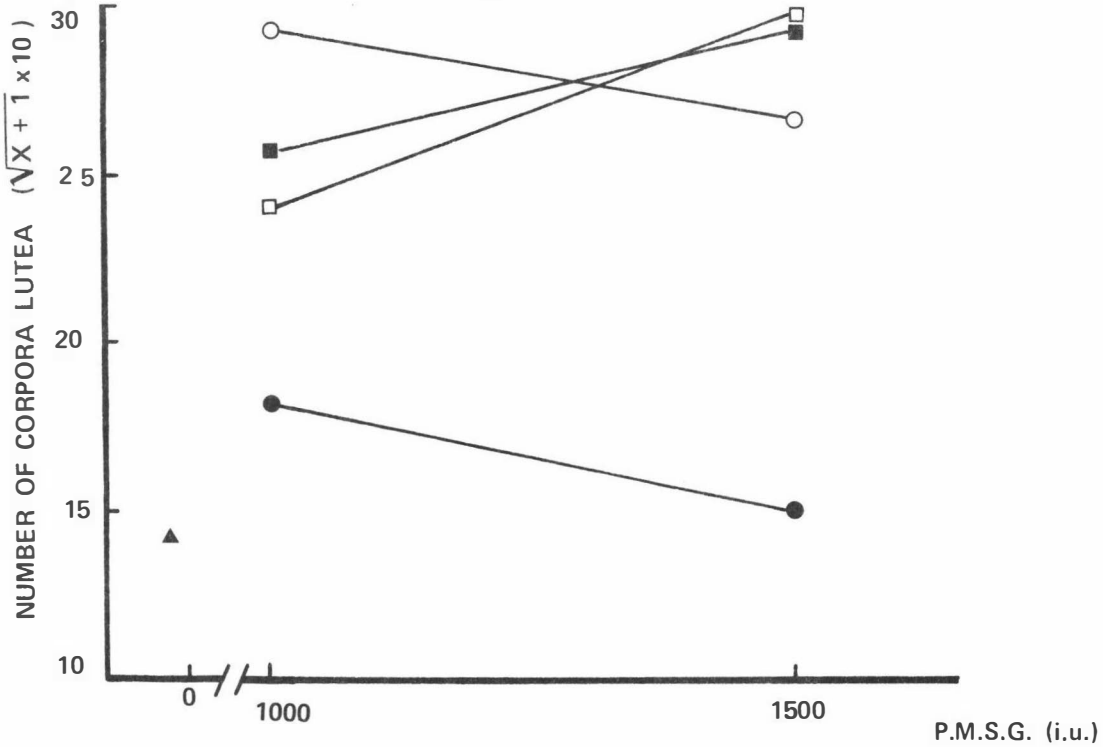


FIGURE 4–11:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON THE NUMBER OF CORPORA LUTEA PLUS FOLLICLES GREATER THAN 3 mm. IN ALL EWES : RAW DATA

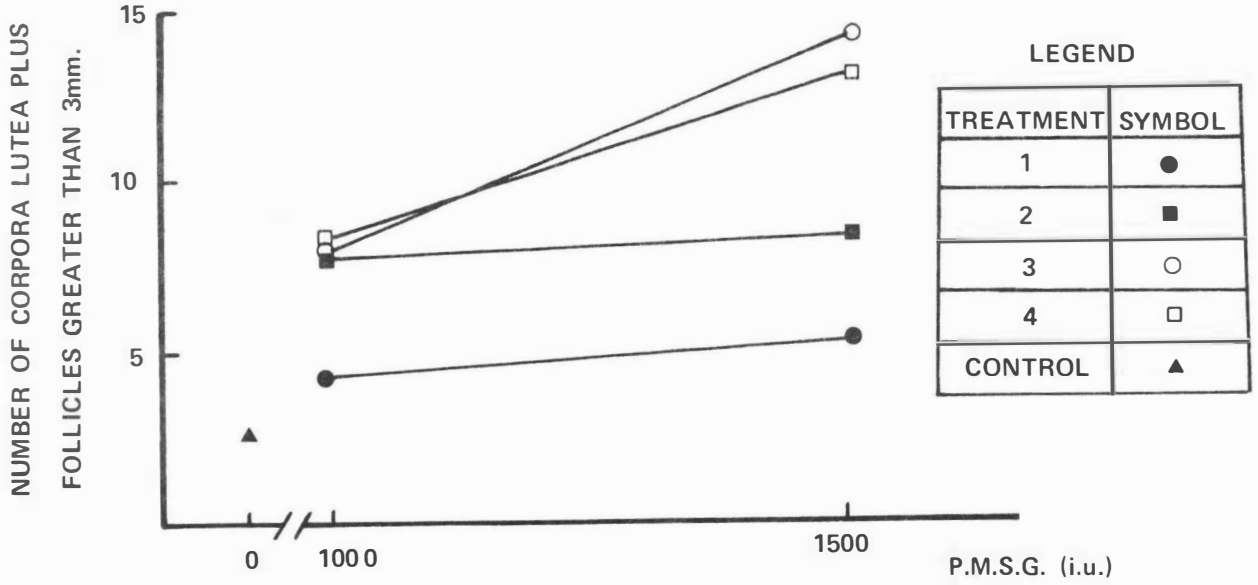


FIGURE 4–12:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON THE NUMBER OF CORPORA LUTEA PLUS FOLLICLES GREATER THAN 3 mm. IN ALL EWES : TRANSFORMED DATA

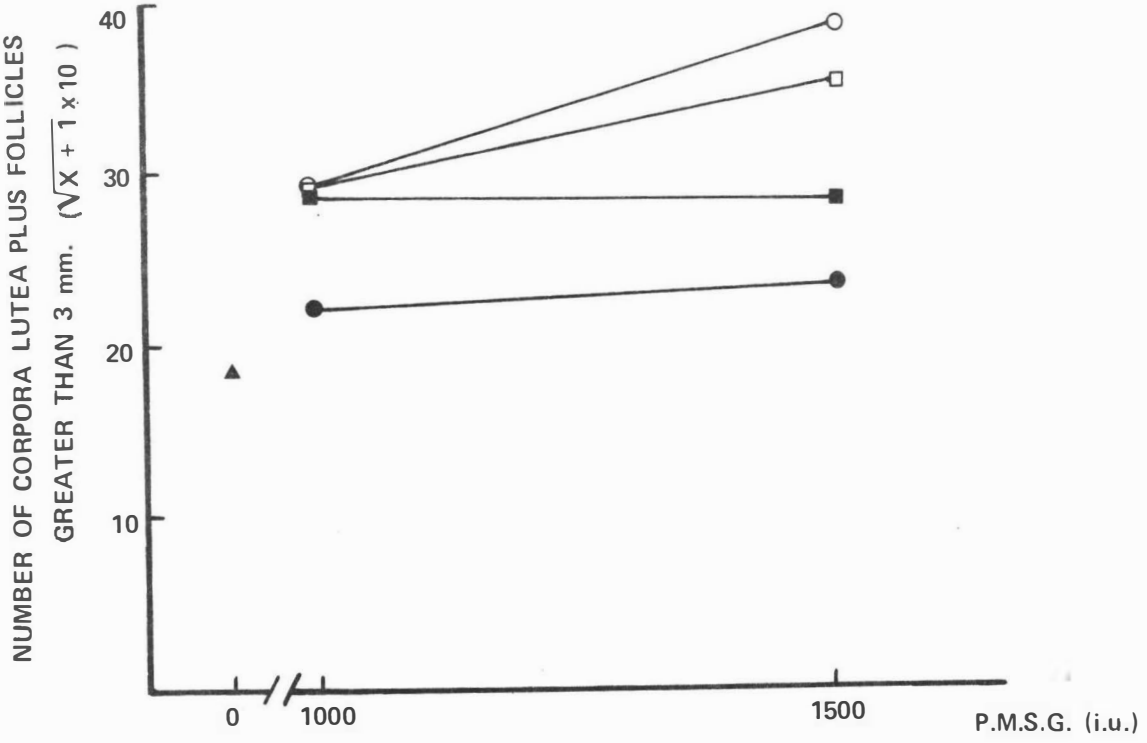


FIGURE 4–13:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON THE NUMBER OF CORPORA LUTEA PLUS FOLLICLES GREATER THAN 3 mm. IN ALL EWES OVULATING : RAW DATA

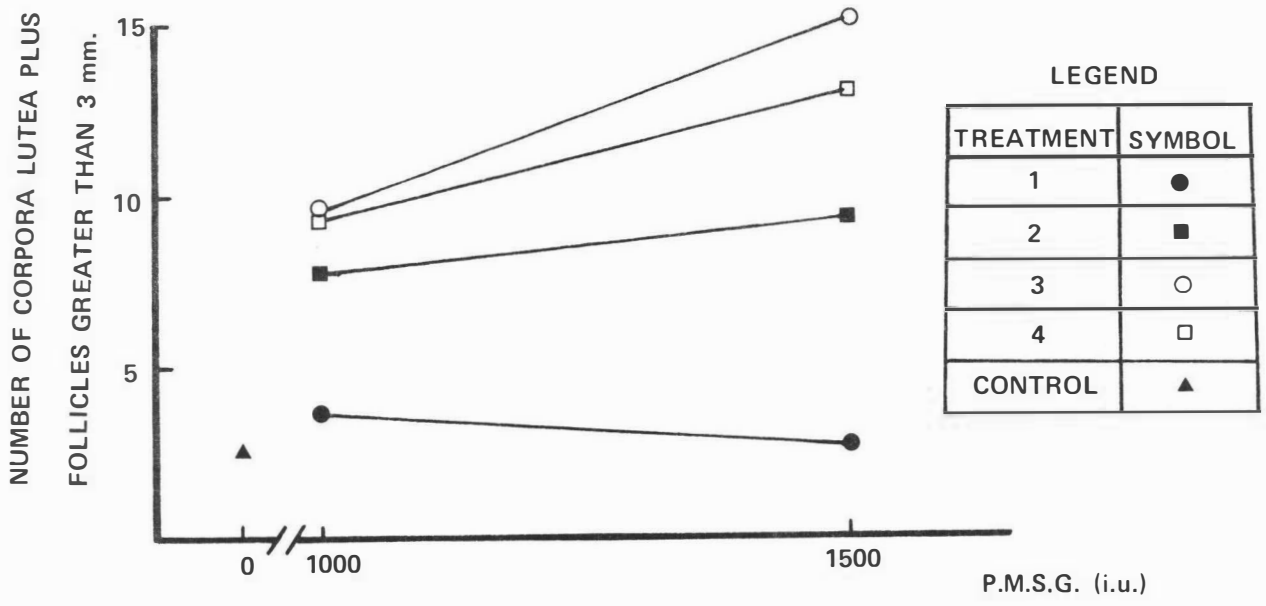


FIGURE 4–14:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON THE NUMBER OF CORPORA LUTEA PLUS FOLLICLES GREATER THAN 3 mm. IN EWES OVULATING : TRANSFORMED DATA

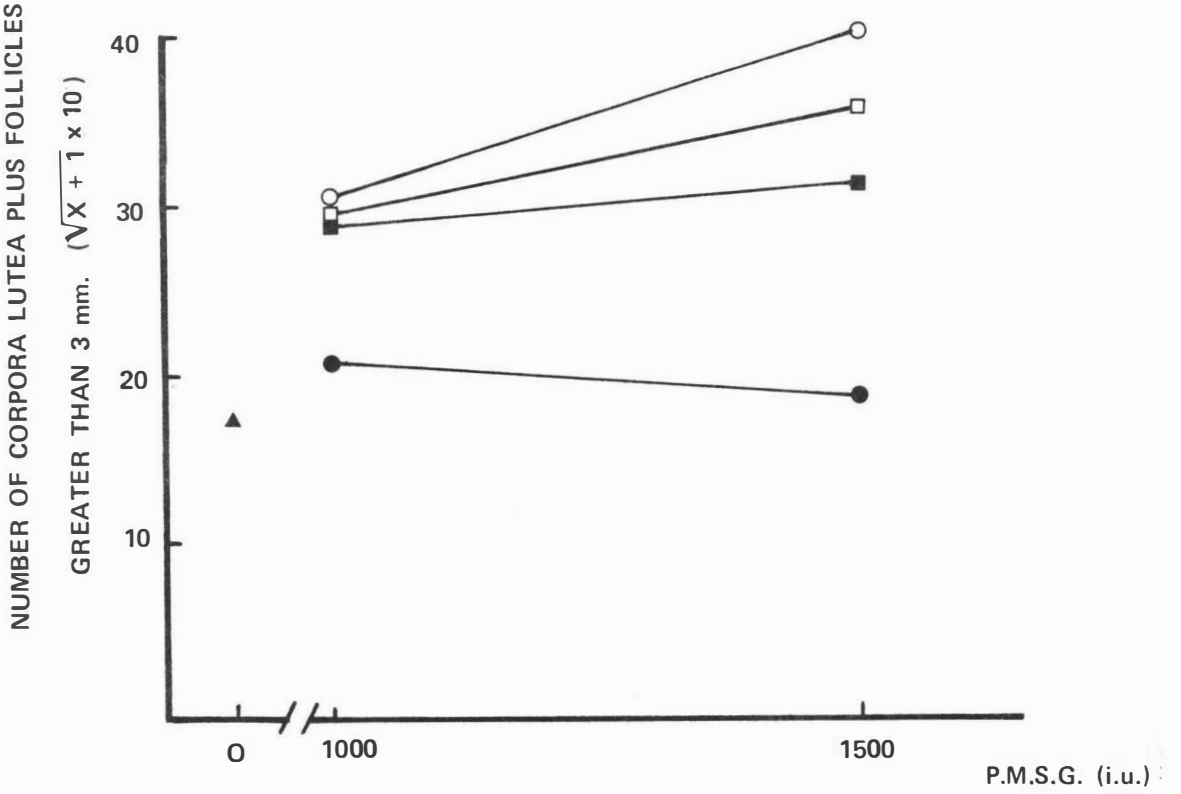


FIGURE 4–15:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON EWE OVARIAN WEIGHT : ALL EWES

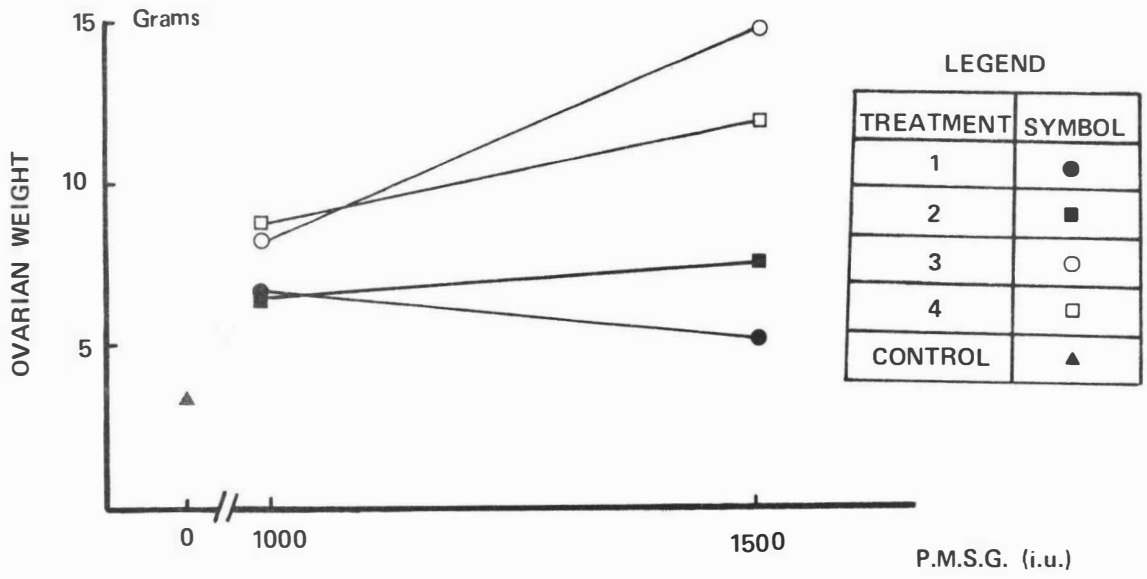


FIGURE 4–16:–

EFFECT OF SEQUENTIAL P.M.S.G. TREATMENT ON EWE OVARIAN WEIGHT : EWES OVULATING

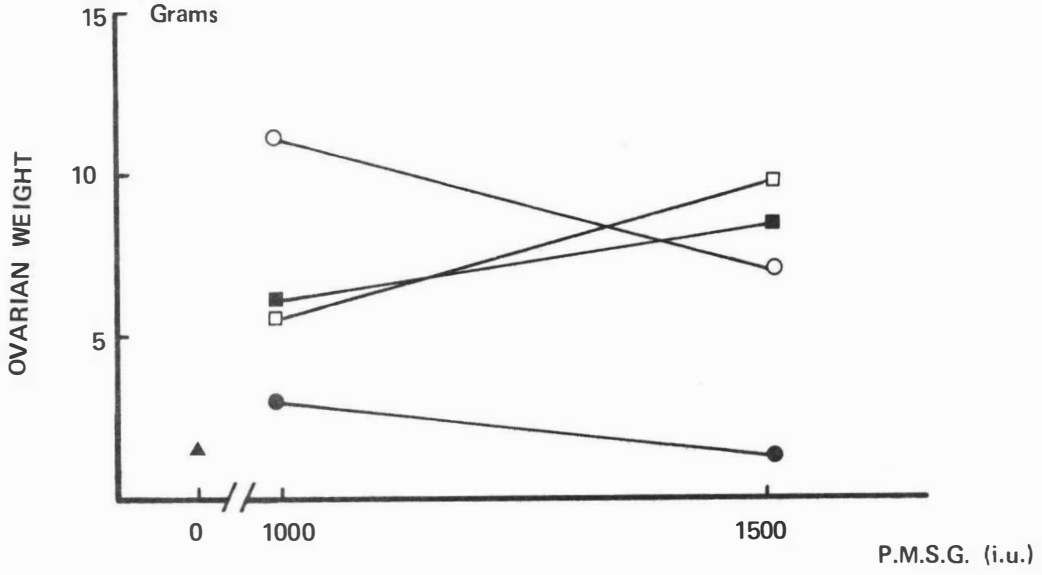


FIGURE 4–17:–

COMPARISON OF EWES SEQUENTIALLY TREATED WITH P.M.S.G. AND CONTROL EWES : OVULATION RATE

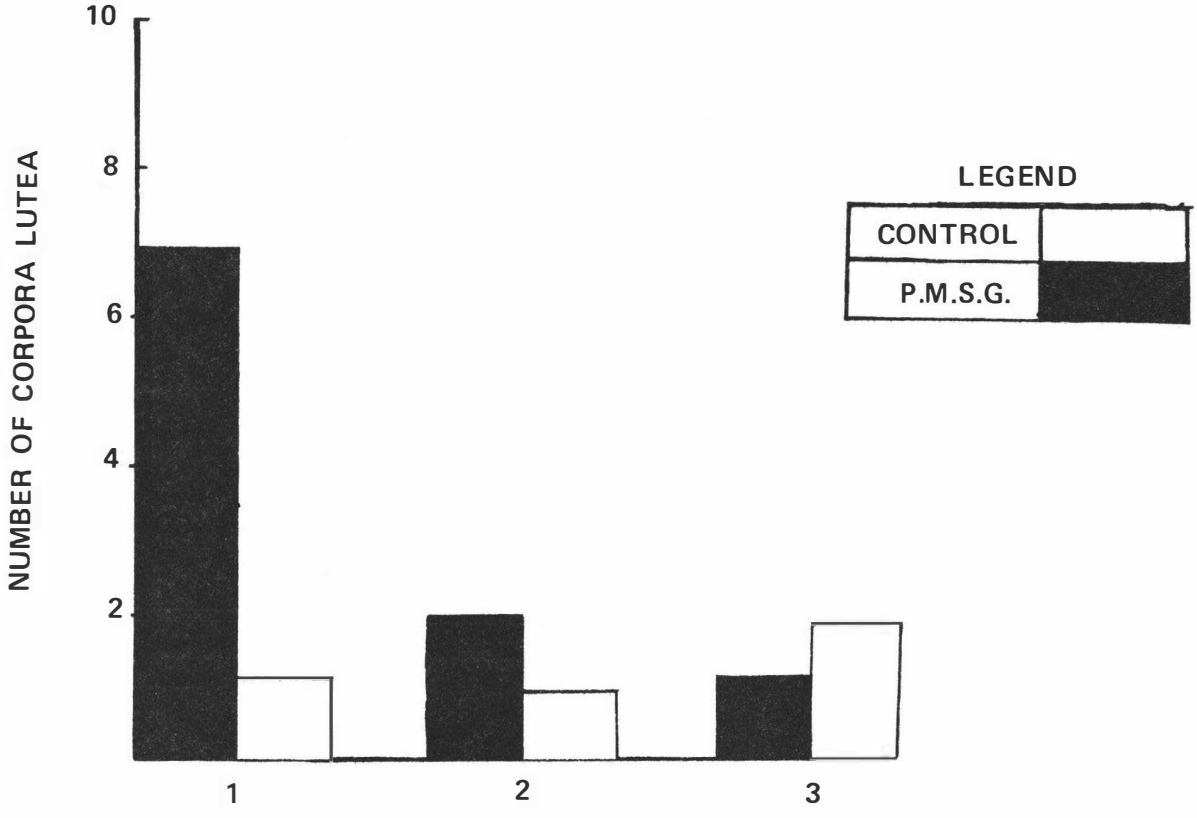
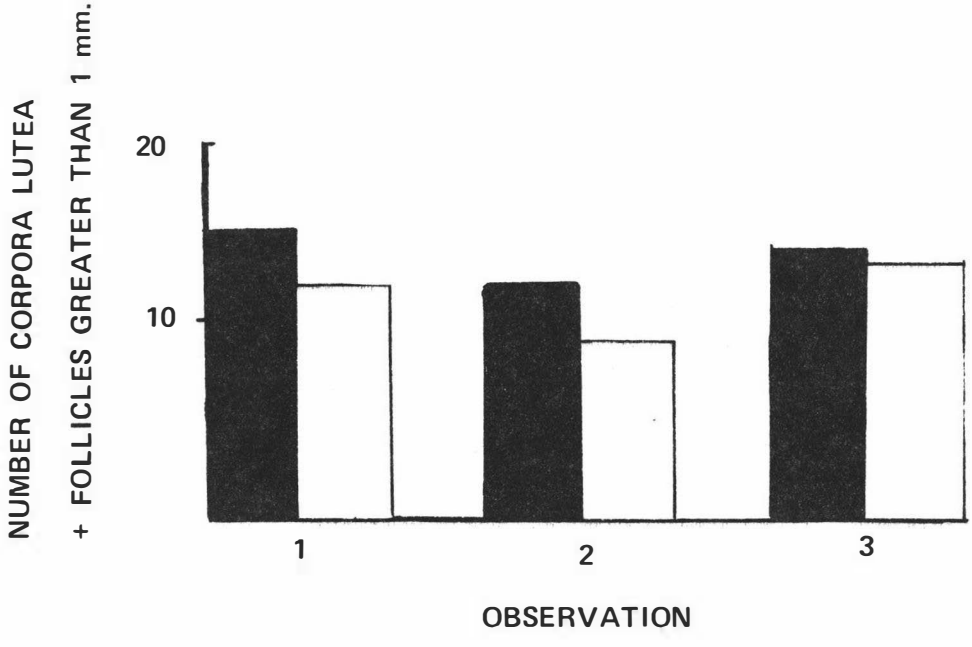


FIGURE 4–18:–

COMPARISON OF EWES SEQUENTIALLY TREATED WITH P.M.S.G. AND CONTROL EWES : CORPORA LUTEA PLUS FOLLICLES GREATER THAN 1mm.



significantly differ from control animals in the number of corpora lutea + follicles $>$ 1 mm. These data are presented in Table 4-8 and Fig. 4-18.

Ovarian Weight

1. Ovarian weight : All ewes (Experiment 1)

Treatments of Experiment 1 exerted no significant effects on ovarian weights (Table 4-3). Dose effects and dose x treatments interaction were also not significant.

2. Ovarian weight : Ewes ovulating (Experiment 1)

By neglecting non-ovulating ewes, significant treatment ($P < 0.01$) and dose ($P < 0.05$) effects were revealed in the analysis of ovarian weights. Ewes of Treatment 1 had lower ovarian weights than ewes of Treatments 3 ($P < 0.01$) and 4 ($P < 0.05$). Treatment 3 produced greater ovarian weights than Treatment 2 ($P < 0.05$) and the high dose of P.M.S.G. increased ovarian weight more than the low dose. Mean ovarian weights of the treatment groups are shown in Fig. 4-16.

3. Dependence of Ovarian Weight on Ovarian Activity

Regression analysis on all ewes in Experiment 1 (Table 4-9a) showed that ovarian weight was significantly affected by the number of corpora lutea ($P < 0.001$) and even more so by the number of corpora lutea + follicles $>$ 3 mm. ($P < 0.001$) The regression analysis presented in this table is for an overall relationship. The individual regression coefficients for each treatment group are given in Table 4-9b.

There was no significant relationship between ovarian weight and the number of corpora lutea, or the number of corpora lutea + follicles > 3 mm., in the control animals. Large positive regression relationships were seen between ovarian weight and number of corpora lutea and between ovarian weight and number of corpora lutea + follicles > 3 mm., in ewes treated once. These counteracted the small, and negative regressions of other single group relationships to produce the overall regression relationship seen in Table 4-9a.

Ewe ovarian weight was not significantly affected by the number of corpora lutea present when ewes were given three sequential injections but if follicle counts were also considered a significant positive regression was obtained.

Difference in Ovulation Rate between the Right and Left Ovaries

Ewes of Experiment 1 ovulated more often on the left ovaries (185 corpora lutea) than on the right ovaries (163 C.L.) (Chi^2 = 1.267,N.S.). This trend was reversed when ewes of Experiment 3 were considered. In these latter ewes the total number of corpora lutea observed on the right ovaries was 147, the left ovaries having 135 (Chi^2 = 0.4290,N.S.). As neither of these trends were statistically significant, there is no evidence that the right ovary is more active than the left.

TABLE 4-9

RELATIONSHIP BETWEEN OVARIAN WEIGHT AND OVARIAN ACTIVITY :

REGRESSION ANALYSIS

A. Overall Regressions

RELATIONSHIP TESTED		REGRESSION ANALYSIS				
DEPENDENT VARIABLE	INDEPENDENT VARIABLE	D.F.	REGR. COEFF.	STD. ERROR	T[A]	P
Ovarian Wt.	Corpora Lutea	69	0.3952	0.0623	6.34	P<0.001
"	Corpora Lutea + Follicles>3mm.	69	0.4793	0.0623	7.69	P<0.001

B. Group Regressions

RELATIONSHIPS TESTED			REGRESSION ANALYSIS				
DEPENDENT VARIABLE	INDEPENDENT VARIABLE	GROUP	D.F.	REGR. COEFF.	STD. ERROR	T[A]	P
OVARIAN WT.	Corpora	L1	8	0.0401	0.2541	0.1598	N.S.
"	Lutea	L2	7	0.2679	0.1091	2.0456	N.S.
"	"	L3	6	0.2831	0.0672	4.2823	P<0.01
"	"	L4	7	0.7812	0.2558	3.0539	P<0.01
"	"	H1	7	0.0236	0.2255	0.1055	N.S.
"	"	H2	7	0.3082	0.0509	6.0550	P<0.001
"	"	H3	5	0.1213	0.4959	0.2448	N.S.
"	"	H4	7	0.6037	0.1055	5.7213	P<0.001
"	"	CC	7	-0.4483	0.3015	1.4868	N.S.
"	Corpora Lutea	L1	8	0.6103	0.1765	3.4597	P<0.01
"	+ Follicles > 3mm	L2	7	0.2556	0.0963	2.6542	P<0.05
"	"	L3	6	0.3247	0.1085	3.0055	P<0.05
"	"	L4	7	0.6539	0.1997	3.2744	P<0.05
"	"	H1	7	0.1591	0.0488	3.2603	P<0.05
"	"	H2	7	0.3097	0.0624	4.9631	P<0.01
"	"	H3	5	0.4090	0.9584	0.4267	N.S.
"	"	H4	7	0.7783	0.0755	10.3986	P<0.001
"	"	CC	7	-0.1758	0.1100	1.4898	N.S.

A - T Test based on Null Hypothesis; Testing whether regression slope differs significantly from zero.

TABLE 4-10

OVULATION RATE OF UNTREATED EWES[A] : EFFECT OF SEASON

Number of Ovulations/Ewe

	OBSERVATION[B]		
	ONE (10)[C]	TWO (9)	THREE (9)
Number of Ewes having 0 C.L.	0	2	0
" " " " 1 "	9	6	3
" " " " 2 "	1	1	4
" " " " 3 "	0	0	2

Means and Standard Errors

	MEAN ± STD. ERROR OBSERVATION		
	ONE (10)	TWO (9)	THREE (9)
Number of Corpora Lutea	14.45	13.57	16.85
(Transformed Counts)	±0.61	±0.64	±0.64

Analysis of Variance

SOURCE OF VARIATION	D.F.	MEAN SQUARES	F
Between Observation	2	26.06	6.98***
Error	25	3.73	

Comparison of Means

COMPARISON OF MEANS	SIGNIFICANCE
OBS 3 vs OBS 2	$P < 0.001$
3 vs OBS 1	$P < 0.001$
2 vs OBS 1	N.S.

A - Ewes of Experiment 3

B - Observations 1,2 and 3 respectively related to the 2nd, 3rd and 4th oestrous cycles of the breeding season.

C - () Number/Group

*** $P < 0.001$

Ovulation Rate of Control Ewes (Experiment 3) : Effect of Season

Table 4-10 shows that the ovulation rate of untreated ewes in Experiment 3 was significantly affected by the stage of the breeding season. These ewes were observed to have significantly more corpora lutea on their ovaries at the fourth oestrus of the breeding season than at the second. This effect was partly due to the animals having more twin and triple ovulations at the fourth oestrus than at the second and third.

CHAPTER SUMMARY

1. Sequential treatment with P.M.S.G. significantly decreases the proportion of ewes ovulating and the ovulatory response to the hormone. Ovulation rate per ewe is reduced by successive treatment whether one considers all ewes or only those ewes ovulating.
2. The number of corpora lutea + follicles $>$ 3 mm. is decreased by sequential treatment but this effect is not apparent if corpora lutea + follicles $>$ 1 mm. are counted.
3. Differences between ovarian weights of ewes treated up to three times with P.M.S.G. are only detected by elimination of anovular ewes from the analysis. Ovarian weight is highly correlated to the number of corpora lutea present when ewes have been superovulated once with P.M.S.G. This relationship lessens with sequential treatment but if corpora lutea + follicles are counted high correlations with ovarian weight are maintained. The relationship between corpora lutea and ovarian weight of control ewes is not significant.
4. There is no evidence to suggest that the right ovary is more active than the left.
5. In untreated ewes, a significant increase in ovulation rate may be seen at the beginning of the breeding season.

CHAPTER V

DETECTION OF ANTI-GONADOTROPHINS

Chapter V

DETECTION OF ANTI-GONADOTROPHINS

Results presented in this chapter involve tests on plasma of sheep from Experiment 1, whose ovarian data were given in Chapter IV.

Immature Mouse Uterine Weight Response to P.M.S.G.

Fig. 5-1 shows the P.M.S.G. dose-uterine weight response curve for immature mice injected with 0.1 - 1.0 i.u. P.M.S.G. Linear increases in uterine weight with log-increments in dose were seen between 0.1 i.u. and 0.8 i.u. P.M.S.G. (Fig. 5-2). The linear increase did not continue above this dosage and 1 i.u. was seen to be slightly supra-optimal for stimulating increased uterine weight.

Doses chosen for the anti-gonadotrophin tests were 0.25 i.u. and 1.0 i.u. P.M.S.G. The lower dose was selected to be mid-way in the log phase of the curve in Fig. 5-2 (where linear increases in response were seen with log-increases in dose). It was thus in the most sensitive region and able to detect small changes in uterine weight, which could be due to antibody inhibition of response. The higher dose, being supra-optimal, would only be counteracted by large amounts of anti-P.M.S.G.

Test for Anti-gonadotrophins in the Plasma of Ewes of Experiment 1

Fig. 5-3 gives the raw means (untransformed) for the uterine weights of mice given P.M.S.G. alone and P.M.S.G. plus plasma from sheep of Experiment 1 and chronically treated ewes. Fig. 5-4 presents the same data after logarithmic transformation. Analysis was performed on the transformed data, using mouse body weight at

FIGURE 5–1:–

IMMATURE MOUSE UTERINE WEIGHT RESPONSE TO P.M.S.G.

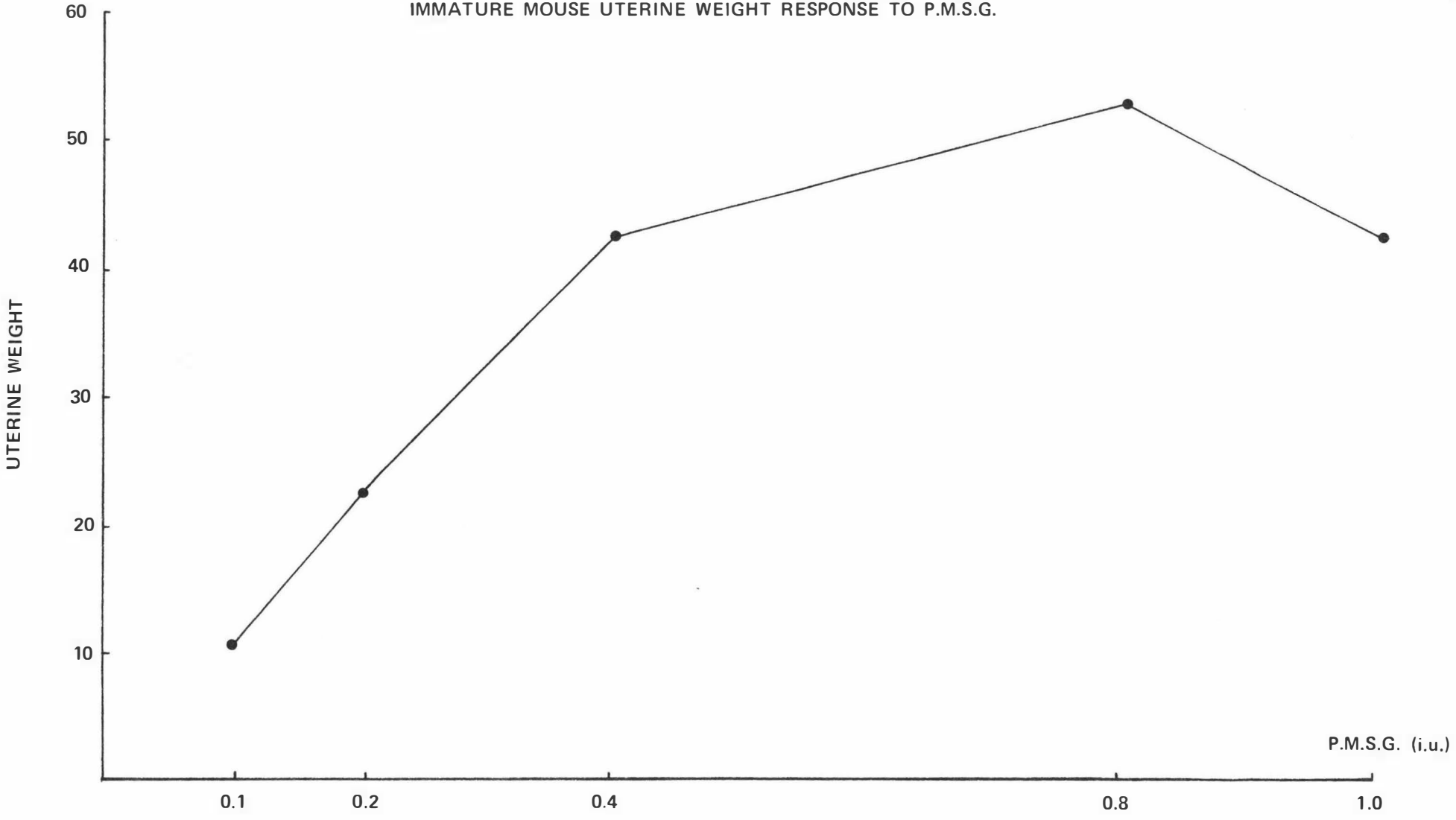
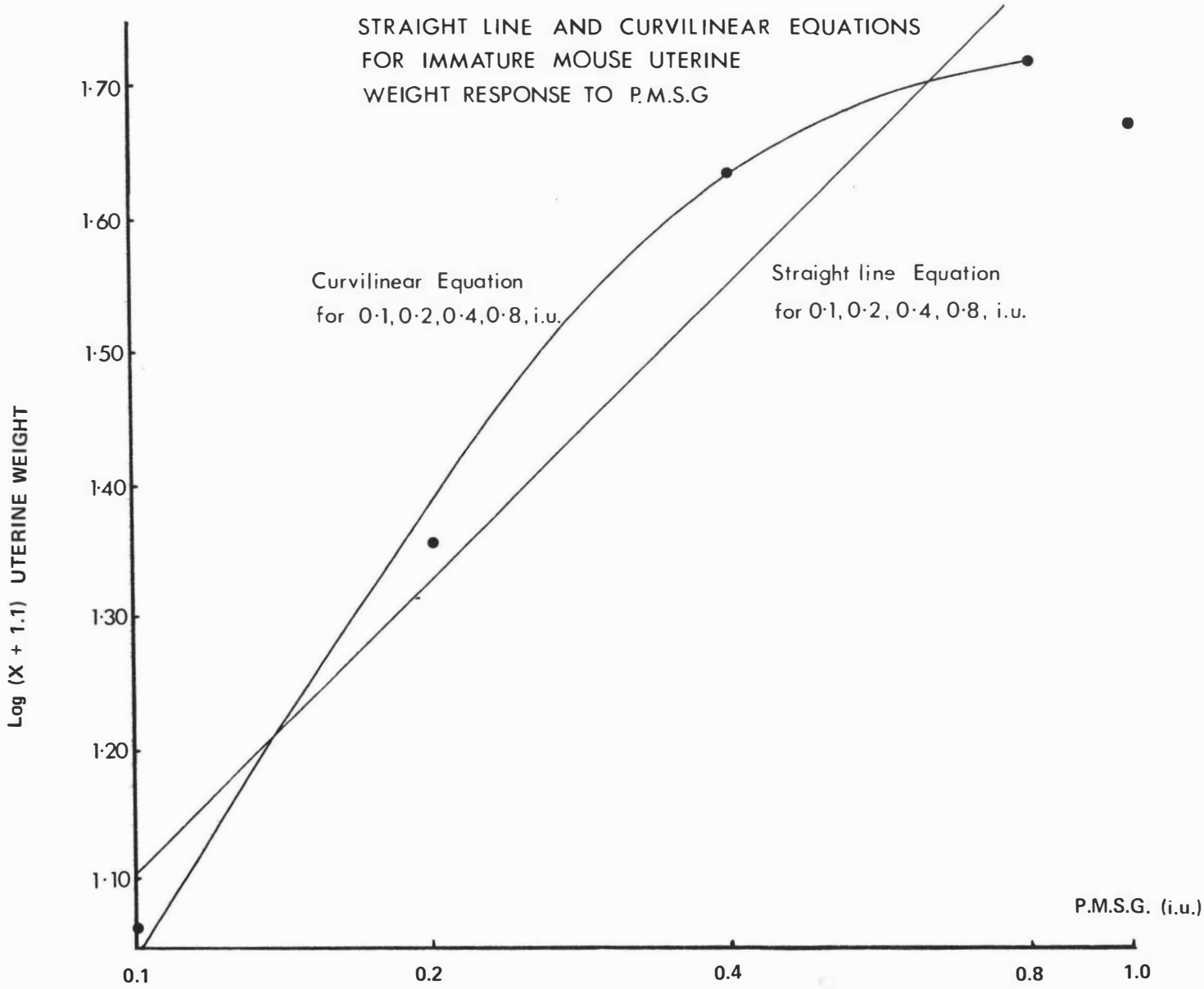


FIGURE 5–2:–


STRAIGHT LINE AND CURVILINEAR EQUATIONS
FOR IMMATURE MOUSE UTERINE
WEIGHT RESPONSE TO P.M.S.G
Curvilinear Equation
for 0·1, 0·2, 0·4, 0·8, i.u.
Straight line Equation
for 0·1, 0·2, 0·4, 0·8, i.u.
Log (X + 1.1) UTERINE WEIGHT
1·70
1·60
1·50
1·40
1·30
1·20
1·10
P.M.S.G. (i.u.)
0.1
0.2
0.4
0.8
1.0

slaughter as a covariate. The use of this covariate reduced the error mean square of uterine weight by 9.77%, compared with the error mean square of analysis of variance. Adjusted means and standard errors of mean uterine weight for each treatment group in Fig. 5-3 and 5-4 are given in Appendix 4.

Sub-cutaneous injection of P.M.S.G. led to significant ($P < 0.01$) increases in mouse uterine weights over control animals. 1.0 i.u. gave significantly greater increases than did 0.25 i.u. ($P < 0.05$). Responses of the mice on the two dose levels were not significantly altered by the injection of 0.25 ml. plasma from ewes of Experiment 1. These results are summarised in Table 5-1.

Test for Anti-gonadotrophins in the plasma of Ewes Chronically Treated with P.M.S.G.

Plasma from ewes chronically treated with P.M.S.G. for 6 weeks inhibited mouse uterine weight response to 1.0 i.u. of the same hormone (Fig. 5-3 and 5-4). The inhibition was not statistically significant when the plasma of ewes given 1000 i.u. P.M.S.G. (twice weekly), was given to the mice. However, the plasma of ewes given 500 i.u. P.M.S.G. under the same regime did inhibit the mouse response to 1.0 i.u. P.M.S.G.

If ewes were given their first injection of P.M.S.G. in Freund's adjuvant, their plasma was able to produce significant ($P < 0.01$) inhibition of mouse uterine weight response to the gonadotrophin.

Whether or not Freund's adjuvant was given, the plasma of ewes given 500 i.u. P.M.S.G. caused greater inhibition (not significant) of mouse uterine weight response than did plasma of ewes given 1000 i.u. (Fig. 5-3 and 5-4).

FIGURE 5-3

MEAN UTERINE WEIGHTS OF MICE TREATED WITH P.M.S.G. AND SHEEP PLASMA: RAW DATA

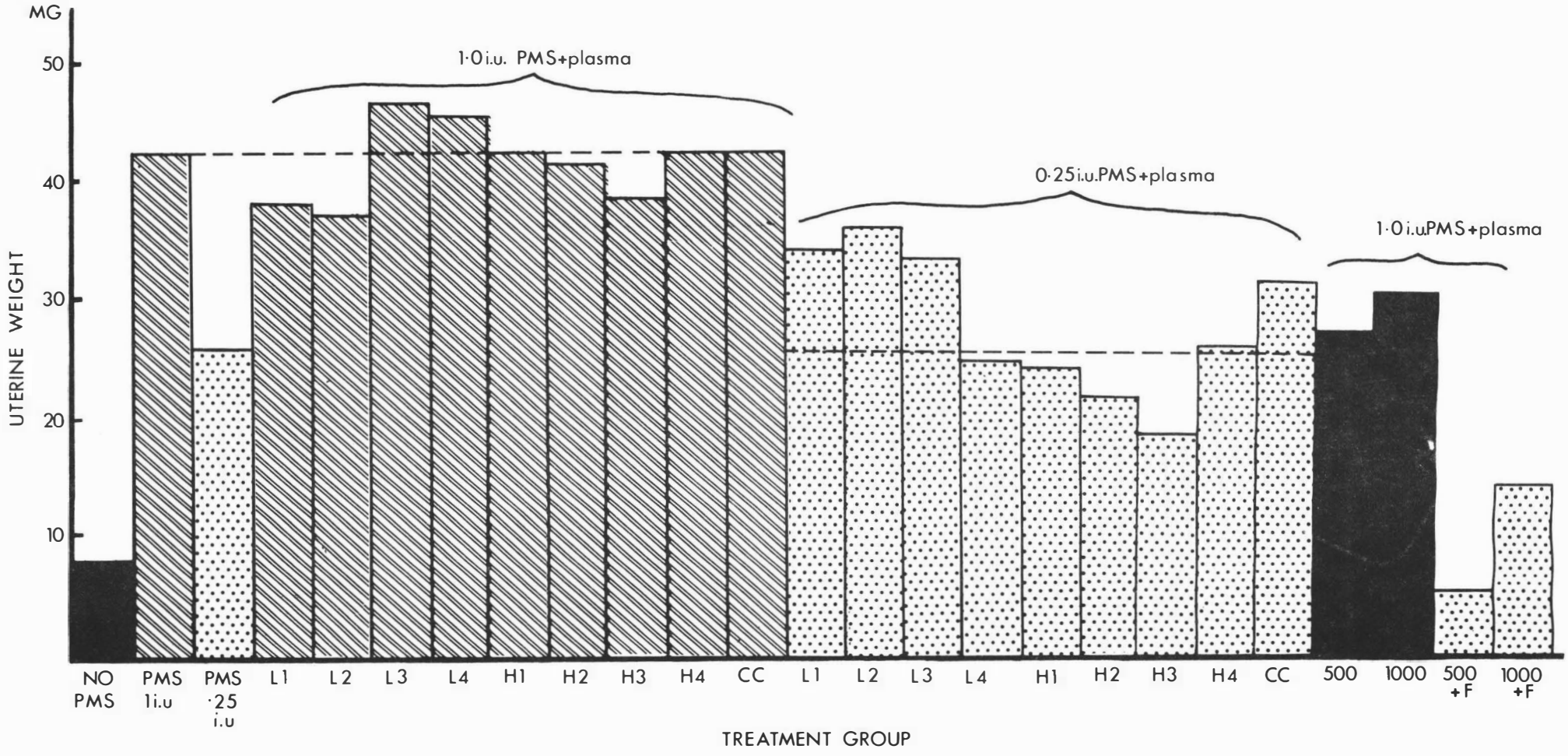


FIGURE 5-4


MEAN UTERINE WEIGHTS OF MICE TREATED WITH P.M.S.G. AND SHEEP PLASMA: TRANSFORMED DATA
1·0 i.u. PMS+plasma
0·25 i.u.PMS + plasma
1·0 i.uPMS+plasma
LOG (x+1·1) UTERINE WEIGHT x 100
180
160
140
120
100
80
60
NO. PMS
PMS 1 i.u.
PMS ·25 i.u.
L1
L2
L3
L4
H1
H2
H3
H4
CC
L1
L2
L3
L4
H1
H2
H3
H4
CC
500
1000
500 +F
1000 +F
TREATMENT GROUP

TABLE 5-1

COMPARISONS OF MEANS AFTER ANALYSIS OF COVARIANCE[A] : MEAN UTERINE WEIGHT OF MICE GIVEN P.M.S.G. ALONE OR WITH PLASMA OF EWES TREATED WITH P.M.S.G.

COMPARISON			P[B]
MOUSE TREATMENT GROUP	VS	MOUSE TREATMENT GROUP	
P.M.S.G. ALONE (0.25 i.u.)	vs	Uninjected	$P < 0.01$
P.M.S.G. ALONE (1.0 i.u.)	vs	Uninjected	$P < 0.01$
P.M.S.G. ALONE	vs	P.M.S.G. (1.0 i.u.) + Plasma of Ewes given 500 i.u. P.M.S.G. alone	$P < 0.05$
P.M.S.G. ALONE	vs	P.M.S.G. (1.0 i.u.) + Plasma of Ewes given 500 i.u. P.M.S.G. + Freund's	$P < 0.01$
P.M.S.G. ALONE	vs	P.M.S.G. (1.0.i.u.) + Plasma of Ewes given 1000 i.u. P.M.S.G. + Freund's	$P < 0.01$

A - Only including comparisons of means that showed significant differences.

B - Level of significance of comparison between two groups.

CHAPTER SUMMARY

1. Uterine growth of immature mice was stimulated by dosages of 0.1 - 1.0 i.u. P.M.S.G. Linear increases in this response were seen with log-increases in dose between 0.1 - 0.8 i.u. P.M.S.G.

2. The injection of plasma from sheep treated at up to three sequential cycles with P.M.S.G. did not inhibit the mouse uterine weight response to P.M.S.G., suggesting that no anti-gonadotrophins had developed in these sheep.

3. Plasma of chronically treated ewes inhibited response to P.M.S.G., suggesting the presence of anti-gonadotrophins.

CHAPTER VI

OVARIAN FOLLICULAR DEVELOPMENT OF EWES SEQUENTIALLY TREATED WITH P.M.S.G.

Chapter VI

OVARIAN FOLLICULAR DEVELOPMENT OF EWES SEQUENTIALLY TREATED WITH P.M.S.G.

Experiment 3 was designed to investigate ovarian follicular development of ewes treated with P.M.S.G. up to three successive times. Laparotomies and recovery of genital tracts after slaughter allowed ovaries of such ewes to be examined at successive cycles. Data on surface follicles were recorded at laparotomy and post-mortem. Cross sectional scores of antral follicles and the calculation of the percentage of these that were normal, provided further information on the treated ewes when the ovaries were recovered at slaughter. Relationships between parameters at successive cycles (repeated observations on the same animals) were also investigated, as were the relationships between cross-sectional follicular counts and surface observations.

Surface Observations

Tables 6-1, 6-2 and 6-3 present analyses on transformed counts of visually appraised follicular development, taken from ewes treated once, twice and three times respectively. As the controls varied in ovulation rate and follicular development from cycle to cycle (see Table 4-10), the treated ewes are compared to control ewes observed at the same time. The tables only show the data for total ovarian follicular response. The right and left ovaries were found to be sufficiently similar to justify pooling the data from both. Data on the counts of corpora lutea and of corpora lutea + total follicles from these sheep are shown in the previous chapter (Table 4-7 and 4-8).

TABLE 6-1

OVARIAN SURFACE ACTIVITY : OBSERVATION ONE

Means and Standard Errors

TREATMENT	N	MEAN NUMBER OF FOLLICLES/SHEEP ± STD. ERROR (TRANSFORMED DATA)					
		SMALL	MEDIUM	LARGE	SMALL + MEDIUM	MEDIUM + LARGE	ALL FOLLICLES
P.M.S.G.	30	22.89	22.53	18.80	31.32	28.01	35.43
		±1.78	±1.60	±1.06	±1.87	±1.88	±1.87
CONTROL	10	19.08	27.63	19.47	33.32	32.64	37.70
		±3.08	±2.78	±1.84	±3.25	±3.26	±3.25

Analysis of Variance

SOURCE OF VARIATION	D.F.	MEAN SQUARES[A]					
		SMALL	MEDIUM	LARGE	SMALL + MEDIUM	MEDIUM + LARGE	ALL FOLLICLES
BETWEEN GROUP	1	108.57	194.64	3.44	38.62	30.27	38.62
ERROR	38	95.63	77.29	34.21	105.91	106.91	105.91

A - F ratios all non-significant

TABLE 6-2

OVARIAN SURFACE ACTIVITY : OBSERVATION TWO

Means and Standard Errors

TREATMENT	N	MEAN NUMBER OF FOLLICLES/SHEEP ± STD ERROR (TRANSFORMED DATA)					
		SMALL	MEDIUM	LARGE	SMALL + MEDIUM	MEDIUM + LARGE	ALL FOLLICLES
P.M.S.G.	20	25.01	18.18	16.56	29.57	21.98	32.15
		±2.02	±1.21	±1.52	±2.18	±1.24	±2.11
CONTROL	9	21.54	18.56	17.28	26.74	24.03	30.56
		±3.01	±1.80	±2.17	±3.25	±1.85	±3.15

Analysis of Variance

SOURCE OF VARIATION	D.F.	MEAN SQUARES[A]					
		SMALL	MEDIUM	LARGE	SMALL + MEDIUM	MEDIUM + LARGE	ALL FOLLICLES
BETWEEN GROUPS	1	74.83	0.97	2.48	49.62	26.15	15.59
ERROR	27	81.63	29.39	46.68	95.36	31.02	89.49

A - F ratios all non-significant

TABLE 6-3

OVARIAN SURFACE ACTIVITY : OBSERVATION THREE

Means and Standard Errors

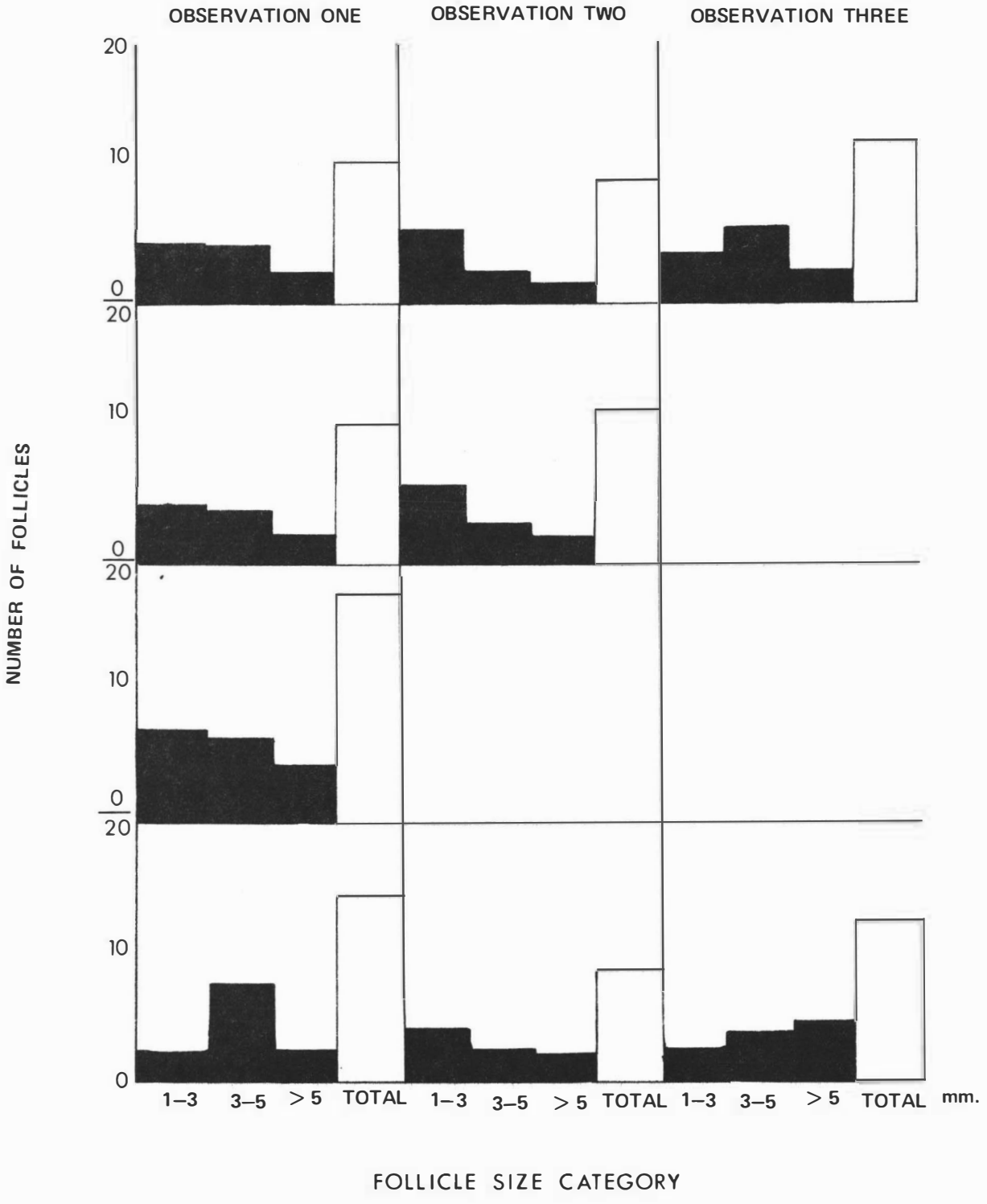
TREATMENT	N	MEAN NUMBER OF FOLLICLES/EWE ± STD ERROR					
		SMALL	MEDIUM	LARGE	SMALL + MEDIUM	MEDIUM + LARGE	ALL FOLLICLES
P.M.S.G.	9	21.54	18.56	17.28	30.58	27.80	30.56
		±2.16	±3.00	±1.65	±3.58	±3.78	±3.51
CONTROL	9	21.46	23.32	17.69	27.06	28.78	12.35
		±2.16	±3.00	±1.65	±3.58	±3.78	±3.51

Analysis of Variance

SOURCE OF VARIATION	D.F.	MEAN SQUARES					
		SMALL	MEDIUM	LARGE	SMALL + MEDIUM	MEDIUM + LARGE	ALL FOLLICLES
BETWEEN GROUP	1	0.03	101.80	0.72	4.61	41.03	53.41
ERROR	16	42.24	81.35	24.56	115.47	129.03	111.22

FIGURE 6–1:–

OBSERVATIONS ON FOLLICLE NUMBERS IN EWES TREATED UP TO THREE TIMES WITH P.M.S.G.



Trends in all parameters, at each of three cycles are shown in Fig. 6-1. There was little variation in the total number of surface follicles present in the ovaries of ewes given either one, two or three sequential treatments. A tendency towards increasing numbers of smaller follicles, and decreasing numbers of larger follicles, with increasing numbers of sequential treatments is not marked. The number of follicles in each different size category for treated animals did not differ from those of the controls at any of the three observations.

Table 6-4 shows the ovarian surface data of the three treated groups at slaughter. These groups differed significantly in ovulation rate ($P < 0.001$). Ewes given one injection had significantly greater ovulation rates than ewes given two or three injections while the latter two groups did not differ. Ewes of Treatment three (1 injection) had significantly more ($P < 0.05$) follicles greater than 3 mm. (med. + lge.) than ewes of treatment two (2 injections). However, ewes of Treatment one (3 injections) did not differ from ewes of Treatment three in this measurement.

When the total sum of corpora lutea and all follicles is considered, ewes given one injection showed a significantly larger score than the other two groups.

The relationships between variates over two immediate oestrous cycles are analysed for control animals in Table 6-5 and for ewes treated up to three times with P.M.S.G. in Table 6-6. These relationships were investigated by regression analysis and the number of corpora lutea at any one observation are related to laparotomy observations at Day 10 of the previous cycle. In the

TABLE 6-4

OVARIAN SURFACE ACTIVITY : OBSERVATION AT SLAUGHTER

Means and Standard Errors

TREATMENT	N	MEANS ± ERRORS OF TRANSFORMED COUNTS							
		CORPORA LUTEA	SMALL FOLL.	MED.FOLL.	LGE.FOLL.	SM.+ MED. FOLL.	MED.+ LGE. FOLL.	ALL FOLL.	C.L. + FOLLICLES
1 (3 Injections)	9	16.44^{B}	21.46	23.32	17.69	30.08	27.80	34.01	37.17^{A}
		±1.73	±3.50	±3.03	±2.38	±4.27	±2.91	±4.03	±3.87
2 (2 Injections)	20	15.45^{A}	26.17	18.87	18.87	31.00	23.37^{A}	34.00	36.30^{B}
		±1.16	±2.34	±2.03	±1.60	±2.86	±1.95	±2.70	±2.59
3 (1 Injection)	30	24.43^{AB}	25.94	22.33	22.33	35.44	32.39^{A}	41.24	47.19^{AB}
		±0.95	±1.91	±1.66	±1.30	±2.34	±1.59	±2.21	±2.12

Analysis of Variance

SOURCE OF VARIATION	D.F.	MEAN SQUARES							
		CORPORA LUTEA	SMALL FOLL.	MED.FOLL.	LGE.FOLL.	SM.+MED. FOLL.	MED.+ LGE. FOLL.	ALL FOLL.	C.L. + FOLLICLES
BETWEEN GROUPS		239.45***	65.70	100.72	56.20	79.87	203.68*	171.57	360.62*
ERROR		27.24	110.41	82.90	51.28	164.69	76.24	146.59	135.16

A,B - Groups with the same subscripts are significantly different means

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

TABLE 6-5

RELATIONSHIPS BETWEEN OVULATION RATE AND OVARIAN ACTIVITY ON DAY 10 OF THE PREVIOUS OESTROUS CYCLE : CONTROL EWES.

RELATIONSHIP[A]		REGR. COEFF.	STD. ERROR	D.F.	T[B]	P
$L.C.L._3$ vs	$L.C.L._2$	-0.1595	0.6986	7	0.2283	N.S.
	$L.Sm.Foll._2$	0.2309	0.2479	7	0.9314	N.S.
	$L.Med.Foll._2$	-0.6930	0.3746	7	1.8499	N.S.
	$L.Lge.Foll._2$	-0.3523	0.3478	7	1.0129	N.S.
	$L.Tot.Foll._2$	0.0028	0.2299	7	0.0121	N.S.
	$L.C.L.+Foll_2$	0.0038	0.2298	7	0.0165	N.S.
$L.C.L._2$ vs	$L.C.L._1$	-0.6667	0.2182	7	3.0554	$P < 0.05$
	$L.Sm.Foll._1$	0.2293	0.1294	7	1.7720	N.S.
	$L.Med.Foll._1$	0.0060	0.0803	7	0.0747	N.S.
	$L.Lge.Foll._1$	0.2525	0.2496	7	1.0116	N.S.
	$L.Tot.Foll._1$	0.0431	0.0836	7	0.5155	N.S.
	$L.C.L.+Foll_1$	0.0234	0.0862	7	0.2714	N.S.
$R.C.L._3$ vs	$R.C.L._2$	1.0116	0.4861	7	2.0810	N.S.
	$R.Sm.Foll._2$	0.4932	0.2572	7	1.9160	N.S.
	$R.Med.Foll._2$	-0.4126	0.4235	7	0.9742	N.S.
	$R.Lge.Foll._2$	0.1705	0.4466	7	0.3817	N.S.
	$R.Tot.Foll._2$	-0.6536	0.3029	7	2.0578	N.S.
	$R.C.L.+Foll_2$	-0.4774	0.3917	7	1.2187	N.S.
$R.C.L._2$ vs	$R.C.L._1$	0.5442	0.1426	7	3.8160	$P < 0.01$
	$R.Sm.Foll._1$	0.0366	0.0914	7	0.3676	N.S.
	$R.Med.Foll._1$	0.0222	0.1010	7	0.2198	N.S.
	$R.Lge.Foll._1$	0.2096	0.0715	7	2.9314	$P < 0.05$
	$R.Tot.Foll._1$	0.0771	0.0744	7	1.0362	N.S.
	$R.C.L.+Foll._1$	0.0862	0.0596	7	1.4463	N.S.

A - L. = Left, R. = Right, C.L. = Corpora Lutea, Subscripts denote Observations 1,2 and 3.

B - T Test based on Null Hypothesis; Testing whether regression slope differs significantly from zero.

TABLE 6-6

RELATIONSHIPS BETWEEN OVULATION RATE AND OVARIAN ACTIVITY ON DAY 10 OF THE PREVICUS OESTROUS CYCLE : TREATED EWES

RELATIONSHIP TESTED[A]		REGR. COEFF.	STD. ERROR	D.F.	T^B	P
$L.C.L._3$ vs	$L.C.L._2$	1.1035	0.4545	7	2.4279	$P<0.05$
	$L.Sm.Foll._2$	-0.2296	0.1919	7	1.1960	N.S.
	$L.Med.Foll_2$	-0.2032	0.6474	7	0.3138	N.S.
	$L.Lge.Foll_2$	0.4326	0.5414	7	0.7990	N.S.
	$L.Tot.Foll_2$	-0.1507	0.1947	7	0.7740	N.S.
	$L.C.L+Foll_2$	-0.1174	0.2345	7	0.5006	N.S.
$L.C.L._2$ vs	$L.C.L._1$	0.2415	0.1965	8	1.2290	N.S.
	$L.Sm.Foll._1$	-0.1352	0.3113	8	0.4343	N.S.
	$L.Med.Foll_1$	0.5026	0.2127	8	2.3629	$P<0.05$
	$L.Lge.Foll_1$	0.4934	0.4413	8	1.1180	N.S.
	$L.Tot.Foll_1$	-0.0675	0.2672	8	0.2526	N.S.
	$L.C.L+Foll_1$	-0.0675	0.2672	8	0.2526	N.S.
$R.C.L._3$ vs	$R.C.L._1$	0.5016	0.6238	7	0.2526	N.S.
	$R.Sm.Foll_2$	-0.4275	0.2762	7	1.5447	N.S.
	$R.Med.Foll_2$	0.1569	0.4257	7	0.3685	N.S.
	$R.Lge.Foll_2$	-0.1768	0.7338	7	0.2409	N.S.
	$R.Tot.Foll_2$	-0.1916	0.2565	7	0.7469	N.S.
	$R.C.L+Foll_2$	0.1046	0.2547	7	0.4106	N.S.
$R.C.L._2$	$R.C.L._1$	-0.0151	0.3628	8	0.0416	N.S.
	$R.Sm.Foll_1$	-0.3483	0.1748	8	1.9523	N.S.
	$R.Med.Foll_1$	0.1838	0.3325	8	0.5527	N.S.
	$R.Lge.Foll_1$	-0.4350	0.3850	8	1.1432	N.S.
	$R.Tot.Foll_1$	-0.1413	0.2311	8	0.6109	N.S.
	$R.C.L+Foll_1$	-0.0981	0.2090	8	0.4693	N.S.

A - L. = Left, R. = Right, C.L. = Corpora Lutea. Subscripts refer to Observations 1,2 and 3.

B - T Test based on Null Hypothesis; Testing whether regression slope differs significantly from zero.

control animals, corpora lutea seen on the right ovaries of ewes at the second observation were significantly related ($P < 0.05$) to the number of large follicles seen 6-8 days before oestrus (observation 1). The corpora lutea seen at the second observation were related to the number of corpora lutea seen at the previous observation ($R.C.L._2$ vs $R.C.L._1$ and $L.C.L._2$ vs $L.C.L._1$)* (Table 6-5). The regression coefficients of these relationships are opposite in sign and hence no real trends are apparent.

Of the P.M.S.G. treated animals, very few significant relationships were found. Ovulation rate of the left ovaries at the third oestrus was positively correlated to the ovulation rate of the same ovaries at the second oestrus and this regression was significant ($P < 0.05$). Ovulation rate of the left ovaries at the second oestrus was significantly related to medium sized follicles (3-5 mm.) At Day 10 of the previous cycle ($P < 0.05$). Again, the significant relationships obtained have little real implication because there are no consistent trends.

Total Ovarian Activity

The number of antral follicles in serial 2 mm. slices gave an estimation of the total ovarian activity and the number of follicles that were potentially able to be stimulated by gonadotrophin. The number of antral follicles per ovary was estimated by counting the number per 2 mm. section and summing them for each ovary. Table 6-7

*Regression of corpora lutea on right/left ovaries at the second ovulation, on corpora lutea of the same ovaries at ovulation one.

TABLE 6-7

ESTIMATES OF TOTAL OVARIAN FOLLICLE POPULATION : NUMBER OF ANTRAL FOLLICLES/OVARY (TRANSFORMED DATA).

Means + Standard Errors

TREATMENT GROUP	NUMBER/GROUP	MEAN NUMBER OF ANTRAL FOLLICLES PER OVARY ± S.E. ($\sqrt{X + 1}$)
1 (3 Injections)	9	41.58 ± 3.53
2 (2 Injections)	10	47.19 ± 3.35
3 (1 Injection)	10	40.97 ± 3.35
4 (Uninjected)	9	36.57 ± 3.53

Analysis of Variance

SOURCE OF VARIATION	D.F.	MEAN SQUARES	F	P
BETWEEN GROUPS	3	181.22	1.61	N.S.
ERROR	34	112.52		

TABLE 6-8

ESTIMATION OF THE PROPORTION OF NORMAL ANTRAL FOLLICLES/TOTAL ANTRAL FOLLICLES

TREATMENT GROUP	NUMBER OF SAMPLES OBSERVED	% NORMAL ANTRAL FOLLICLES/TOTAL ANTRAL FOLLICLES (CORRECTION FACTOR)
1 (3 Injections)	10	31.2%
2 (2 Injections)	10	31.2%
3 (1 Injection)	10	13.9%
4 (Uninjected)	10	33.0%

TABLE 6-9

ESTIMATES OF THE TOTAL NORMAL ANTRAL FOLLICLE POPULATION :
NUMBER OF NORMAL ANTRAL FOLLICLES/OVARY (TRANSFORMED DATA).

Means ± Standard Errors

TREATMENT GROUP	NUMBER/GROUP	MEAN NUMBER OF NORMAL ANTRAL FOLLICLES PER OVARY ± S.E. ($\sqrt{X+1}$)
1 (3 Injections)	9	24.83 ± 1.63
2 (2 Injections)	10	27.71 ± 1.55
3 (1 Injection)	10	17.87 ± 1.55
4 (Uninjected)	9	22.59 ± 1.63

Analysis of Variance

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES	F	P
BETWEEN GROUPS	3	171.41		
ERROR	34	24.18	7.1	$P < 0.001$

Comparison of Means

TREATMENT	vs	TREATMENT	SIGNIFICANCE
1	vs	2	N.S.
1	vs	3	$P < 0.001$
1	vs	4	N.S.
2	vs	3	$P < 0.001$
2	vs	4	N.S.
3	vs	4	$P < 0.01$

presents the data obtained from these counts. There was no significant difference between treatment groups in the number of antral follicles per ovary.

Histological examination of 10 of the 2 mm. slices per treatment group enabled estimation of the number of normal antral follicles per total antral follicles. An average figure was obtained for each group and these are given in Table 6-8. The figures (correction factors) presented in this table were used to correct the data of Table 6-7 (mean number of antral follicles per ovary).

Photomicrographs showing various stages of follicular atresia are seen in Fig. 6-2 to 6-7.

The proportion of normal antral follicles/ total antral follicles of ovaries recovered from ewes on Treatment 3 (1 injection) was lower than that of the other three groups (Table 6-8). The other three groups were comparable in this measure.

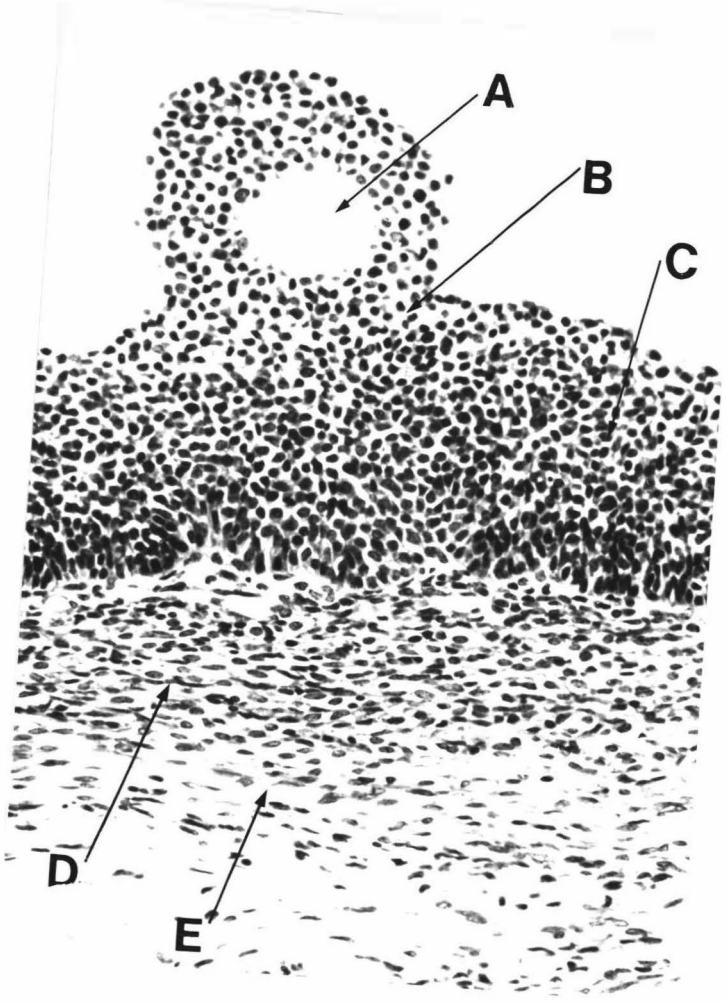
Analysis of follicle populations corrected for the degree of atresia i.e. normal follicles per ovary, is given in Table 6-9 and the application of the correction factor reveals that ewes of Treatment 3 had a significantly lower ($P < 0.001$) number of normal follicles per ovary than the other groups.

Relationships Between Total Ovarian Data and Surface Data

Relationships between estimated total ovarian activity and ovarian surface observations were tested by regression analysis. The estimated total number of antral follicles per ovary and the estimated total number of normal antral follicles per ovary were related to the number of surface follicles per ovary and the

Fig. 6-2:- Normal follicle sectioned through the ovum :
a - ovum; b - cumulus cells; c - granulosa cells;
d - theca interna; e - theca externa (x 168).

Fig. 6-3:- Cross section of a normal tertiary follicle:
a - granulosa; b - theca interna; c - theca
externa (x 65).


A
B
C
D
E

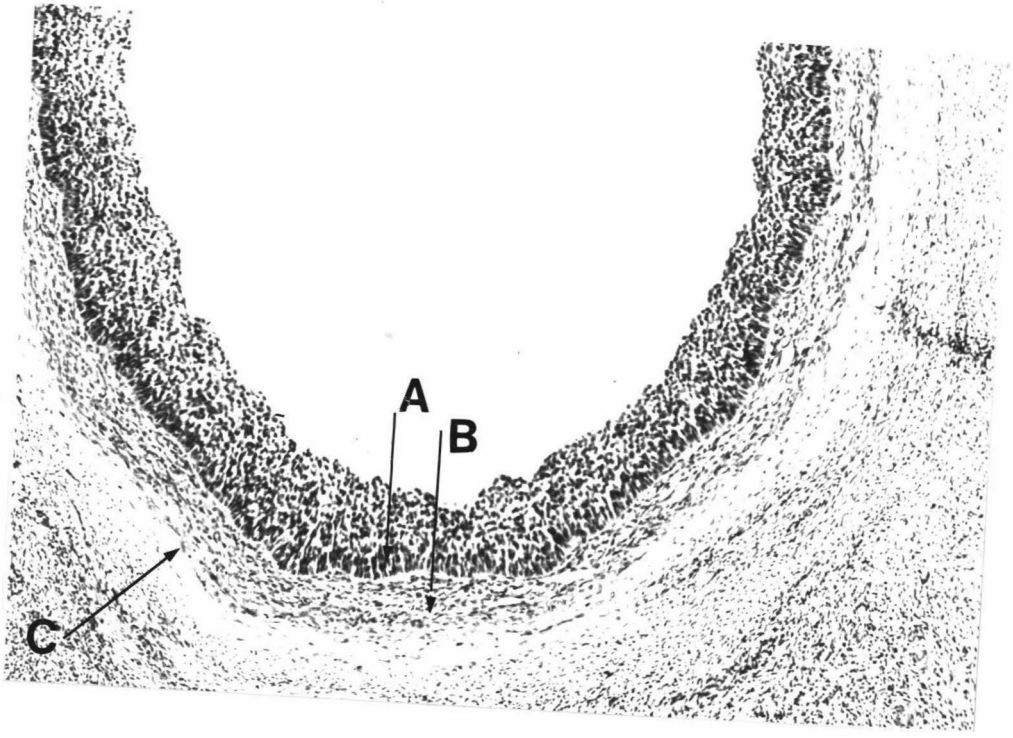
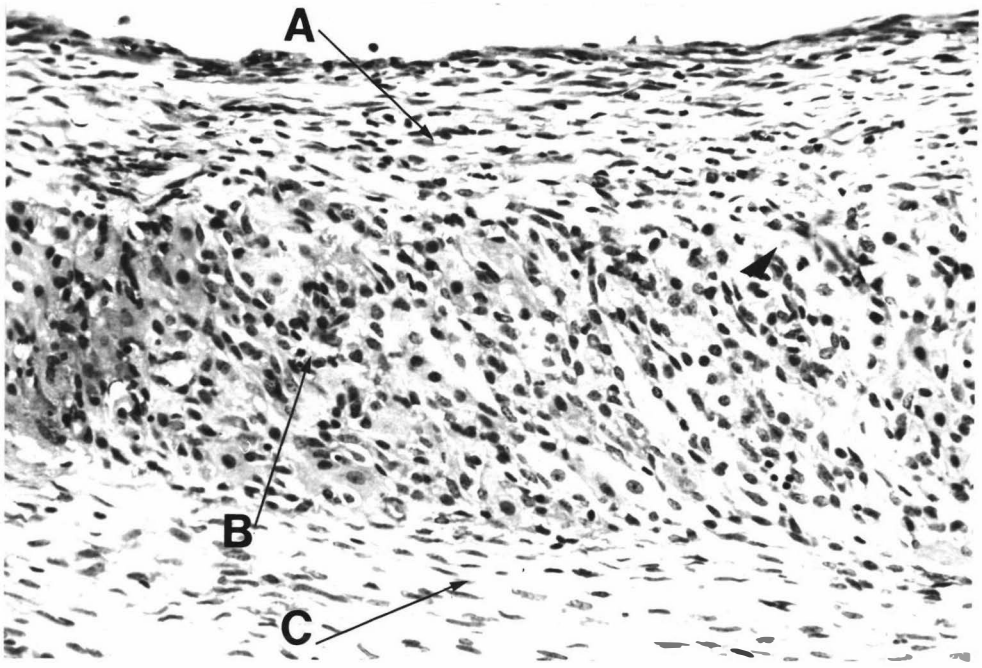

A
B
C

Fig. 6-4:- Cross section of the luteinised wall of a cystic follicle: a - theca interna; b - lutein cells; c - theca externa (x 168).

Fig. 6-5:- Early atresia of a tertiary follicle: a - granulosa cells moving into the antrum (x 168).


A
B
C

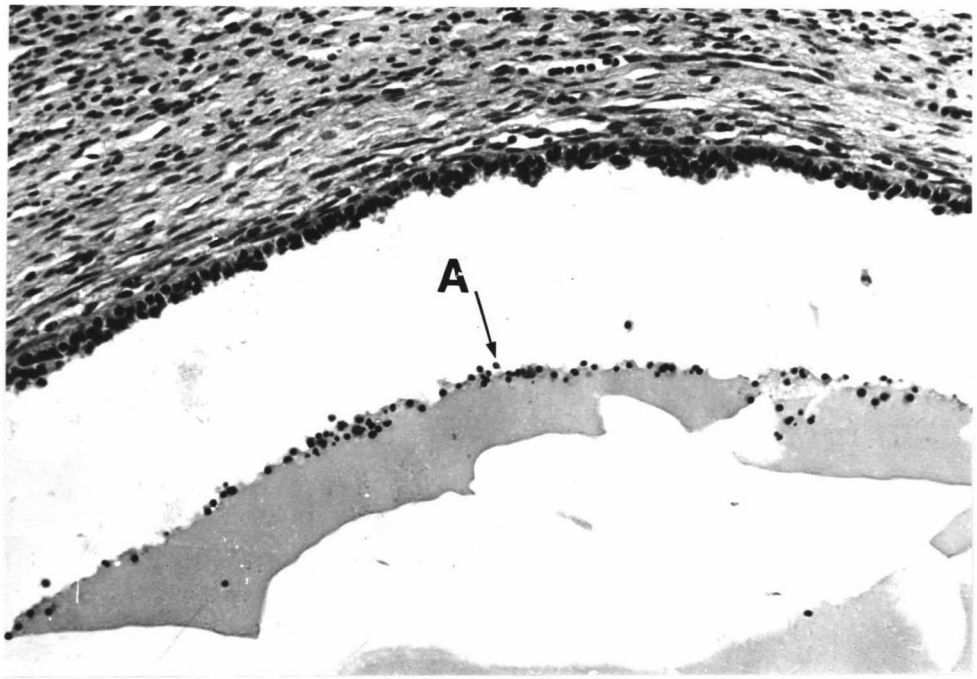
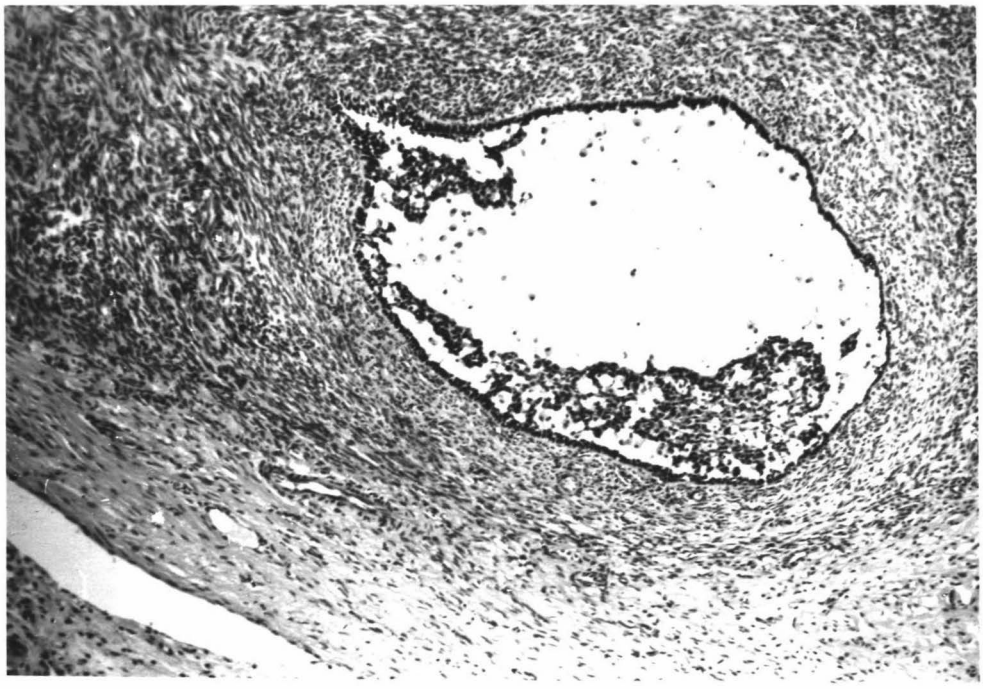
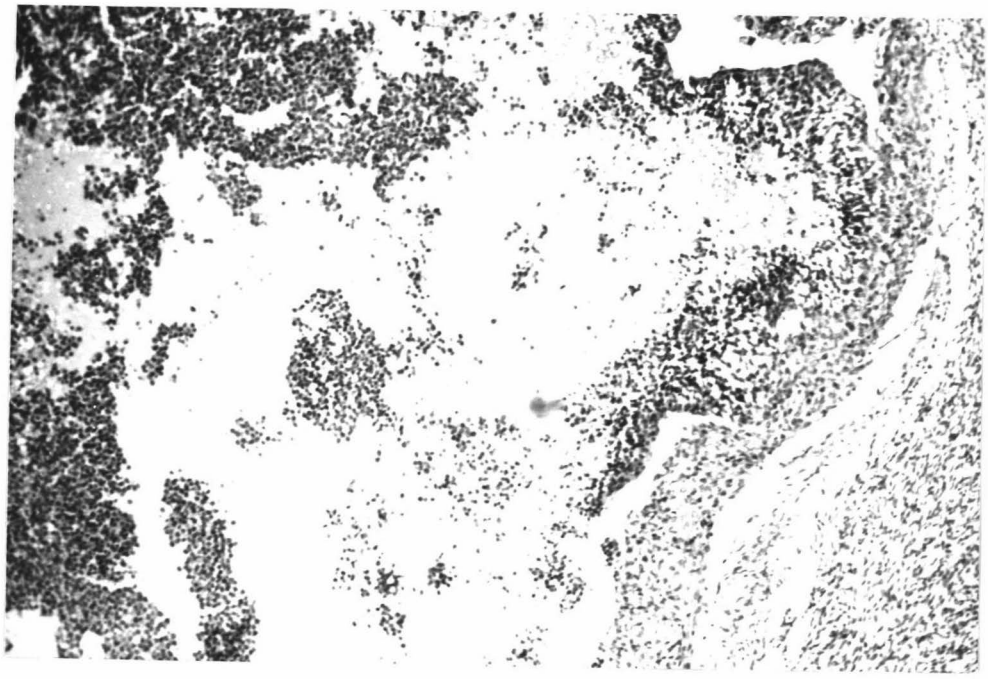

A

Fig. 6-6:- Atresia of a tertiary follicle; disintegration of the granulosa (x 65).

Fig. 6-7:- Late atresia of a tertiary follicle; granulosa cells dispersed throughout the antrum (x 65).





number of corpora lutea per ovary. None of these regression relationships were found to be significant. The regression coefficients are given in Appendix 5.

CHAPTER SUMMARY

1. Although there is no evidence of a reduction in total ovarian population with increasing numbers of sequential treatments with P.M.S.G., there is a trend towards increasing numbers of small follicles and decreasing numbers of large follicles. None of these trends were shown to be statistically significant.
2. The number of follicles greater than 3 mm. was seen to be less in ewes treated twice with P.M.S.G. than in ewes treated once (observation 10 days after the onset of oestrus following injection).
3. Relationships between ovulation rate and ovarian activity (surface) observed on Day 10 of the previous cycle are poor. However, relationships between estimated total ovarian activity and observed surface activity are not significant, indicating that surface observations do not represent total ovarian activity.
4. The number of total normal antral follicles per ovary was seen to be significantly lower 10 days after one injection with P.M.S.G. than at a similar time after a second and third injection.

CHAPTER VII

DISCUSSION

Chapter VII

D I S C U S S I O N

Synchronisation and Oestrous Phenomena

Oestrous cycles were synchronised with progestagen sponges to assist the conduct of the experiments.

After 14 days intravaginal administration of M.A.P. to cyclic ewes (Expt. 1), injection of P.M.S.G. was observed not to affect the time after sponge withdrawal in which ewes came into oestrus. Neither did this injection of P.M.S.G. reduce the number of ewes showing silent oestrus.

Robinson and Smith (1967) have shown the administration of P.M.S.G. after sponge withdrawal to increase the number of anoestrous ewes brought into oestrus with progestagen sponge treatment. Furthermore, ewes were brought into heat earlier when P.M.S.G. was given. Attempting to induce early breeding, Gordon (1971a) showed that more ewes were mated after progestagen treatment when P.M.S.G. was given. With ewes given progesterone treatment early in the season, Robinson (1955) found that P.M.S.G. would give an earlier and a more predictable onset of oestrus, but later stated (Robinson, 1961) that this effect was more marked in younger ewes. Ford (1966) derived no advantage in giving gonadotrophin to cyclic ewes treated with progestagens. The indications are therefore, that P.M.S.G. may effect a greater oestrous response in ewes treated with progestagens in the anoestrous season or early in the breeding season. The effect seems less marked when mature ewes are treated, late in the breeding

season. The synchronisation obtained in ewes of Experiment 1 substantiates this thought.

Injection of 10 mg. progesterone in oil, on Day 12 of a 14 day intravaginal M.A.P. treatment, may provide a more effective synchronisation of early-bred ewes than M.A.P. treatment alone.

Robinson et al. (1968) showed a linear decline, with time of insertion, in the amount of residual progestagen in an inserted intravaginal sponge. Progestagen treatment early in the season is thought to prime oestrogen receptors (Raeside and McDonald, 1959; Gibson and Robinson, 1971). It was therefore thought that injection of progesterone in the latter stages of an intravaginal treatment may supplement the depleted supply in the sponge and give a more effective priming of oestrogen receptors.

Bindon and Roberts (1964) decreased the variability in time of onset of oestrus following synchronisation by the combined use of short- and long-acting progestagens.

Gordon (1971b) suggested that minor modifications in progestagen treatments may improve the oestrous response of early bred ewes and that this was probably related to the amount of progestagen absorbed. However, Gordon (1971d) found that the addition of 400 mg. of progesterone, to an intravaginal sponge already impregnated with progestagen, did not improve the oestrous response in such ewes and only gave marginal superiority in cyclic ewes. Robinson et al. (1968) and Robinson (1971) indicated that this effect may be achieved by modifications in preparation of progestagen sponges.

Although synchronisation in Experiment 3 was better than that achieved in Experiment 1, comparison between the two trials is limited. Lamond and Bindon (1962), Lamond (1964c) and Robinson (1971) have demonstrated seasonal variation in ewe response to progestagens and for this reason, comparisons in Tables 3-2 and 3-4 are not strictly valid. No control ewes were available to guage the effectiveness of a progesterone injection *per se* but the given comparisons were carried out in order to obtain some indication of whether such a practice holds any future promise.

Ewes came into oestrus over a more compact period when ewes were given an injection of progesterone and M.A.P. rather than M.A.P. alone. A greater number of ewes were in oestrus on Day 2 following withdrawal of sponges. The progesterone injection appears to have produced a better synchronisation. The results of Robinson *et al.* (1968) indicate that there is considerable between-ewe variation in the amount of progestagen absorbed from progestagen impregnated sponges. Administration of progesterone on Day 12 of treatment may overcome this variation and ensure that circulating levels of administered progestagens, at the termination of treatment, are more uniform between animals.

The number of ewes showing overt oestrus was not improved by the administration of progesterone, compared with progestagen treatment late in the breeding season. It appears as though the injection did not aid the priming of oestrogen receptors. Cumming (1965) showed that 32-38% of Romney ewes experienced silent oestrus when given a constant daily dose of progesterone during

February. Larsen (1971) found this figure to be 19% with intravaginal M.A.P. treatment of similar ewes during March. It is known that ewes often experience cyclic activity and silent oestrus at the end of the anoestrous season (McKenzie and Terrill, 1937 and Robinson, 1950). Ewes not experiencing oestrus after M.A.P./progesterone treatment probably experienced silent oestrus (confirmed by laparotomy in two ewes). Maybe those ewes coming into oestrus had previously experienced silent oestrus and those showing silent oestrus post-treatment had not. This animal variation in the time of natural silent oestrus may be responsible for the inability of hormonal treatment to affect the incidence of silent oestrus at this time of the year. There is scope for further study in this respect.

Thorough investigation of the effect of a progesterone injection, as given in this study, may be fruitful. With the use of appropriate control animals, the true value of the practice could be revealed.

Effect of Sequential P.M.S.G. Treatment on Synchronisation

Synchronisation in ewes given M.A.P. only (control ewes: Expt. 1) remained effective over three oestrous cycles. The distribution of the number of ewes in oestrus on any particular day, following M.A.P. synchronisation is typically skewed (see Clarke et al., 1966) in these ewes. However the distribution of the day of onset of oestrus becomes more dispersed in nature during the subsequent two oestrous periods. Ewes coming into oestrus over three days at oestrous periods 1 and 2 were distributed over 4 days

at their third oestrus (Fig.3-3). Edey and Thwaites (1966) have reported that once oestrus is synchronised in ewes then the degree of dispersion at the next cycle is small. This has been substantiated by Robinson et al. (1967) who maintained that the second oestrus after synchronisation would be convenient for artificial insemination as the depressed fertility seen in ewes inseminated immediately following synchronisation is not evident one cycle later. Hancock and Hovell (1962) and Larsen (1971) were able to use the second synchronised cycle as a defined period over which ovum transfer could be carried out.

The results of Experiment 1 show that oestrus of ewes given progestagen will remain effectively synchronised for three oestrous cycles. Laparotomy at each of the three cycles (on Day 10 of the cycle) does not alter this synchronisation markedly).

Synchronisation is not well preserved in ewes treated with gonadotrophin (Table 3-9). The administration of P.M.S.G. affects the synchronisation to different degrees, depending on the cycle(s) at which it is given. Ewes superovulated immediately after synchronisation (Trts. 1 + 2 - Expt.1) show a greater dispersion in their oestrous periods at the second cycle than ewes not treated at this point. Ewes treated with P.M.S.G. over three cycles came into heat over a period of 16 days after the third injection and thus synchronisation was completely destroyed by this time. The staggering of injections with P.M.S.G. (Trt.2 - Expt.1) also caused considerable dispersion in the onset of oestrus when compared with ewes given two or one injections (Trts.3 and 4 - Expt.1).

The influence of sequential P.M.S.G. injections on synchronisation does not facilitate ovum transfer work. The dispersing influence of the gonadotrophin, as compared with ewes given progestagen alone, would mean that superovulated donors and synchronised recipients would become progressively asynchronous with successive treatment.

Variation in superovulatory response may be responsible for the dispersion of once synchronised oestrous periods, in animals treated with gonadotrophin. Extreme variation in response to P.M.S.G. is well documented (e.g. Robinson 1951) and was also evident in this study (see Appendix 1). Excessive populations of corpora lutea and large follicles on the ovaries of stimulated animals may cause the variation in time taken to display oestrus at a later cycle.

Increasing numbers of silent heats in ewes treated over three cycles would have rendered any statistical analysis of cycle length to be suspect (in Expt.3 6 ewes came into oestrus after three injections). For this reason, oestrous cycle lengths were thought not to be a valid indicator of any real trend e.g. effect of sequential superovulation as discussed in the above paragraphs. Unless some account of silent oestrus was taken, conclusions based on oestrous cycle length could be misleading.

Results of Experiment 1 showed the treatments to have no effect on the number of ewes showing overt oestrus (Table 3-7). However, in Experiment 3 increasing numbers of sequential treatments with P.M.S.G. reduced the number of ewes displaying overt oestrus (compared with control ewes). The inconsistency between these two

experiments may be seasonal. Ewes could be expected to show silent heat more readily during the earlier part of the breeding season. Nevertheless, all the control ewes of Experiment 3 exhibited oestrus, and the trend seen in the sequentially treated ewes of this experiment is probably real.

Development of Ovarian Refractoriness in Ewes Sequentially Treated with P.M.S.G.

Studies on ovarian refractoriness to P.M.S.G. in sequentially treated ewes involved investigation into more than one aspect of the condition. The results obtained will be discussed in the following order:- 1. Ovarian response and the development of refractoriness. 2. Development of anti-gonadotrophins

3. Follicular dynamics of the ovary

4. General discussion on refractoriness to P.M.S.G.

The first three sections will discuss the results of Experiments 1 to 3 and the fourth section will integrate all information on ovarian refractoriness.

1. Ovarian response and the development of refractoriness to P.M.S.G.

Ovarian refractoriness to P.M.S.G. has been demonstrated in the ewes of both Experiments 1 and 3.

In Experiment 1, ewes given three sequential injections (Trt. 1) had significantly lower terminal ovulation rates (numbers of corpora lutea) than ewes given a single injection (Trt.4) or two spaced injections (Trt.2). Ewes given two sequential injections (Trt.3) also had significantly lower ovulation rates than those given one injection but the difference between treatments 2 and 3 failed to attain significance ($0.05 < P < 0.10$).

When a period of one oestrous cycle separated two injections then the ewe's terminal response to the hormone did not differ from that of ewes given one injection.

When treated ewes were compared with control ewes (Expt.3), the first stimulation with P.M.S.G. was able to superovulate animals but the ovulation rates of ewes treated a second and third did not differ from those of control animals observed at the same time.

The results of this investigation indicate that refractoriness to P.M.S.G. superovulation in ewes is rapidly attained. The mean response of the ovaries to a second injection during the cycle immediately following the first injection will be considerably lower than the normal superovulatory response. This very rapid development of refractoriness was not previously noted by Hulet and Foote (1967, 1969) but was by Larsen (1971). In one experiment, the former authors treated ewes 4 times with 1000 i.u. P.M.S.G., the interval between the second and third injections varying between 33 and 340 days and the intervals between the first and second and third and fourth injections being 16 days. In all cases refractoriness was attained between the third and fourth injections but became less marked as the period between second and third injections increased. They therefore suggested that there was a gradual dissipation of the refractoriness with time. The results of Experiment 1 indicate that the refractoriness may be dissipated more rapidly than this after a single injection. A space of one oestrous cycle between injections is sufficient to enable ewes to respond to a second injection to the same extent as ewes superovulated once.

In a second experiment, Hulet and Foote (1969) treated ewes up to 6 times with P.M.S.G. and showed that ewes became progressively more refractory with increasing numbers of treatments. In the present investigation, there was little difference in the number of corpora lutea of ewes treated twice and ewes treated three times. This further reinforces the idea that animals rapidly lose sensitivity to the exogenous hormone and responses to successive treatments will be low compared to the initial treatment. Furthermore, it appears as though the condition persists.

Hulet and Foote (1967 and 1969) showed that the first injection, given on withdrawal of progesterone synchronisation, always produced lower superovulatory responses than P.M.S.G. injection of the same ewes at the next cycle. A similar situation could have existed in ewes of Treatments 1 and 2 of Experiment 1 in this study. The effect has been attributed to an interaction between progesterone and P.M.S.G. (Hulet and Foote, 1969) but it must be noted that these authors used progesterone and the present study employed progestagen sponge treatments. Moore and Holst (1967) have shown that there is no significant difference in ovulation rates between ewes given either intrvaginal or intramuscular treatments of progesterone or synthetic progestagen, when anoestrous ewes were given P.M.S.G. after treatment. Averill (1958) reported that the ovulation rates of ewes given P.M.S.G. treatments during the breeding season were not significantly different to those of ewes given progesterone-P.M.S.G. treatment during the anoestrous season. Many authors have shown good responses to P.M.S.G. immediately after progestagen sponge treatment (e.g. Holst, 1969)

and it is therefore difficult to decide whether ewes of Treatments 1 and 2 (Expt.1) would have had lowered superovulatory responses to P.M.S.G. because of the prior administration of progestagen. The interaction reported by Hulet and Foote may not be so extreme if progestagen is used in preference to progesterone. If it is accepted that ewes experienced some degree of superovulation, when injected with P.M.S.G. immediately following synchronisation, then the lack of apparent refractoriness in ewes of Treatment 2 (Expt.1) is a real effect. The possibility of an interaction between P.M.S.G. and the progestagen was avoided in Experiment 3 by commencing injections one cycle after synchronisation. In this Experiment it was confirmed that refractoriness was established after only one injection of the gonadotrophin.

Jainudeen et al. (1966) gave cows a total of 4 injections of P.M.S.G. at intervals of 5-7 months, 31-34 days and 18-21 days (first injection = 2000 i.u. and subsequent injections were 3000 i.u.) The second injection produced a superovulatory response equivalent to the first, in spite of the increased dosage. The final two injections failed to produce ovulation rates greater than in untreated ewes. Again, a refractory condition was evident at the second sequential treatment. Willet et al. (1958) used more intensive gonadotrophin treatments than are normally used to superovulate domestic animals. They also showed that animals will very rapidly develop a refractoriness in response to exogenous gonadotrophins and this condition persists for a long period of time. Hafez et al. (1964) and Laster et al. (1971) have shown that refractoriness in cows is evident at a second sequential

superovulation. Larsen (1971) obtained a similar effect in ewes regardless of increasing dose levels of P.M.S.G. All the observations of the above workers substantiate the results of this investigation, in that refractoriness is established after only one injection. But there are varying opinions as to the period of time required to dissipate the condition. The long period stated by Hulet and Foote (1969) may have been because initial treatment was in fact 2 sequential injections.

The response of ewes treated with a single injection of P.M.S.G. (Trt.4; Expt.1; Fig.4-3) is comparable to results plotted in Fig.1-1. The ewes were of comparable natural fecundity, and of the same breed, as those used by Wallace (1954). The dose response curve of ewes in this study is slightly steeper than that of Wallace's ewes, but may be regarded as similar. The responses to 1000 i.u. and 1500 i.u. P.M.S.G. are greater than those obtained by Tervit (1967) and Larsen (1971) but are similar to those of Cumming (1965).

Differences in superovulatory response to a single injection of P.M.S.G. between treatments in this study and treatments imposed by other workers in the same environment may be due to age factors (e.g. Larsen, 1971) and seasonal differences in response (e.g. Cumming, 1965). The latter of these two factors is the more likely, as there is little evidence of differential responses in ewes of different ages (Robinson, 1951 and Averill, 1958).

The range in ovulatory response of ewes given 1500 i.u. was greater than in ewes given 1000 i.u. P.M.S.G. (Appendix 1). Robinson (1951) Wallace (1954) Averill (1958) and Larsen (1971)

have all noted that there is an increase in individual ewe variation with increasing dose of P.M.S.G.

Dose level and treatment x dose interactions did not have significant effects on the numbers of corpora lutea observed in ewes of Experiment 1. Fig. 4-4 shows that, although these effects were not statistically significant, some discussion on these trends may be justified providing the limitations of significance are appreciated. With sequential treatments (Trts.1 and 3; Expt.1), lower doses (1999 i.u.) produced higher terminal ovulation rates than higher doses (1500 i.u.). The reverse trend was seen in less intensively treated ewes (Treatments 2 and 4; Expt.1). With sequential treatments, high doses of gonadotrophin may enhance refractoriness by producing greater ovarian responses at the first injection. This suggestion may in part explain why ewes given spaced injections (Trt.2) have a dose response curve of similar slope to that of ewes given a single injection. The space of one oestrous cycle possibly allows the hypertrophied ovaries to regain normal size and function before restimulation.

Although there was no incidence of cystic ovaries in Treatments 2 and 4 (Expt.1) while there was in sequentially treated ewes, statistical significance was not attained. Larsen (1971) has indicated that successive stimulations are likely to cause cystic and luteinised follicles to be more prevalent. However, Robinson (1951) and Wallace (1954) report that similar effects are obtainable with administration of a single large dose of P.M.S.G. The two may be similar in that they are both over-stimulating the ovaries. The development of cystic ovaries is

thus unlikely to be a primary causal factor of refractoriness but may enhance its effect.

Hulet and Foote (1969) consider that refractoriness (lowering of ovulatory response to P.M.S.G.) is in part due to ewes becoming anovular. They were able to partially negate the effect of refractoriness by considering ovulation rates of only ewes ovulating. A similar effect was apparent in the present study when numbers of corpora lutea of ovulating ewes only were analysed. Ovulating ewes given three sequential injections had significantly lower terminal ovulation rates than all other ewes in Experiment 1. This result implies that the failure to ovulate may be the first demonstrable cause of apparent refractoriness as the elimination of non-ovulating ewes rendered the terminal ovulation rates of ewes on treatment 3 (two sequential injections) similar to those of ewes given one injection. This effect was reproduced to a lesser extent in Experiment 3 where 2/20 and 2/9 ewes failed to ovulate with a second injection and third injection respectively. Averill (1958) has given some evidence of a similar anovular condition where ewes being treated with P.M.S.G. for a second and third time within one breeding season, failed to ovulate.

P.M.S.G. is primarily follicle stimulatory in action (Lamond, 1960) and may stimulate the growth of many follicles that do not ovulate (Lamond, 1964). Scanlon et al. (1968) noted that only 63% of mature follicles ovulate when cows were stimulated with the hormone. It is therefore likely that counts of corpora lutea + large follicles would provide a more accurate idea of total ovarian response to gonadotrophic stimulation than corpora lutea

alone. Lamond (1964) and Larsen (1971) have found larger standard errors associated with means of corpora lutea + follicles than with means of corpora lutea only. In Experiment 1 this effect was not evident when corpora lutea + follicles greater than 3 mm. were considered. The mean for these measurements was significantly less for ewes on Treatment 1 than for all other groups of ewes. Adding these follicle counts to corpora lutea causes Treatment 3 (two sequential injections) to differ from Treatment 1 and therefore the difference between these two groups is in the number of large follicles. It is interesting to note that in Experiment 3 there was no difference between control ewes and ewes given one, two or three sequential treatments with P.M.S.G. when the number of corpora lutea + follicles greater than 1 mm. were counted. Smaller follicles were taken into account in this experiment whereas measurements in Experiment one neglected any follicles less than 3 mm. in size. It appears as though the population of 'larger' follicles is more sensitive than their 'smaller' counterparts to gonadotrophic stimulation.

Over all treatments of Experiment 1, the higher dose of P.M.S.G. (1500 i.u.) stimulated a greater number of corpora lutea + follicles greater than 3 mm. than did the low (1000 i.u.) dose. This result conforms to that of Holst (1969) which shows that increasing doses will increase the number of large follicles as well as the count of corpora lutea + large follicles. Lamond (1964) and Larsen (1971) have also reported increases in follicle growth with increasing levels of administered P.M.S.G.

Regression analyses given in Table 4-9 show that ovarian weight is dependent on the number of corpora lutea per ovary when ewes are treated once with P.M.S.G. This relationship does not hold for uninjected ewes and does not always apply when ewes are given successive injections. Robinson (1951) has shown the ovarian weights of treated ewes to be significantly correlated with the number of corpora lutea present in ewes superovulated with P.M.S.G. and Allen and Lamming (1961) have derived relationships for untreated, flushed ewes.

Regression relationships for ovarian weight on corpora lutea plus large follicles are better than if only corpora lutea are considered. This is consistent with the earlier suggestion that this composite figure is a better indication of total ovarian activity than is "corpora lutea alone".

Slopes of curves in Figs.4-15 and 4-16 are, in majority, similar to those of Figs.4-4 and 4-5 showing that ovarian weight is related to the amount of luteai tissue. The high figure for group H3 in Fig.4-15 is a consequence of the number of ewes on this treatment that had cystic ovaries (see Table 4-2). This effect is eliminated in Fig.4-16 because such ewes failed to ovulate.

No evidence of a difference in activity between the left and right ovaries was revealed in these studies. McKenzie and Terrill (1937) and Wallace (1954) reported that the right ovaries of ewes were significantly more active than the left but Hutchinson and Robertson (1966) were unable to verify this.

In Experiment 3, untreated ewes were observed at each of three successive oestrous cycles (2nd to 4th oestrous cycles of the breeding season) (Table 4-11). The ovulation rate was significantly greater at the third observed oestrus than at the first and second. That ovulation rate increases from the beginning of the season, has been noted by Averill (1964) and McDonald and Ch'ang (1966), using Romney ewes. Observations on control ewes of the present study conform to the pattern suggested by these authors.

Detection of Anti-gonadotrophins

Lin and Bailey (1965) have shown strain differences in mouse response to P.M.S.G. Bell (1969) has also shown that some strains of mice may be relatively insensitive to gonadotrophic stimulation whereas others are very sensitive. The mice used in this study were known to be oestrogen sensitive (Anon 1965) and for this reason, only low levels of P.M.S.G. were required to elicit a uterine weight response in immature females. Suehiro (1955) and Lamond and Bindon (1966) showed response curves of mice to plateau at 60 i.u. and 10 i.u. respectively. The plateau obtained in the mice of the present study was between 0.8 i.u. and 1.0 i.u. and the sensitivity of these mice is thus exemplified. Supra-optimal doses frequently yield lower ovarian and uterine weight responses than sub-optimal doses (Suehiro et al., 1955; Lamond and Bindon, 1966) and this was also noted in the mice of Experiment 3 (Fig.5-1).

Responses of mice given 1.0 i.u. gave more uniform (between group) response than mice given 0.25 i.u. P.M.S.G., when injected with plasma (Figs.5-3 and 5-4). The mice given the lower dose

were in a far more sensitive dose range than those given the high dose and the greater variation of the former may be a consequence of this.

Standard errors associated with mean uterine weights of mouse treatment groups were comparable whether mice were given 1.0 i.u. or 0.25 i.u. P.M.S.G. (Appendix 4). Increased dosage, producing greater increases in uterine weight, often increase the standard error associated with the mean response (Lamond and Bindon, 1966 and de la Lastra, 1972). Administration of an antiserum may (Sasamoto et al., 1972), or may not (Flux and Li, 1965) decrease standard errors of mean response to P.M.S.G. at a particular dose level. This is maybe due to the inhibition in response, which gives lower mean values, and lower standard errors could be associated with these. No reduction in the standard errors of the mean uterine weight response to P.M.S.G. by the administration of plasma was seen in this study. This is explainable in the mice not showing inhibition of response as the mean response is not lowered. However, it may have been reasonable to expect a decrease in standard errors associated with the uterine weights of mice receiving plasma from chronically treated ewes but this was not evident.

Failure to obtain any reduction in immature mouse uterine weight response to P.M.S.G., by injecting plasma of ewes from Experiment 1, implies that no anti-P.M.S.G. factors were present in the blood of these ewes. The credence of concluding that the observed ovarian refractoriness was not due to antibody production, depends on the sensitivity and validity of this test. Detection of anti-gonadotrophins in the plasma of chronically treated ewes

confirms that the test used was able to pick up antibody activity and to indicate the relative potencies of the same. Similar tests have been used by Cole et al. (1957), Pigon et al. (1960), Johnson et al. (1962), Nakahari et al. (1964) and Jainudeen et al. (1966) to successfully demonstrate antibodies to P.M.S.G. Cole et al. (1957) have shown that anti-gonadotrophins in cattle behaved in a manner similar to serological antibodies.

For the present study, a biological method of anti-gonadotrophin determination was selected in preference to a chemical method. Biological inhibition would indicate in vivo neutralisation of hormone action. It is this consequence of antibody reaction that is being investigated when one considers refractoriness. Geshwind (1963) points out that the various methods used to detect and quantitate antibody production may not necessarily parallel one another. Cole et al. (1957) exemplified this when obtaining better indications of anti-gonadotrophic activity when using biological neutralisation tests than with precipitation tests.

Rees-Midgely (1969) expresses some scepticism about the inferences drawn from tests which rely on neutralisation of biological activity. He states that neutralisation of a hormone is a good indication of the presence of antibodies, but failure to detect antibodies by this method does not automatically mean that they do not exist. Antibodies may be produced in too low a titre to be detected by inhibition tests and he suggests that they should be combined with immunochemical procedures. However, Johnson (1962) presents evidence to indicate that an antibody to a hormone may be present yet not produce precipitins in immuno-chemical analysis and that the P.M. S.G.-anti-P.M.S.G. complex

may be of this type.

Ouchterlony double-diffusion tests (Ouchterlony, 1949) were attempted on samples of plasma obtained from sheep in this study. Failure to observe any precipitation between antigen and antibody implied no evidence of anti-gonadotrophins whereas inhibition was obtained when similar samples were injected into mice. The Ouchterlony tests were abandoned because it was thought the antigen (P.M.S.G.) was of too low a potency for this method.

Chronic treatments of sheep with P.M.S.G. (Expt.3) produced results similar to those of Pigon et al. (1960). It appeared as though sheep produced antibodies to P.M.S.G. more readily with a lower dose of the hormone. In the present study sheep injected twice weekly with 500 i.u. P.M.S.G. had greater antibody titres (not significant) than those given 1000 i.u., at the end of 6 weeks of treatment. This is contrary to results obtained in cattle (Cole et al., 1957) where lower doses are seen to produce lower titres of antibody.

The P.M.S.G. preparation used in this study was of low potency (approx. 40 i.u./mg.) which is similar to that used by Pigon et al. (1960) (37.6 i.u./mg.) and much lower than other workers have used to develop antisera (e.g. Flux and Li, 1965 used a preparation with a potency of 2500 i.u./mg.).

Before detection of anti-P.M.S.G. in sheep, Pigon et al. injected approx. 46 mg. (at a dose of 200 i.u.), approx. 80.4 mg. (at a dose of 500 i.u.) and approx. 267 mg. (at a dose of 1000 i.u.). The amounts of hormone injected into sheep receiving three injections of 1500 i.u. (e.g.Trt.H1) in this study, would be

approx. 30 mg. Of chronically treated sheep, those injected with 500 i.u. received a total of approx. 150 mg. and those dosed at 1000 i.u., approx. 300 mg.

If the study of Pigon et al. (1960) presents a typical picture of anti-P.M.S.G. production is sheep, if the sheep in that study were of the same weight as those used in the present study and if the amount of antigen introduced has an effect on the rate of antibody production, then certain deductions may be made. It appears as though sheep on all treatments in Experiment 1 did not receive sufficient antigen to produce an immune reaction, whereas chronically treated ewes were supplied with adequate amounts. Both Cole et al. (1957) and Nakahari et al. (1964) have stated that treatment with gonadotrophins must be excessive and unphysiological to obtain immune responses. One limitation of this argument is that the amount of hormone introduced may not be as important as other factors in enhancing antibody formation (see Cole et al., 1957).

Time factors are also important in the development of antisera. Jainudeen et al. (1966) show that if cows are dosed once with P.M.S.G. and then left for 188 days, a second injection will produce rapid formation of antibodies. This typical effect is also displayed in cows by the study of Cole et al. (1957) and in rabbits (Leathem, 1947). It is likely that if sheep in this study were left for a period of months and then given an injection, or a second series of injections, appreciable antibodies titres would have been detected. In contrast to studies where anti-gonadotrophins are obtained by retreatment of animals some time after the first injection(s), some workers have shown that short term treatments will do little

to produce anti-P.M.S.G. factors. Cole et al. (1957) showed that 1500 i.u. given every week, or 3000 i.u. given every third week, results in very little formation of antibodies in cattle (treatment for about three months). Polge and Rowson (1973) reported that gonadotrophic stimulation of pigs at each of three sequential oestrous cycles did not produce a refractory condition in these animals and no evidence of anti-gonadotrophins was obtained.

In conclusion, it appears that the injection of sheep over a short space of time (2 oestrous cycle lengths separating three injections : 34 days) is unlikely to produce serological antibodies against P.M.S.G. The present study ratifies this statement.

Follicular Dynamics of the Ovary

Discussion in this section relates primarily to results obtained in Experiment 3 and deals with observations on follicular dynamics in the ovaries of ewes sequentially treated with P.M.S.G.

The choice of Day 10 as a reference point to monitor follicle development could have an important bearing on any conclusions drawn from observations that were made. If the pattern of growth suggested by Smeaton and Robertson (1971) is accepted, then the time of observation would have coincided with the waning of the second wave of follicular growth and the beginning of the third (see Chapter 1). If Brand and de Jong (1973) have revealed a more likely pattern of follicular growth, then the observations would have been made after the first wave and mid-way during the wave that terminated in ovulation. In this latter case observations would have given some indication of follicles that would respond to gonadotrophic stimulation 2 days after observation (Day 12 of

the oestrous cycle). Brand and de Jong killed 39 ewes at different stages of the oestrous cycle and studied microscopic changes in follicles, accounting for follicular atresia. Smeaton and Robertson injected Indian ink into follicles and followed development in a much smaller number of ewes. The work of Brand and de Jong is probably more informative than that of Smeaton and Robertson because of the more sophisticated techniques and the larger numbers of ewes employed by the former authors.

It has been shown that there was very little difference in the total number of corpora lutea plus follicles greater than 1 mm. when ewes treated with P.M.S.G. (once, twice or three times) are compared with control animals (Table 4-8). In ewes superovulated once, about half of this composite figure is accounted for by the count of corpora lutea. In the appropriate control animals, the figure is mainly derived from a count of follicles. Apparently, the injection of P.M.S.G. was able to ovulate follicles that were analogous to those observed on the surface of ovaries in the control animals. However, the injection must have stimulated extra follicle growth as well since the total number of follicles seen in treated ewes was not significantly different from the number in control ewes (Table 6-1).

In animals stimulated a second and third time, ovulation rates were not significantly greater than those of control animals, indicating that a refractory condition had been attained. Neither did the count of corpora lutea plus follicles greater than 1 mm.

differ between control and treated animals at these two observations. The first injection seems to stimulate ovulating and non-ovulating follicles. The latter are enlarged by the gonadotrophic stimulation and then become atretic. At the second and third injections neither ovulating follicles nor non-ovulating follicles were stimulated by the gonadotrophin. The follicle stimulatory action of P.M.S.G. seems to have become ineffective as well as the ovulatory capacity.

Although P.M.S.G. is often thought of an an F.S.H.-like hormone, it may also display properties similar to L.H. Lamond (1959) and Schmidt-Emendorf et al. (1962) have suggested that approximately one-third of the endocrinological action of P.M.S.G. may be L.H.-like. It is not known to what extent the L.H. fraction of a P.M.S.G. preparation may contribute to the superovulatory response of ewes. However, there is evidence to suggest that the exogenous gonadotrophin may exert an effect on the pituitary gland and thus the ovulatory capacity may not be by direct influence on the ovary. This mediation of response via the pituitary will be discussed in more detail later.

Inference from the data presented in Tables 6-1 to 6-4 is difficult. Error mean squares (EMS) for comparisons involving surface follicle counts are large and thus, large standard errors are associated with the treatment group means. This indicates extreme between-animal-within group variation and has been encountered by other authors (e.g. Brand and de Jong, 1973) when measuring similar variates. These large standard errors make comparisons between treatment groups unlikely to be of statistical

significance. The likelihood of apparent real differences between groups is further decreased by the small numbers of animals per group that were on some of the treatments. For these statistical reasons, any real trends in Fig. 6-1 are of little significance.

From Fig. 6-1 there is some suggestion that treated ewes consistently show follicles in the 'large size' category to be a lesser proportion of the total follicular population than they are in the control ewes. This trend is not statistically significant. Even if the number of large follicles per ovary is slightly diminished in treated ewes this is of little import to the development of a refractory condition. There are still follicles present on the ovaries that would be capable of responding to gonadotrophic stimulation. Smeaton and Robertson (1971) show that follicles of approx. 2 mm. in diameter may grow rapidly over the last 36-48 hours before ovulation. Gonadotrophins should therefore be able to enhance the growth of such follicles. Refractoriness seems to be the inability of the exogenous gonadotrophin to stimulate such follicles.

All relationships between ovarian surface data and estimated total ovarian activity were found to be statistically not-significant. It therefore appears as though inference from results on surface follicular data are not representative of total ovarian activity. Land (1973) has recently presented evidence which suggests that the number of follicles destined to ovulate from ewe ovaries is not determined until within 3 days before ovulation. Maybe the observations of the present study were made too early to give informative indications of the imminent

effect of a gonadotrophic stimulation on the follicles observed at Day 10. This suggestion is supported by the fact that ovulation rates were very rarely and inconsistently related to the observations made on Day 10 of the cycle (Tables 6-5 and 6-6). Cross-sectional and histological observations may be more informative than the surface follicular data.

Elimination of the proportion of antral follicles that were atretic accounted for a very important unknown from the estimations of total ovarian activity. The proportion of atretic follicles that were included in surface counts was not known and for this reason, estimated total number of normal follicles per ovary is probably a more accurate assessment of ovarian follicular status. The percentage of follicles in the ovaries of control ewes that were classed as being atretic compared agreeably with observations of Brand and de Jong (1973). This is encouraging as the assessment of atresia may often suffer from the subjective nature of descriminations between normal and atretic follicles. The early stages of atresia are difficult to discern (Ingram 1962). Brand and de Jong were able to use more elaborate methods to differentiate between normal and atretic follicles as they sectioned each follicle serially. This was not done in the present study where samples were drawn from many ovaries and the state of a single follicle was assessed from only one microscopic section per ovary, although criteria of follicle atresia were basically similar to those used by Brand and de Jong. It is debatable whether serial section of a small number of ovaries is more informative than limited sectioning of a larger number.

The proportion of antral follicles that were atretic was larger in animals given one injection than in animals on other treatments of Experiment 3. The number of normal follicles of ewes in this group was thus lowered to become significantly less than for ewes on the other three treatments. Although ewes treated once with P.M.S.G. had significantly greater numbers of follicles > 3 mm. (Table 6-4) when compared with ewes treated two or three times, the majority of these follicles were probably atretic.

The low number of normal antral follicles observed in the ovaries of ewes treated once with P.M.S.G. may be of pertinence to the development of a refractory condition. These ewes had similar numbers of antral follicles to ewes treated two or three times (Table 6-7) but a larger proportion were atretic. The atretic follicles were likely to have been stimulated by the previous injection and have persisted to the point of observation. Their persistence may impair the growth of smaller, 'healthier' follicles which would be capable of responding to a further injection of gonadotrophin. Greenwald (1963) has surmised that the persistence of such follicles might increase steroid production which would in turn decrease the release of endogenous gonadotrophin. If P.M.S.G. acts synergistically with endogenous gonadotrophin (see Chapter 1) then it's action could be impaired by this sequence of events. Furthermore, the ovary at this point is supporting corpora lutea and persistent follicles. Stimulation with P.M.S.G. 2 days after observation may not be effective because normal follicles have little space in which to grow. Hulet and Foote (1969) have indicated that sequential P.M.S.G. treatment may have a luteotrophic effect and if this caused persistence of corpora lutea,

the ovary may be less sensitive to gonadotrophins on two counts; hormonal status of the animal (high circulating levels of progesterone) and persistence of corpora lutea which may absorb the blood supply of L.H. (or P.M.S.G.) and inhibit follicular growth.

Suggestions in the last paragraph are unsubstantiated by any convincing experimental evidence but should be considered by any workers who further investigate the refractoriness of ewes to P.M.S.G. The state of the ovaries in ewes over the oestrous cycle subsequent to P.M.S.G. injection has not been studied in detail. Such a study could utilise laparoscopic techniques and would be valuable in trying to assess the effects of gonadotrophic stimulation. The relationships between endocrine status of ewes and concomitant changes in follicle growth have not been studied and much information on these related events is required before a true understanding of the pattern of follicular growth in ewes is obtained.

General Discussion on Refractoriness to P.M.S.G.

It has been shown that refractoriness is rapidly attained and much lower ovulation rates will be achieved by a second injection of P.M.S.G., given one cycle after the first. Anti-gonadotrophic activity is unlikely to account for this rapid decline in response, probably because of a lack of time for the establishment of an immune reaction and also because of the insufficient quantity of antigen injected.

The most prominent result obtained in a study of ovarian follicular development was that a significantly lower number of

normal follicles per ovary are observed 10 days after superovulatory response to one injection of P.M.S.G. compared to the number in animals on all other treatments. Lower ovulation rates in response to a second injection of P.M.S.G. are likely to be due to this lowered number of normal antral follicles that are potentially able to respond to gonadotrophin. However, an apparent restoration of the follicular population caused the estimated number of normal follicles in ovaries of ewes stimulated a second and third time not to differ from similar counts in the ovaries of control animals. The lack of normal, potentially ovulating, antral follicles is therefore unlikely to be a persistent causal factor of refractoriness.

Observation at Day 10 of the second cycle (following a second injection) suggests that ovarian follicular populations have been restored and yet the third stimulation does not produce effective superovulation. It is at this point that a third factor is suggested as likely to cause refractoriness. A review of literature on the mode of action of P.M.S.G. is outlined in Chapter One and it is apparent that P.M.S.G. may have a direct effect on the pituitary by eliciting the release of endogenous gonadotrophin. This evidence has mainly been gained from polytocous animals (e.g. mice) but there is now some suggestion of similar effects in sheep (monotocous). Pigon et al. (1960) have shown that chronic treatment of ewes with P.M.S.G. will markedly lower the pituitary L.H. content of such animals. The increasing prevalence of ewes becoming anovular, and the increasing incidence of cystic and persistent luteinised follicles, in ewes treated sequentially

with P.M.S.G. further suggests that the L.H. supplies of these animals are becoming exhausted. The L.H.-like moeity of P.M.S.G. does not seem able to exert an ovulatory effect in sequentially treated ewes and it may be that this fraction of the exogenous hormone acts synergistically with endogenous gonadotrophin, which would be released on administration of P.M.S.G.

Polge and Rowson (1973) have recently reported that pigs will not become refractory to gonadotrophic stimulation if P.M.S.G. is used in conjunction with H.C.G., over three oestrous cycles. Although there may be important differences between polytocous and monotocous animals, this observation of Polge and Rowson (1973) may have important implications with respect to the development of ovarian refractoriness in ewes. Varied results have been obtained with the use of H.C.G. in ewes but Hunt et al. (1971) have shown good responses with purified F.S.H. and L.H. extracts. It is suggested that these results should be examined in greater detail in an effort to see whether such hormonal techniques would help to overcome refractoriness in ewes.

If refractoriness observed in ewes treated with P.M.S.G. over three oestrous cycles is related to the depletion of endogenous L.H., a hypothesis of the chain of events which causes the condition could be proposed. The condition may be precipitated by an initial exhaustion of normal antral follicles which would reduce response to a second injection. However, due to sequential treatment, L.H. reserves (pituitary) may also become depleted so that when follicular populations are restored (i.e. at the second observation in Experiment 3) the ovaries are unable to respond to

further stimulation because of a lack of L.H. to enhance the effect of the exogenous hormone.

The staggering of superovulatory doses was seen to partially overcome refractoriness. This may be explained with reference to the proposal in the preceeding paragraph. With a space of one oestrous cycle between injections, follicular populations have a sufficient period of time to be restored and atretic or cystic follicles would be given time to regress. Furthermore, pituitary reserves of L.H. could be replenished without any exogenous interference. A second injection of gonadotrophin is thus able to restimulate the ovaries to superovulatory responses.

Further work on the development of a refractory condition in ewes treated with P.M.S.G. over a short period is probably justified. If the condition were overcome, the short term yields of large numbers of ova could be possible from a small number of ewes. It is unlikely that anti-gonadotrophic factors are responsible for the condition and limited information would be obtained from studies on follicular status of such ewes until further work is done on animals in the normal state. Investigation of endogenous L.H. depletion seems a promising line of study. It should be established whether such a depletion does occur with short term treatment. It may then be feasible to rectify the condition by supplementation with reliable L.H. extracts.

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APPENDICES

APPENDIX I

RAW DATA : MEANS AND RANGES OF OVARIAN RESPONSE DATA (EXPERIMENT 1)

GROUP	CORPORA LUTEA : MEAN NUMBER/ EWE (AND RANGE)	CORPORA LUTEA + FOLLICLES > 3 mm. : MEAN NUMBER/ EWE (AND RANGE)
L1	1.8 (0-10)	3.7 (1-11)
L2	6.1 (1-12)	7.8 (2-12)
L3	5.6 (0-20)	9.6 (2-20)
L4	4.9 (0-18)	9.6 (2-29)
H1	0.9 (0-3)	2.7 (1-18)
H2	7.4 (0-10)	9.4 (0-18)
H3	2.0 (0-12)	16.0 (9-20)
H4	9.6 (1.36)	13.0 (5-40)
CC	1.1 (1-2)	2.6 (1-4)

APPENDIX II

BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE OF OVULATION RATE DATA (EXPERIMENT 1)

a. Raw data

Group[A]	D.F. (n-1)	Variance S^2	Coded S^2	Log coded S^2
L1	9	9.070	90.70	1.95761
L2	8	15.027	150.27	2.17696
L3	7	51.387	513.87	2.71079
L4	8	29.112	291.12	2.46404
H1	8	0.861	8.61	0.93505
H2	8	37.528	375.28	2.57438
H3	6	21.143	211.43	2.32531
H4	8	127.500	1275.50	3.10551

D.F. = 7 $\quad Chi^2 = 39.7535^{**}$

The variance is significantly heterogeneous

b. Transformed data[B]

Group	D.F. (n-1)	Variance S^2	Coded S^2	Log coded S^2
L1	9	0.4862	48.62	1.6868
L2	8	0.7219	72.19	1.8585
L3	7	2.0776	207.76	2.3176
L4	8	0.9465	94.65	1.7961
H1	8	0.1083	10.83	1.0346
H2	8	0.9507	95.07	1.9780
H3	6	1.1077	110.77	2.0322
H4	8	2.2244	222.44	2.3471

D.F. = 7 $\quad Chi^2 = 17.8210^{**}$ $(0.05 < P < 0.01)$

Variance approaches homogeneity.

A Group abbreviations are described in Chapter II.

B Data transformed to $\sqrt{x+1}$.

* $P < 0.05$

** $P < 0.01$

APPENDIX III

STUDENT-NEWMAN-KEULS TEST TO COMPARE MEANS : OVULATION RATE, EXPERIMENT 1

1. Differences between means when these means are ranked from smallest to largest

Rank	1	2	3	4
Mean	14.612	18.416	25.807	26.436
No./group	19	15	18	18
Group	1	3	4	2

Rank	Mean	No./group	Group			
1	14.612	19	1	-		
2	18.416	15	3	3.084[A]	-	
3	25.807	18	4	11.195	7.391	-
4	26.436	18	2	11.824	8.020	0.629 -

$M.S._{within}$ = 109.4343 $M.S._{within}$ = 10.461

Degrees of freedom = 62 Use D.F. = 60

2. By consulting a table giving the critical values of the Studentized range the following table may be drawn up

	K^{B} =	2	3	4
	$Q^{C}_{10(k,60)}$ =	2.363	2.959	3.312
$\sqrt{M.S._{within}}$	$Q_{10(k,60)}$ =	24.719	30.954	34.647
	$Q_{0.05(k,60)}$ =	2.829	3.399	3.737
$\sqrt{M.S._{within}}$	$Q_{0.05(k,60)}$ =	29.594	35.557	39.093
	$Q_{0.01(k,60)}$ =	3.762	4.282	4.595
$\sqrt{M.S._{within}}$	$Q_{0.01(k,60)}$ =	39.354	44.794	48.068

3. For a difference between means to be significant at the α level of significance it must be equal to or greater than:-

$$LSR = Q_{\alpha(k,60)}\sqrt{M.S._{within}}\sqrt{\frac{n_1 + n_2}{2n_1 n_2}}^{D}$$

A Difference between means

B k = 1+ difference in ranks of two means.

C $Q_{0.10(k,60)}$ = Value from studentized range for k items when the error variance has 60 degrees of freedom. 0.01 refers to a test being carried out at the 10% level of significance.

D n_1 and n_2 are the sample sizes of the two means.

4. Using the Q values from the above table the following ranges may be computed:-

a. 10% level of significance

$$LSR^{A}_{1\rightarrow 4} = 34.647 \times \frac{6.0828}{26.155}$$

$$= 8.058 \text{ (sign)}^{B}$$

$$LSR_{1\rightarrow 3} = 30.954 \times \frac{6.0828}{26.155}$$

$$= 7.199 \text{ (sign)}$$

$$LSR_{1\rightarrow 2} = 24.719 \times \frac{5.831}{23.875}$$

$$= 6.037 \text{ (N.S.)}^{C}$$

$$LSR_{2\rightarrow 4} = 30.954 \times \frac{5.745}{23.2379}$$

$$= 7.652 \text{ (sign)}$$

$$LSR_{2\rightarrow 3} = 24.7193 \times \frac{5.745}{23.2379}$$

$$= 6.111 \text{ (sign)}$$

$$LSR_{3\rightarrow 4} = 24.719 \times \frac{1}{4.243}$$

$$= 5.826 \text{ (N.S.)}$$

b. 5% level of significance (testing only those ranges which were

$LSR_{1\rightarrow 4}$ = 9.092 (sign) significant at $P < 0.10$)

$LSR_{1\rightarrow 3}$ = 8.269 (sign)

$LSR_{2\rightarrow 4}$ = 8.790 (N.S.)

$LSR_{2\rightarrow 3}$ = 7,316 (sign)

A $LSR_{1\rightarrow 4}$ = Least significant range for comparing means with ranks of 1 and 4 (Note this is not comparing treatments 1 and 4.

B Indicates that these two means are significantly different at this level of significance, i.e. the difference between the means is greater than the LSR.

C Indicates that these two means are not significantly different at this level of significance, i.e. the difference between the means is less than the LSR.

c. 1% level of significance (only testing those ranges which were significant at $P < 0.05$)

$LSR_{1 \rightarrow 4} = 11.176$ (sign)

$LSR_{1 \rightarrow 3} = 10.043$ (sign)

$LSR_{2 \rightarrow 3} = 9.728$ (N.S.)

5. A table indicating the difference between means may now be constructed:-

Ranks of the mean pairs	group pairs	difference of means	significance of mean difference
1 and 4	1 and 2	11.824	$P < 0.01$
1 and 3	1 and 4	11.195	$P < 0.01$
1 and 2	1 and 3	3.804	$P > 0.10$
2 and 4	2 and 3	8.020	$P < 0.10$
2 and 3	2 and 4	7.391	$P < 0.05$
3 and 4	4 and 2	0.629	$P > 0.10$

If comparison of means was not significant at the 5% level then means were said not to be significantly different. However those which fell into the $P < 0.10$ category were reported as N.S. ($P < 0.10$), and those of $P < 0.10$ were reported as N.S.

APPENDIX IV

MEAN UTERINE WEIGHTS AND STANDARD ERRORS FOR MOUSE GROUPS OF BIOLOGICAL INHIBITION TEST (EXPERIMENT 2)

MOUSE GROUP	SHEEP GROUP	MEAN (± STD.ERROR) *
Uninjected	-	86.51 (± 5.07)
P.M.S.G. Alone : 1.0. i.u.	-	163.60 (± 4.99)
P.M.S.G. Alone : 0.25 i.u.	-	137.60 (± 5.00)
1.0 i.u. P.M.S.G. + plasma	L1	162.98 (± 5.09)
" " "	L2	159.73 (± 4.99)
" " "	L3	167.05 (± 4.98)
" " "	L4	165.55 (± 4.91)
" " "	H1	159.87 (± 5.02)
" " "	H2	159.93 (± 4.99)
" " "	H3	159.95 (± 5.02)
" " "	H4	160.46 (± 5.00)
" " "	CC	161.39 (± 4.99)
" " "	500 i.u. P.M.S.G.	84.27 (± 4.99)
" " "	500 i.u. P.M.S.G. + adjuvant	137.60 (± 4.99)
" " "	1000 i.u. P.M.S.G.	106.35 (± 4.98)
" " "	1000 i.u. P.M.S.G. + adjuvant	146.06 (± 4.99)
0.25 i.u. P.M.S.G. + plasma	L1	152.87 (± 4.99)
" " "	L2	151.53 (± 5.02)
" " "	L3	150.93 (± 4.98)
" " "	L4	143.55 (± 4.99)
" " "	H1	137.86 (± 5.00)
" " "	H2	131.72 (± 5.07)
" " "	H3	127.09 (± 4.99)
" " "	H4	137.50 (± 5.00)
" " "	CC	150.14 (± 4.07)

* Transformed data ; transformation = log (X + 1.1) x 100.

APPENDIX V

RELATIONSHIP BETWEEN ESTIMATED TOTAL OVARIAN FOLLICULAR DEVELOPMENT AND OVARIAN SURFACE OBSERVATIONS (EXPERIMENT 3)

RELATIONSHIP TESTED			REGRESSION	STANDARD		
CROSS SECTIONAL COUNTS	VS	SURFACE OBSERVATION	COEFFICIENT	ERROR	t	p
LEFT OVARY						
ANTRAL FOLLICLES	vs	C.L.	0.17	0.41	0.41	N.S.
	vs	Sm. Fol.	0.26	0.25	1.04	N.S.
	vs	Med. Fol.	0.06	0.21	0.29	N.S.
	vs	Lge. Fol.	0.31	0.41	0.76	N.S.
	vs	All Fol.	0.13	0.16	0.81	N.S.
	vs	C.L. + Fol.	0.18	0.18	1.00	N.S.
NORMAL ANTRAL FOLLICLES	vs	C.L.	-0.12	0.09	1.33	N.S.
	vs	Sm. Fol.	0.09	0.05	1.80	N.S.
	vs	Med. Fol.	-0.04	0.06	0.66	N.S.
	vs	Lge. Fol.	0.04	0.11	0.36	N.S.
	vs	All Fol.	0.04	0.05	0.80	N.S.
	vs	C.L. + Fol.	0.02	0.05	0.40	N.S.
RIGHT OVARY						
ANTRAL FOLLICLES	vs	C.L.	0.48	0.68	0.70	N.S.
	vs	Sm. Fol.	0.19	0.43	0.44	N.S.
	vs	Med. Fol.	0.07	0.34	0.21	N.S.
	vs	Lge. Fol.	0.68	0.69	0.98	N.S.
	vs	All Fol.	0.13	0.27	0.48	N.S.
	vs	C.L. + Fol.	0.22	0.30	0.73	N.S.
NORMAL ANTRAL FOLLICLES	vs	C.L.	-0.27	0.67	0.40	N.S.
	vs	Sm. Fol.	1.11	0.32	3.47	N.S.
	vs	Med. Fol.	0.21	0.47	0.45	N.S.
	vs	Lge. Fol.	-0.32	0.82	0.39	N.S.
	vs	All Fol.	0.72	0.33	2.18	N.S.
	vs	C.L. + Fol.	0.66	0.34	1.94	N.S.