EFFECT OF COFACTORS AND LH-FSH ON THE SYNTHESIS OF PROGESTERONE BY THE PORCINE OVARY OF EARLY PREGNANCY

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CHAPTER I

INTRODUCTION

For many years researchers have known that progesterone is one of the major products of the corpus luteum in pregnant and normal cyclic animals. Considerable knowledge has been gained concerning the formation of the corpus luteum but many questions concerning the control, development, maintenance, and function of this transient organ in different species remain to be answered.

One method used to elucidate some of the answers has been the *in vivo* method of study with the organ remaining intact in its normal physiological environment. Technical problems such as anesthesia, changes in blood flow, and surgical stress have prevented this method from being used effectively to bring forth answers to questions concerning the ovary and corpus luteum.

A second method of study is the *in vitro* method. There are two forms of this method currently in use. They are the incubation of luteal slices or minces and the perfusion of isolated organs. Perhaps the isolated organ perfusion method, though least used, is the preferred of the two forms. The organ is removed intact from the donor and placed in an artificial environment. It is then perfused with a medium, natural or artificial, while the function of the organ is studied. A distinct advantage of this *in vitro* method is that organ and cell compartmentalization is maintained and rates of synthesis and

secretion can be measured more precisely.

After studying several *in vitro* models, an experiment was designed utilizing the isolated organ perfusion technique to determine the effect of pituitary gonadotropins and cofactors on the rate of progesterone synthesis.

Ovaries were removed from animals less than three weeks pregnant at the time of slaughter at an abattoir and were perfused with citrated whole blood from a castrated male donor. The blood was allowed to pass through the ovary only once (monocyclic); gonadotropins and cofactors were added to the perfusate mid-way through the perfusion. Samples of perfusate collected before and after the treatment provide pretreatment control values and treatment effect respectively.

CHAPTER II

LITERATURE REVIEW

In Vitro Synthesis of Progesterone

The biogenesis of ovarian hormones takes place in a setting of heterogeneous cell populations regulated primarily by pituitary gonadotropins and secondarily by the neuro-endocrine control of the hypothalamus (56). Follicular development followed by ovulation and then corpus luteum formation are the recurring phenomena of the porcine ovary. Two layers of cells are found in the follicle, the granulosa layer and the theca interna layer (14). The site of formation of progesterone has been debated, some saying it is in the granulosa lutein cells while others favor the theca lutein or special K cells as the formation site. Upon formation of the corpus luteum, the granulosa becomes vascularized and the progesterone which is formed can enter the blood stream directly without passing through the theca interna layer. This results in much less being converted to estrogens. Most of the steps in steroid biosynthesis require a reducing agent in the form of reduced triphosphopyridine nucleotide (TPNH) or a TPNH generating system. The sequence for the biosynthesis of progesterone seems to follow the conversion of acetate to cholesterol, to pregnenolone and eventually to progesterone.

Progesterone, once synthesized, has several biological actions in swine (22, 50). It is essential for ovum implantation, acts as an inhibitor of ovulation during pregnancy, and is essential for maintenance of pregnancy. Progesterone also aids in maintenance of the fetus, and affects ovarian function, gonadotropin elaboration, and oxytocin and relaxin actions.

The porcine ovary has not been as widely studied as the ovary of other species. Evidence for progesterone synthesis by the ovary has come from three sources (56). One source is the isolation of progesterone from the corpus luteum (42, 47). Another source of evidence is de novo synthesis during perfusion or by incubation (43, 48, 51, 12), and another source of evidence is the isolation of progesterone from ovarian vein blood (7, 23, 59). Tissue slices, tissue homogenates, and intact isolated perfused ovaries have all been shown to synthesize steroids and have been used as experimental models.

Armstrong et al. (3) utilized rat ovary slices to study the effects of luteinizing hormone (LH) on progesterone synthesis. It was shown that LH significantly increased the synthesis of progesterone when added to the incubation media. The postulation was made that LH acted to convert some preformed percursor to progesterone because of the decrease in progesterone synthesis observed in ovarian slices from rats that had been administered LH four days before removal of the ovaries. Kidwell et al. (32) also found that addition of LH to superovulated rat ovaries incubated in vitro resulted in increased activity of glucose-6-phosphatase and 20-alpha-hydroxysteroid dehydrogenase enzymes. In another study, Major and Armstrong (35), using superovulated rats, concluded that LH stimulated the conversion of

cholesterol to progesterone and 20-alpha-hydroxypregnen-4-en-3-one.

Armstrong and Black (4) utilized the bovine corpus luteum of the estrous cycle to show that the greatest synthetic activity occurred in the youngest corpora lutea and that LH had a stimulatory effect upon the synthesis of progesterone from the active corpora lutea. Addition of nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate had a minimal effect upon progesterone synthesis in the young corpora lutea, but became more effective in the older corpora lutea. Corpora lutea from day 18 of the cycle possessed very little ability to synthesize progesterone.

Cook et al. (12) incubated slices of porcine corpora lutea in the presence of acetate-1-^{14}C. It was shown that incorporation of the acetate into progesterone and 20-alpha-hydroxypregnene-3-one occurred. Addition of ovine, bovine, or porcine LH resulted in increased synthesis of progesterone 15% above the controls although there was considerable variation. Addition of follicle stimulating hormone (FSH) and prolactin had no stimulatory effect. Corpora lutea slices from animals of mid-cycle or of early pregnancy showed the greatest amount of synthesis. Bjersing and Carstensen (6) employed the same technique but incubated the granulosa cells from porcine ovarian follicles. Incubation for three hours showed the granulosa cells to be weak in 17-alpha-hydroxylase and in side chain cleaving enzymes which are necessary for steroid synthesis. Cook et al. (13) also found that LH stimulated progesterone synthesis in the opossum. Addition of LH to luteinized opossum ovaries being incubated <u>in vitro</u> resulted in an increased synthesis of progesterone. Addition of FSH was shown to have no stimulatory effect.

Dorrington and Kilpatrick (15) utilized rabbit ovarian tissue to study the effects of pituitary hormones on progesterone synthesis. Ovine LH, ovine FSH, and growth hormone stimulated progesterone synthesis. Ovine prolactin had no stimulatory effect. The stimulatory effect of FSH was probably due to LH contamination. Luteinizing hormone administered at the rate of 2 ug/ml of incubation medium increased progesterone production five fold.

Duncan et al. (16) also utilized porcine corpora lutea to study the synthesis of progesterone. Tissue slices incubated for two hours synthesized progesterone. Pregnenolone added to the incubation medium increased progesterone synthesis 208% above that of the incubated controls. Nicotinamide adenine dinucleotide (NAD) added to the incubation medium also increased progesterone synthesis. It was thought that NAD furnished some of the energy needed by the steps in the biosynthetic pathways. It was also shown that progesterone synthesis increased from 21 ug/gm luteal tissue on day 4 to a peak of 74 ug/gm luteal tissue on day 16 and then fell to an almost undetectable level on day 18 of the estrous cycle. Progesterone synthesis was higher in pregnant animals on days 16, 24, and 48 of gestation than in normally cycling gilts. Progesterone synthesis by corpora lutea late in pregnancy was found to be decreased. The authors concluded that morphologic development and regression of the corpora lutea paralleled their capacity to synthesize progesterone. Duncan et al. (17) in a later experiment analyzed the major factors influencing <u>in vitro</u> synthesis of progesterone. Pituitary homogenates from gilts of various stages of the estrous cycle had no influence upon progesterone synthesis. Pregnant mare serum (PMS), human chorionic gonadotropin (HCG), and ovine lactogenic hormone showed no stimulatory

effect. Endometrial filtrates from days 12 and 13 of the estrous cycle had a stimulatory effect upon progesterone synthesis while filtrates from days 16 and 18 had an inhibitory effect. Subtotal hysterectomy was found to prolong the functional life span of the corpora lutea.

Hall and Koritz (25) devised an experiment to study the effects of LH and cyclic 3'5'-adenosine monophosphate (cyclic 3'5'-AMP) on steroidogenesis in the bovine corpus luteum. Addition of LH and 3'5'-AMP to the incubating slices of bovine corpus luteum increased the rate of conversion of 7-alpha-^{3}H cholesterol to progesterone. Luteinizing hormone caused a marked stimulation of synthesis of progesterone as well as the incorporation of 7-alpha-^{3}H cholesterol into progesterone. It was concluded that LH stimulated progesterone synthesis in the corpus luteum at some step beyond the formation of cholesterol and that 3'5'-AMP played some role in the response of the corpus luteum to LH.

As a follow-up of the research showing the stimulatory effects of LH, Hall and Young (26) performed an experiment to study the site of action of a tropic hormone. Using slices of bovine adrenal cortex and bovine corpus luteum, they were able to show that LH and adrenocorticotropic hormone (ACTH) performed a similar role in their respective organs. The site of action of LH and ACTH was postulated to be in the conversion of cholesterol to pregnenolone. It was stated that <u>in vitro</u> LH and ACTH stimulated steroidogenesis by stimulating the 20-alpha-hydroxylation of cholesterol which appeared to be the slow step of steroid biosynthesis. These results were in agreement with those of Ichii et al. (31) who found that gonadotropins stimulated the 20-alpha-hydroxylation of cholesterol <u>in vitro</u>. Using microsomal and soluble cell fractions of bovine corpora lutea they were able to bring

about a two fold increase in the rate of side chain cleavage by adding LH, FSH, and HCG. No cleavage occurred in the absence of cofactors but when nicotinamide adenine dinucleotide phosphate (NADP) and NAD were added, cleavage of the side chain occurred. A NADPH generating system composed of glucose-6-phosphate, NADP, and glucose-6-phosphate dehydrogenase also induced cleavage. The authors concluded that the integrity of cellular structure was not essential for tropic hormone action on target organs though a compartmentalized in vitro model should more nearly mimic the in vivo model.

As a result of the finding that LH could stimulate the conversion of cholesterol to progesterone, other sites of action for LH were studied. Marsh and Savard (36) using incubated bovine corpus luteum slices were able to show that LH stimulated the activity of luteal phosphorylase which resulted in the enhancement of progesterone synthesis. Addition of 3'5'-AMP and adenosine triphosphate (ATP) did not result in an increase of phosphorylase activity. The hypothesis was formed that there is a relationship between the phosphorylase activity and progesterone synthesis and that possibly the phosphorylase activity needs to be stimulated critically to trigger progesterone synthesis. Marsh and Savard (37) performed a second experiment to study the mode of action of LH. Bovine corpus luteum slices were incubated in Kreb's Ringer bicarbonate solution. It was observed that LH addition brought about an increase in the accumulation of 3'5'-AMP. It was then postulated that 3'5'-AMP then acted to mediate the action of LH in progesterone biosynthesis. Addition of the cofactors NADP and glucose-6-phosphate also brought about a marked increase in the amount of progesterone synthesized. A third experiment by Marsh and Savard (38) gave

further evidence for the action of LH and 3'5'-AMP. Addition of 3'5'-AMP at a concentration of .02 M to incubating slices of bovine corpus luteum significantly increased steroid synthesis. Addition of LH to a preparation maximally stimulated by 3'5'-AMP did not result in further increase of progesterone synthesis. Addition of the cofactors 3'-AMP, 5'-AMP, and ATP did not result in stimulation of progesterone synthesis. The authors concluded that the action of LH was possibly mediated through 3'5'-AMP. Marsh et al. (39) in a fourth experiment demonstrated again that LH caused the accumulation of 3'5'-AMP. The increased accumulation of 3'5'-AMP could have been due to the activation of the adenyl cyclase system or to a decrease in destruction of 3'5'-AMP because of an inhibition of cyclic 3'5'-nucleotide phosphodiesterase by LH.

Progesterone synthesis <u>in vitro</u> was found to be increased in an experiment conducted by Mason and Savard (40). Incubated bovine corpus luteum slices responded to additions of LH, HCG, bovine and equine pituitary gonadotropins, and highly purified ovine LH by an enhanced synthesis of progesterone. The results showed that the bovine corpus luteum showed no specificity toward LH. Upon addition of the cofactors glucose-6-phosphate and NADP and glucose-6-phosphate dehydrogenase to the incubation media a marked increase in progesterone synthesis occurred. It was postulated that NADP might be the cofactor through which the gonadotropins exerted their steroidogenic action.

The action of the cofactor NADP in relation to LH was investigated by Mason and Savard (41). Results of their experiments, based upon the incorporation of 7-^{3}H cholesterol into progesterone, showed that LH and NADP have different modes of action and that each exerts its

action on a different pool of cholesterol in the ovary. Both NADP and LH increased the in vitro synthesis of progesterone. It was concluded that NADP might act to mobilize a pool of cholesterol not ordinarily involved in steroidogenesis.

Savard and Casey (57) found acetate-1-^{14}C to be incorporated into progesterone by bovine corpus luteum and adrenal gland slices. Luteinizing hormone increased the amount of progesterone synthesized and the incorporation of acetate. The addition of NADP also increased progesterone synthesis but failed to increase the incorporation of acetate. Luteinizing hormone was postulated to have activated luteal phosphorylase which then helped generate NADPH which in turn increased progesterone synthesis. It was also suggested that biosynthesis of progesterone in the presence of NADPH involved an entirely different precursor pool than that drawn upon by LH. The authors concluded that the action of LH in the corpus luteum and ACTH in the adrenal gland points toward a common biosynthetic pathway for the two organs.

Tamaoki and Pincus (63) incubated tissue homogenates of bovine corpus luteum to study the side chain cleavage of cholesterol. Five ml of corpus luteum homogenate were incubated in flasks containing cholesterol-26-^{14}C and the results showed side chain cleavage with the cleavage being unaffected by the addition of HCG. Also it was shown that a 710 µci quantity of cholesterol-4-^{14}C incubated with bovine corpus luteum homogenates in a 20 ml volume was converted to progesterone. When 20-alpha-hydroxycholesterol-22-^{14}C was used as a substrate for incubation of corpus luteum homogenates, side chain cleavage also occurred. The addition of FSH and PMS had no effect upon the side chain cleavage. Incubation of 20-alpha-hydroxycholesterol with corpus

luteum-containing and corpus luteum-free tissue showed that the corpus luteum-containing tissue contained the greatest side chain cleaving enzyme activity. Incubation of corpus luteum homogenates with delta^5-pregnenolone resulted in an increased yield of progesterone over samples to which no delta^5-pregnenolone was added. Follicle fluid and follicle tissue homogenates were also incubated in the presence of pregnenolone with only the follicle tissue homogenates showing a small production of progesterone. Addition of the gonadotropins HCG, PMS, purified sheep FSH and LH alone or combined, and prolactin yielded no increase in progesterone production from delta^5-pregnenolone by ovarian tissue homogenates. Corpus luteum and follicular tissue homogenates were found to be steroidogenic as shown by their ability to cleave the side chain of cholesterol. The limited enzymatic activity of the follicular tissue was postulated to have been due to the smaller amount of secretory cells represented. The role of the corpus luteum as the primary site of progesterone synthesis was substantiated.

As a result of the observation that the corpus luteum could synthesize progesterone in vitro, Brinkley and Young (7) conducted an experiment to study the in vivo rate of progesterone synthesis by the porcine corpus luteum. They found that the right ovary secreted an average of 1.41 µg progesterone/ml of plasma on day 7, 2.23 µg progesterone/ml of plasma on day 11, and 1.41 µg progesterone/ml of plasma on day 13 of the estrous cycle. Their results showed that the corpus luteum synthesized progesterone and that a peak is reached on about day 12 of the estrous cycle.

The technique of isolated organ perfusion was utilized by Cardeilhac et al. (9) to study the synthesis of progesterone. Freshly

collected luteal phase swine ovaries were perfused with a blood substitute for 1-3 hours. Synthesis of progesterone was found to occur in the absence of HCG. Of the de novo progesterone synthesized, 88% ± 12% was found in the corpus luteum and the remainder in the perfusate. It was concluded that luteal phase porcine ovaries can synthesize progesterone and release it into the perfusate when perfused with a blood substitute.

Mills (48) and Mills et al. (49) also used the perfusion technique to study progesterone synthesis in the bovine ovary of early and late pregnancy. Ovaries of late pregnancy synthesized progesterone as efficiently as ovaries of early pregnancy when perfused with homologous blood. Addition of LH resulted in an increased synthesis of progesterone by both the early and late pregnancy ovaries. The incorporation of acetate-1-^{14}C during these experiments was inconsistent and erratic. These experiments seemed to indicate that the waning of corpus luteum function in late pregnancy in the bovine is due to a reduction in circulating gonadotropins. Ainsworth (1, 2) has shown that ovine and equine placenta preparations of late pregnancy can form progesterone from pregnenolone in vitro. These results indicate that there may be some factor lacking in the ovary during late pregnancy to cause the site of final progesterone synthesis to be changed. The results of the work of Mills (48) are in agreement with those of Romanoff (55) in perfusion of bovine ovaries. Both studies indicate that LH had a stimulatory effect upon the synthesis of progesterone. Armstrong (5) postulated that the effect of LH was to stimulate the conversion of accumulated precursors to progesterone. Only after these accumulations have been depleted to a critical level are secondary mechanisms

stimulated and latent precursor pools depleted. The increased incorporation of acetate-1-^{14}C after considerable unlabeled progesterone was synthesized was an indication of these secondary mechanisms.

Stimulation of increased progesterone synthesis by gonadotropins in vitro could not be shown by Mathis (43) in perfused luteal phase porcine ovaries of early pregnancy. The ovaries were perfused for periods up to $2\frac{1}{2}$ hours with homologous gilt blood. During this period the organs remained viable and synthesized progesterone at a constant rate with and without gonadotropins. It was postulated that the ovaries may have been maximally stimulated at this stage of pregnancy and could not respond further to the added gonadotropins.

In vitro synthesis of progesterone was studied by Mehdi (46) using a mince incubation technique. Incubation of porcine luteal minces in Krebs-Ringer bicarbonate of Waymouth's cell culture medium had no effect on the ability of porcine luteal tissue to synthesize progesterone. Additions of porcine LH, LH plus serum, and LH plus serum previously incubated together showed no effect upon the synthesis of progesterone. It was thought that as in the case of the experiments reported by Mathis (43) degenerative changes in the tissue and loss of some essential factors in luteal tissue, along with maximal luteal tissue stimulation at the time of collection were the reasons for the lack of response.

From the preceeding reports and observations it can be hypothesized that cofactors play an important role in the synthesis of progesterone. White et al. (69) and Cantarow and Schepartz (8) have outlined the following scheme for the synthesis of progesterone and the role of the cofactors (Figure 1). Acetyl Co-A and acetoacetyl

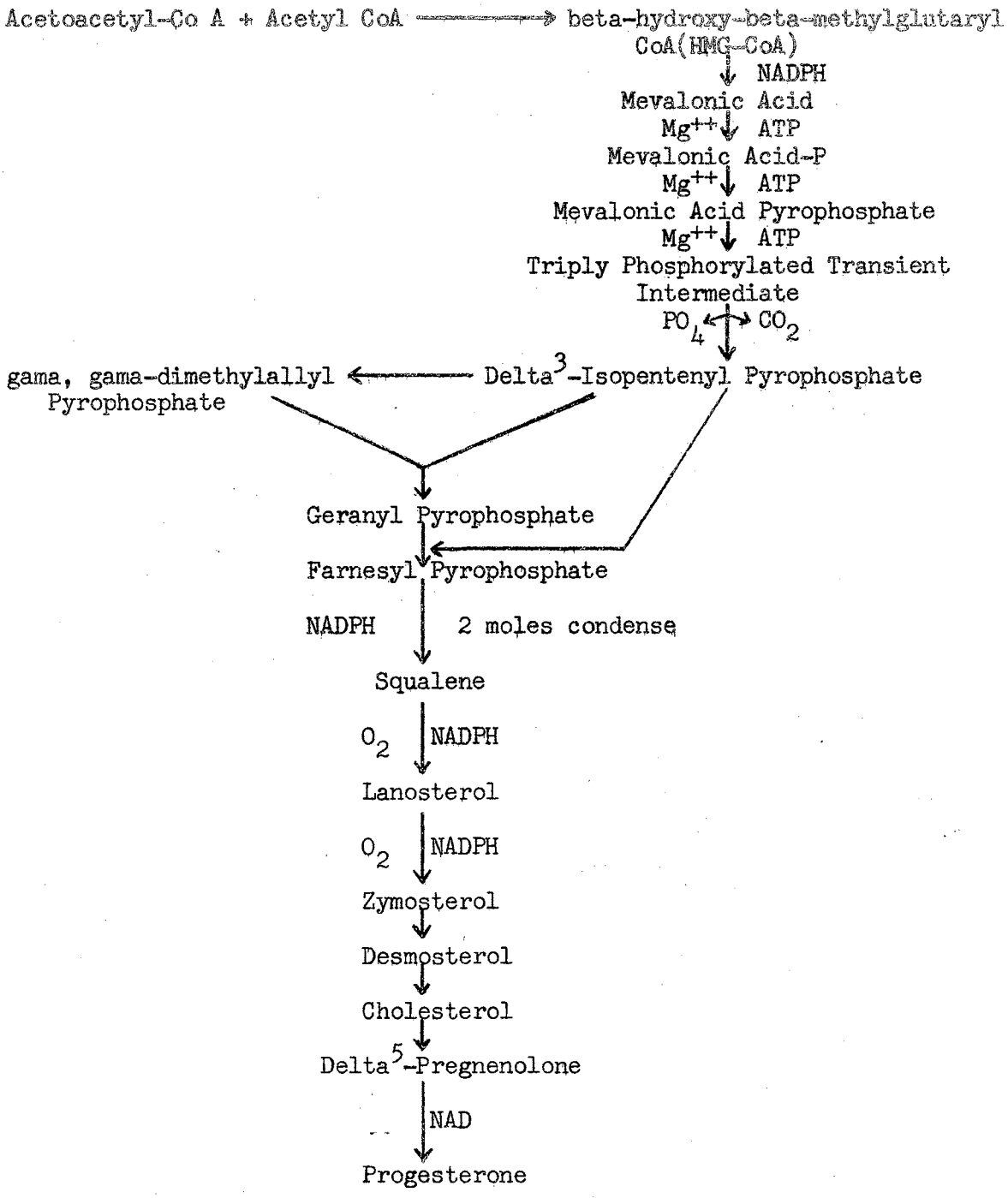

Figure 1. Biosynthesis of Progesterone

condense to form beta-hydroxy-beta-methylglutaryl CoA (HMG-CoA). The latter compound is reduced by two moles of NADPH to form mevalonic acid. Mevalonic acid is then phosphorylated three times by ATP in the presence of Mg++. This triply phosphorylated intermediate then loses a mole of phosphate and is decarboxylated producing delta^{3}-isopentenyl-pyrophosphate. This compound isomerizes by a shift of a double bond to give gamma-gamma-dimethylallyl pyrophosphate. One mole of each of the last two compounds condense to form geranyl-pyrophosphate. Addition of another mole of isopentenyl-pyrophosphate results in the formation of farnesyl-pyrophosphate. Two moles of farnesyl-pyrophosphate are reduced and condensed in the presence of NADPH to form squalene. An enzyme, oxidocyclase, in the presence of NADPH converts squalene to lanosterol. Three methyl groups are then removed from lanosterol by oxidation in the presence of NADPH resulting in the formation of zymosterol. Migration of the double bond in the ring system of zymosterol and reduction of the double bond in the side chain results in the formation of cholesterol. Oxidative cleavage of the side chain on C-17 of cholesterol results in the formation of delta^{5}-pregnenolone which is then converted to progesterone. The conversion is catalyzed by a 3-beta-dehydrogenase enzyme in the presence of the cofactor NAD. Throughout the entire sequence the cofactor NAD acts as an electron transfer agent while the cofactors fumarate and glucose-6-phosphate play a role in the generation of NADPH which seems to be necessary for the enzyme reactions.

Several investigators have conducted research to determine the roles of cofactors in <u>in vitro</u> synthesis of progesterone other than those described above. Seifart (58) in describing optimal incubation

conditions for bovine corpora lutea noted that $NADPH_2$ caused an increase in progesterone synthesis when added to the incubation medium. The addition of nicotinamide also increased progesterone synthesis presumably by maintaining the integrity of NADPase and NADase which are obligatory cofactors in progesterone biosynthesis. White et al. (69) suggested that cyclic AMP controlled steroid synthesis by maintaining the glucose supply while glucose-6-phosphate was found to play a role in the activation of glycogen synthetase enzymes. Adenosine monophosphate (AMP) was found to play a role in the activation of phosphorylase activity while ATP was found to be required for the phosphorylation reactions. Constantopoulous and Tchen (11) found that ATP, NAD, and fumarate could constitute a NADPH generating system to provide NADPH for utilization in the side chain cleavage involved in the conversion of cholesterol to progesterone. Haynes and Berthet (38) used bovine adrenal cortex preparations to study cofactor activity. Addition of fumarate and NADP to this system resulted in an increased production of corticosteroids. It was thought that fumarate stimulated the 11-beta hydroxylase activity of the enzymes by maintaining NADP in the reduced form (NADPH), the reduction being mediated by a transhydrogenase. The mechanism of corticosteroid synthesis was thought to proceed as follows: (1) ACTH increased phosphorylase activity; (2) glycogen was converted to glucose-1-phosphate; (3) glucose-1-phosphate was rapidly converted to glucose-6-phosphate which was metabolized by dehydrogenation; and (4) reduced NADP was generated which stimulated corticosteroid synthesis. Luteinizing hormone was thought to follow the same mechanism in the corpus luteum which means that a common biosynthetic pathway possibly exists in both organs. Haynes (30)

described another similarity between ACTH and LH. He reported that ACTH when added to adrenal slices caused an increase in the tissue level of cyclic 3'5'-AMP and also stimulated adrenal phosphorylase activity. Tchen and Block (64) demonstrated the essentiality of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP in the conversion of squalene to lanosterol in vitro. Koritz et al. (33) utilized rat adrenal homogenates to demonstrate an inhibition of synthesis of corticoids by cyclic 3'5'-AMP. Nicotinamide adenine dinucleotide added at sufficiently high levels was able to prevent the inhibition. Evidence was obtained indicating that cyclic 3'5-AMP may have a species specificity for its stimulating action.

Chemical Methods for Progesterone Analysis and Values Reported

Progesterone was first isolated from tissues and identified in 1934 (60). Since that time numerous investigators have sought chemical methods to detect progesterone in circulating body fluids. The first procedure for the chemical determination of progesterone was described by Haskins (27). Plasma volumes of 5-10 ml were extracted three times with equal volumes of ether and the ether extracts evaporated to dryness. The residue was dissolved in ethanol and progesterone determinations were made by ultraviolet analysis in a Beckman spectrophotometer based upon absorption at 240 mμ. The analyses of plasma from gravid women failed to show the presence of progesterone. Because this technique was not sensitive enough to detect progesterone in the blood of pregnant women, Edgar (18) developed a more sophisticated technique to detect and isolate progesterone in biologic materials. Oxalated

blood samples (40 ml) were added to 100 ml of a 3:1 ethanol-ethyl ether mixture. The mixture was centrifuged and the plasma decanted. The precipitate was washed twice with 50 ml portions of the 3:1 ethanol-ethyl ether mixture and centrifuged. The supernatant was decanted and added to that removed previously. Twenty ml volumes of the extracts were diluted with 40 ml of distilled water and extracted three times with 60 ml of ethyl acetate. The combined extracts were evaporated to dryness and the residue transferred to a centrifuge tube by washing the flask three times with 3 ml portions of 70% aqueous methanol. The mixture was stored for 18 hours at -15°C and then centrifuged at -10°C. The supernatant was decanted, diluted with 20 ml distilled water, and extracted three times with 30 ml of light petroleum ether. The combined extracts were washed twice with 30 ml portions of water, reduced in vacuo to 1 ml, and transferred with two washings of .5 ml light petroleum ether to a glass evaporating dish. When dry, the residue was dissolved in .1 ml benzene and transferred to chromatography paper with two further portions of benzene. Chromatography was performed in tanks containing benzene-chloroform as solvents and alumina paper, or in tanks containing light petroleum ether-80% aqueous methanol as solvents and filter paper. The latter chromatography system was maintained at a temperature of 34°C. Reverse phase chromatography in a solvent system of aqueous ethanol-chloroform in which the non-polar phase was held stationary and the more polar phase was the moving one, was also used to separate the progesterone. The portion of the paper corresponding to the progesterone standard chromatogram was cut out and the progesterone eluted with 96% ethanol (2-3 ml). Amounts of progesterone varying from 60 µg to 3.75 ug were detected by

absorption in a spectrophotometer. The results showed that the extraction procedure yielded good recoveries, that paper chromatography in light petroleum ether-80% aqueous methanol gave better purification than reverse-phase chromatography, and that elution with 96% ethanol gave progesterone recovery in quantities that can be measured by use of a spectrophotometer. Concentrations of progesterone ranging from 1-20 µg/ml ethanol could be readily measured which agreed with the results obtained by Haskins (27).

Raeside and Turner (53) described another technique to determine progesterone levels in the blood of cattle, sheep, and goats. Progesterone was extracted from heparinized blood plasma samples with an ethanol-ether mixture. After fractionation between organic solvents, the extracts were dried in vacuo. The residue was dissolved in .1 ml benzene and the .1 ml volume then transferred to the filter paper. The flask was then washed twice more with .1 ml volumes of benzene and each volume also transferred to the filter paper for chromatography. Paper chromatography was carried out in a glass tank with petroleum ether-80% aqueous methanol as the solvent system at a constant temperature of 34°C. The progesterone in the extracts was identified by comparing the chromatogram of the sample with a reference chromatogram after treatment of the reference chromatogram with dinitrophenylhydrazine. That portion of the chromatogram containing the progesterone was cut out and eluted with 2-3 ml of 96% ethanol. The quantity of progesterone was determined by measuring the U.V. absorption of the eluates at 240 mu in a spectrophotometer. The results showed the chromatography system to be adequate for separation of progesterone. The addition of 2-40 µg of pure progesterone to 20 ml samples of plasma

resulted in recovery rates of 70-80%. No progesterone could be detected in the peripheral blood from four goats but the ovarian venous effluent of a pregnant goat was found to contain 2.3 µg/ml of blood. In heifers injected with 1 gm of progesterone, 1 µg progesterone/ml of blood was detected after 2 hours and no progesterone could be detected after 24 hours.

As a result of the observations that progesterone could be detected in blood, Melampy et al (47) described a procedure for analyzing the progesterone content of reproductive organs and blood during pregnancy in the bovine. Reproductive tracts were obtained from cows at different stages of pregnancy as determined by the crown to rump length of the fetus. Tissue samples as well as blood samples were collected. Extracts of the tissues and plasma were subjected to paper chromatography for separation of progesterone. Further purification was obtained by counter-current distribution. Final quantitation of the sample was by infra-red analysis. Progesterone determinations were made on corpora lutea tissue, residual ovarian tissue, placenta, allantoic and amniotic fluids and adrenal glands. The corpus luteum was found to contain the highest concentration of progesterone. Their results showed that on days 10-49 of pregnancy the corpus luteum contained 2.3 µg progesterone/gm of fresh weight of tissue, on days 90-129, 5.0 µg progesterone/gm tissue, and on days 250-280, 1.1 µg progesterone/gm tissue. The average progesterone content of whole blood increased from 0.9 µg progesterone/100 ml of blood on days 10-49, to 3.1 µg progesterone/100 ml blood on days 250-280 of pregnancy.

Motivated by the foregoing, McCracken (45) described a procedure for analyzing progesterone concentrations in the non-pregnant cow after

removal of the corpus luteum during the estrous cycle. Plasma progesterone concentrations were determined in 1 liter blood samples taken at mid-cycle after removal of the corpus luteum. The method of Short (59) was used for progesterone determination except for the addition of progesterone-4-^{14}C to the plasma before extraction to facilitate calculation of recovery rates. The results showed that removal of the corpus luteum resulted in a rapid fall in plasma progesterone concentration and that the corpus luteum was the major endogenous source of progesterone. Quantities of 1.0 ug progesterone/100 ml plasma before removal, 0.4 µg progesterone/100 ml plasma 30 minutes after removal, and 0.2 µg progesterone/100 ml plasma 180 minutes after removal were observed.

Techniques also were described to determine plasma progesterone concentrations in other species. A technique to determine plasma progesterone concentration in the non-pregnant sow was described by Gomes et al. (23). Daily blood samples were collected from the previously cannulated ovarian vein. The blood samples were centrifuged at 4°C and the plasma stored at -15°C until assayed. The plasma samples were then extracted with ether and the extracts subjected to paper chromatography for separation of the progesterone. The endogenous progesterone was converted to 20-beta-hydroxy-delta-4-pregnene-3-one (20 beta-ol) with sodium borohydride-^{3}H and partially reoxidized with manganese dioxide. The 20-beta-ol was then purified by double paper chromatography and quantitated by absorption in a spectrophotometer. The results showed that the progesterone concentration increased from 0.03 ug/ml of plasma on day 0 to a peak of 1.07 ug/ml of plasma on days 10-12, and then fell to a level of 0.04 ug/ml of plasma on days

19-21. This method used by Gomes was the first that was sensitive enough to detect progesterone levels on day 0.

By utilizing portions of the procedures that had been previously described for the individual species, Short (59) developed another technique to chemically determine progesterone concentration in the venous blood of women, horses, cattle, sheep, and pigs. Blood samples were collected in an anticoagulant of sodium oxalate and centrifuged immediately. The plasma was removed and treated with sodium hydroxide pellets. The plasma was then extracted twice with ether, the pooled extracts washed with distilled water, and evaporated to dryness. The residue was dissolved using three 10 ml portions of light petroleum ether. This solution was then extracted six times with 10 ml portions of 70% methanol and the combined extracts evaporated to dryness under vacuum. The residue was transferred to a test tube using 1, 1, and .5 ml portions of absolute methanol and then spotted on chromatograph paper for progesterone separation. The solvent system used was 80% methanol and ligroin. The portions of the chromatogram that corresponded to a standard progesterone sample when viewed under ultra-violet light was cut out and eluted with absolute methanol. The methanol was evaporated and the residue redissolved in absolute ethanol. Quantitation was accomplished by absorption in a spectrophotometer. The results showed the procedure to yield good recoveries of progesterone. The level of sensitivity was found to be about 0.3-0.5 µg of progesterone/ml of plasma.

In view of the findings by Melampy et al. (47) that the corpus luteum was the primary site of progesterone concentration in the ovary, Loy et al. (34) described a more rapid technique to determine

progesterone concentration in luteal tissue. Corpora lutea (0.9-4.7 gm) from sows and cows were chopped finely and placed in 150 ml of ethanol. The solution was transferred to a flask with an additional washing of 150 ml ethanol and refluxed for one hour over a steam bath. The supernatant was decanted and the tissue extracted twice more using 100 ml ethanol. The combined extracts were evaporated to dryness and the residue subjected to column absorption chromatography. A solution of 20% chloroform-Skellysolve B was used to elute the progesterone. The fraction of the eluate containing the progesterone was evaporated to dryness and subjected to counter-current distribution in a solvent system of petroleum ether-methanol. Separatory funnels were used to separate the petroleum-ether-methanol portions from the Skellysolve B portions. The petroleum ether-fraction containing the progesterone was evaporated to dryness, the residue was dissolved in absolute ethanol, and the progesterone quantitated by absorption in the spectrophotometer. Recovery rates averaged 77-92%. The use of the column adsorption chromatography shortened the analysis considerably.

Edgar et al. (19) described a technique for determining blood levels of progesterone in the ewe. Blood samples were collected from the jugular vein, the ovarian vein, and the uterine vein. Twenty ml blood samples were extracted three times with equal volumes of ethyl acetate. To the combined extracts were added 5 ml ethyl alcohol and two drops of acetic acid and the resulting mixture was evaporated to dryness. The residue was dissolved in petroleum ether and partitioned with 70% ethyl alcohol (three times). The combined extracts were then partitioned three times with chloroform and the resulting extract evaporated to dryness. The residue was then transferred to a chromato-

gram for paper chromatography in a solvent system of benzene-chloroform in a 3:1 ratio (volume). Final quantitation of progesterone was accomplished by ultraviolet absorption spectroscopy. The results showed that blood from the jugular vein contained no detectable progesterone. The progesterone concentration in ovarian venous blood was undetectable until day 2 of estrous. The progesterone concentration then rose to 1.8 µg progesterone/ml of plasma on day 7 and remained at this level until day 16 when the progesterone concentration fell to 0.15 µg progesterone/ml of plasma on day 17 of the cycle. During pregnancy the progesterone concentration of the ovarian venous blood remained at 1.8 µg progesterone/ml of plasma until the 17th week and then fell to 0.15 ug progesterone/ml of plasma just prior to parturition. The limit of sensitivity was 0.15 µg progesterone/ml of plasma and the recovery rate was 60.8%.

Progesterone concentrations in swine blood have been determined by a technique developed by Masuda et al. (42). Ovarian venous blood was collected on days 1, 4, 8, 12, 14, 16, and 18 of the estrous cycle. Ovarian venous blood was also obtained from animals, hysterectomized on days 5-7 of the cycle, on days 14, 18, and 25 of pregnancy. Ovarian venous blood was also collected during early pregnancy (days 14, 18, and 25) and late pregnancy (days 102, 110, and 112). Luteal tissues were also collected and analyzed for progesterone concentrations. The blood samples were centrifuged, the plasma removed and 5N sodium hydroxide added to it. The mixture was then extracted five times with ether. Progesterone-4-^{14}C was added to each sample before extraction to facilitate estimation of recovery rates. The combined extracts were evaporated to dryness and the residue subjected to descending

paper chromatography in a solvent system of 80% methanol-n-heptane. Portions of the chromatogram corresponding to the RF of standard progesterone were cut out and eluted and quantitated by absorption in a spectrophotometer. The luteal tissues were extracted three times with 95% ethanol after the addition of 0.05 μc of progesterone-^{14}C. The extracts were evaporated to dryness, dissolved in n-hexane, and subjected to column adsorption chromatography. The chloroform fraction from the column was evaporated to dryness, redissolved in n-hexane, evaporated to dryness under nitrogen, redissolved in benzene-dichloromethane, and applied to a thin layer plate of silica gel G. The sample was chromatographed in a solvent system of n-hexane-ethyl acetate and the area corresponding to the standard progesterone was scraped from the plate and eluted with ethanol. Progesterone concentration was then determined by absorption in a spectrophotometer. The results showed that during the estrous cycle corpora lutea weights were greatest on day 8 and this weight was maintained until day 16 when the weight began to decrease. Ovarian venous plasma progesterone concentrations increased until day 8 (237 μg) and declined thereafter to day 18 (209 μg) of estrus. During pregnancy, total luteal tissue increased from a mean corpora lutea weight of 448 mg for one ovary on day 14 to a mean corpora lutea weight of 522 mg for one ovary on day 112. Ovarian venous progesterone concentration decreased from 2.18 μg/ml of plasma on day 14 to 2.63 μg/ml of plasma on day 18, and then decreased steadily to 0.89 μg/ml of plasma on day 112 of pregnancy. The ovarian venous plasma progesterone concentrations in the hysterectomized animals were found to be 3.43 μg/ml of plasma on day 14, 2.50 μg/ml of plasma on day 18, and 3.41 μg/ml of plasma on day 25 of pregnancy. The results also showed that measurement

progesterone concentrations in luteal tissues provided an indication of the relative concentrations in the ovarian venous blood.

Isolated Organ Perfusion

Perfusion of isolated organs is one of the methods currently used to investigate and find solutions to problems concerning ovarian physiology. According to Carrel (10) the idea of perfusing an organ was first expressed by Le Gallois, a French physiologist, in 1812. Le Gallois expressed his belief that a fluid could be circulated through an organ or an anatomical region. Sixteen years later in 1828, the first artificial circulation was performed by Kay. He used the perfusion method to restore irritability to a dying muscle. Twenty-one years later in 1849, Lobell used a crube perfusion apparatus to perfuse a kidney. The first successful perfusion apparatus was developed to Ludwig's laboratory in 1868. Noting the benefits obtained by infusing media via a syringe, Ludwig developed a pulsatile element to accompany the perfusion. This proved to be a forerunner of the presently used peristaltic pump. Several variables such as temperature, osmotic pressure, pH, oxygenation, and optimal pressure were recognized as being essential to a successful perfusion. The early workers thought that it was necessary to remove all the viscera from the organism for a successful perfusion. As a result, removal of the intestine, the stomach, the esophagus, the kidneys, and the other glands accompanies the perfusions. In 1935, the first successful whole organ perfusion occurred when Lindberg successfully perfused the right thyroid gland of a cat. The perfusion lasted for 18 days. As techniques improved, organs cultivated in vitro were found to remain alive

and to modify their structure and their function according to the composition of the circulation fluid.

Continual development of perfusion apparatus was the result of work performed during the years following Ludwig's discovery. Werthessen (67), in describing techniques of organ culture for metabolic studies, emphasized that control of infection was necessary for prolonged organ perfusion and that the length of a perfusion would be determined by the development of an uncontrollable infection. Four years later, Werthessen (68) was able to control infection and the result was a perfusion of sow ovaries for 44 hours. By perfusing the ovaries with sodium acetate labeled with ^{14}C in the carboxyl group, he was able to demonstrate the synthesis of labeled estrone, beta-estradiol, and cholesterol by the ovaries. No apparent degeneration in the organs was noted as a result of the perfusion.

Haynes et al. (29) and Stone et al. (62) perfused bovine adrenal glands to study the possible site of action of the adrenocroticotropic hormone (ACTH) on the biosynthesis of steroids. They perfused a gland for one hour with homologous citrated blood and then for three hours in a recycling process with radioactive substrates added to the blood. They concluded that the organ remained alive and viable and that ACTH aided in the conversion of cholesterol to progesterone.

The testis has been utilized extensively for protracted perfusion studies. Ewing and Eik-Nes (20) and Van Demark and Ewing (65) demonstrated the feasibility of perfusion as a method to demonstrate testosterone biosynthesis. Labeled cholesterol and acetate were incorporated into testosterone by the rabbit testis and the testosterone appearing in the venous effluent was shown to be newly synthesized and not due

to passive leakage from the organ. Perfusion periods of up to seven hours demonstrated that the enzyme systems were maintained and that HCG or LH could increase the testosterone secretion rate. The authors noted that the perfusion technique provided information on the testosterone secretion rate, the blood flow needed for optimal hormone production, and the need for removal of intermediate and hormone end products from the biosynthetic organ, and other facts not provided by the incubation technique. They also noted the feasibility of using the perfusion technique to study physiological alterations such as stress, maturation, and aging upon testosterone secretion.

Nyman et al. (52) perfused the testis of a stallion which had been primed with gonadotropins several hours before the perfusion. The testis was perfused with homologous blood and sodium acetate-1-^{14}C for 24 hours. It was observed that the stallion testis was capable of synthesizing and releasing estrogens into the circulating medium. Acetate was also shown to be a precursor of the estrogens.

Considerable attention has been devoted to the isolated perfused ovary in order to elucidate some of the answers of corpus luteum physiology. Romanoff (55) perfused bovine ovaries with bovine blood and was able to demonstrate that the addition of LH, FSH, or prolactin stimulated the synthesis of progesterone. Cholesterol was also shown to be a precursor of progesterone synthesis. The gonadotropins were found to cause a rapid turnover of the cholesterol in the corpus luteum. The gonadotropins were thought to act on the biosynthetic pathways functioning in the ovary rather than stimulating development of new biosynthetic pathways. In a later perfusion study, Romanoff and Pincus (54) perfused bovine ovaries with homologous blood and continued

their study on the mode of action of gonadotropins. Other uses of the perfusion technique were to illustrate the accumulation of acetate-1-^{14}C in the perfusate, and to illustrate the effect of gonadotropins upon acetate incorporation. Cardeilhac et al. (9), Morrissette et al. (51), Mills (48), Mills and Morrissette (49), Mathis (43), and Mathis and Morrissette (44) have all successfully perfused ovaries and observed progesterone synthesis.

The results of porcine luteal tissue incubations by Duncan et al. (11) and ovarian perfusions by Mathis (43) indicated that LH-FSH have little or no effect on the progesterone synthesis in swine corpora lutea in vitro. It was suggested that the loss of cofactors during collection and preparation of the porcine tissue rendered the tissues refractory to gonadotropins. It was postulated, therefore, that adding selected cofactors to the perfusion medium would allow the porcine corpus luteum to respond to gonadotropins in vitro and thus provide a readily available reconstituted model for the study of steroidogenesis and ovarian physiology.

CHAPTER III

MATERIALS AND METHODS

Perfusion

Apparatus. The perfusion device employed in the current investigation (Figure 2) was a modification of the apparatus designed by Mills (48) for the perfusion of the bovine ovary. The apparatus was constructed to allow continuous collection of perfusate samples without recirculation. An 8 liter container served as a reservoir for the perfusion medium, which was gently agitated by a magnetic stirrer to provide a continuous hematocrit with a minimum of hemolysis. The perfusion medium was continuously oxygenated in the reservoir with prehumidified 95% oxygen and 5% carbon dioxide. The medium was withdrawn from the reservoir and pumped through a glass wool filter to the organs by means of two peristalic pumps.[1] The two pumps provided arterial pressure adjusted to 100 mm Hg with a thumbscrew clamp on the oxygen by-pass. A three-way valve was located just prior to the point of attachment of each organ which permitted the injection of a vasodilator when necessary. Four stainless steel pans were held in place by means of clamps which were fastened to a supporting rod. One organ was placed in each pan and connected to the arterial blood supply by means of a cannula which

[1] New Brunswick Scientific Co., Inc., New Brunswick, New Jersey.

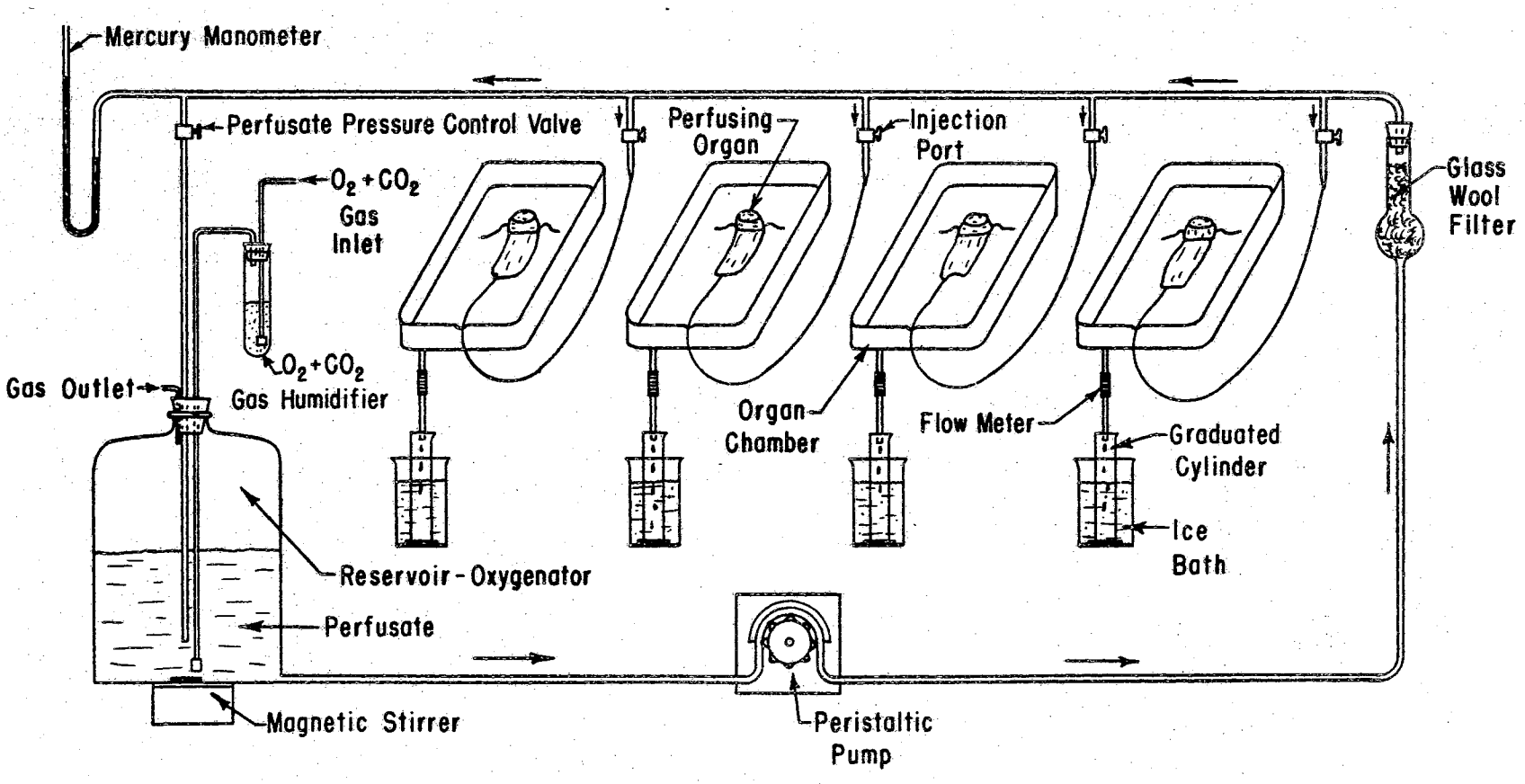

Figure 2. Multiorgan Monocyclic Perfusion Apparatus (48)

had been placed in the ovarian artery of the organ at the time of slaughter of the donor. As the venous effluent passed from the cut ovarian vein and drained from the organ chambers, a 1 mm flow meter allowed the periodic determination of the perfusate flow rate for each organ. Rubber tubing extended from each flow meter into a 100 ml graduated cylinder into which the venous effluent flowed prohibiting recirculation of the medium and thus establishing the monocyclic system. Clamps were attached to the rubber tubing allowing separate collections to be made. The apparatus was designed to allow four organs to be perfused with the same medium simultaneously. The entire apparatus was housed in a cabinet which was maintained at a constant temperature of 39°C. Glass doors on the cabinet allowed continuous observation and easy access for making necessary sample collections and adjustments.

Blood Collection and Perfusion Medium Preparation. Homologous blood was obtained from a healthy barrow at an abattoir. Approximately 2000-2400 ml of citrated blood were prepared by mixing the freshly drawn blood with 240 ml of concentrated ACD solution (see Appendix). Procaine penicillin (200,000 IU) and 250 mg of dihydrostreptomycin base were added to the citrated blood to prevent bacterial growth, and the final mixture was stored on ice until used 3-4 hours later.

In the laboratory the citrated blood was filtered through glass wool to remove debris and collected in flasks. The flasks were placed in hot water and the citrated blood was warmed to 37°C. After the blood was warmed, 1.7 gm of pilocarpine hydrochloride, 0.6 gm of sodium acetate, and 5.7 gm of dextran (Mol. Wt. 75,000) dissolved in 25 ml of 0.85% saline were added to a 2200 ml volume of citrated blood. The

dextran was added to correct for the reduction in the oncotic pressure caused by dilution with ACD, and the sodium acetate served as a steroid precursor. Each perfusion of four ovaries each required 2200 ml of medium for a complete experiment.

Organ Collection and Preparation. The procedure used for organ collection was that of Morrissette et al. (51). Intact reproductive tracts of pregnant gilts and sows were obtained from the abattoir processing line 25 minutes postslaughter. The tracts were chosen from apparently healthy sows and gilts less than 40 days pregnant based upon the embryo length. After selection, the transected ovarian artery was located and clamped with a hemostat to prevent retraction. The ovarian artery was stripped of connective tissue for about 2 inches. A ligature was placed around the ovarian ligament and tightly tied. The ovary was then separated from the rest of the tract leaving the ligature affixed to the portion to be perfused. The ovarian artery was cannulated approximately 1 cm distal to the point of ramification with a 12" x .034" ID x .050" OD polyethylene tubing attached to a 20 gauge hypodermic needle. With a syringe, via the cannula, the entire ovarian vascular system was flushed three times with 20 ml of dilute ACD solution to remove all blood from the vascular system and to chill the ovary. Whenever the corpora lutea appeared pale and chilled, the organ was considered to be adequately flushed. The organ was gently massaged during flushing to ensure removal of slugged blood. The oviduct and infundibulum were ligated and excised from the ovary and ovarian ligament. The flushed organ, with cannula, was placed in a plastic bag containing dilute ACD, sealed, and placed on ice in an ice chest and

transported to the laboratory.

In the laboratory, while the perfusion medium was being prepared and the perfusion apparatus primed, each flushed organ was flushed with chilled citrated blood to check for leaks in the ligated portions of the transected vascular system. Major leaks were doubly ligated with string. The organ was flushed again with 30 ml of dilute ACD to remove the citrated blood and held in a refrigerator until all four organs could be prepared in a similar manner. An organ was placed in each of the perfusion pans and the hypodermic needle affixed to the ovarian artery cannula was then attached to the perfusion medium outlet, and the perfusion began. Each perfusion was started at the same time. Four organs were perfused simultaneously in the same apparatus with a medium prepared from a single donor.

The Monocyclic Perfusion. During the first 15-20 minute warm-up period, the venous effluents were collected and later discarded. The perfusion pumps were adjusted to 100 mm Hg by adjusting the perfusate pressure control valve and maintained at this level throughout the perfusion period. As the organs began to metabolize, there was an obvious color difference between the veins and the arteries. The venous effluent was distinctly darker than the oxygenated perfusate. The organs which did not function properly within the first 15-20 minutes (low flow rates or no arterial-venous color difference) were replaced. The organs were observed continuously, and if vasoconstriction occurred, as indicated by a reduction in flow rate, $\frac{1}{2}$ to 2 ml or more of a 3% pilocarpine-hydrochloride solution was administered through the injection port.

Following the initial warm-up period, perfusates were collected for $2\frac{1}{2}$ hours. A sample was collected from each organ every 30 minutes. Following the addition of the treatments (see Appendix), samples were again collected from each organ every 30 minutes. Samples were collected in graduated cylinders which were placed in an 800 ml beaker and packed with ice. Each sample volume was recorded and all samples were stored at $4^{o}C$ until the experiment was completed.

Upon termination of the perfusion, the hematocrit and pH were determined for each sample to evaluate the quality of the perfusion. Each sample was then centrifuged[2] for 20 minutes at 9500 rpm at $5^{o}C$. The clear plasma supernatant was placed in sterile milk cartons and stored at $-20^{o}C$ until analyzed for progesterone. The cellular component was discarded. The perfused ovaries were placed in plastic bags and stored frozen until the corpora lutea were removed and weighed.

Progesterone Analysis

Plasma samples were extracted using analytical grade solvents, purified by thin layer chromatography, and quantitated by gas liquid chromatography. Percent recovery was determined by adding $3x10^{-3}$ µci of progesterone-4-^{14}C[3] to each sample before extraction and then measuring the amount of labeled progesterone recovered using liquid scintillation counting techniques.

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[2]International High-Speed Centrifuge Model HR-1, International Equipment Co., Needham Heights, Massachusetts.

[3]New England Nuclear Corporation, Boston, Massachusetts.

Extraction. A modification of the technique described by Stabenfeldt (60) was used for extraction of the plasma samples. Twenty ml of plasma from each sample were extracted, unless the volume of the sample was less than 20 ml in which case the entire sample was extracted. Each sample was extracted three times using $2\frac{1}{2}$ volumes of dichloromethane each time, and each sample was centrifuged at 10,000 rpm for a period of 12 minutes at 5°C. The solvent was then removed and placed in a round bottom flask. The solvent from combined extracts was removed from the flask by flash evaporation.[4] The residue was removed from the flask after being dissolved in ether and placed in a 35 ml conical test tube. Each flask was rinsed three times with 5 ml of absolute ether. The ether solution was saponified with 1N NaOH (5 ml) and the NaOH layer was removed. Centrifugation at 1500 rpm was used to break any emulsion that formed. The ether layer was washed two times with 5 ml distilled water, centrifuged, and the water layer removed each time. The final ether solution was dried under nitrogen and stored at -4°C until purified by thin layer chromatography in a benzene-ethyl acetate (4:1) solvent system.

Thin Layer Chromatography. Each sample was purified by thin layer chromatography using silica gel HR/UV[5] and a solvent system of benzene-ethyl acetate (4:1). Authentic repurified progesterone[6] was spotted in an isolated lane on the plate along with three samples, each in an

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[4]Rotovapor Model VE-50, Rinco Instrument Co., Inc., Greenville, Illinois.

[5]Machery, Nagel, and Co., Brinkman Instrument Inc., Westbury, N.Y.

[6]Sigma Chemical Co., St. Louis, Missouri.

isolated lane. Each plate remained in the solvent system until the solvent had ascended to within $\frac{1}{2}$ inch of the top of the plate. Each plate was then observed under ultraviolet light and the nonfluorescent bands corresponding to the nonfluorescent progesterone standard band were outlined on the plate. These bands were then scraped from the plate and eluted with diethyl ether. Each sample was then dried under nitrogen and stored at -20°C until subjected to gas liquid chromatography.

Gas Liquid Chromatography. One-tenth of the extracted sample was chromatographed on 3 ft glass columns packed with 1% QF-1 liquid phase on 100/200 mesh gas chrom Q[7] solid support. A Barber-Coleman Gas Chromatograph[8] with hydrogen flame detectors was used to quantitate the progesterone. The injector temperature was set at 250°C and the column bath temperature was set at 220°C. A nitrogen gas carrier was also used. A progesterone retention time of 11 minutes was attained. The attenuation settings were 30 and 100. The detector temperature was set at 230°C. The areas of the peaks were determined by means of a planimeter. Standard progesterone samples of known quantities were chromatographed and the peak areas measured. The peak areas were then plotted to provide a standard curve. The quantity of progesterone in each unknown sample was then determined by comparing the peak area units of the unknown with the standard curve. Each sample, standard and unknown, was dissolved in tetrahydrofuran (THF) prior to injection into the gas chromatograph.

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[7]Applied Science Laboratory, College Station, Pennsylvania.

[8]Barber-Coleman Co., Industrial Instrument Div., Rockford, Ill.

Liquid Scintillation Counting. One-tenth of each extracted sample was placed in a scintillation vial at the time of gas chromatography and counted for estimation of recovery rate. A Packard Tri-Carb Model 314-E liquid scintillation counter[9] was employed. The fluor (see Appendix) in which the samples were counted was 4.0 gm PPO and 0.3 gm POPOP per liter of toluene. Each sample was counted twice for a period of 10 minutes.

Experimental Design

It has been reported in the literature that cofactors play a role in steroidogenesis and the response of the bovine corpus luteum to gonadotropins. Furthermore, the objective of this study was to determine whether or not these same seven combined cofactors[10] could produce these results in the perfused porcine corpus luteum in view of reports that the corpus luteum of this species is reluctant to respond to gonadotropins in vitro. The experiment was designed to meet this objective. The study was divided into four divisions with a total of 44 ovaries (Figure 3).

Experiment I was designated as a control. Nine porcine ovaries were perfused monocyclically for 2½ hours with samples being collected every 30 minutes. No treatment was added to the perfusing medium in order that a basic level of progesterone synthesis could be determined to compare with the perfusions to which treatments were added.

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[9]Packard Instrument Co., Inc., La Grange, Illinois.

[10]Sigma Chemical Co., St. Louis, Missouri.

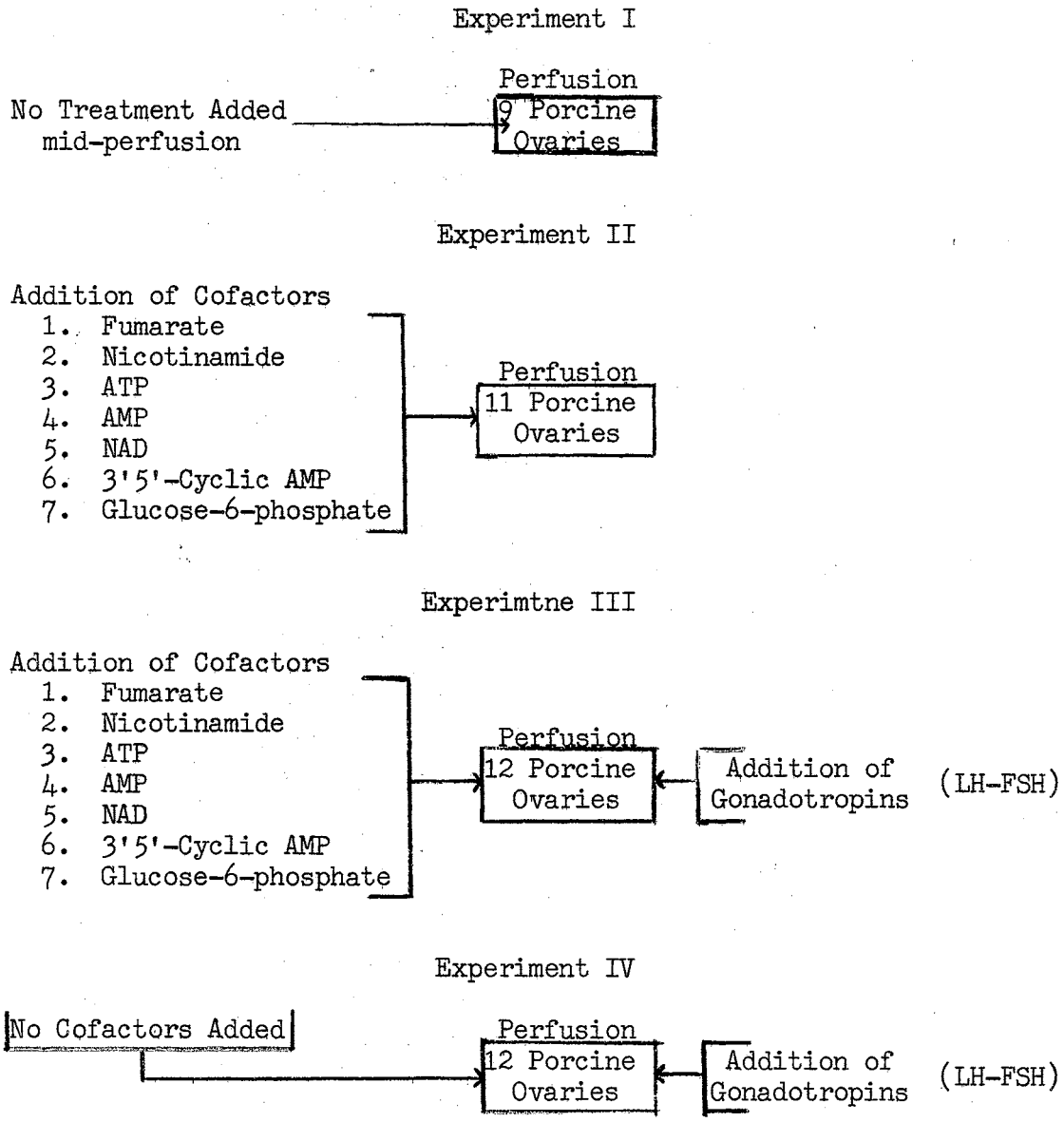

Figure 3. Experimental Design

Experiment II of the study was designed to study the effect of a combination of seven different cofactors upon the synthesis of progesterone. Eleven porcine ovaries were perfused exactly as in Experiment I. The cofactors were added to the perfusion medium after the ovaries had been perfused for 1½ hours including the warm-up period. Two samples were collected preceeding the addition of the cofactors, thus allowing control samples to which the treatment samples could be compared.

Experiment III of the study was designed to study the effects of LH-FSH[11] and the same seven cofactors upon the synthesis of progesterone in the perfused corpus luteum. The primary objective was to determine if the cofactors would make the luteal tissue responsive to gonadotropins. Twelve ovaries were perfused monocyclically with the sample collections and treatment occurring at the same time intervals as in Experiments I and II.

Experiment IV of the study was designed to study the effects of LH-FSH without the cofactors added on the synthesis of progesterone. Twelve porcine ovaries were perfused monocyclically as in Experiments I, II, and III.

Statistical Analysis

The statistical test used was an analysis of variance based upon the split plot design (61). A computer, IBM 360 Model 50, was employed to calculate the means. The data were expressed in two forms, percent of the total ug progesterone estimated to have been synthesized and

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[11]Endocrinology Study Section, National Institutes of Health, Bethesda, Maryland.

μg progesterone/gm luteal tissue. Each experiment of the study consisted of three trials. Each trial for each treatment was analyzed separately and then analyzed as combined data. For each treatment the data were analyzed both before and after the treatment. The analysis tested the differences between trials, the differences that occurred with time within the trials, and the differences that occurred among the ovaries with time among the three trials. The significance or nonsignificance was determined by comparing the F value to a table. Slopes of the line for pretreatment and posttreatment were used as indicators of the treatment effect. The Computer Program was written by the Statistics Laboratory at Oklahoma State University.

CHAPTER IV

RESULTS AND DISCUSSION

Previous reports by Duncan et al. (11) and Mathis (43) have indicated that a combination of LH and FSH has little or no effect on progesterone synthesis in swine corpora lutea *in vitro* when compared to other species. They suggested that the loss of cofactors during collection and preparation of the porcine tissues rendered them refractory to gonadotropins. It was postulated, therefore, that adding a combination of selected cofactors to the perfusate would allow the intact compartmentalized isolated organs to respond to gonadotropins.

A total of 44 ovaries was successfully perfused with citrated whole blood from a barrow donor as described in Chapter III. All 44 of the ovaries were from gilts or sows in early pregnancy (less than 40 days) determined by the crown-rump measurement of the embryos.

The ovarian and corpus luteum weights of ovaries perfused in Experiments II, III, and IV are shown in Table I. The weights of ovaries perfused in Experiment I were not recorded. The average ovarian weight for all ovaries weighed was 10.48 gm and the average corpus luteum weight was 5.18 gm.

The data from Experiments I, II, III, and IV expressed as μg progesterone/gm luteal tissue are shown in Tables II, III, IV, and V. When these data were subjected to an analysis of variance, the analysis indicated that no significant alteration in progesterone

TABLE I

OVARIAN AND CORPUS LUTEUM WEIGHTS FROM SOWS AND GILTS OF EARLY PREGNANCY*

Ovary Number	Total Ovarian Weight (gm)	Corpus Luteum Weight (gm)	Ovary Number	Total Ovarian Weight (gm)	Corpus Luteum Weight (gm)
2	11.28	5.96	24	18.90	8.12
3	9.16	4.61	25	11.73	4.45
4	9.49	4.49	26	14.19	6.23
9	11.10	4.58	27	13.93	6.94
10	13.27	7.47	28	12.31	4.94
11	7.49	3.90	29	9.84	4.89
12	10.28	6.08	30	9.42	3.86
13	9.33	4.15	31	11.47	6.22
14	9.06	4.42	32	9.75	4.78
15	11.01	5.92	33	11.35	5.00
16	7.26	3.67	34	13.36	7.55
17	8.90	5.08	35	10.63	5.33
18	11.64	6.98	36	10.08	5.56
19	11.30	5.72	37	7.87	4.23
20	9.79	3.91	38	11.33	6.28
21	8.99	4.51	39	6.19	3.69
22	6.79	2.76	40	7.92	3.58
23	10.21	5.70			
*Average for all Ovaries	10.48	5.18			

synthesis occurred with the addition of any treatment. The lack of a significant alteration was perhaps due to the great variance, of C.V., between organs. However the lack of a significant response does mean that, because of the great variance among organs, many more trials in each experiment would need to be performed in order to obtain a dependable F value. When data from individual organs were plotted, a subjective evaluation of each plot seemed to indicate that most of the organs responded to treatment, though each organ was synthesizing progesterone at a different basic level. Therefore, it was postulated that if the sample data from all the trials in a particular experiment were recorded on the basis of percent of the total ug progesterone estimated to be synthesized by the ovary, then the great variation between organs would be minimized. However, data recorded in this manner could not be analyzed statistically because the total progesterone synthesized by each organ added up to 100% and, therefore, the experimental error could not be determined. Because of the foregoing, the data for all experiments recorded as percent of total synthesized (Tables VI, VII, VIII, and IX) will be discussed subjectively after the statistical analysis of data recorded in µg progesterone/gm luteal tissue is first discussed.

Evaluation of Data Expressed in µg Progesterone/gm Luteal Tissue

The amounts of progesterone synthesized by the ovaries in Experiment I expressed as µg progesterone/gm luteal tissue is shown in Table II and in Figure 4. These data showed that the ovaries started at a high level of synthesis (15.97 µg progesterone/gm luteal tissue)

and decreased in synthesis during the second 30 minutes of perfusion. At the end of 90 minutes of perfusion, the ovaries had regained some of their synthesizing ability, but decreased in synthesizing ability during the final hour of perfusion reaching a value of 11.36 µg progesterone/gm luteal tissue at the end of the perfusion period. The statistical analysis showed that there were no significant differences among the times of collection within the trials, that there were no significant differences between the trials, and that there were no significant differences among the times between the trials. These findings agreed with the expected results since no treatment had been added at mid-perfusion.

The amounts of progesterone synthesized by the ovaries in Experiment II expressed as µg progesterone/gm luteal tissue are shown in Table III and Figure 5. When Table II and Table III are compared, it can be seen that the level of synthesis for the ovaries in Experiment II was lower than for the ovaries of Experiment I. This difference could have been due to the great variation among the organs. From Table III it can be seen that the ovaries began synthesis at a low level (3.74 µg progesterone/gm luteal tissue) and reached their peak ability to synthesize progesterone at the end of one hour of perfusion (4.09 µg progesterone/gm luteal tissue). This increase in ability to synthesize progesterone was not significant which agreed with the expected results. Following the addition of the treatment, the ovaries decreased in their ability to synthesize progesterone (2.56 µg progesterone/gm luteal tissue) during the first 30 minutes of perfusion but regained their ability to synthesize progesterone during the next 30 minutes of perfusion (3.14 µg progesterone/gm luteal tissue). During

TABLE II

PROGESTERONE SYNTHESIZED BY UNTREATED PORCINE OVARIES OF EARLY PREGNANCY*†

Trial	Ovaries/Trial	Time of Sample Collection (min)**				
		30	60	90	120	150
I	3	17.35	11.75	10.34	9.72	9.63
II	3	16.26	10.20	14.18	12.19	11.95
III	3	14.29	11.73	15.73	13.91	12.49
Mean		15.97	11.23	13.42	11.94	11.36

*Expressed as μg progesterone/gm luteal tissue.

†No treatment added in this experiment (control); therefore, no pretreatment or posttreatment values.

**Not statistically significant.

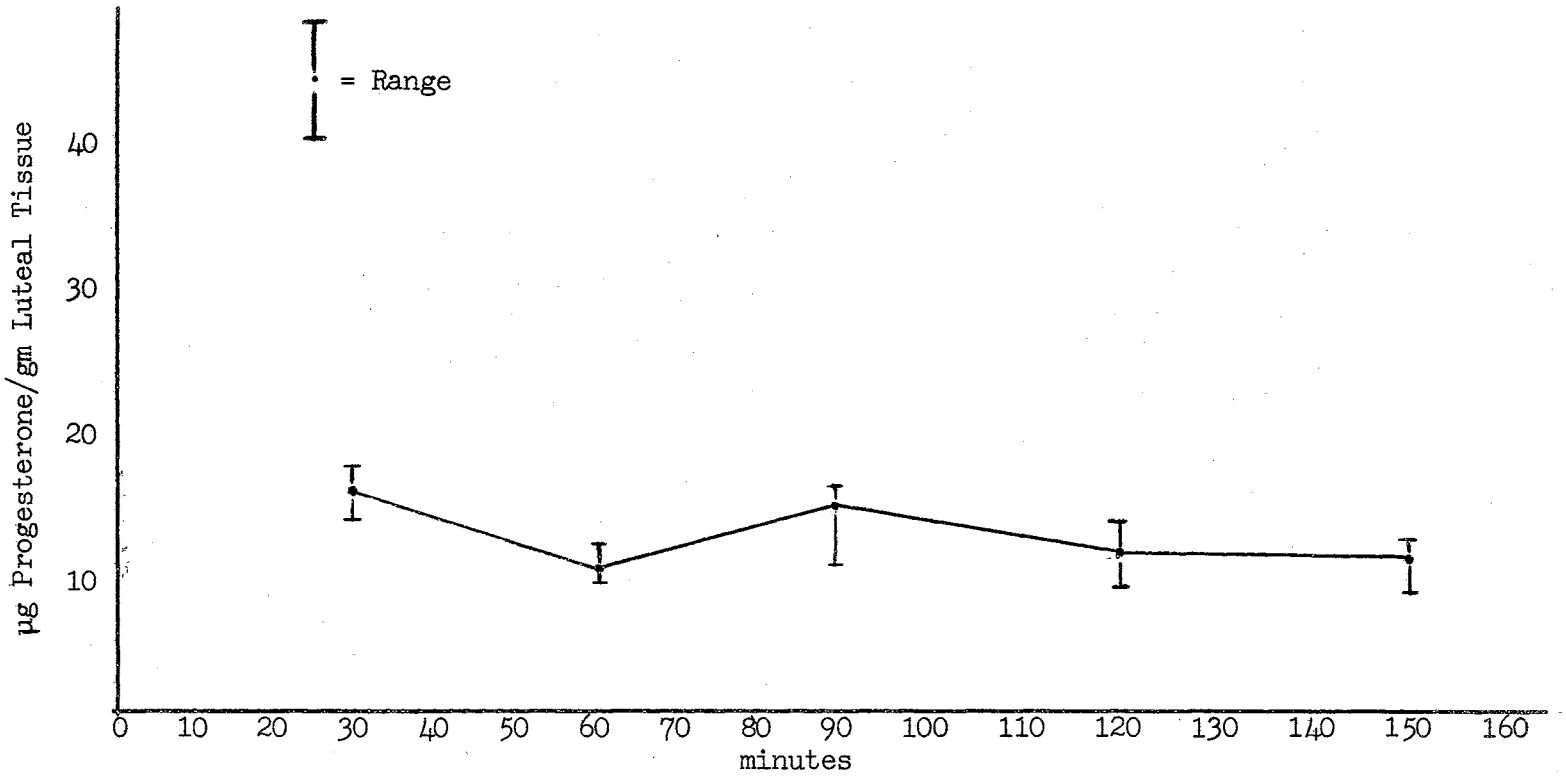

Figure 4. µg Progesterone/gm Luteal Tissue in Each Sample in Experiment I (Average of 9 Ovaries). No Treatment Added.

the final 30 minutes of perfusion, the ovaries decreased slightly in their ability to synthesize progesterone (2.99 µg progesterone/gm luteal tissue). It appeared that the treatment stimulated progesterone synthesis. However, the changes which occurred following the addition were not statistically significant. The lack of significance was probably due to the large variation among the organs (28%). Comparison of the slopes of the line of Figure 5 before and after the treatment showed no treatment effect because the slopes were equal. It appeared that the cofactors stabilized progesterone synthesis at a constant level rather than allowing a decrease in progesterone synthesis.

The amounts of progesterone synthesized by the ovaries in Experiment III are shown in Table IV and Figure 6. The level of synthesis was also lower than that of the controls in Table II. From Table IV, it can be seen that synthesis began at a high level of 3.18 µg progesterone/gm luteal tissue and then decreased in their ability to snythesize throughout the remainder of the experiment. The difference between the pretreatment samples was not significant which agreed with the expected results. The changes in rate of synthesis which occurred following the addition of the treatment were also statistically insignificant. Although there appeared to be an increase in progesterone synthesis following the addition of the treatment (Figure 6) this change was not statistically significant. Comparison of the slope of the line before and after the treatment showed no treatment effect. The ovaries appeared to respond to the treatment but the lack of a significant response was due to the large variation (51%) among the ovaries. The cofactors and LH-FSH appeared to stabilize progesterone synthesis.

TABLE III

PROGESTERONE SYNTHESIZED BY PORCINE OVARIES OF EARLY PREGNANCY TREATED WITH COFACTORS*†

Trial	Ovaries/Trial	Time of Sample Collection (min)** (Pretreatment) 30	60	(Posttreatment) 90	120	150
I	3	0.87	1.47	2.54	2.33	2.92
II	4	2.86	3.92	2.34	2.73	1.97
III	4	6.77	6.23	2.80	4.14	4.08
Mean		3.74	4.09	2.56	3.14	2.99

*Expressed as µg progesterone/gm luteal tissue.

†Treated with a group of seven cofactors at mid-perfusion thus allowing for pretreatment and posttreatment values.

**Not statistically significant.

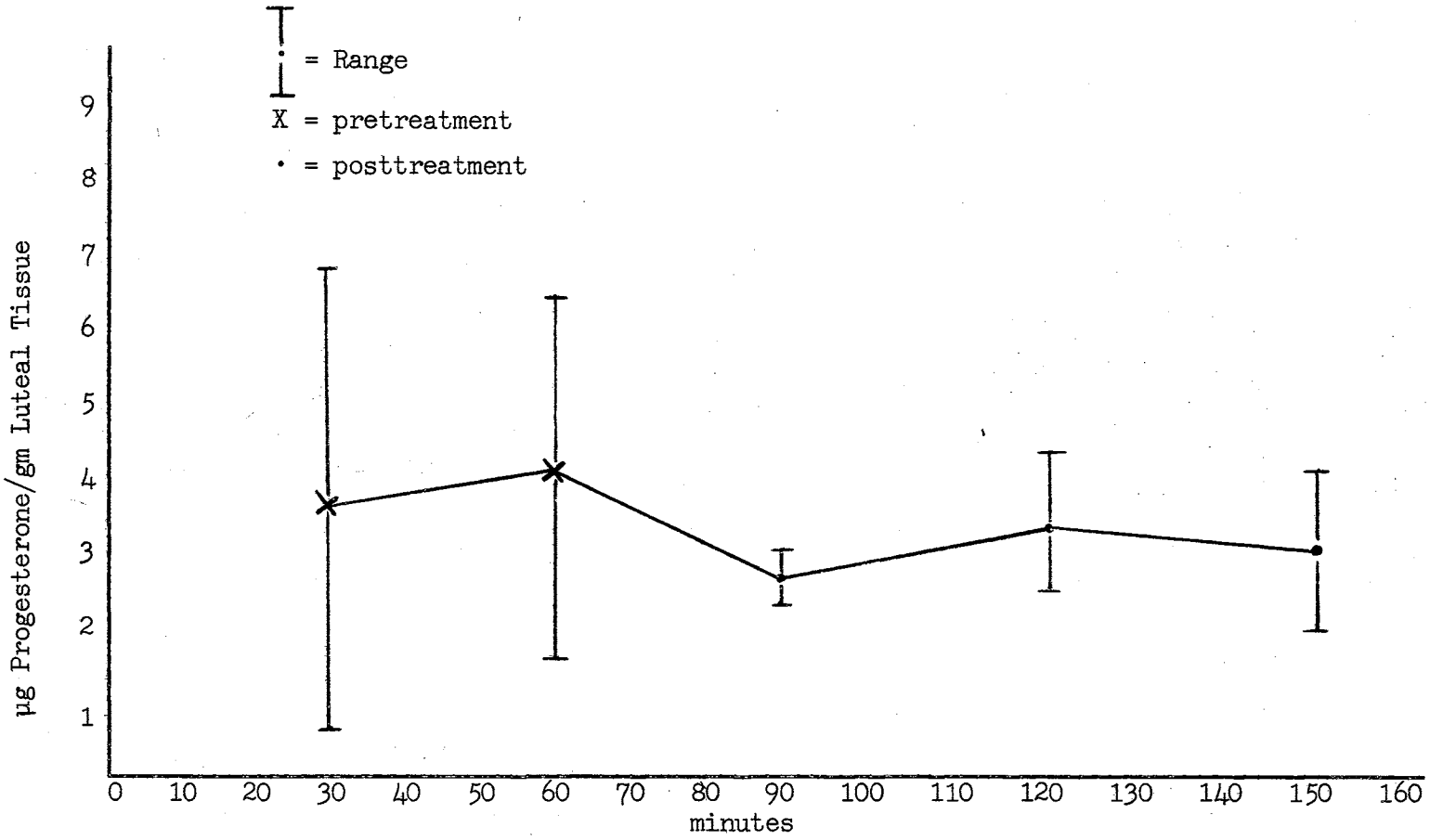

Figure 5. µg Progesterone/gm Luteal Tissue in Each Sample in Experiment II (Average of 11 Ovaries). Treated with Cofactors.

TABLE IV

PROGESTERONE SYNTHESIZED BY PORCINE OVARIES OF EARLY PREGNANCY TREATED WITH COFACTORS AND LH-FSH*†

Trial	Ovaries/Trial	Time of Sample Collection (min) (Pretreatment)**		(Posttreatment)**		
		30	60	90	120	150
I	4	3.01	2.49	2.99	3.26	2.93
II	4	2.36	3.37	3.15	4.25	3.72
III	4	4.18	2.76	1.89	1.36	1.20
Mean		3.18	2.87	2.68	2.96	2.62

*Expressed as μg progesterone/gm luteal tissue.

†Treated with a group of seven cofactors and LH-FSH at mid-perfusion thus providing pretreatment and posttreatment values.

**Not statistically significant.

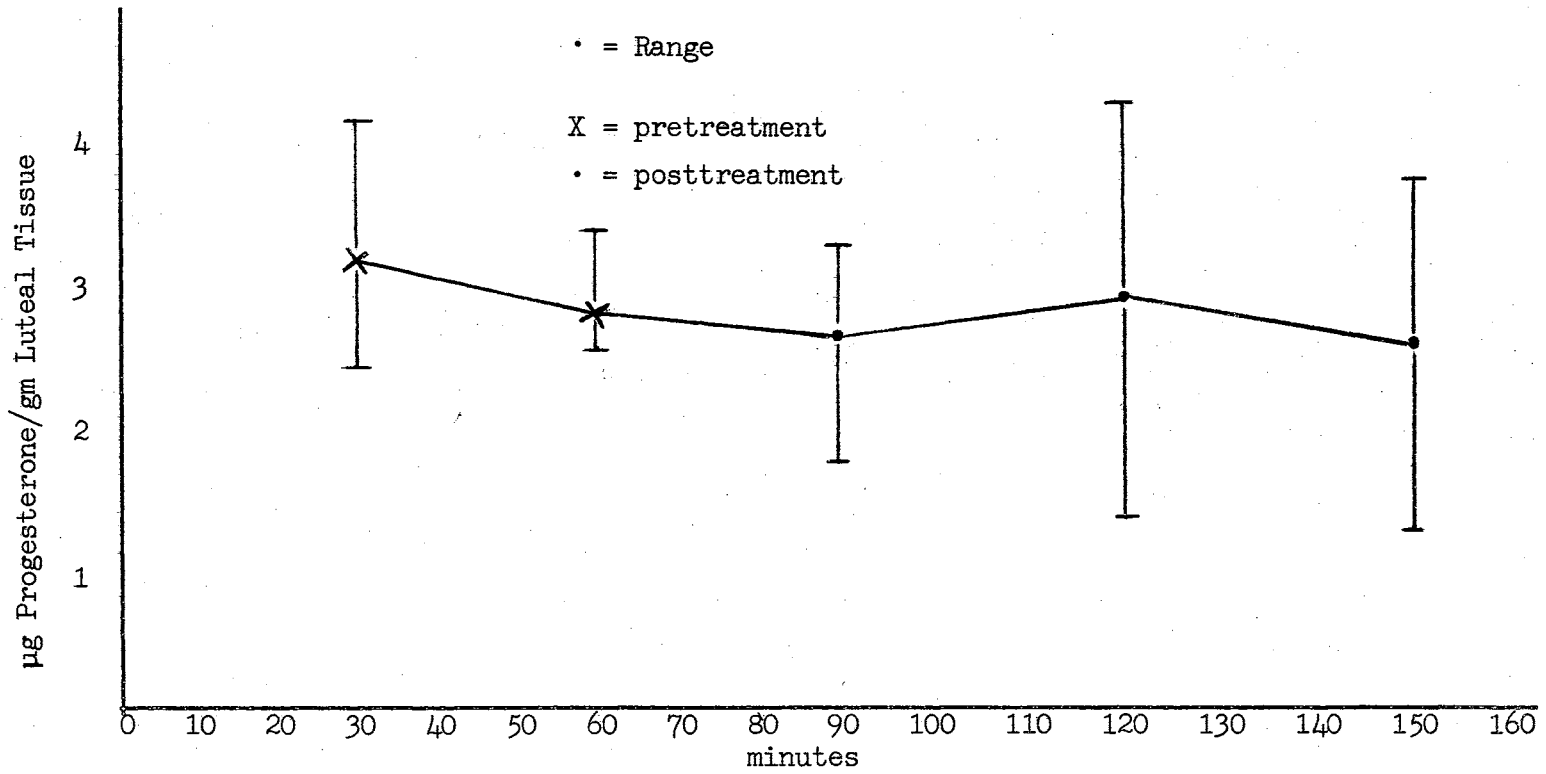

Figure 6. µg Progesterone/gm Luteal Tissue in Each Sample in Experiment III (Average of 12 Ovaries). Treated with Cofactors and LH-FSH.

The amounts of progesterone synthesized by the ovaries in Experiment IV expressed as µg progesterone/gm luteal tissue are shown in Table V and Figure 7. The level of progesterone synthesis was lower than that for the controls (Table II). From Table V it can be seen that the ovaries began synthesis at a high level (10.46 µg progesterone/gm luteal tissue) and decreased steadily throughout the perfusion period. The difference which occurred between the two pretreatment collections was significant ($P < .025$) which indicated that something happened to the synthesizing ability of the ovaries before the treatment was added. It was postulated that an immunological reaction occurred between the donor's blood and the ovaries. The addition of the treatment, LH-FSH, failed to cause a change in progesterone synthesis. It appeared that the treatment was inhibitory to progesterone synthesis but the inhibition was not significant. Again, the lack of significance can be explained on the basis of the great variation among the ovaries (51%). Comparison of Figures 4, 5, 6, and 7 also showed that LH-FSH appeared to be inhibitory to progesterone synthesis.

Evaluation of Data Expressed as Percent of Total Progesterone Synthesized

The amounts of progesterone synthesized by the ovaries in Experiment I, expressed as percent of total µg of progesterone estimated to have been synthesized, are shown in Table VI and Figure 8. Comparison of Table VI and Table II shows somewhat the same general trend of synthesis. The ovaries synthesized 27% of the total during the first 30 minutes of perfusion and then decreased in synthesizing ability during the remainder of the perfusion period except for an increase at

TABLE V

PROGESTERONE SYNTHESIZED BY PORCINE OVARIES OF EARLY PREGNANCY TREATED WITH LH-FSH*†

Trial	Ovaries/Trial	Time of Sample Collection (min)				
		(Pretreatment)**			(Posttreatment)***	
		30	60	90	120	150
I	4	9.21	5.01	3.28	3.47	2.93
II	4	12.20	4.33	3.08	1.93	2.63
III	4	9.97	7.90	5.68	2.80	2.45
Mean		10.46	5.75	4.01	2.73	2.67

*Expressed as μg progesterone/gm luteal tissue.

†Treated with LH-FSH at mid-perfusion to allow for pretreatment and posttreatment values.

**Statistically significant ($P<.025$).

***Not statistically significant.

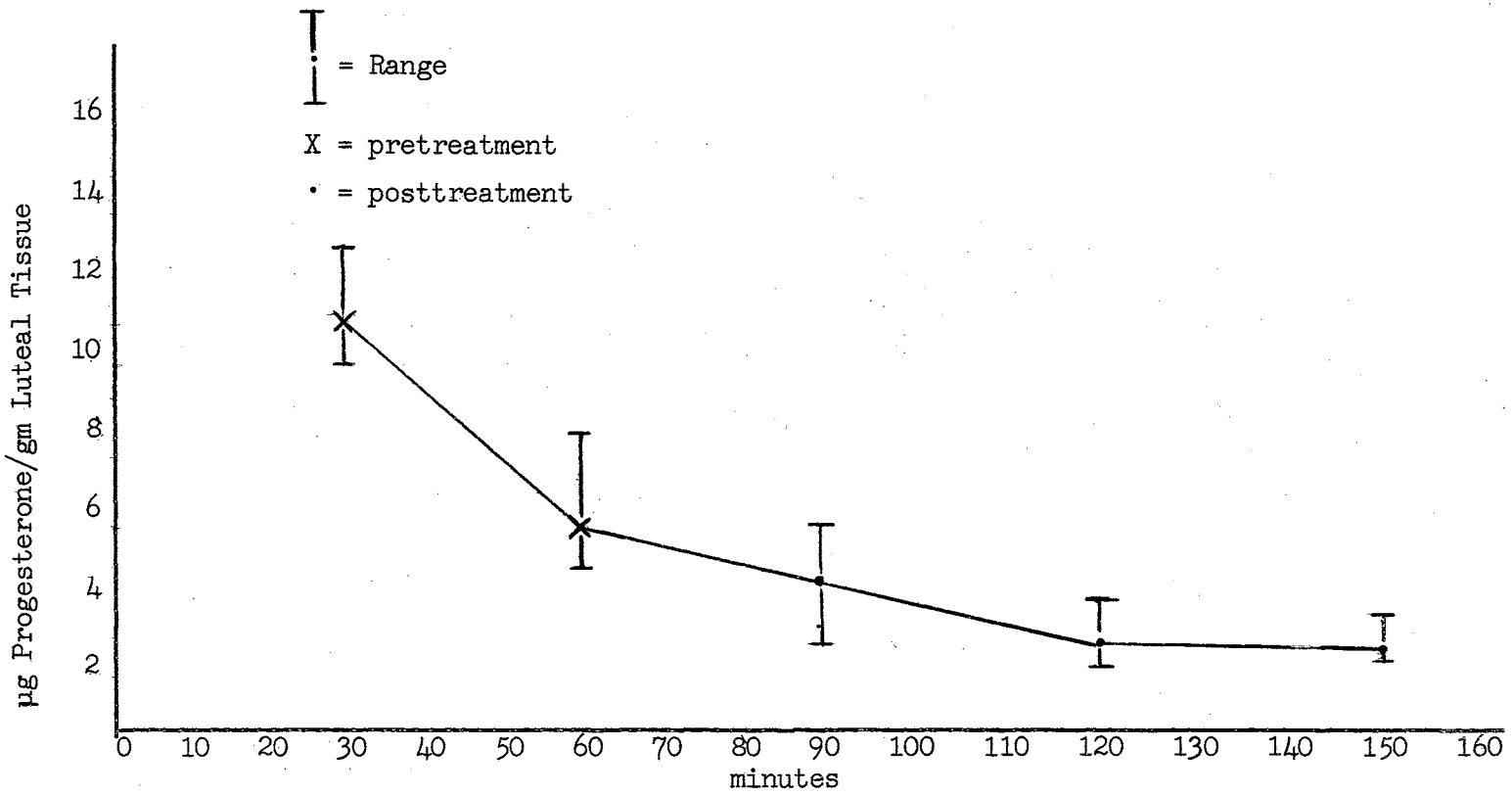

Figure 7. µg Progesterone/gm Luteal Tissue in Each Sample in Experiment IV (Average of 12 Ovaries). Treated with LH-FSH.

the end of 90 minutes of perfusion. Comparison of Figures 8 and 4 also shows this trend of synthesis.

The amounts of progesterone synthesized by the ovaries in Experiment II expressed as percent of total ug of progesterone are shown in Table VII and Figure 9. The ovaries synthesized 20.85% of the total during the first 30 minutes of perfusion and increased to 21.8% of the total during the next 30 minutes of perfusion. Following the addition of the treatment the synthesizing ability of the ovaries decreased during the first 30 minutes of perfusion (14.6%) and increased during the final hour of perfusion. Comparison of Figures 9 and 5 show the same type of synthesis pattern and that the ovaries appeared to respond to the treatment. Progesterone synthesis also appeared to be stabilized.

The amounts of progesterone synthesized by the ovaries in Experiment III expressed as percent of the total ug progesterone are shown in Table VIII and Figure 10. The data in Table VIII shows that the ovaries began synthesis at a low level of 16.93% of the total progesterone synthesized and steadily increased throughout the perfusion period. It appeared that the treatment increased and stabilized progesterone synthesis.

The amounts of progesterone synthesized by the ovaries in Experiment IV expressed as percent of the total ug progesterone are shown in Table IX and Figure 11. The ovaries synthesized 27.9% of the total progesterone synthesized during the first 30 minutes of perfusion and then decreased in synthesizing ability throughout the remainder of the perfusion period. Comparison of Tables IX and V shows the same general trend of synthesis. Comparison of Figures 11 and 7 also

TABLE VI

PROGESTERONE SYNTHESIZED BY UNTREATED PERFUSED PORCINE OVARIES OF EARLY PREGNANCY*†

Trial	Ovaries/Trial	Time of Sample Collection (min)				
		30	60	90	120	150
I	3	29.20	20.00	18.93	17.00	16.33
II	3	25.70	16.90	21.07	17.43	18.73
III	3	26.10	17.33	20.13	19.37	16.80
Mean		27.00	18.08	20.04	17.93	17.29

*Expressed as percent of total μg of progesterone estimated to have been synthesized.

†No treatment added in this experiment (control); therefore, no pretreatment and posttreatment values.

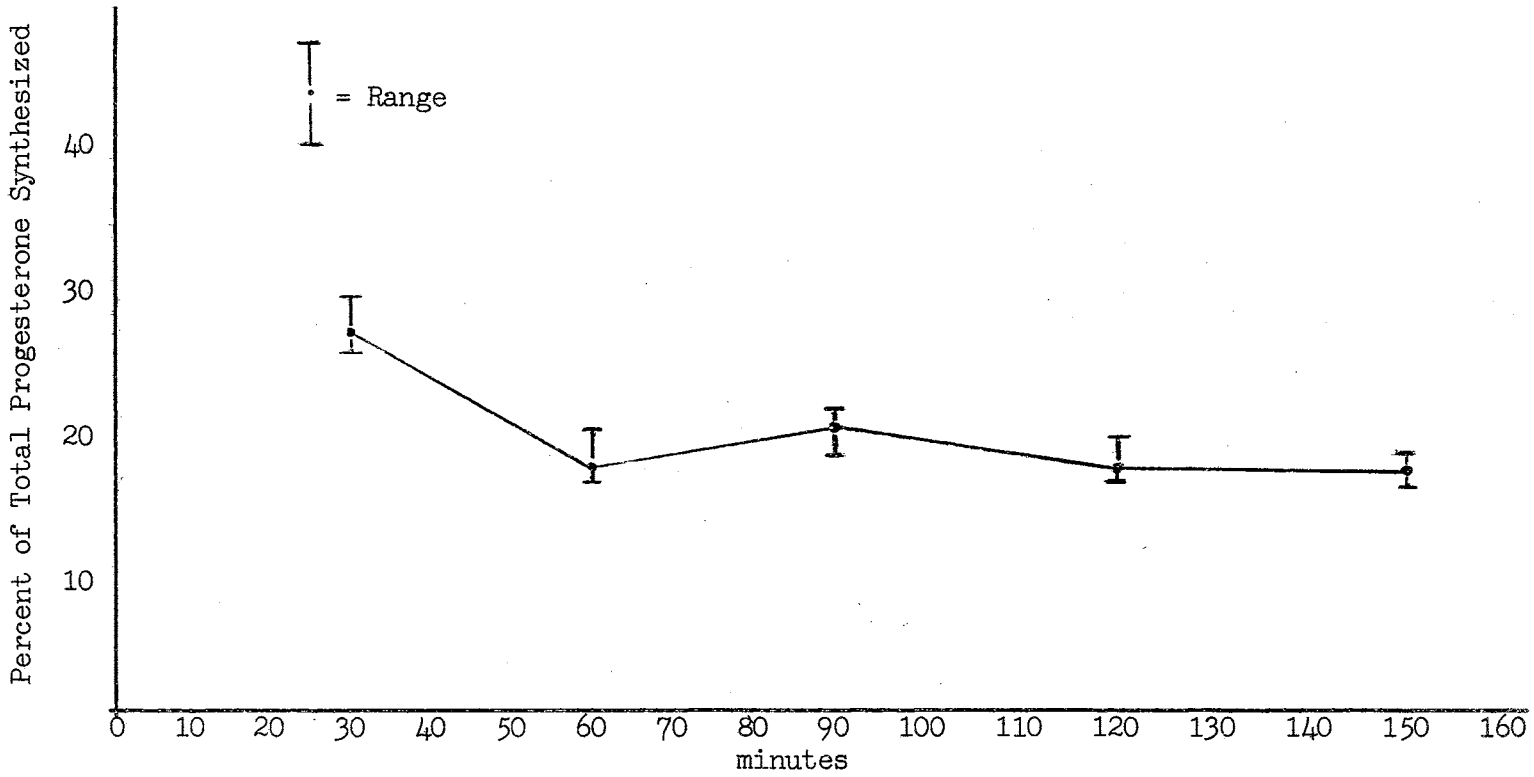

Figure 8. Percent of Total Progesterone in Each Sample In Experiment I. (Average of 9 Ovaries). No Treatment Added.

TABLE VII

PROGESTERONE SYNTHESIZED BY PERFUSED PORCINE OVARIES OF EARLY PREGNANCY TREATED WITH COFACTORS*†

Trial	Ovaries/Trial	Time of Sample Collection (min) (Pretreatment) 30	60	(Posttreatment) 90	120	150
I	3	14.80	16.03	19.53	20.60	24.77
II	4	24.48	22.80	14.78	15.30	16.75
III	4	20.95	25.13	10.73	14.73	14.73
Mean		20.55	21.80	14.60	16.54	18.20

*Expressed as percent of total µg of progesterone estimated to have been synthesized.

†Treated with a group of seven cofactors at mid-perfusion to allow for pretreatment and posttreatment values.

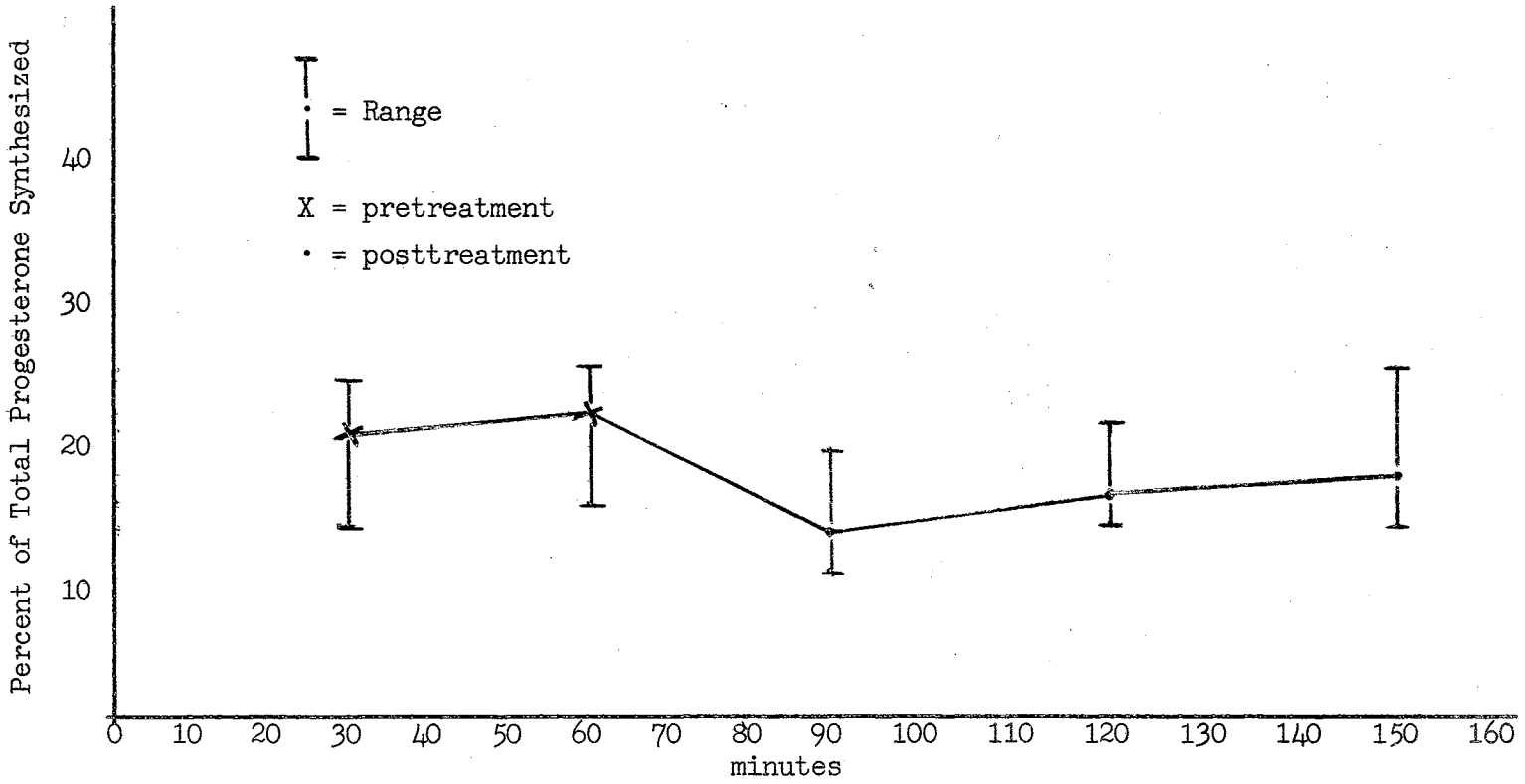

Figure 9. Percent of Total Progesterone in Each Sample in Experiment II (Average of 11 Ovaries). Treated with Cofactors.

TABLE VIII

PROGESTERONE SYNTHESIZED BY PERFUSED PORCINE OVARIES OF EARLY PREGNANCY TREATED WITH COFACTORS AND LH-FSH*+

Trial	Ovaries/Trial	Time of Sample Collection (min) (Pretreatment) 30	60	(Posttreatment) 90	120	150
I	4	18.30	16.33	19.28	21.30	19.18
II	4	11.85	18.75	17.20	21.93	23.48
III	4	20.65	18.88	15.35	10.24	13.43
Mean		16.93	17.98	17.28	17.82	18.69

*Expressed as percent ot total µg of progesterone estimated to have been synthesized.

+Treated with cofactors and LH-FSH at mid-perfusion to allow for pretreatment and posttreatment values.

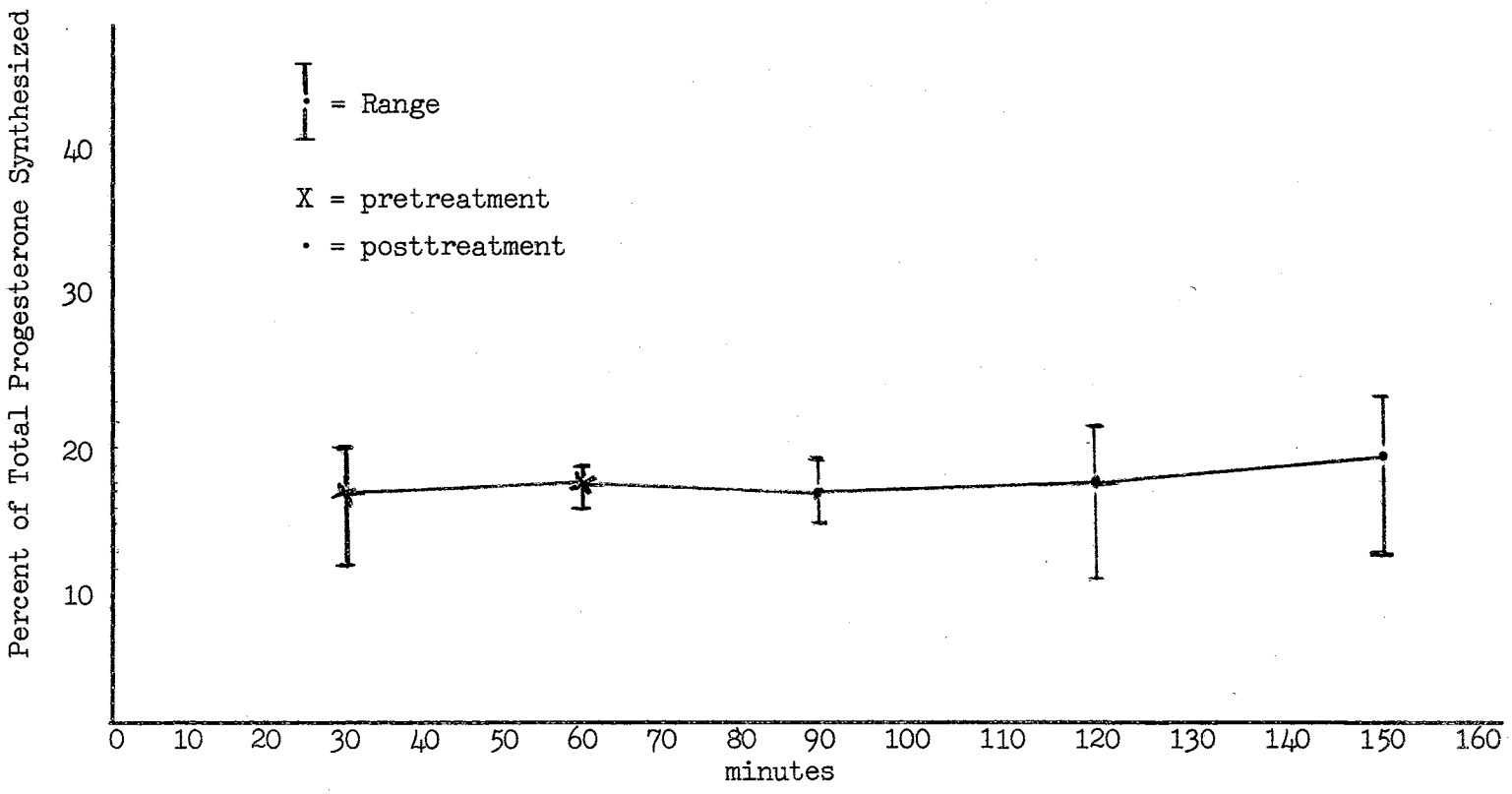

Figure 10. Percent of Total Progesterone in Each Sample in Experiment III (Average of 12 Ovaries). Treated with Cofactors and LH-FSH.

shows the same general trend of synthesis. The treatment appeared to be inhibitory to the synthesis of progesterone.

The ovaries of all four experiments appeared to remain viable throughout the perfusion period. However, edema began to appear in some organs during the terminal phases of the experiments. The organs in Experiment IV showed more signs of tissue deterioration and loss of synthesizing ability than the other organs. This is in agreement with the decrease in progesterone synthesis observed in Experiment IV.

Several factors could have contributed to the results that were obtained. The primary factor thought to alter the results was organ variation. The normal C.V. or variation for biological assays should be about 15%-20%. In the present study, the C.V. or variance values for the organs ranged from 29%-54%. Had time and physical facilities permitted the perfusion of a larger number of ovaries, this variation may have been reduced. Considerable variation was found to be present from one ovary to the next throughout all the perfusions. This variation is an inherent characteristic of each individual ovary. Mathis (43) reported the same difficulty in perfusing porcine ovaries. The percentage of error in response of porcine luteal tissue to gonadotropins has been found to be twice that of sheep and cows (12). Great variation has also been found in perfused rabbit testes (20) as well as in perfused bovine ovaries (48). A second factor to be considered is the possibility that the ovaries were maximally stimulated at the time of collection. Masuda et al. (42) found that on day 14 of pregnancy there was a progesterone concentration of 2.18 ug/ml plasma and that a peak of 2.63 µg/ml plasma was reached on day 18. The

TABLE IX

PROGESTERONE SYNTHESIZED BY PERFUSED PORCINE OVARIES OF EARLY PREGNANCY TREATED WITH LH-FSH*†

Trial	Ovaries/Trial	Time of Sample Collection (min) (Pretreatment) 30	60	(Posttreatment) 90	120	150
I	4	27.00	13.10	8.70	9.10	7.75
II	4	33.38	13.80	8.73	7.80	8.25
III	4	23.40	18.48	12.48	6.17	5.55
Mean		27.93	15.13	9.97	7.69	7.18

*Expressed as percent of total µg of progesterone estimated to have been synthesized.

†Treated with LH-FSH at mid-perfusion to allow for pretreatment and posttreatment values.

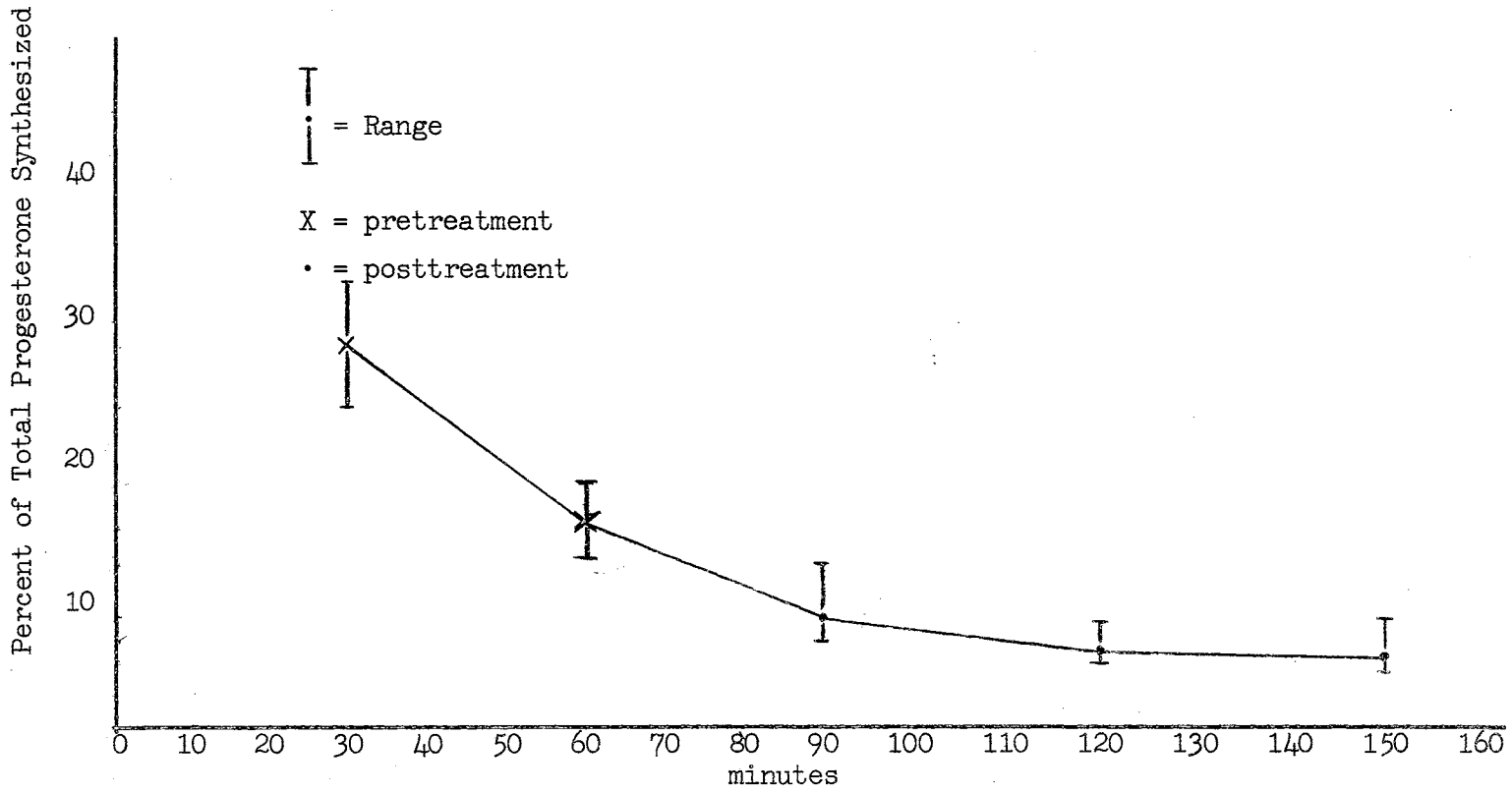

Figure 11. Percent of Total Progesterone in Each Sample in Experiment IV (Average of 12 Ovaries). Treated with LH-FSH.

concentration of progesterone then decreased until parturition. It can be seen from these findings that the ovary of early pregnancy could be highly stimulated. A third factor to be considered is the possible loss of some essential factor at the time of slaughter. Removal of the ovaries from their natural environment, or the collection process, could have resulted in the loss of essential factors other than those added as treatment. A fourth factor affecting the results could have been tissue alteration due to anaerobic degradition. A time lapse of approximately 25 minutes occurred from the time of slaughter until the removal of the ovaries. Armstrong and Black (4) have shown that the handling of bovine donors before and after slaughter and anaerobic conditions of 30-40 minutes greatly influenced the ability of the bovine corpus luteum to respond to the addition of LH in vitro. A fifth factor considered is the possibility of an immunological reaction between the blood and ovaries of the different donors. Vasoconstriction was an ever present problem throughout the course of this investigation.

CHAPTER V

SUMMARY

Since the isolation of progesterone in 1934, numerous studies concerning the synthesis of progesterone by the adrenal glands, ovaries, testes, and the placenta have been conducted. Duncan et al. (11) and Mehdi (46), using porcine luteal slices and minces, reported that a combination of LH and FSH had little or no effect on progesterone synthesis *in vitro*. Mathis (43) studied progesterone synthesis in isolated, intact perfused porcine ovaries and reported similar results. The aforementioned investigators suggested that the loss of cofactors during the collection of the ovaries rendered them refractory to gonadotropins. *In vitro* studies by Duncan et al. (11), with porcine luteal tissues, and studies by Hall and Young (26) and Mason and Savard (36, 37, 38) with bovine luteal tissues in which cofactors were added, all resulted in increased progesterone synthesis.

Because of the foregoing, an experiment was designed in which isolated intact organs were perfused with citrated barrow blood to which a group of selected cofactors, cofactors and LH-FSH, and LH-FSH alone were added. It was postulated that the addition of cofactors plus the advantage of a system with a complete microcirculation and undisturbed compartmentalization would be more steroidogenically responsive to gonadotropins. The combined techniques of Mills (48), Mathis (43), Morrissette (51), and Cardeilhac and Morrissette (9) were used.

A total of 44 porcine ovaries from sows or gilts of early pregnancy was perfused in a multi-organ monocyclic perfusion apparatus. The effects of the addition of cofactors, cofactors and LH-FSH, and LH-FSH alone were observed.

The addition of the cofactors to the perfusion medium at mid-perfusion did not cause a significant increase in progesterone synthesis. The cofactors appeared to stablize progesterone synthesis, however, and to keep the organs viable for a longer period of time.

The addition of the cofactors and LH-FSH caused organ responses comparable to those observed when cofactors alone were added. The treatment appeared to stabilize progesterone synthesis and to maintain a nearly constant level of progesterone synthesis for a longer period of time.

The addition of LH-FSH to the perfusion medium at mid-perfusion appeared to be inhibitory to the synthesis of progesterone. The true effect of the treatment may not have been seen, however, due to a reaction which apparently occurred between the barrow blood and several of the ovaries before the addition of the treatment.

When the data from the three experiments were plotted, it appeared that the organs were responding to the treatment. The lack of a statistically significant response was probably due to the great variation found to be present among the individual ovaries. It was concluded that if a larger number of perfusions had been performed for each trial, the factor of organ variation may have been overcome and a more dependable F value attained.

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APPENDIX

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Concentrated ACD Solution

Sodium Citrate	77.0 gm
Citric Acid	28.0 gm
Dextrose	85.7 gm

Dilute to 3500 ml with distilled water

pH adjusted to 7.4

Dilute ACD Solution

Dilute 480 ml of concentrated ACD solution with 2520 ml of 0.85% NaCl.

Buffer with 60 ml of phosphate buffer.

pH adjusted to 7.4.

Phosphate Buffer

Solution #1. KH_2PO_4 (M.W. 136). Dissolve 9.08 gm in 1 liter of distilled water.

Solution #2. Na_2HPO_4 (M.W. 141). Dissolve 9.47 gm in 1 liter of distilled water.

For 60 ml of phosphate buffer, mix 11.5 ml of Solution #1 with 48.5 ml of Solution #2.

Cofactors and Amount of Each Added

Fumarate	6.5 mg
Nicotinamide	6.6 mg
ATP	30.9 mg
AMP	16.5 mg

Cofactors and Amount of Each Added (continued)

NAD	7.5 mg
3'5'-Cyclic AMP	.27mg
Glucose-6-Phosphate	9.3 mg

LH-FSH

FSH	3 mg/1500 ml blood
LH	.08 mg/1500 ml blood

Scintillation Fluid

PPO	4.0 gm
POPOP	0.3 gm

Mix in 1 liter of spectro-quality Toluene in an Amber jar.

VITA

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Master of Science

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