PROGESTERONE SYNTHESIS BY THE PERFUSED BOVINE OVARY

By

Roy Clayton Mills II

Bachelor of Science

Southwest Texas State College

San Marcos, Texas

1964

Submitted to the faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
July, 1967

PROGESTERONE SYNTHESIS BY THE PERFUSED

BOVINE OVARY

Thesis Approved:

M. C. Marrisett

Thesis Adviser

L E McDonald

Dennis D. Goetsch

A A Gerhan

Dean of the Graduate College

ACKNOWLEDGMENTS

The author expresses sincere gratitude to Dr. M. C. Morrissette for his guidance, as well as his patient understanding, throughout the period of this study. This feeling of gratitude is extended to the entire faculty of the Department of Physiology and Pharmacology, who have all given knowledgable advice when approached with various technical problems. The author also expresses sincere appreciation to his wife for her patience, sacrifices, and encouragement during the period of this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The need to study living tissues isolated from the influences of neural and humoral factors in the body has led to the development of *in vitro* techniques. *In vitro* studies have been performed using models representing different levels of cellular or tissue organization such as isolated enzyme systems, subcellular fractions, homogenate and tissue slice preparations, and isolated organ perfusions. These studies have indicated that anatomical structure, compartmentalization, and microcirculation are important in the normal physiological function of organs. Therefore, a high level of tissue organization is desirable and justifies isolated intact organ studies.

Many of the functions of bovine luteal tissue have been based on corpus luteum slice incubation techniques. The question arises whether this *in vitro* model is representative of the *in vivo* system. Therefore, it was postulated that the isolated ovarian perfusion technique would provide a more nearly physiologic approach to the study of bovine luteal function. In this report, isolated bovine ovaries were perfused with a blood substitute of known composition in an attempt to develop a technique for the study of intact bovine luteal tissue in the absence of not only neural but humoral factors contained in donor blood.

CHAPTER II

LITERATURE REVIEW

Isolated Organ Perfusion

Perfusion of the whole organ appears to provide a more nearly physiologic approach to the study of metabolic and synthetic activities than homogenate, slice, or subcellular fraction preparations. Isolated organ perfusion eliminates interferences by *in vivo* systems but does not interrupt normal histological arrangement of the organ. Another advantage of organ perfusion over slice and homogenate preparations is the transport of substrates and metabolites by the intact microcirculation. With both slice and homogenate preparations, substrate concentration declines and the products of reaction accumulate. Hechter et al. (12) state,

> Perfusion without recirculation of the perfusate provides an *in vitro* metabolic system wherein substrates can be administered at a constant concentration during the course of experimentation and products of reaction may be removed continuously in a manner comparable to the *in vivo* situation.

If the products of reaction are not removed they may inhibit the rate of reaction or may be converted into secondary or tertiary products. Hechter et al. (12) compared the perfused organ to an organized collection of tissue slices wherein the circulatory apparatus was employed to permit entry of substrates into the cells. Factors which were thought

to influence the rate of biochemical reactions in a perfused organ were capillary hydrostatic pressure, permeability of substrate through the capillary wall, and a possibility of change in the number of vascular channels.

The use of the perfusion technique for the study of biochemical processes in an isolated organ is based on the assumption that the organ under investigation is carrying out, when supplied with adequate nutrients and substrates, most of its normal biochemical reactions. The isolated organ when perfused with blood cannot be expected to be in the same physiological state as in the body, for the integrating and coordinating action of nerves and endocrine glands are completely absent, and homeostasis on which the proper functioning of each individual organ depends is difficult to achieve and maintain. Despite these limitations one would expect that the normal biochemical reactions of the tissues are proceeding and that at least qualitative information regarding these can be obtained by the use of this technique (16) along with the true unmodulated physiology of the organ.

Ewing (8) has reviewed the literature concerning isolated organ perfusions prior to 1960 and reported that the idea of organ perfusion dates back to some time before 1813 when "LeGallois expressed the notion of organ culture via artificial perfusion". Ewing's report indicated that among the organs that had been perfused were the mammalian and Elasmobranch hearts, rabbit kidneys, dog and rat livers, tibias of the dog and cat, dog brains, cat spleens, bovine udders, and stallion testes.

Other organs that have been perfused include the pancreases of rat (3), rumens of goat (5), rabbit testes (8), bovine and sheep adrenals (12), rabbit spleens, thymi, livers, and lungs (11), placentae of guinea

pig (7), sheep (1), and human (28), and the ovaries of the sow (6,33) and the cow (23).

Ewing and Eik-Nes (9) evaluated perfusion in vitro as a method of investigating testosterone biosynthesis and secretion in the rabbit testis. They were able to prove the feasibility of using a perfusion method to demonstrate testosterone biosynthesis and secretion. It was shown that 1) accumulation of testosterone in the venous effluent of the perfused testis was the result of biosynthesis and secretion rather than passive leakage from the organ; 2) the secretion rate of testosterone in their system was independent of testis size, venous blood flow, and glucose supply to the testis; 3) integrity of the enzymes involved in testosterone biosynthesis were maintained during perfusion for up to 7 hours; 4) both acetate-1-^{14}C and cholesterol-7α-^{3}H were incorporated into testosterone; 5) no solubilizing agents were needed in their preparation for the biotransformation of cholesterol-7α-^{3}H to testosterone-^{3}H; 6) addition of HCG or ICSH to the perfusate resulted in increased testosterone secretion and alterations in rates of incorporation of acetate acetate-1-^{14}C and cholesterol-7α-^{3}H into testosterone; 7) slices of rabbit testis produced radioactive testosterone when incubated with cholesterol-7α-^{3}H and acetate-1-^{14}C, and when gonadotropins were added to such incubations, the specific radioactivity of testosterone-^{14}C increased whereas that of testosterone-^{3}H decreased; and 8) the ICSH may have its predominant effect in promoting biosynthesis of cholesterol from acetate both in perfused and in slices of rabbit testis.

Kumar et al. (16) felt that the perfusion technique offered the best means to investigate several problems connected with milk secretion in the mammary gland of cows. They found that perfusion of the bovine

mammary gland with blood hyperventilated with pure oxygen produced an apparent constriction of blood vessels resulting in a pronounced lowering of the rate of blood flow through the gland. The use of 5% carbon dioxide in oxygen or the addition of chloral hydrate prevented this vasoconstriction. It was observed that the time lapse between the shedding of blood and the beginning of the perfusion, the method of suspending the gland, and the pumping mechanism had definite effects on the rate of blood flow. Thorough filtration of the blood and passage of the blood through a lung appeared to have a beneficial effect.

Miller et al. (20) perfused the isolated rat liver and reported that perfusion of the liver with oxygenated blood permitted the liver to synthesize plasma proteins, to separate them from its own tissue proteins, and to contribute them to the circulating plasma in a manner closely approximating the physiological observed in intact normal animals. Their perfusion procedure afforded a general method for the in vitro study of the factors affecting protein synthesis in mammalian tissue. Comparison of perfusion methods to homogenate and slice methods revealed that the perfused liver repeated its action quantitatively when a second dose of C^{14}-labeled substrate was given, when measuring 1) the removal of amino acids, 2) the oxidation of amino acids to CO_2 and 3) the synthesis of the plasma proteins even after a four hour perfusion.

> This is in distinct contrast to the failure of a liver homogenate to respond to a second dose of substrate at any time in spite of the addition of numerous potentially involved factors, or indeed after about 1 hour to respond to anything.(20)

Werthessen and Schwenk (32) perfused the isolated swine liver with defibrinated blood and found that damage to the organ, such as would be encountered during tissue slice studies, played an important part in the

efficiency of the biosynthesis of C^{14}-cholesterol from C^{14}-acetate. Livers mounted in the perfusion system with a minimum of damage converted a minimum of labeled acetate to labeled cholesterol. Additional damage, such as scission of the organ, multiple puncturing of its surface, increased the isotope concentration in the C^{14}-cholesterol. There were two concepts offered to explain the observed results: 1) Damage to the liver may damage the mechanisms which convert newly formed cholesterol into other products or which destroy it. 2) Damage to the liver may stimulate the mechanisms which synthesize cholesterol from simpler substances such as acetate.

In 1953, Hechter et al. (12) described the techniques employed in the perfusion of bovine adrenals. It was pointed out that complexities of the perfusion technique, which are difficult to control quantitatively, limit the usefulness of the perfusion methods in general. However, it was stated that the conversion of cholesterol to corticosteroid hormones had not been achieved in simpler systems. Therefore, studies with perfused glands would continue to be useful until conversions such as this could be achieved in homogenates or slices.

Troen and Gordon (28) studied the effects of human chorionic gonadotropin (HCG) and estradiol-17β upon citrate metabolism in the isolated perfused human placenta and found that estradiol-17β alone had no effect upon citrate utilization. Addition of HCG and estradiol-17β enhanced citrate utilization after a five hour lag period. The addition of HCG alone appeared to stimulate citrate utilization to a lesser degree and after a longer lag period. These results were in conflict with studies in a particulate-free cytoplasmic preparation of human placental tissue. Other workers have reported that estradiol-17β increased citrate

utilization and alpha-ketoglutarate production in the purified preparation. Cellular permeability to estrogen was considered to be responsible for the discrepancy and it was proposed that HCG increased the permeability of the cell membrane to estrogen.

Hems et al. (14) have reported that attempts to study gluconeogenesis in rat liver slices met with major difficulties: The rates of gluconeogenesis obtained with substrates such as succinate, malate, fumarate, glutamate, and aspartate were unexpectedly low or even nil. Lactate and pyruvate formed glucose but less rapidly than expected. They concluded that liver slices had a limited scope in the study of gluconeogenesis and therefore decided to use the isolated perfused rat liver. In the isolated perfused rat liver the rate of gluconeogenesis was considerably greater than that observed in the slice studies and in respect to some parameters was even greater than that observed in the live intact animal.

These examples indicate that where possible, the highest level of tissue organization should be used in physiological and biochemical studies. Indeed, perfusion studies, slices, homogenates, subcellular fractions and isolated enzymes all have their place in research but one must use utmost care in interpretation of data obtained from these deviations from the in vivo situation.

Perfusion Mediums

Ewing (8) listed some of the various perfusates that have been used and has given their composition where feasible. Those listed were heparinized blood, Rosenfeld's artificial medium, White's solution, the perfusion medium of Young, Prudden and Stirman, Long and Lyon's medium,

Gunberg's perfusate, Ringer's solution, Locke's solution, and Tyrode's solution.

Heparinized blood has usually been considered the most physiologic medium (12) but due to the large amounts of blood needed in perfusion techniques and the high cost of heparin, other, less expensive, non-physiological anticoagulants have been used. A glucose-citrate solution (12) has often been used as an anticoagulant. Other investigators, such as Ewing (8) and Werthessen and Schwenk (32), preferred to perfuse with blood defibrinated by shaking with glass beads.

When heparinized or citrated blood was used as a perfusate, it was usually filtered in some manner to remove clots and debris that were present. In some cases the blood was perfused through a rat liver (12) or a rat lung (11) for the purpose of removing small thrombi and foreign material that might have passed through the previous filters. Hechter (11) indicated that perfusing the blood through a lung removed "vasoconstrictor substances" which interfered with the isolated organ perfusion.

When blood was used as the perfusate, homologous blood was generally preferred. Brodie (4), in 1903, reported that use of heterologous blood resulted in toxicity to perfused organs and recommended that blood from the organ-donor animal be used if possible. Hechter et al. (12) have more recently reported that bovine blood was as effective as sheep blood in ACTH-induced steroidogenesis in sheep glands. They indicated that the use of heterologous blood in organ perfusion represented a solution of those problems which arise when the organs perfused are derived from animals which cannot easily supply adequate volumes of homologous blood.

Using labeled progesterone as a substrate, Kitchen et al. (15) perfused the human term placenta <u>in situ</u> with whole blood. They were able

to present evidence for the rapid metabolism of progesterone by the human term placenta. The major metabolites isolated were 20α-hydroxypregn-4-en-3-one and 6α-hydroxypregn-4en-3,20-dione. Neither radioactive androgens nor estrogens were found since little, if any, 17α-hydroxylation occurred under the conditions of these experiments.

Sereni et al. (27) perfused the livers of newborn rabbits using heparinized blood containing labeled cortisol as the perfusate to study the metabolism of cortisol in these tissues. Their results suggested a relative deficiency in the liver of newborn rabbits of the enzymes reducing the A ring and the 20-oxo group of cortisol.

Blood substitutes, also referred to as "synthetic" or "defined" mediums, probably found a place in perfusion studies because of the difficulty in maintaining a successful blood flow through the organ as a result of vasoconstriction when blood was used as the perfusion medium (16). Blood substitutes have also attained wide usage due to the variability and multiplicity of the constituents of blood, many of which are believed to play regulatory roles in the synthesis and/or release of certain products (24).

Rosenfeld (24) perfused bovine adrenals with homologous citrated whole blood and with an artificial perfusion medium. He reported that, upon addition of ACTH, the corticoid output of adrenals perfused with the artificial perfusion medium was approximately the same (less than 7% difference) as that of the blood-perfused contralateral organ.

In 1949, Werthessen (31) reported that it was necessary to use erythrocytes in his perfusion medium (White's solution) to obtain growth as evidenced by healing of inflicted wounds in the perfused human ovary and skeletal muscle. Werthessen (31) perfused the human ovary for

periods up to 5 days in length and stated, "The extent of time during which the organ can be maintained is dependent only on the development of an uncontrollable infection".

Macchi and Hechter (18) found that in the absence of erythrocytes, there was little or no steroidogenesis in perfused bovine adrenals. However, the artificial perfusion medium of Rosenfeld (24), referred to previously, contained no erythrocytes and Rosenfeld stated that although erythrocytes are not needed for maximal stimulation of the glands, their presence lowers the concentration of ACTH required. Rosenfeld used a one-hundred fold concentration of ACTH in comparison to the amount of ACTH used by Macchi and Hechter.

Arnold and Rutter (2) perfused the rat liver to determine if the liver was a source of amylase. Perfusions with whole-blood were unable to provide the answer because of the possibility that an inactive form of the enzyme in the whole-blood may be converted to the active form by the liver. In vivo systems were not dependable since the rat has a very diffuse pancreas making it difficult to isolate liver secretions without pancreatic amylase as a contaminant. The specific activity of pancreatic amylase is three times that of liver amylase. Perfusion of the isolated rat liver with a perfusion medium containing washed erythrocytes allowed them to avoid the problems mentioned and prove conclusively that the liver was a source of amylase.

Dancis (7), in perfusing the guinea pig placenta in situ, used 6% dextran in isotonic saline and found that the placenta rapidly became "leaky" as measured by the transfer rate of ^{22}Na. Heparinized whole blood frequently clotted during the perfusions. Dancis found it simpler to use guinea pig plasma either undiluted or diluted with 1 or 2 volumes

of isotonic saline. Dancis reported that placental perfusion makes possible 1) manipulation of the "fetal circulation", 2) serial sampling from the perfusate, 3) maintenance of constant levels of test substances thus facilitating quantitative studies, and 4) reduction of complications in interpretation resulting from fetal metabolism.

Troen and Gordon (28) perfused the human placenta with a perfusion solution consisting of the following in millimoles per liter: NaCl, 129.5; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 0.5; NaH_2PO_4, 0.5; $NaHCO_3$, 11.9; and citric acid, 5.0. To each liter of perfusing fluid were added 100,000 units of crystalline penicillin and 1 gram of streptomycin. They demonstrated that placental metabolic activity was present for at least eight hours of perfusion of the intact gland and that this activity could be enhanced and/or inhibited.

From these various studies, it appears that maintenance of tissue and of its functions can be accomplished through perfusion of the organ with citrated or heparinized whole blood, or with a blood substitute. Erythrocytes may or may not be essential for this maintenance of tissue and function but are apparently beneficial.

Ovarian Perfusion

In 1949, Werthessen (31) reported on a "technique of organ culture for protracted metabolism studies". In this publication Werthessen indicated that he had perfused and maintained the human ovary as well as skeletal muscle for periods up to five days in length. The primary objective of the study was the metabolism of the steroid hormones. It was felt that findings from dead or dying tissue would be of small value and considerable work was done to prove that the organs were viable. To

demonstrate viability the tissues were cut and almost complete healing of the cut surfaces occurred when the perfusion lasted for sufficient time. A portion of the ovary was also removed for section prior to the perfusion and compared with a specimen obtained at the end of the experiment. These comparisons were favorable in spite of bacterial infection and damage in most cases. Further physiological evidence of function was available in the response of the ovaries to gonadotropin. Addition of 500 R. U. of a gonadotropin caused an approximate three-fold increase in total estrone concentration in the perfusate.

In a later experiment, Werthessen et al. (33) perfused sow ovaries with acetate-1-^{14}C. One ovary was perfused for 44 hours and during this time 1000 R. U. of gonadotropin and 0.1125 mc of acetate-1-^{14}C were added at 20 minutes, 9 hours, and 25 hours after circulation in the perfusion apparatus had started. In a second experiment, which lasted 13 hours, one injection of 1200 R. U. of gonadotropin and 0.225 mc of labeled acetate were added. A mixture of pig's blood and White's solution was used as the perfusate. They concluded that isolated sow ovaries, when perfused with acetate-1-^{14}C, produced labeled estrone, β-estradiol, and cholesterol. The experiments as such did not allow any conclusion as to whether the hormones were derived from synthesized cholesterol-^{14}C, or whether cholesterol and the hormones were produced from a common precursor.

In 1962, Romanoff and Pincus (23) described a technique for the perfusion of the bovine ovary with homologous citrated blood. Perfusion was via the ovarian artery which was cannulated at the abattoir where the organs were obtained and the ovary was flushed with a chilled citrate glucose saline solution. The ovary was then transported to the

laboratory where it was placed in the perfusion apparatus and perfused for 4 hours. During this period it was demonstrated that the ovaries responded to horse pituitary gonadotropin by increasing the rate of accumulation of labeled acetate into various extractable fractions. Considerable counts were incorporated into the progesterone fraction.

The luteal phase swine ovary has been perfused through the ovarian artery with a blood substitute by Morrissette et al. (21). The blood substitute was used to reduce or remove stimulation of the organ by luteotropins which would be present in whole blood. This then would allow addition of known amounts of various luteotropins and/or lysins for critical studies. Ovaries perfused in this manner were found to utilize oxygen for several hours and incorporate acetate-1-^{14}C into ether soluble material. As a follow-up, Cardielhac et al. (6) offered evidence that the labeled acetate in the perfusate was also incorporated into progesterone. There was a net synthesis of progesterone in twelve out of sixteen perfusions. Addition of human chorionic gonadotropin did not increase progesterone synthesis. It was concluded from these experiments that the isolated luteal phase swine ovary perfused with a blood substitute could synthesize progesterone and release it into the perfusate. Addition of gonadotropins was not necessary to obtain progesterone synthesis.

Romanoff (22) reported on steroidogenesis in the perfused bovine ovary using techniques modified from those previously mentioned. In this series of experiments, the perfusate (citrated bovine blood containing acetate-1-^{14}C) passed through the organ only once and the perfusion was therefore termed "monocyclic". Each ovary was perfused for a given period of time before the desired gonadotropin was added. This

was to provide a control period of hormone secretion. Samples were obtained at 15 minute intervals and analyzed for various steroids. Addition of FSH (2.89 µg/ml) to the perfusate caused progesterone synthesis to be increased 3.3 times. This increase could have been due to the LH content of the FSH. Addition of LH (2.5 µg/ml) caused consistent increases in the rate of synthesis of progesterone from perfused luteal ovaries. Infusion of LTH (18 µg/ml) into the perfusate increased the incorporation of acetate-1-^{14}C into progesterone 2.3 times. Following the LTH infusion LH was infused at a concentration of 2.5 µg/ml of blood. The amount of progesterone formed was doubled under the influence of LH and LTH over that of LTH alone. It was concluded that FSH (probably due to its LH content), LH, and LTH all stimulate the production of progesterone in the perfused bovine ovary. The levels of gonadotropin present in the perfusion medium prior to the perfusion were not known.

From the foregoing, it was concluded that isolated organ perfusion afforded the most desirable method of studying progesterone synthesis by the bovine ovary of pregnancy. Blood substitute perfusion medium appeared to have certain advantages over whole-blood perfusate. It appeared feasible to combine the technique of Romanoff and Pincus (23) for the perfusion of the bovine ovary and the technique of Morrissette et al. (21) for the perfusion of ovaries with a blood substitute. The blood substitute would allow exact knowledge of the amounts and/or ratios of gonadotropins administered to the perfused ovary and would therefore provide more meaningful data regarding the responses of the bovine ovary to the various gonadotropins individually, or in combination, and other unmodulated physiologic functions. It was therefore proposed that the techniques of these two groups of workers be combined

and applied to the perfusion of the bovine ovary.

CHAPTER III

MATERIALS AND METHODS

Several bovine ovaries were collected at an abattoir, disected, flushed, and perfused to establish routine techniques. Corpora lutea and perfusates from these trials were extracted and assayed for progesterone to establish analysis techniques. No data were collected from these preliminary studies. The techniques that were developed are described.

Collection of Ovaries

The uteri of cows with unknown reproductive histories were observed as they passed through the processing line about thirty minutes after the cows had been killed. Ovaries were taken only from cows which appeared normal and in early pregnancy as determined by embryo length (less than 25 cm, crown to rump). It was necessary to remove a large portion of the broad ligament along with the reproductive tract to allow proper cannulation of the ovarian artery. The uterine artery was located and traced to a point of bifurcation where the ovarian artery originated. A hemostat was placed on the ovarian artery and the uterus and excess tissues were trimmed away. The ovarian artery is highly convoluted in this area and was difficult to cannulate. Best results were obtained by removing the connective tissue which held the artery in its convolutions and straightening the artery as much as possible.

Cannulations closer to the ovary usually resulted in only partial flushing of the ovary due to subsequent branching of the ovarian artery as it approached the ovary. Cannulas were made of polyethylene tubing (I.D. .034" X O.D. .050") about ten inches long, beveled on one end, and the other end attached to a ten gauge hypodermic needle.

Following cannulation, the ovary was flushed with a cold (12° C) sodium citrate, citric acid, sodium chloride, and dextrose solution (ACD solution) adjusted to pH 7.4 (see appendix). Flushing was continued until all superficial blood vessels appeared clear and the ovary was blanched and chilled. Leaks that became apparent were tied off during the flushing process. Gentle massaging sometimes aided flushing of vessels that did not clear readily. If the ovary did not take on a blanched appearance, it was discarded and another collected since it was assumed that some of the blood vessels were occluded.

The cannulated, flushed ovaries were placed in plastic bags and covered with cold dilute ACD solution. The bags were closed and placed in an ice chest with several layers of wrapping paper placed over the ice to prevent excessive chilling of the ovaries. The ovaries were transported to the laboratory in the ice chest.

Perfusion Mediums

The blood for the whole-blood perfusions, and the blood from which the erythrocytes were taken for the blood substitutes, was collected by bleeding a mature Holstein cow via the jugular vein. The area on the neck was first washed with 70% alcohol and ischemia was produced by pinching to provide an area of pressure anesthesia. A small incision was made through the skin to allow easy insertion of a large trocar into

the jugular vein. The blood then drained through the trocar equipped with a two-foot section of rubber tubing into quart jars containing 140 ml of concentrated ACD solution (see appendix), 400,000 units of penicillin, and 0.5 grams of dihydrosteptomycin sulfate. The trocar was previously coated inside with paraffin and the trocar and rubber tubing were soaked in concentrated ACD solution to minimize the possibility of clotting of the blood during collection. The jars were stirred to ensure quick and thorough mixing of the blood with the anticoagulant. The blood was used immediately or stored over-night at approximately 5°C.

Prior to a citrated blood perfusion, the blood was filtered through several layers of sterilized glass wool. Three-hundred milliliters of blood were used for each perfusion and 50 ml were used to flush the ovary prior to placing it in the organ chamber. One gram of dextran (av. mol. wt. 75,000), previously dissolved in 10 ml of saline, was added to the 350 ml of blood.

Perfusion with a blood substitute necessitated that the blood be collected two days prior to the scheduled perfusion to allow preparation of the blood substitute on the day prior to the scheduled perfusion. Seven-hundred milliliters of medium were prepared for each pair of perfusions allowing 100 ml for flushing purposes and 300 ml for each perfusion. Two-hundred and forty-five milliliters of erythrocytes were needed to obtain a hematocrit of 35 in 700 ml of medium. Therefore, 900 ml of citrated blood (average hematocrit of 33) were centrifuged[1] at 7500 times gravity for 30 minutes at 5°C. The plasma was removed with an aspirator and discarded and the erythrocytes were washed three

---

[1]International High-Speed Refrigerated Centrifuge Model HR-1, International Equipment Company, Needham Heights, Massachussetts.

times. Washing was accomplished by restoring to the original volume with cold (5° C) dilute ACD solution adjusted to pH 7.4 with NaOH and centrifuging as before. The supernate was removed with an aspirator and discarded after each wash. The hematocrit of the resulting washed erythrocytes when divided into 245 ml yielded the volume of erythrocytes needed in 700 ml of medium to attain the desired hematocrit of 35.

Waymouth's nutrient medium[2] (30) was used as the base for the blood substitute. Four-hundred thousand units of penicillin and 0.5 grams of dihydrostreptomycin sulfate were added to 400 ml of the nutrient medium to prevent microbial growth. Fourteen grams of dextran and 7 grams of egg albumin (to provide oncotic pressure), 360 mg of pilocarpine hydrochloride (vasodilator), and 21 mg of sodium acetate (steroid precursor) were dissolved in 50 ml of nutrient medium. The pH was adjusted to 7.4 with NaOH and the solution was added to the washed erythrocytes. Nutrient medium was added to provide a final volume of 700 ml. The medium was mixed thoroughly with the erythrocytes and the hematocrit and pH were determined to ensure quality. The medium was kept 24-48 hours at 5° C in a stoppered Erlenmeyer flask. The perfusions were done in pairs and after 100 ml were removed for flushing purposes, the remaining 600 ml were divided into two 300 ml portions. Hormones and other additives were added just prior to perfusion.

Perfusion Apparatus

The organs were perfused in an apparatus described by Vandemark and Ewing (29) and modified by Morrissette et al. (21) as shown in

---

[2]Waymouth Medium (Medium 752) without serum, Colorado Serum Company, Denver, Colorado.

Figure 1. During the first eight perfusions a flow-through oximeter was included in the apparatus to record evidence that the perfused ovary was viable and utilizing oxygen. When the ovary was viable and utilizing oxygen there was an obvious difference in the colors of the arterial and venous perfusates when viewed directly. The oximeter was therefore omitted from the perfusion apparatus used in the remaining perfusions.

The organ chamber, oxygenation reservoir, and gas bubble trap were wrapped and autoclaved individually. The assembled tubing and all remaining pieces were wrapped together and autoclaved. The apparatus was then assembled and sealed until used.

The perfusions were performed in a constant temperature room maintained at a temperature of 38°C.

Perfusion pressures were monitored throughout each of the perfusions with a Bourdon type pressure transducer and rectilinear polygraph.[3]

Perfusion of the Ovary

After returning to the laboratory with the flushed and chilled ovaries, each of the 300 ml fractions of chilled medium was placed in an oxygenation reservoir of a perfusion apparatus. The medium was oxygenated with 95% oxygen and 5% carbon dioxide until it attained a bright red color. The peristaltic pump was turned on and the medium was allowed to circulate through the already warm (38° C) apparatus. While the medium was circulating and warming one of the ovaries was flushed with 20 ml of the perfusion medium using a 10 ml syringe. The ovary was observed carefully and all leakages appearing before the medium reached

---

[3]E & M Instruments Co., Houston, Texas.

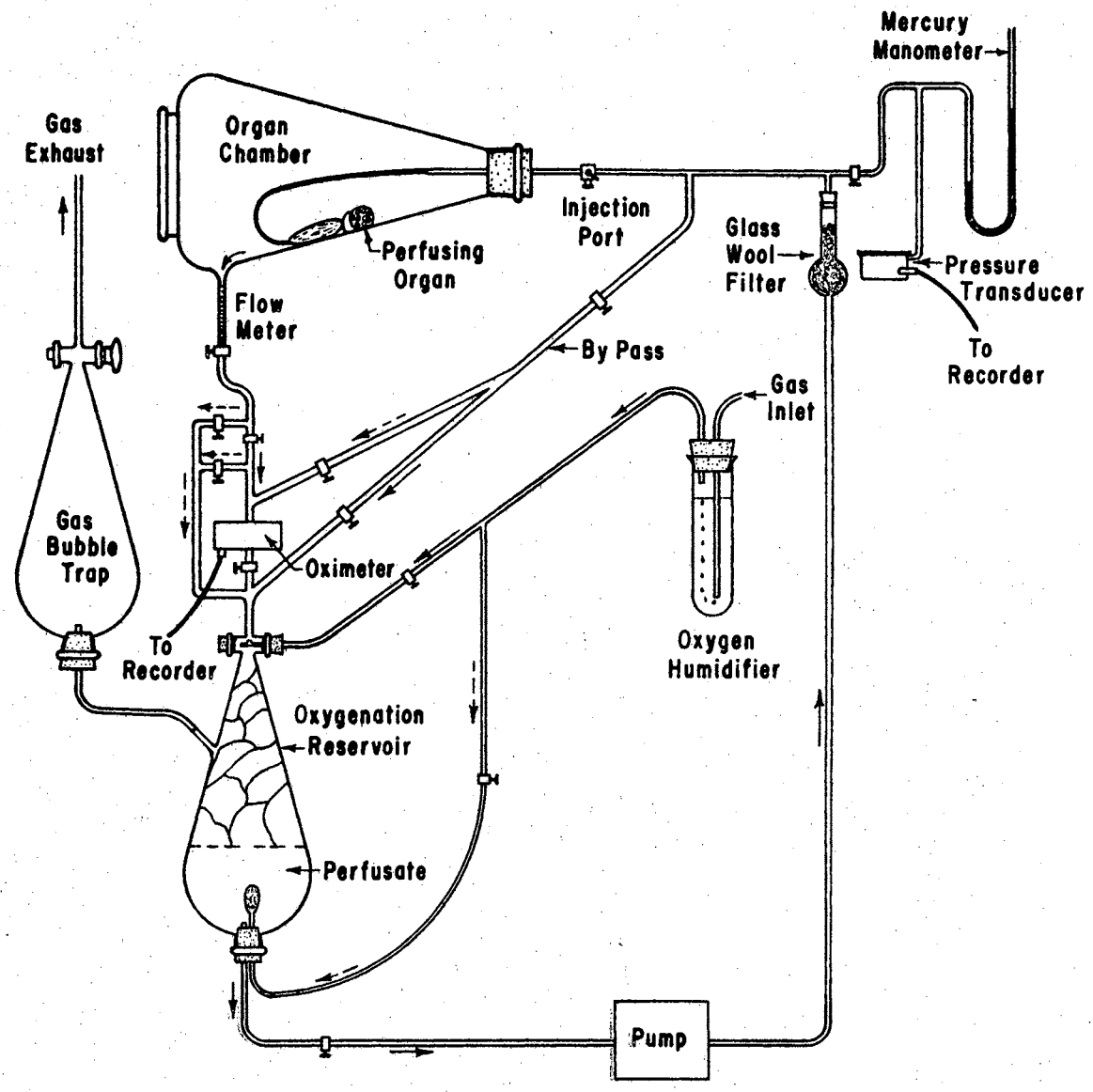

Figure 1. Perfusion Apparatus

the ovary were tied off. The superficial vessels of the ovary and corpus luteum were checked to ensure complete circulation. If the medium flowed through all the superficial vessels and the ovary lost its blanched appearance it was then placed in the organ chamber and its cannula was connected. The pressure was immediately adjusted to 100 mm Hg and maintained at this pressure throughout the perfusion period by adjusting the screw clamp on the by-pass. The organ and perfusate were allowed to warm together to the desired temperature of 38° C. The flow rate of the venous effluent was measured and recorded repeatedly for a five-minute period to determine if the ovary was perfusing properly. Flow rates were measured by timing the filling of the flow meter after closing the clamp below the flow meter. If the ovary appeared to be perfusing satisfactorily the second ovary was prepared in the same manner as the first. If either of the ovaries showed signs of vasoconstriction, as indicated by a rise in perfusion pressure accompanied by a decrease in venous flow rate, 5 ml of pilocarpine hydrochloride (12 mg/ml) were injected into the ovary via the injection port. If an ovary did not appear to be perfusing satisfactorily, it was discarded and replaced. Perfusion pressures were monitored continuously and the venous flow rates were checked frequently during all perfusions.

At termination of each perfusion, the ovary was removed from the organ chamber, all connective tissue was trimmed away, and the ovary was frozen at -20° C to await analysis. The perfusion medium was removed from the apparatus, centrifuged at 7500 times gravity at 5° C, and the erythrocytes were discarded. The supernatant was then frozen at -20° C to await analysis.

Progesterone Extraction and Analysis

Each of the frozen ovaries was partially thawed and the corpus luteum was extirpated from its connective tissue capsule. Working in a cold room (10° C), each ovary and corpus luteum was weighed individually and the corpus luteum was then homogenized in enough cold (5° C) 0.85% NaCl solution to make a 20% homogenate (wt/vol). The homogenate was extracted immediately or stored at -20° C.

Progesterone was isolated from the corpora lutea and perfusates using a combination of methods described by Ryan and Short (25) and Hellig and Savard (13) and modified by Cardeilhac et al. (6). After thawing, 13.2×10^{-3} µc of 7 α-^{3}H-progesterone[4] were added to each 8 ml of the homogenate and 100 ml of the blood substitute which were then extracted with 15 volumes of hot (54-55° C) acetone. The labeled progesterone was added to allow estimation of the recovery of progesterone.

After addition of the 15 volumes of hot acetone, the temperature was maintained at 54-55° C and the mixture was stirred for 5 minutes. The mixture was filtered through #40 Whatman filter paper with suction. The acetone was removed under reduced pressure at 50° C and the residue was evaporated to dryness in vacuo.

To the dry residue were added 5 ml of distilled water and 0.25 ml of 10% NaOH. This solution was extracted 3 times with 10 ml portions of diethyl ether. The ether extract was washed three times with 2 ml of water and the volume was reduced by evaporation under nitrogen to 5 ml which was then transferred to a 15 ml conical centrifuge tube and evaporated to dryness under nitrogen.

---

[4]Nuclear-Chicago Corporation, Des Plaines, Ill.

The dried extract was reconstituted with 2 ml of 90% aqueous methanol. This was passed twice through a three-tube counter-current distribution system using legroin as the upper phase. The three lower phases remaining were combined, evaporated to dryness, and reconstituted to 0.5 ml with distilled water which was then extracted three times with 1.5 ml portions of diethyl ether. The ether was evaporated to dryness under nitrogen and half of the sample was chromatographed by thin-layer chromatography[5] using a chloroform-methanol (99:1) solvent. A progesterone standard[6] was chromatographed on the same plate in the same solvent system. The separated bands were detected by use of an ultraviolet lamp and were marked. The bands most closely corresponding to the progesterone standard in retention time were scraped off the plate and extracted by washing three times with 1 ml portions of diethyl ether. Sedimentation of the silica was hastened by centrifugation. The ether was evaporated and the residue was rechromatographed using a 95:5 chloroform-methanol system. The residues obtained from the bands matching the progesterone standard were subjected to liquid scintillation counting[7] to determine which band contained the tritiated progesterone and the per cent recovery of progesterone.

The quantity of progesterone in each extract was determined by gas-liquid chromatography using a Barber-Coleman Series 5000 instrument equipped with a Model 5121 hydrogen flame detector. Samples were chromatographed on a 3-foot column packed with 1% XE-60 on 80/100 mesh

---

[5]Silica Gel G-HR/UV, Machery, Nagel & Co, Brinkman Instruments, Inc.

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

[7]Packard Tricarb Model 314-B, Packard Instruments, La Grange, Ill.

Gas-Chrom Q.[8] Nitrogen was used as the carrier gas with a flow rate of 48 ml/min from the column. The injection port, column bath, and detector temperatures were 270°, 240°, and 265°, respectively. Peak areas were measured by planimetry and quantitation was accomplished by comparing peaks of unknowns with peaks of known amounts of injected standards.

Experimental Design

Because no previous reports describing perfusion of bovine ovaries with a blood substitute were found in the literature it was not practical to formulate a precise experimental design. Therefore, the results from each series of perfusions determined the design of subsequent perfusions. The sequence of experiments performed are shown in Table I which was formulated in retrospect.

[8]Applied Science Laboratories, College Station, Penn.

TABLE I

EXPERIMENTAL DESIGN

Perfusion Number	Perfusate	Additives
1	Citrated Blood	1 gram dextran, 250 units HCG
2	Citrated Blood	1 gram dextran
3	Blood Substitute	250 units HCG
4	Blood Substitute	None
5	Blood Substitute	Bovine serum replaced 10% of the nutrient medium, 250 units HCG
6	Blood Substitute	Bovine serum replaced 10% of the nutrient medium
7	Blood Substitute	100 mg of porcine α-globulins*, 250 units HCG
8	Blood Substitute	100 mg porcine α-globulins
9	Blood Substitute	100 mg bovine β-lipoprotein*, 250 units HCG
10	Blood Substitute	100 mg bovine β-lipoprotein
11	Blood Substitute	100 mg bovine β-lipoprotein, 1500 units HCG
12	Blood Substitute	100 mg bovine β-lipoprotein
13	Blood Substitute	100 mg bovine β-lipoprotein, 1500 units HCG
14	Blood Substitute	100 mg bovine β-lipoprotein
15	Blood Substitute	100 mg bovine β-lipoprotein, 1500 units HCG
16	Blood Substitute	100 mg bovine β-lipoprotein
17	Blood Substitute	100 mg bovine β-lipoprotein, 1500 units HCG
18	Blood Substitute	100 mg bovine β-lipoprotein

TABLE I (Continued)

Perfusion Number	Perfusate	Additives
19	†Blood Substitute	200 mg bovine β-lipoprotein, 1 mg LH
20	†Blood Substitute	200 mg bovine β-lipoprotein, 1 mg LH
21	†Blood Substitute	200 mg bovine β-lipoprotein, 1 mg LH
22	†Blood Substitute	200 mg bovine β-lipoprotein, 1 mg LH
23	Blood Substitute	Bovine serum replaced 10% of the nutrient medium
24	Blood Substitute	Bovine serum replaced 10% of the nutrient medium
25	Citrated Blood	1 gram dextran
26	Citrated Blood	1 gram dextran

*Nutritional Biochemicals Corp.

†Monocyclic perfusions--all others multicyclic

CHAPTER IV

RESULTS AND DISCUSSION

The preliminary perfusions, performed to develop techniques, indicated that bovine ovaries perfused with citrated bovine blood synthesized progesterone whereas ovaries perfused with the blood substitute synthesized only small amounts of the steroid. Morrissette et al. (21) and Cardeilhac et al. (6) reported progesterone synthesis by swine corpora lutea perfused with a similar blood substitute. Since all techniques were essentially the same as those described by these workers, it was postulated that the differing results were due to different requirements for progesterone synthesis by the ovaries of the two species.

Postulations explaining the lack of progesterone synthesis by ovaries perfused with the blood substitute are: 1) the citrated bovine blood contained specific factors, such as "carrier proteins" (10), which were essential for progesterone synthesis and which were not present in the blood substitute, 2) the citrated bovine blood contained species specific gonadotropins not added to the blood substitute, 3) the blood substitute contained anti-steroidogenic material not present in the citrated blood, or 4) citrated blood provided nutrients not provided by the blood substitute.

As indicated in Chapter III, 26 perfusions (in 3 series) were performed in a probing attempt to find the physiological factors or techniques that would allow progesterone synthesis with a blood substitute.

The results of the first series of 18 perfusions are recorded in Table II.

Prior to the perfusions, control corpora lutea were extracted and analyzed for progesterone. The mean progesterone concentration was 5.1 ± 0.4 µg/gram of luteal tissue. Net synthesis of progesterone for this series was calculated by subtracting the endogenous luteal progesterone, assumed to be 5.1 µg/gram, from the total postperfusion progesterone.

The first pair of ovaries (1 and 2) perfused with citrated bovine blood synthesized 22.5 and 58.1 µg of progesterone per gram of luteal tissue. This level of net synthesis provided a reference level for the blood substitute perfusions that followed.

The second pair of ovaries (3 and 4) were perfused with a blood substitute. The postperfusion progesterone concentrations were lower than the assumed endogenous level and were therefore referred to as negative syntheses. Whenever negative synthesis occurred it was postulated that synthesis did not occur and that the endogenous progesterone was converted to other compounds by the ovarian tissue.

Replacement of 10% of the nutrient medium with bovine serum resulted in net syntheses of 8.7 and 14.2 µg of progesterone per gram of luteal tissue in a third pair of ovaries (5 and 6). These values, though lower than when citrated blood was used, gave support to the postulations previously proposed.

Pursuing the postulation that plasma protein fractions, such as α-globulins and β-lipoproteins, might contribute to steroidogenesis, perfusions were performed containing each of these protein fractions. One pair of perfusions (7 and 8), with α-globulins added to the blood substitute, provided net syntheses of 2.9 and 3.7 µg of progesterone per gram of luteal tissue. These levels were not considered significant but

TABLE II

PROGESTERONE SYNTHESIS BY PERFUSED BOVINE OVARIES

Perfusion Number	Perfusate	Progesterone in Corpora Lutea (µg/gm)	Progesterone in Perfusate (µg/100 ml)	Total Progesterone (µg/gm)	Net Progesterone* Synthesis (µg/gm)
1	†Citrated Bovine Blood	10.6	31.5	27.6	22.5
2	Citrated Bovine Blood	11.8	89.4	63.2	58.1
3	†Blood Substitute	1.2	2.8	2.4	-2.7
4	Blood Substitute	0	4.0	1.4	-3.7
5	†Blood Substitute + Serum	7.9	16.0	13.8	8.7
6	Blood Substitute + Serum	5.0	33.6	19.3	14.2
7	†Blood Substitute + α-Globulins	3.0	19.8	8.0	2.9
8	Blood Substitute + α-Globulins	2.0	22.4	8.8	3.7
9	†Blood Substitute + β-Lipoprotein	2.7	5.2	3.5	-1.6
10	Blood Substitute + β-Lipoprotein	19.2	14.9	24.0	18.9

TABLE II (Continued)

Perfusion Number	Perfusate	Progesterone in Corpora Lutea (µg/gm)	Progesterone in Perfusate (µg/100 ml)	Total Progesterone (µg/gm)	Net Progesterone Synthesis* (µg/gm)
11	ψBlood Substitute + β-Lipoprotein	21.5	16.6	25.3	20.2
12	Blood Substitute + β-Lipoprotein	2.4	13.6	8.8	3.7
13	ψBlood Substitute + β-Lipoprotein	17.0	2.9	18.4	13.3
14	Blood Substitute + β-Lipoprotein	21.3	4.0	23.9	18.8
15	ψBlood Substitute + β-Lipoprotein	4.2	3.2	5.4	0.3
16	Blood Substitute + β-Lipoprotein	3.5	2.1	4.2	-0.9
17	ψBlood Substitute + β-Lipoprotein	2.8	4.6	4.2	-0.9
18	Blood Substitute + β-Lipoprotein	4.2	1.0	4.4	-0.7

*Total progesterone in excess of an endogenous level of 5.1 µg/gm of luteal tissue.

†Contained 250 Units of HCG.

ψContained 1500 Units of HCG.

indicated that a steroidogenic factor could be in the α-globulins. Because β-lipoproteins seemed to be the most likely fraction in α-globulins to provide the steroidogenic factor (10), ten perfusions were then performed with β-lipoproteins added to the blood substitute (9 through 18). Six of the ten perfused ovaries provided net progesterone synthesis. Three of the 6 levels of net synthesis were of questionable significance, whereas the other 3 levels of progesterone synthesized were comparable to the blood substitute-serum perfusions.

Although the number of perfusions performed thus far did not allow firm conclusions, it appeared that the β-lipoprotein may have enhanced progesterone synthesis. The lack of synthesis (negative synthesis) observed could be explained in light of a report by Savard et al. (26). In their studies, using slices of luteal tissues from cows in early pregnancy, they found a number of corpora lutea which they termed "refractory" to gonadotropic stimulation. This refractoriness was attributed to "the inadequacy of the slaughterhouse material".

All of the 18 ovaries just discussed appeared to be viable at the end of each perfusion although net synthesis of progesterone was not observed in all of them. Therefore, the nutrient medium apparently supplied all of the essential nutrients for metabolism. Criteria used to determine viability were: 1) the obvious utilization of oxygen as indicated by arterial-venous perfusate color differences, 2) appearance of cyanosis if the flow rate through a perfusing organ was reduced, and 3) gross appearance of the ovarian tissue at the termination of perfusion. Tissues that had remained viable during the perfusion were not distinguishable from ovarian tissue freshly removed from the donor, whereas non-viable tissues appeared dark and discolored when incised and

examined. It was concluded that tissue maintenance and steroid synthesis were separate entities.

It was recognized from the beginning that one of the major problems inherent in the methods employed thus far was the lack of adequate control. It was evident that an average endogenous level of progesterone may not be a true control value. The wide variability between endogenous levels of two different ovaries could be responsible for an apparent lack of progesterone synthesis or could also account for an apparent net progesterone synthesis. Morrissette et al. (21) circumvented this problem in the perfusion of swine ovaries by using the contralateral ovary as the control. It had been reported prior to their experiments that progesterone concentration in right and left ovaries taken from the same animal was nearly the same (17). No such means of control exists for the perfusion of the bovine ovary because only one of the two ovaries contains a functional corpus luteum at any one time. Romanoff (22) reported that a control could be provided for the perfusion of the bovine ovary by using a "monocyclic" method of perfusion and measuring only plasma (perfusate) progesterone. A control period of progesterone synthesis may be obtained prior to the addition of gonadotropins by permitting the perfusate to pass through the ovary only once. His report was published after the experiments just discussed were completed.

The monocyclic method was utilized for the perfusion of a second series of four bovine ovaries using blood substitute containing bovine ß-lipoprotein to determine if the lack of adequate control was responsible for the inconsistency in progesterone synthesis. The results of these four perfusions are shown in Table III. Progesterone was synthesized by only one ovary during the control period and no progesterone

iii was synthesized by any of the ovaries after the addition of 1 mg of bovine NIH-LH to the remaining blood substitute.

TABLE III

PROGESTERONE SYNTHESIS DURING MONOCYCLIC PERFUSION* OF BOVINE OVARIES

Perfusion Number	Control Sample† (µg)	Treatment Sample† (µg)
19	0	0
20	0	0
21	0	0
22	7.1	0

*Blood substitute perfusate + bovine β-lipoprotein.

†1 mg of bovine NIH-LH was added to the 300 ml of remaining perfusate after 300 ml had been collected for a control.

Romanoff (22) reported control levels ranging from 2.28 to 11.23 µg of progesterone per minute of perfusion time and levels of 3.72 to 29.18 µg/minute after infusion of LH into the perfusate (citrated bovine blood). He did not report flow rates (ml/min.) for the perfusate.

It was concluded that the lack of adequate control was not entirely responsible for the inconsistency of progesterone synthesis when the perfusate was recirculated through the perfused ovaries. This also allowed the conclusion that the bovine β-lipoprotein may not have

provided the factor essential for progesterone synthesis when the blood substitute was used, as was assumed earlier.

A third series of four ovaries was then perfused using the original multicyclic technique to demonstrate that the techniques employed earlier had been satisfactory. Two of the four were perfused with blood substitute to which bovine serum was added as previously described and two were perfused with citrated bovine blood. The results of these four perfusions are recorded in Table IV. A control value for this series was obtained from the analysis of a pooled homogenate of fifteen bovine corpora lutea. A level of 15.1 µg of progesterone per gram of luteal tissue was found and was used as the endogenous control level. Both of the ovaries perfused with citrated bovine blood provided net syntheses of progesterone. One of the two ovaries perfused with blood substitute and serum provided a net progesterone synthesis and the other did not.

Regrouping of the data according to the type of perfusate employed allowed a better evaluation of the results. Table V lists the average net progesterone synthesis of the different groups of perfusions. All four ovaries perfused with citrated bovine blood provided net progesterone syntheses with an average of 54.2 µg/gram of luteal tissue. Three out of four ovaries perfused with blood substitute containing serum yielded net progesterone syntheses with an average of 25.6 µg/gram of luteal tissue for the three. Only eight out of eighteen ovaries perfused with blood substitute alone or with α-globulins or β-lipoproteins added provided net progesterone syntheses. The average synthesis by these eight ovaries was 10.2 µg/gram of luteal tissue and may not be significant.

From these data it was concluded that the blood substitute employed

TABLE IV

PROGESTERONE SYNTHESIS DURING MULTICYCLIC PERFUSION OF BOVINE OVARIES

Perfusion Number	Perfusate	Progesterone in Corpora Lutea (µg/gm)	Progesterone in Perfusate (µg/100 ml)	Total Progesterone (µg/gm)	Net Progesterone Synthesis* (µg/gm)
23	Blood Substitute + Serum	6.4	2.2	7.4	-7.7
24	Blood Substitute + Serum	53.9	31.3	69.0	53.9
25	Citrated Bovine Blood	42.5	116.2	98.4	83.3
26	Citrated Bovine Blood	51.6	56.6	54.0	38.9

*Total progesterone in excess of a control endogenous level of 5.1 µg/gm.

TABLE V

AVERAGE NET PROGESTERONE SYNTHESIS BY OVARIES
PERFUSED WITH DIFFERENT PERFUSATES

Perfusate	Number of Perfusions	Number of Perfusions Providing Net Synthesis	Average Net Progesterone Synthesis (µg/gm)
Citrated Bovine Blood	4	4	54.2
Blood Substitute + Serum	4	3	25.6
Blood Substitute + α-Globulins or β-Lipoproteins	18	8	10.2

by Morrissette et al. (21) for the perfusion of the sow ovary was not adequate for progesterone synthesis by the perfused bovine ovary unless serum was added to the perfusate.

Addition of gonadotropins (HCG or LH) to 13 of the 26 perfusions apparently had no effect on progesterone synthesis even though Romanoff (22) has shown that the bovine ovary perfused with citrated blood is responsive to FSH, LH, and LTH. Further, Marsh and Savard (19) have reported that bovine luteal slices respond to HCG with an increase in progesterone synthesis. The apparent lack of response of ovaries to gonadotropins when perfused with citrated whole blood may be due to the wide variability of function of bovine ovaries (19), or due to metabolism of the synthesized progesterone upon recirculation through the ovary or both. No monocyclic citrated bovine blood perfusions were performed in these probes. Studies of ovarian responses to gonadotropins appear to be restricted to 1) slice techniques where part of the corpus luteum may serve as a precise endogenous level of progesterone or 2) monocyclic perfusion with citrated bovine blood where a reference level of progesterone secretion into the perfusate can be obtained prior to the addition of gonadotropins.

CHAPTER V

SUMMARY AND CONCLUSIONS

The intact bovine ovary of early pregnancy was perfused with homologous citrated blood and with a blood substitute containing homologous erythrocytes. Ovaries perfused with citrated blood synthesized progesterone whereas those perfused with blood substitute did not. Addition of serum to the blood substitute allowed progesterone synthesis but additions of serum protein fractions (α-globulins and β-lipoproteins) had questionable effects upon steroidogenesis. Addition of gonadotropins (HCG and LH) did not stimulate increased progesterone synthesis in any of the perfusions to which they were added. There was adequate evidence that all ovaries were viable after 4 hours of perfusion.

It was concluded that the blood substitute without serum was not adequate for the synthesis of progesterone by the perfused bovine ovary of early pregnancy although tissue viability was maintained.

SELECTED BIBLIOGRAPHY

1. Alexander, D. P., R. D. Andrews, A. St. G. Huggett, D. A. Nixon, and W. F. Widdas. "The Placental Transfer of Sugars in the Sheep; Studies with Radioactive Sugar." J. Physiol. 129:352, 1955.

2. Arnold, Marjorie, and W. J. Rutter. "Liver Amylase III. Synthesis by the Perfused Liver and Secretion into the Perfusion Medium." J. Biol. Chem. 238:2760, 1963.

3. Babkin, B. P., and E. H. Starling. "A Method for the Study of the Perfused Pancreas." J. Physiol. 61:245, 1926.

4. Brodie, T. G. "The Perfusion of Surviving Organs." J. Physiol. 29:266, 1903.

5. Brown, R. E., C. L. Davis, J. R. Staubus, and W. O. Nelson. "Production and Absorption of Volatile Fatty Acids in the Perfused Rumen." J. Dairy Sci. 43:1788, 1960.

6. Cardielhac, P. T., M. C. Morrissette, and J. D. Calle. "Progesterone Synthesis in Perfused Corpora Lutea." Proc. Soc. Exptl. Biol. Med. 123:343, 1966.

7. Dancis, Joseph. "The Perfusion of Guinea Pig Placenta in Situ." Fed. Proc. 23:781, 1964.

8. Ewing, L. L. "A Simplified Technique for Maintaining Isolated Rabbit Testes by Perfusion." (unpub. M. S. thesis, University of Illinois, 1960).

9. Ewing, L. L., and K. B. Eik-Nes. "On the Formation of Testosterone by the Perfused Rabbit Testis." Canadian J. Biochem. 44:1327, 1966.

10. Haurowitz, Felix. in Functions of the Blood. R. G. Macfarlane and A. H. T. Robb-Smith, eds. New York: Academic Press, 1961. 527-547.

11. Hechter, O. "Lymphocyte Discharge from the Isolated Rabbit Spleen by Adrenal Cortical Extract." Endocrinol. 42:285, 1948.

12. Hechter, O., R. P. Jacobsen, V. Schenker, H. Levy, R. W. Jeanloz, C. W. Marshall, and G. Pincus. "Chemical Transformation of Steroids by Adrenal Perfusion: Perfusion Methods." Endocrinol. 52:679, 1953.

13. Hellig, H. R., and K. Savard. "Biosynthesis of Progesterone, Sterols, and Squalene-1-^{14}C and Mevalonate-2-^{14}C by the Bovine Corpus Luteum in Vitro." J. Biol. Chem. 240:1957, 1965.

14. Hems, R., B. D. Ross, M. N. Berry, and H. A. Krebs. "Gluconeogenesis in the Perfused Rat Liver." Biochem. J. 101:284, 1966.

15. Kitchen, J. D., R. J. Pion, and S. H. Conrad. "Metabolism of Progesterone by the Term Human Placenta Perfused in Situ." Steroids, 9:263, 1967.

16. Kumar, Soma, S. Lakshmanan, E. A. Corbin, and J. C. Shaw. "Hyperventilation and Other Factors Affecting Rate of Blood Flow in Perfusion of Excised Bovine Mammary Glands." Am. J. Physiol. 173:82, 1953.

17. Loy, R. G., W. H. McShan, H. L. Self, and L. E. Casida. "Interrelations of Number, Average Weight and Progesterone Content of Corpora Lutea in Swine." J. Anim. Sci. 17:405, 1958.

18. Macchi, I. A., and O. Hechter. "Studies of ACTH Action Upon Perfused Bovine Adrenals: Corticosteroid Biosynthesis in Isolated Glands Maximally Stimulated with ACTH. Endocrinol. 55:387, 1954.

19. Marsh, J. M., and K. Savard. "Studies on the Mode of Action of Luteinizing Hormone on Steroidogenesis in the Corpus Luteum in Vitro." J. Reprod. Fert., Suppl. 1:113, 1966.

20. Miller, L. L., C. G. Bly, M. L. Watson, and W. F. Bale. "The Dominant Role of the Liver in Plasma Protein Synthesis." J. Exptl. Med. 94:431, 1951.

21. Morrissette, M. C., P. T. Cardielhac, and L. E. McDonald. "Perfusion of the Isolated Ovary with a Blood Substitute." Fed. Proc. 25:251, 1966.

22. Romanoff, E. B. "Steroidogenesis in the Perfused Bovine Ovary." J. Reprod. Fert., Suppl. 1:89, 1966.

23. Romanoff, E. B., and Gregory Pincus. "Studies of the Isolated Perfused Ovary: Methods and Examples of Application." Endocrinol. 71:752, 1962.

24. Rosenfeld, George. "Steroidogenesis by Inctact Calf Adrenals Perfused in Vitro." Endocrinol. 56:649, 1955.

25. Ryan, K. J., and R. V. Short. "Formation of Estradiol by Granulosa and Theca Cells of the Equine Ovarian Follicle." Endocrinol. 76:108, 1965.

26. Savard, K., J. M. Marsh, and B. F. Rice. "Steroid Sex Hormones: Gonadotropins and Ovarian Steroidogenesis." Progr. Horm. Res. XXI:285, 1965.

27. Sereni, F., E. Castegnaro, L. Perletti, and G. Sala. "Cortisol Metabolism by the Isolated and Perfused Newborn Rabbit Liver." J. Endocrinol. 35:13, 1966.

28. Troen, P., and E. E. Gordon. "Perfusion Studies of the Human Placenta. I. Effect of Estradiol and Human Chorionic Gonadotropin on Citric Acid Metabolism." J. Clin. Invest. 37:1516, 1958.

29. Vandemark, N. L., and L. L. Ewing. "Factors Affecting Testicular Metabolism and Function. I. A Simplified Perfusion Technique for Short-term Maintenance of Rabbit Testis." J. Reprod. Fert. 6:1, 1963.

30. Waymouth, Charity. "Rapid Proliferation of Sublines of NCTC Clone 929 (Strain L) Mouse Cells in a Simple Chemically Defined Medium (MB 752/1)." J. Natl. Cancer Inst. 22:1003, 1959.

31. Werthessen, N. T. "A Technique of Organ Culture for Protracted Metabolism Studies." Endocrinol. 44:109, 1949.

32. Werthessen, N. T., and E. Schwenk. "Biosynthesis of Cholesterol. Factors Regulating Biosynthesis of Cholesterol-Like Substances in Isolated Whole Livers." Am. J. Physiol. 171:55, 1952.

33. Werthessen, N. T., Erwin Schwenk, and Cyril Baker. "Biosynthesis of Estrone and β-Estradiol in the Perfused Ovary." Science. 117:380, 1953.

A P P E N D I X

Concentrated ACD Solution

Sodium Citrate	77.0 gm
Citric Acid	28.0 gm
Dextrose	85.7 gm

Dilute to 3,500 ml with distilled water

Dilute ACD Solution

Dilute 480 ml of concentrated ACD solution with 2,520 ml of 0.85% NaCl. Buffer with 60 ml of phosphate buffer.

Phosphate Buffer

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

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

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

Scintillation Cocktail

PPO	4.0 gm
POPOP	0.3 gm

Dilute to 1 L with spectro-quality toluene.

VITA

Roy Clayton Mills

Candidate for the Degree of

Master of Science

Thesis: PROGESTERONE SYNTHESIS BY THE PERFUSED BOVINE OVARY

Major Field: Physiology

Biographical:

Personal Data: Born near Sutherland Springs, Texas, January 15, 1942, the son of Willard B. and Viva L. Mills.

Education: Attended grade school in Sutherland Springs and Stockdale, Texas; graduated from Stockdale High School in 1959; received the Bachelor of Science degree from the Southwest Texas State College, with a major in Agriculture, in May, 1964; completed requirements for the Master of Science degree in July, 1967.

Professional experience: Employed as a graduate teaching assistant from September, 1964 to June, 1965, as a graduate research assistant from June, 1965 to February, 1966, and as a NIH Predoctoral Fellow from February, 1966 to July, 1967, in the Department of Physiology and Pharmacology at Oklahoma State University.