70-14,413

BEALL, James Robert, 1940-
LIPID BIOSYNTHESIS BY UTERINE TISSUES DURING NORMAL REPRODUCTIVE CYCLES.

The University of Oklahoma, Ph.D., 1970
Physiology

University Microfilms, Inc., Ann Arbor, Michigan

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

LIPID BIOSYNTHESIS BY UTERINE TISSUES DURING NORMAL REPRODUCTIVE CYCLES

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1969

LIPID BIOSYNTHESIS BY UTERINE TISSUES DURING

NORMAL REPRODUCTIVE CYCLES

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

I wish to thank Dr. N. T. Werthessen for having extended his friendship and enduring patience to me during the last several years. His wisdom and guidance have contributed immeasurably to my personal and my scientific development.

I am also indebted to Dr. A. Kurt Weiss for his valuable suggestions which directed my graduate program.

In addition, I am thankful to the members of the Departments of Physiology and Biochemistry for generously giving their time and efforts to further my education.

TABLE OF CONTENTS

TABLE OF CONTENTS - continued

LIST OF TABLES

LIST OF TABLES - continued

LIST OF FIGURES

LIST OF FIGURES - continued .

LIPID BIOSYNTHESIS BY UTERINE TISSUES DURING NORMAL REPRODUCTIVE CYCLES

CHAPTER I

INTRODUCTION

General Concepts

The basic premise of the studies to be described is that uterine lipid metabolism is important to uterine function. Specifically, this dissertation attempts to show the relation between uterine lipid metabolism and the reproductive cycle of two mammals.

The prime functions of the uterus of any mammal are the maintenance of the life of the preimplantation embryo and the supply of tissue in which implantation and full fetal development can occur. (85) Nutrition of the embryo requires donations from the maternal system to the offspring. It begins when the ovum starts to develop in the ovary and ends when the offspring is weaned. The role of the uterus is limited from tubular fertilization of the ovum to birth of the offspring (i.e. the period of gestation).

The development of a preimplantation embryo is (as with any living organism) under control of two factors; its environment and its inherent growth properties. Embryonic growth must be in harmony with its environment (namely uterine growth) or implantation will not occur.

Harmony is requisite because as endometrium changes in preparation for implantation, it enters a period of transient sensitivity when and only when the blastocyst can implant. If the blastocyst is not developed and is not ready for implantation when the endometrium is in the sensitive period, implantation will not occur. For example, progesterone administration in large doses (5 mg per day) to a female rabbit for three days following mating will accelerate endometrial development.(76) Blastocyst growth, however, will proceed normally. Consequently, the sensitive period will be over before the blastocyst develops and implantation will not occur. Conversely, if the blastocyst is fully developed but the endometrium has not entered the sensitive period, implantation will not occur.(14,57,58,59,73,76)

Obviously, growth and development of the endometrium is under the direction of the ovarian hormones. In many mammals (for example, the mouse, rat, and rabbit), the copulatory act initiates, via hypothalamic reflexes, the hormonal sequelae necessary for environmental development.(85) In other mammals (for example, the rhesus monkey, baboon, and man), the hormonal sequelae necessary for endometrial development is built into the reproductive cycle (with no dependence upon a copulatory act). In both cases, while the endometrium is being prepared for implantation the preimplantation embryo is also developing rapidly.

Since the guiding purpose of this study is to further an understanding of the blastocyst as well as its environment, the origin of a mammalian blastocyst is briefly described.

Following ovulation, the fimbriae of the infundibulum sweep the

ovum from the peritoneal cavity (in a manner not fully understood) into the Fallopian tube. The movements of the tube and its villi propel the ovum along the lumen toward the uterus. The rate of migration is species dependent. Sperm penetration in mammals normally takes place in the ampullae of the Fallopian tube some 20 to 30 hours following ovulation. Sperm penetrates the cumulus oophorus to enter the oocyte and effect fertilization. Exactly how the penetration occurs is not known. Early studies revealed the presence of hyaluronidase in sperm. This enzyme dispersed the cells of the cumulus and therefore offered a logical mechanism for sperm penetration.(85) However, dog sperm does not contain hyaluronidase and yet it can penetrate the cumulus. Hence, the value of hyaluronidase as a necessary enzyme for cumulus penetration is doubtful. Dispersion of the follicular cells does occur during fertilization and is followed by penetration of the corona radiata. Sperm then penetrate the zona pellucida and enter the perivitelline space. According to Shettles (1953) 18 minutes were required for one human spermatozoon to traverse the zona pellucida and enter the perivitelline space.(62) Penetration of the vitelline membrane then places the nuclear material from the sperm in the cytoplasm of the ovum forming a zygote. Next the polar bodies appear and cell replication follows. Some 78 hours later the blastocystic stage of embryonic development is reached.

The entire process of growth and development of a fetus is obviously a continuity. However, gestation can be superficially divided into three phases corresponding to physiological events of nurture. As mentioned above, the uterus first prepares a receptive

environment following ovulation, for the blastocyst. During this phase, fertilization occurs and is followed by cell replication. Secretions into the lumen of the reproductive tract supply the nutritional material necessary for growth and development of the preimplantation embryo. This phase lasts about 5 to 8 days in most mammals but can extend to weeks or longer under special conditions such as delayed implantation. (76,85)

A second phase of nurture exists at the time the blastocyst is in contact with its final implantation locus. Theoretically it becomes possible for the invading trophoblast to derive embryonic nutriment directly from the uterine tissue and blood (which now engorges the endometrial arterioles). This phase permits embryonic nurture as decidua formation occurs and lasts only approximately 24 hours.

Following decidual formation, vascular exchange of materials from maternal blood through placental tissue occurs. This exchange (which defines the third phase) is from the maternal arterioles to the decidual blood lake through the placental membranes to the fetal circulatory system.

The life maintaining nutrition of many fetuses comes from two sources. As mentioned above, the uterus plays an important role. In addition, a portion of the nutritional material is in the ovum (yolk) as it has developed in the ovary. The quantity of yolk is species dependent. Oviparous animals, like some reptiles, produce eggs containing abundant quantities of yolk. The nutritional material for full embryonic development in these animals is supplied entirely by the yolk. No additional material need be supplied by the maternal system.

For example, in the tortoise (Clemmys Marmorata) ovaries fill most of the abdominal cavity after the eggs have acquired their yolk.(49) Yet other reptiles, notably snakes and lizards (again depending upon the species), are viviparous. The maternal system must supplement the yolk with additional nutritional material as the embryo grows. Aves (which are ovoviviparous) also produce eggs with sufficient nutritional material to maintain the life of the embryo without further maternal support. In fowl, the yolk develops in the preovulatory ovum and by the time of ovulation is complete. At this time, the yolk contains large amounts of lipid material and cholesterol.(85) The role of spermatozoa as nutritional donors to the embryo in these animals is not known.

A review of the literature indicates that in mammals the mature ovum and spermatozoa do not contain abundant supplies of reserve nutrient material.(85) Consequently, after fertilization the zygote does not contain sufficient nutrient material to permit development through the blastocyst stage, gastrulation, and implantation. Obviously then, the normal preimplantation embryo, if it is to survive, must receive nourishment from material in its environment. In the first stages of gestation, secretions into the lumen from the Fallopian tubes and the uterus provide most extensively to the nurture of the preimplantation embryo.(45,85) Perhaps the ungulates best demonstrate the necessity of uterine secretions. In the cow, for example, the trophoblast is "loosely attached" to the uterus for some 45 days. Flushing or washing will separate it from the uterus. During this period the blastodermic vesicle is elongating to fill both uterine horns. Only after some 45 days do the caruncles attach to the cotyledons

establishing a direct maternal supply of nutriment.(82) In other mammals, the maternal secretions are also necessary. For example, the acquisition of the mucolemma (a noncellular mucopolysaccharide envelope) by the rat blastocyst is known to be from maternal secretions.(85)

In vitro studies have demonstrated that a blastocyst must reside in an environment containing more than simply a balanced salt solution to remain viable.(69) In vitro development of mammalian ova ceased early in the blastocyst stage (near day 4) in media supplemented with serum. Older blastocysts may grow in vitro for several days, but growth is at a reduced and declining rate.(35,56) If the incubation medium utilizes homologous serum which is continually circulated, regular expansion of the blastocyst is obtained.(56,85) Still, according to Krishnan and Daniel, it has "Not yet been possible to get continued growth of blastocysts, nor growth of cleavage-stage embryos beyond initial cavitation, comparable to the normal."(35) Uterine proteins ("Blastokinins") may be necessary as endogenous blastocystic development regulators.(35) However, aside from its enzymatic needs, the blastocyst also requires nutritional factors for normal growth. (7,39,72,73,83)

For example, when rabbit blastocysts are incubated in vitro, cock serum or chick embryo extract is often used for extended viability. In one study, the cock serum had a glucose concentration of 240 mg per 100 ml and a lactate concentration of 7 mg per 100 ml. During a 24 hour incubation, the glucose concentration decreased by 20 mg per 100 ml as the lactate concentration doubled.(39) Such studies demonstrate that the environment supplies the blastocyst with a portion of its nutriment.

Thus, they support the concept that lumen fluid is a source of nutritional material.

Semen could conceivably donate some nutritional material to the embryo in its earliest stages. The relationship between semen composition and physiology of the female genital tract is largely unexplored. Energy substrates of seminal plasma are introduced into the tubes of intrauterine ejaculatory animals.(82) The concentrations of fructose, citric acid, and ergothioneine were measured along the reproductive tract of the gilt and the mare following copulation.(43) In 40 minutes after mating, the uterine horns of the gilt were filled with semen and with high concentrations of the three compounds. In the mare, 50 minutes after mating, the lumen concentrations of the three compounds equaled semen concentrations. In the gilt only traces of fructose and citric acid were present after 6 hours. Rapid disappearance of the semen components indicated its role as nutritional material would be, at best, limited. However, the possible role of this material as nutriment for a preimplantation embryo was not discussed.(43)

Quantities of semen are also present in the uteri of rats following copulation.(85) However, the import of seminal plasma as nutritional material for the preimplantation embryo in the intravaginal ejaculators is also questionable. In the uterine lumen of rabbits, very little, if any, hexose compounds or phospholipids could be detected following copulation (semen has a high fructose concentration).(85)

To say the least, information of this area of reproduction is inadequate. More work must be done before the role of semen as nutrient material for the preimplantation embryo is known.

Knowledge of fluids of the reproductive tract is needed because the viability of the blastocyst is dependent upon the mater al uterine environment. Hormonally controlled implantation studies substantiate the dependence.(39,56,57,58,72) Such studies are plentiful and are well documented. They demonstrate (as stated earlier) that in most mammals, normal development and implantation of a blastocyst depends upon a harmonic relation between the embryo and the endometrium. Such a relation depends on precise biological timing coupled with proper maternal estrogen and progesterone titres.(14,72,73,74) For example, in the rat, implantation requires a high titre of progesterone for at least 48 hours followed by a surge of estrogen.(73,84) Species differences in hormonal requirements do exist. For example, in the rabbit, only progesterone is requisite for implantation.(29)

One can define the period that the unimplanted blastocyst remains unattached and viable <u>in utero</u> as the "neutral state." The neutral state is created by the hormonal status of the mother and results in delayed implantation.(73,76) In normal sequelae of events, the blastocyst is traversing the Fallopian tubes while the uterine environment is proper for a neutral state to exist. Normally, by the time the blastocyst reaches the uterus, changing hormonal titres are preparing the uterus for implantation precluding a neutral state. (14,73,84)

There are several experimental procedures which will prolong the neutral state (hypothalamic lesions, ovariectomy, hypophysectomy, etc.). A review article by Psychoyos (1964) indicates that all such procedures act through a common mechanism; namely interruption of the hormonal sequence necessary for inducing implantation. (57)

Yechim and Fea reproduced the timing of onset of the period of sensitivity and its duration in intact pseudopregnant rats by controlled daily injections of estrone and progresterone.(73) Their data suggested that "no major alteration in secretory activity need occur to account for both the transient uterine sensitivity and resulting decidual growth." Thus, the study revealed that once the ovary has produced the proper environment for implantation, no further hormonal changes are required for decidualization. If either the progesterone preparation is insufficient or the estrogen surge is postponed, delayed implantation will be induced and normal development will not continue.(76)

While viability of the preimplantation embryo is dependent upon its environment, direct studies of the fluids bathing the embryo in the reproductive tract lumen are few. During fertilization and for 60 hours thereafter, the Fallopian tubes are the key fluid producers. Metabolic studies of the Fallopian tubes of humans reveal that this tissue has a lactate production.(44) Lactate is also found in high concentration in the lumen. Mastroianni (et al.) state that lactate production is the result of the Fallopian tissues' "preference" for anaerobic glycolysis. (44) Pyruvate is not a substrate for growth of a zygote to the 4 cell stage. As the eight cell stage is reached, pyruvate can become a substrate.(7) While the biochemical relation between lactate and pyruvate is well established, the meaning of lactate and pyruvate as regards nurture of the blastocyst is unknown.

After some 3 days, the developing embryo enters the uterine lumen where endometrial growth and secretion are of maximal importance.

Fluid studies of both the uterus and the Fallopian tubes are

limited because only small quantities of fluid are available for analysis.(15,33,45) Blandau, et al. analyzed rat uterine fluid pH at various periods in the estrous cycle.(9) They state that small fluid volumes were present and they were able to perform the study because the technique permitted pH measurements of 0.001 ml of fluid. During estrus in the rat, larger volumes of uterine fluid can be collected. This varies from 0.1 ml to 1.5 ml.(9) Pooled rat uterine fluid samples (from 19 to 45 rats per pooled sample) have been studied for electrophoretic properties. Such a study could obviously not yield information on any single rat. (It did demonstrate that the protein patterns of rat uterine fluid were characteristic only of this fluid and were unlike the distribution patters of rat serum.)(33) As stated by Lutwak-Mann, a biochemist, "It has often been vaguely assumed - generally on the basis of microscopic, instead of direct observation - that there is ample 'uterine milk' available, but when an actual attempt is made to assess the quantity of secretion available per rabbit uterus little of it is found."(40) In a non-pregnant rabbit, there are from 20-80 milligrams of fluid per uterus at any given time.(40) Because of the small quantities of these fluids produced by rats and rabbits, ligation of the uterus is usually a necessary step for fluid collection. Clewe and Mastroianni used ligation in the development of a method of continuous collection of rabbit uterine milk.(15) They found secretion rates to range from 0.02 ml to 0.13 ml per hour.

Lipid analysis of uterine fluid on individual rats or rabbits has not been done. Difficulties of such a study are greatly increased by the limited material available.

Lipids are the major mode of energy storage.(78) The question could well be asked if the uterus produces and stores lipids which are later used as an energy source for the preimplantation embryo and for nidation. One indirect approach to the study of this question would be to observe the effects of environmental lipids on blastocyst growth and implantation. In vitro incubation of blastocysts with various lipid solutions would seem to be an appropriate method of study. Aside from the technical problems involved in obtaining an homogeneous solution of lipid material in an aqueous solution, the problem becomes one of determining which lipid or lipid mixture should be placed in the incubation solution. The size of the problem is demonstrated when one considers that the triglycerides comprise only one class of neutral lipids and yet there are some 10 different fatty acids which are commonly esterified to the glycerol fraction. With three sites for esterification, one could have some 10^3 possible triglyceride compounds. In addition, there are other neutral lipid classes (i.e. sterol esters, free fatty acids, etc.) plus phosphate containing lipids. The choice of which lipid or lipid mixture should be used would be from an astronomically large number of possibilities. Studies of uterine lipid metabolism would most likely yield the most useful information as regards the role of lipids produced by the uterus and utilized by the preimplantation embryo.

Uterine lipid biosynthesis has been studied previously under normal and experimental conditions. While many such studies have been reported over the years, no studies have been published which correlate uterine lipid changes with development of a preimplantation embryo and with nidation.

Historical Review

Historically, three methods of analyzing lipids present in uterine tissue have been developed. The first studies used histological methods. Later, lipid chemical analysis became feasible and was used as a method of analysis. In recent years, the availability of radioisotopes has permitted study of lipid metabolism by tracer techniques.

As early as 1896, Westphalen used histological techniques to first study the presence of lipid in human endometrium.(70) He reported that osmiophilic lipids were found in the uterine glands during every phase of the menstrual cycle. Froboese (1924) thoroughly described the stainable fats in human endometrium throughout the menstrual cycle.(22) He found the greatest increase in stainable fats occurred between the 21st and 25th day of the menstrual cycle. The total histologic data relating hormones to endometrial lipids were so conclusive that Black in 1941 proposed that the stainable fats be used as the basis of a bioassay method for determining progesterone activity.(8) He showed that no optimum balance between estrogen and progesterone was necessary in the human for stainable fat deposition to occur. Page, et al. used histological techniques to describe a pattern for human endometrial fat deposition in relation to progesterone activity.(55) According to them, the stainable fat deposits increased with an increase in progesterone activity. Elftman presented a histological description of fluctuations in the Golgi apparatus (which histologically appears to contain a large portion of phospholipids) and in the stainable phospholipids during the rat estrus cycle.(20) He showed that the Golgi apparatus increases during estrus and decreases during other periods of the cycle. He also

demonstrated that the pattern for stainable phospholipids was inversely related to the patterns of the Golgi apparatus. At that time, the function of the Golgi apparatus was not known. Consequently, the significance of these changes was not discussed by him. Recently the Golgi apparatus has been implicated in the production of glycogen.(50)

As indicated by Alden in 1947, osmiophilic epithelial fat most likely does not represent the total intracellular uterine fat since protein bound fat is not visibly colored by osmication or fat-soluble dyes.(2) Berg later confirmed this finding.(6) He used the benzphrene-caffein technique for "masked" lipids and found, in the mouse uterus, nearly as much lipid material in the endometrial stroma as in the epithelium. In 1959, Davis and Alden measured rat uterine fat by both quantitative techniques and histochemical techniques. They studied spayed rats treated with estrogen and progesterone and concluded that stainable fat is different from total uterine fat in its response to the hormones.(19)

There have been several electron micrograph studies in recent years of mouse uterine lipids.(23,52) These too are based on histological techniques. Fuxe and Nilsson used fluorescence microscopy, histochemistry, and electron microscopy techniques to study the ultra-structure of the ovariectomized mouse uterine epithelium.(52) They also extracted lipids from corresponding unfixed tissue sections by one of the following three methods: (1) hot pyridine or (2) ethanol-ether (3:1) for easily extractable lipids, or (3) chloroform-methanol (2:1) for lipids difficult to extract. By correlating data obtained by these methods, three types of lipid granules were identified. (A) Dark

granules which correspond to easily extractable lipids, (B) membrane enclosed lipid bodies which were too few in number to be seen with the light microscope, and (C) dense lipid bodies which are less easily extractable and which they thought may be highly unsaturated fatty acids esterified as phospholipids. Histochemically, glycerides formed the most numerous granules in the basal portion of the epithelial cells. Their electron micrographs demonstrated the presence of granules which supported the histochemical glyceride data. Apically, (again using histochemical analysis) the granules contained a high degree of unsaturated fatty acids. No attempt was made to correlate these findings with the function of the uterus.

One of the earliest quantitative lipid studies of uterine material was performed by Okey, Bloor, and Corner who used histological and quantitative analytical techniques to measure fat deposits in the pig uterus.(54) They found that the amount of cholesterol esters in the pig uterus was small and did not alter during the estrus cycle. In addition, they observed that the lecithin concentration increased slightly from day 7 to day 14 post estrus. This increase averages about 120% above the concentration observed during the rest of the cycle. Six years later in 1940, Van Dyke used chemical analysis to observe changes in the endometrial lipid distribution during the menstrual cycle of the monkey.(6) A rise in total neutral lipid concentration was seen during the follicular phase.

McLennon and Loets (1953) showed, by analysis of endometrial tissue for total lipid and water content, that the lipids were most concentrated during the secretory and menstrual phases in human

endometrium. Total lipid content increased by 150%.(46)

In 1959, two quantitative studies were reported.(19,37) Leathem analyzed for total lipid concentration, cholesterol concentration, and glycogen, in the immature mouse uterus following estradiol and progesterone injections.(37) He found estradiol reduced uterine lipid concentration but increased total lipids. Cholesterol concentration was unaffected by estrogen; progesterone had no effect on total lipids or on cholesterol. Davis and Alden studied quantitative changes in neutral fats and phospholipids in the rat uteri.(19) Their results were expressed as milligrams of lipid per gram of uterine wet weight. They found the phospholipids to be 12.263 (mg/gm) and the neutral fats to be 0.791 (mg/gm) during proestrus. During estrus, both concentrations increased, phospholipids to 12,591 (mg/gm) and neutral fats to 1.279 (mg/gm). In diestrus, the neutral fat concentration increased even further while the phospholipid concentration decreased to 11.479 (mg/gm). Goswami, et al. in 1963, extracted and quantitatively analyzed the total lipids for triglycerides, sterol esters, free sterols, and phospholipids from the mouse uterus during the estrus cycle.(28) They also studied the presence of the unsaturated fatty acids from the triglyceride fractions. They found that the phospholipid concentrations increased during estrus probably as a result of estrogen stimulation. These results correlate well with the results of Davis and Alden. Associated with the increased phospholipid concentrations was a reduction in the triglyceride concentration. This finding did not substantiate the data of Davis and Alden. Differences of results might be attributed to the fact that triglycerides measured by Goswami don't

comprise total neutral fats (as measured by Davis and Alden).

Isotopic tracer studies opened new areas of investigation in uterine lipid metabolism.

Borrell used estradiol benzoate and progesterone treated ovariectomized rabbits to study incorporation of P^{32} into phospholipids. (10) In the endometrium, the largest increase in specific activity of phospholipids followed the progesterone treatment. Estrogen caused no change in specific activity. In the myometrium, estradiol benzoate caused the most rapid incorporation of P^{32}.(10)

The endometrium consistently showed higher incorporation activity than the myometrium. Since PO_4 is incorporated during lecithin biosynthesis, his work supported the data of Okey, Bloor and Corner, who saw increased uterine lecithin in the post estrous phase of the pig estrous cycle.

Emmelot in 1954 used <u>in vitro</u> incubations of acetate-1-C^{14} to study lipid biosynthesis in the mouse uterus.(21) He was interested in the relative abilities of 17-β-estradiol estrone, and estriol, to cause a change in uterine lipid biosynthesis. He used ovariectomized mice pretreated with <u>in vivo</u> dosages of estrogens. Emmelot's results compared the C^{14} incorporation into cholesterol and fatty acids. He found that conversion of acetate-1-C^{14} was promoted to the greatest extent by 17-β-estradiol and to the least extent by estriol. (For later discussion, the reader should note that the uteri in the above study were undergoing regeneration.)

Aizawa and Mueller performed an <u>in vitro</u> experiment using both P^{32} and C^{14} to evaluate the effect of estrogens on lipid synthesis in

the castrated rat uterus.(1) They analyzed for changes in isotope incorporation into fatty acids, cholesterol, and phospholipids. Estrogen was shown to cause "rapid" acceleration of all pathways for lipid production. Following this experiment, there were a series of studies utilizing similar techniques.(25,26,27,51) These papers, which were primarily concerned with elucidation of the mechanism of estrogenic control of cellular function (i.e. DNA activation and RNA synthesis), all have supported the fact that estrogens stimulate uterine lipid biosynthesis. These studies did not attempt to evaluate progesterone effects on uterine lipid metabolism.

Uterine metabolic changes, which are induced by the presence of estrogen, are indeed quite rapid. Szego and Davis have shown that there is a 100% increase in 3'5' cyclic AMP concentration in the ovariectomized rat's uterus in the first 15 seconds following intravenous injection of 17-β-estradiol.(64) The cyclic AMP concentration increase occurs just prior to the increased uptake of specific protein precursors. Other workers, using isotope incorporation techniques, have demonstrated increased incorporation of P^{32} and C^{14} into uterine lipids within 15 minutes following estrogen administration to ovariectomized rats.(1,10,25,26,27,51,63) Uterine growth induced by 17-β-estradiol administration to castrated rats is antagonized by some hormones. Such growth has been shown by Velardo, Hisaw, and Bever to be inhibited by deoxycorticosterone, cortisone acetate, and testosterone.(67)

As demonstrated by the literature cited, there are changes in uterine lipid metabolism. These changes can be induced by ovarian hormones. Few studies of normal uterine lipid biosynthesis were

reported. Consequently, the meaning of the lipid changes as regards the reproductive processes of the uterus is not known. In an attempt to discover the relation between normal uterine lipid metabolism and the reproductive cycle, the following studies, which form the basis of this dissertation, were performed during the past five years.

CHAPTER II

MATERIALS AND METHODS

Descriptions of the methods are best divided into three categories. First, a general introduction and description will be presented. This will be followed by discussion of (2) methods of obtaining the tissue and procedures for incubation and lipid extraction, and (3) methods used in analysis of the lipids extracted from the tissues.

General Introduction

The description of the methods for 3 separate experiments (one human and two animal studies) will follow. Chemical analysis in these studies ranged from standard methods of separation and analysis to the newly developed methods of lipid analysis which are presented in APPENDICES I and II. In order that easy reference can be made to each experiment during the following discussions, a brief description and reference number is presented below. These are listed in chronological order as a function of the time which the studies were started.

> Study I (Normal Human Endometrial Lipids). Analysis of lipids from endometrial tissue incubated *in vitro* with acetate-C^{14}. Preliminary data from this study were presented in my Master's thesis and detailed data were published by Merrill and Werthessen.(5,47)
>
> Study II (Rat Uterine Lipids During the Estrous Cycle). A

study of lipids and C^{14} incorporation during the normal estrous cycle. In this study, vaginal cytology, ovarian and uterine morphology were all used to establish phases in the cycle.

Study III (Rat Uterine Lipids Following Copulation). The final study which investigated uterine lipids in the post-copulatory period. All technological improvements were applied in this study. Both in vivo and in vitro incubations were performed. This study forms the major portion of the dissertation and is therefore presented in the greatest detail.

During the entire period of investigation, many methods were tried, modified and used, or discarded. Consequently, the methods which will be described were in a constant state of flux and improvement. For example, Study I used column chromatography in the beginning. Yet, as thin-layer chromatography became feasible, due to technological advances in other lipid laboratories, these procedures were also applied to Study I and II.

As is elaborated upon in APPENDIX I, the analytical limitation permitted only study of C^{14} incorporation in Studies I and II. Following development of the new analytical methods, Study III included lipid concentration and C^{14} incorporation measurements.

All three studies utilized the following general methods. Tissues were incubated with acetate containing C^{14}. Following incubation, enzymatic processes were stopped by saturating the tissue with chloroform-methanol. An extract containing total lipids was obtained by repeated washings of the tissue with the solvent. This extract was purified by changing the solvent system to chloroform and by removing insoluble contaminants by filtration. Thus, a "pure" total lipid extract was obtained.[1] The phospholipids were then separated from

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[1] A lipid is defined in these studies as being any compound which remains soluble in chloroform and is not removed by the filtration process.

the neutral lipids by precipitation of the phospholipids in acetone and their subsequent filtration. The neutral lipids were then separated into components by various techniques of chromatography.

The complete methodology will be considered in the next two sections: The first section will describe the preparation, incubation and tissue extraction procedures. The second section will present the methods of lipid separation and analysis. Each study will be discussed individually.

Methods of Tissue Preparation and Extraction

Study I, (Normal Human Endometrial Lipids)

The tissues which were used in this experiment were removed at surgery usually via hysterectomy primarily performed on women with carcinoma *in situ* or uterine prolapse. Immediately following surgical removal and opening of the uterus, the endometrial tissue was scraped from the inner cavity with a knife. The endometrial fragments were placed into a test tube containing 5 ml of Hank's solution (a balanced salt solution containing glucose) to best preserve their normal biosynthetic properties. Endometrium removed by dilatation and currettage was not preferentially used in this study because the amount of blood mixed with the tissue fragments was usually extensive. It was virtually impossible to effectively separate the blood from the tissue.

Histological examination was utilized to segregate abnormal from normal endometrium and to "date" the normal specimens.

Fifty ml of blood were removed from the patient (1 to 16 hours) prior to surgery and were centrifuged. The serum was decanted and stored at $4^{o}C$ until needed.

Immediately after its removal, the endometrial tissue was brought to the laboratory and examined in a Petri Dish containing Hank's solution. Blood clots and tissue fragments were separated grossly. The tissue was then cut into strips approximately 1 mm square and 2 to 3 mm long. Two to three representative pieces were placed in Bouin's fixative for histological study.

Ten ml of serum were removed from storage and brought to room temperature in the incubation flask (a 50 ml Erlenmeyer flask). The remaining tissue was blotted on filter paper to remove excess amounts of Hank's solution and placed in the incubation flask. Acetate-1-C^{14} was added to the serum (in concentration of 1 mc per liter).[1] The total incubation system (tissue, serum and acetate) was placed in a Dubnoff Metabolic Shaking Incubator for 4 hours.[2] A gas mixture of 95% O_2; 5% CO_2 was bubbled through water (to saturate the gas with water) and into the incubator at a rate of 2 liters per minute. The water which surrounded the lower 1/3 of the incubation flask was maintained at 37°C.

The tissue was removed from the serum at four hours and was blotted dry on filter paper. The tissue was then placed in a tared beaker and weighed to obtain a wet weight. Then about 2 ml of

---

[1]The stock concentration of the acetate permitted adding 0.1 ml of solution to 10 ml of serum. At this 1 to 100 ratio, no correction was needed for osmolality. In later studies, this ratio was even greater.

[2]Since serum has antibacterial properties which can slow the growth of bacteria within it for a period of time and since bacteria can produce lipids which could not be separated from those produced in the tissues by the methods employed in this experiment, the incubation time was established as four hours. It was thought that four hours would be long enough to allow the tissue to produce measurable quantities of C^{14} activity in lipids and short enough to minimize bacterial growth.

chloroform-methanol (CM; 2:1 vol/vol) were added to the tissue to stop enzymatic processes. A dry weight was obtained by the following method; the beaker containing the tissue and the CM was placed in a desiccator containing P_2O_5. After each 24 hour period, it was reweighed until a constant weight had been recorded for three consecutive days.

It was hoped that some information could be gained about (1) dry weights, and (2) percentage of fat in a tissue, and (3) substrate incorporation. However, measurement of the dry weight of a tissue often required two to three weeks.

After obtaining preliminary data, the long tissue drying process was abandoned for two reasons. The first and most important reason was the lack of any significant yield of information. The second reason the process was deleted was to avoid accidental oxidation of lipids: Oxidation could occur even in a desiccator containing P_2O_5 if the vacuum was temporarily lost.

Following incubation, the serum was decanted into a second Erlenmeyer flask and approximately 50 ml of CM were added to the tissue to stop enzymatic processes and to initiate lipid extraction.

Since the number of steps involved in the lipid analysis were extensive and since the quantitative amounts of lipids that could be produced by such small fragments of tissue were so small, the lipids present in the serum were extracted with CM to serve as carrier lipids. That is, the lipids from the tissue and the serum were extracted and combined to yield a larger quantity of lipids with which to work.

The water which was present in the serum extract was removed in a rotating evaporator. During water removal, CM was added to the serum

extract periodically to maintain the lipids in solution and to facilitate the evaporation of water. In the last series of tissues, the enzyme activities of the tissue were stopped immediately following incubation while the tissue was in the serum, by quick freezing in dry ice and acetone. The water was then removed by lyophilization (which has been shown by Bottcher et al. to have no effect on lipids within the system).(69) The lipids from both the serum and the tissue were then extracted simultaneously with CM.

The extraction of lipids from both the serum and the tissue was done in all cases with CM, regardless of which of the three preparatory procedures was followed. Bottcher's five day extraction technique with CM was used.(11) Approximately 25 ml of CM were added to the tissue and the serum each day. The following day, the CM extract was removed from the tissue and passed through a filter into a 100 ml round bottom flask. Twenty-five ml of CM were then again added to the tissue and serum. The filter paper was then placed in a second flask and saturated with 50 ml of CM. On the second day, the CM extracts were transferred through another filter paper into the same round bottom flask holding the first extract. Following the extractions each day, the excess solvents were evaporated from the round bottom flask by use of a rotating vacuum evaporator. During evaporation, the lower 1/3 of the round bottom flask was submerged in water from a constant temperature bath kept at 40°C. The processes, outlined above, were repeated every day for five days.

Following the removal of the last (fifth) extraction, the tissue was placed in a tared weighing flask. The flask and tissue were then placed in a vacuum desiccator over P_2O_5 until weights within 0.0002

gms of each other were recorded. The dry defatted weight of the tissue was thus determined.

The CM extract included not only lipids which were to be analyzed, but many additional compounds. Only lipids in the following categories, (1) phospholipids, (2) sterol esters, (3) triglycerides, and (4) other neutral lipids, were analyzed. Consequently, chloroform, a solvent which will dissolve all lipids in these classes, was used to define the lipids for the remainder of the experiment. The other compounds were, by the following procedure, eliminated from the extract to be studied. The extract solvents were evaporated to 2-3 ml. Twenty-five ml of chloroform were added and again the solvents were reduced to 2-3 ml. The process was repeated three times. After three repetitions, any compounds which were not chloroform soluble had precipitated. Care was taken to keep the soluble lipids in solution, thus reducing oxidation and possible precipitation losses (due to failure to redissolve any lipids which might precipitate).

The entire process described above of changing lipid solvents is referred to in this dissertation as "slipping over."

All chloroform soluble products were passed through a micro-filter into a round bottom flask.[1] This material was quantitatively transferred to a 25 ml flask and labeled total lipid (TL). Chemical

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[1]Micro-filtration was used several times in the analysis, the technique was the same each time. A micro-filter was placed on a round bottom flask. Minced filter paper (about 10 ml), saturated with the same solvent which was to be passed through the filter, was placed in the filter. Vacuum was applied to the system to remove excess solvent from the filter paper and press the paper to the bottom of the filter. The solution to be filtered was then quantitatively passed through the filter into the round bottom flask (using vacuum).

analysis of the TL fractions will be considered in the second section of methods.

Study II (Rat Uterine Lipids during the Estrous Cycle)

White female virgin rats from Holtzman Co. (approx. wt. 200 gms.) were used in this study. The estrous cycles of 21 animals were recorded for five cycles (by vaginal cytology). At specific times, the animals were sacrificed and the uteri were removed for incubation. Following their excision, the uterine horns were slit longitudinally and placed in 10 ml of Hank's solution for incubation.[1] Acetate-1-C^{14} was added to the medium and the uteri were incubated for two hours under the same conditions as described for Study I. Enzymatic processes were terminated by quick freezing in dry ice and acetone. Water was then removed by lyophilization. Extraction and definition of the lipids utilized CM and chloroform (respectively) and followed the same procedure as did the human study.

Carbon 14 incorporation studies have indicated that a damaged liver will produce cholesterol faster than normal liver.(69) Consequently, uterine trauma was minimized during the entire procedure. In addition, a liver specimen from each rat was incubated to serve as a "control" tissue.

A specimen of the uterus and both ovaries was preserved in Bouin's fixative for later histological examination. Both gross and histological examination of the ovaries and the uterine specimen were

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[1]Hank's solution was the medium of incubation for all animal studies. As evaluation of biosynthesis under extant physiological conditions was desired, no cofactors were added to the medium.

considered with vaginal cytology to establish as accurately as possible the exact period in any given estrous cycle that a rat was sacrificed. The criteria and stage designation used were those described by Long and Evans.(38) Incubation and extraction procedures were unchanged from those described in other rat studies.

Study III (Rat Uterine Lipids Following Copulation)

In an attempt to increase the maximum data yield, all technological improvements resulting from earlier experiences were incorporated into this study.

Virgin female rats were used in this study. Daily vaginal smears were used to establish the estrous cycles of the animals. When a female was in heat, she was placed with a male for mating. Copulation was observed (in most cases). Vaginal smears for sperm were taken frequently (10 minutes to 3 hours) to confirm that a successful mating had occurred. If mating had not been seen, a sperm positive smear was utilized as evidence that copulation had occurred. The time of copulation was designated as time zero (with a maximum error of ± 1.5 hours). The female rats were then sacrificed by cranial fracture at 24 hour intervals, starting with time zero and ending with day 7 postcopulation.

This study contained two parts; the uteri were either (1) incubated in vitro or (2) excised after intraperitoneal administration of acetate-1-2-C^{12} (in vivo incubations).

In vitro incubations. After the animals were sacrificed, the ovaries and the uterine horns were removed and placed in chilled Hank's solution during tissue manipulation. The horns were then minced (with

scissors) and the tissue particles were dried on filter paper. Approximately 200 mg wet weight of tissue were then placed in an incubation flask which contained 10 ml of Hank's solution. Acetate-1-2-C^{14} was added to the incubation mixture in concentrations of C^{14} equal to 0.1 mc per liter of incubating solution. The tissues were then incubated for 2 hours at 37°C under a 95% O_2; 5% CO_2 atmosphere. At the time of sacrifice, a 200 mg liver specimen was also removed and incubated under identical conditions. The ovaries and uterus were examined for number and size of corpora lutea and gross morphology, respectively.

After the tissues were removed from the incubation medium they were washed five times (10 ml each) with normal saline over a Buchner funnel and filter paper to remove excess substrate. They were then placed in 5 ml of CM to stop enzymatic processes and to effect the first step of lipid extraction. The CM and tissue were kept in a 15 ml capped (teflon lined) culture tube at 0°C to await further processing.

In vivo incubations. Sixteen rats (two from each time period post-copulation) were injected intraperitoneally with acetate-1-2-C^{14} (0.1 mc per kg). Two hours after injection, they were sacrificed and the ovaries, uterine horns, a liver specimen, and a blood sample were removed.

The liver specimen and uterine tissues were minced, dried on filter paper and weighed. They were then placed in 5 ml of CM. All samples were stored at 0°C in stoppered culture tubes to await further processing. The ovaries were examined for number and size of corpora lutea. In addition, the uterine horns were examined grossly.

In vitro and in vivo. Lipid extraction of the tissues was

effected by five daily washings with 5 ml each of CM. During the extraction procedure, tissue extracts were stored at $0^{o}C$ and tissues being extracted were stored at room temperature in stoppered tubes.

The CM extract was then slipped over to chloroform by repeated evaporations. The CM extract solution was evaporated to 1 ml (not 2-3), then 10 ml (not 25 as in earlier studies) of chloroform were added to the solution. Following the third such evaporation, the chloroform soluble material was filtered to remove contaminants. The solution was transferred to a 25 ml volumetric flask and raised to volume by the addition of CM. This procedure defined the total lipid extract and was designated the TL fraction.

Methods of Lipid Analysis

Study I (Normal Human Endometrial Lipids)

From each TL sample, four aliquots (1 and 1/2 ml, with and without acetic acid) were taken for liquid scintillation counting of C^{14}.[1] The samples with acetic acid were evaporated three times following three acetic acid washes. This procedure removed contaminating acetate-1-C^{14} from the sample.

The counts per minute (CPM) as recorded by the machine when corrected for background, machine fluctuations, and quench represented an accurate quantitative description of the radioactivity in a sample.

The CPM in the TL fraction were calculated and recorded. The first major separation of lipid classes was effected following determination of the radioactivity in the TL fraction.

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[1] In all studies, multiple samples were used to permit detection of quench effects in C^{14} analysis.

The remaining 22 ml of TL in the 25 ml flask were quantitatively transferred, using CM, to a round bottom flask.[1] The excess CM was then evaporated from the lipids. The solutes were slipped over from CM to acetone in the same manner as the CM soluble products were slipped over to chloroform. Approximately 95% of the phospholipids (as members of a class, not concentration) are insoluble in acetone.(69) Consequently, these precipitate when the solvent is changed from CM to acetone. Following the last evaporation, approximately 50 ml of acetone were added to the round bottom flask. The flask was allowed to stand 12 hours to insure complete precipitation of the insoluble phospholipids.[2] Next, the acetone soluble contents of the round bottom flask were quantitatively transferred through the micro-filter system. This fraction contained the lipids to be separated by chromatography and was labeled "total lipids for chromatography (TLC)."

The round bottom flask, from which the acetone soluble products had been removed, was then placed under the micro-filter. The precipitated phospholipids were quantitatively recollected by washing the filter with CM. This fraction was labeled PL since it contained the phospholipids. Excess solvent was evaporated from both the TLC and PL

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[1] There were some variations in the quantities removed from the various analytical steps in the different studies. In every case, the quantities taken were recorded and calculation back to the total extract was performed during analysis of the data. All reported data, therefore, have been corrected for any such variations in sample volumes.

[2] $MgCl_2$ was added to the total lipids in acetone (in the later part of this study) following removal of the phospholipids which had precipitated. The addition of $MgCl_2$ should cause further precipitation of phospholipids, if any were present.(17) The results were never greatly changed by the addition of $MgCl_2$ indicating that nearly all phospholipids had precipitated in acetone before the $MgCl_2$ was added.

fractions which were then quantitatively transferred to separate 25 ml volumetric flasks and raised to volume.

Four aliquots labeled: (1) 1 ml, (2) 1/2 ml, (3) 1 ml plus acetate, and (4) 1/2 ml plus acetate, were removed from both the PL and TLC fractions and were placed into scintillation vials. The samples were prepared and counted in the same fashion as described for the TL. The counts were corrected for the amount of sample that had been removed in determination of TL activity.

The next step was the separation of the TLC into classes of neutral lipids. Separation was started by evaporating the remaining 22 ml of TLC and "slipping over" to hexane three times. Following the third addition of hexane, the excess solvent was evaporated to 3-5 ml and the TLC fraction was ready to be placed on the column.

The chromatographic column employed was that described by Crider.(18) It was packed with Silica Gel G which had been humidified to 10%. The silica grain size was from 5-25μ in diameter. Thirty grams of silica gel were used per column. A column of silica gel about 26 cm high, obtained from the 30 gms, was used to give the effective separation. The silica gel was never allowed to dry after the hexane was added.[1]

The 3-5 ml of hexane containing the TLC were then placed on the column, taking care to get an even distribution of the liquid on the surface of the silica gel. The first of the five solvents was added to the column. The five eluates were:

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[1] If the silica gel has been saturated with hexane and allowed to dry, it has a much greater attraction to the lipids and consequently they are harder to remove from the column.(81)

Eluate Number	Solvent System
1	60 ml of hexane only
2	228 ml hexane + 12 ml ethyl ether
3	136 ml hexane + 24 ml ethyl ether
4	150 ml hexane + 50 ml ethyl ether
5	200 ml methanol only

As the meniscus of the first eluate reached the surface of the silica gel, the second wash was added. Each successive wash was added just as the meniscus of the preceding wash reached the surface of the silica gel. Solvent flow rates through the column were not accurately controlled.

Constant volume fractions of lipids were collected from the base of the column without regard for the solvent which was passing through the column at that time. After the first four washes had passed through the column, the fifth solvent (methanol) was received *in toto* in a round bottom flask marked "methanol strip." This fraction was evaporated to 2-3 ml and quantitatively transferred to a 25 ml flask and raised to volume.

A one ml sample was taken from the aliquots (numbers 5 through 10 and 21 through 25) and placed in a properly marked test tube.

A "Liebermann Burchard" spot test for cholesterol type compounds was then performed on each sample.[1] Following determination of the volumetric flasks that contained cholesterol compounds, samples from each volumetric flask were taken in varying sizes. Those with

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[1]The Liebermann-Burchard test was performed as follows: The sample solvent was evaporated and the sample was placed in a heated (40°C) vacuum desiccator for 2 hours. One ml of acetic anhydride was added to each sample and time for solubilization was allowed. Lastly, one ml of concentrated sulfuric acid was added slowly to the acetic anhydride. A green color at the interphase indicated the presence of cholesterol type compounds.(16)

cholesterol had a 1 ml and a 2 ml sample taken. The two aliquots prior to and following these fractions had 2 ml and 4 ml samples taken. Five and 10 ml aliquots were taken from all remaining samples. All samples were prepared for counting as described earlier except that the glacial acetic acid treatment was omitted from all but the methanol strip samples.[1]

The C^{14} titres in the samples that reacted with a positive Liebermann Burchard spot test, during the first ten aliquots (usually number 7) to be removed from the column, were totaled. This total represented the activity present in the SE fractions. The C^{14} titres that were found in aliquots 13-15 or 17, depending upon the activity present in them (a definite peak was seen in all cases), were totaled. This total represented the C^{14} present in the TG_3 fraction.

In 90% of the analyses performed, fraction 7 contained all or most of the sterol esters; in the other 10% the sterol esters were found in either fraction 6 or 8. Similarly, the free sterols were usually in fraction 24, and never out of the range of fractions 23-25.

Following the chromatographic analysis, an audit was performed to evaluate the percent recovery of lipids. Any separation in which 85% of the material was not recovered was inadequate and was not used in data analysis.

Study II (Rat Uterine Lipids during the Estrous Cycle)

Analysis of the TL fractions and subsequent separation of the

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[1]Repeated tests revealed no acetate contamination in the SE and TG_3 fractions; however, acetate contamination was found on occasion after fraction 21.

TLC fractions and PL fraction all used the methods described in Study I. However, analysis of the TLC fraction utilized thin-layer techniques. The plates were made with silica gel G. Two plates (one for charring tracer compounds and the other containing the unknown) were developed simultaneously in 7% ethyl ether in petroleum ether. Tracer fractions were then identified by charring with sulfuric acid. Utilization of thin-layer techniques permitted an evaluation of C^{14} incorporation into the free fatty acid fractions in addition to the fractions evaluated in earlier studies.

Study III (Rat Uterine Lipids Following Copulation)

Experiments leading to Study III. These pilot experiments defined the special manner of (1) observing copulation time and (2) controlling incubation conditions and other biological variables. The precise biological conditions described in Study III evolved from these studies. However, the specific analytical techniques used in these experiments were fully comparable to those employed in the final study. In addition, remains of various fractions from the pilot experiments were subjected to several analytical procedures.

Through the courtesy of Dr. Ahluwalia, preliminary studies using gas liquid chromatography were performed in two instances. (1) Samples from the 4 fractions (i.e., SE, TG_3, FS and FFA) from rats (for each day pre-implantation) were evaluated for concentration distribution of individual free fatty acids from the classes. (2) Pooled fractions from 2 rats at day 1 and 2 rats at day 4 were analyzed for specific activity in the various individual free fatty acids.

In addition to these analyses, others were performed. A

thin-layer technique was developed to permit fractionation of the free sterol fraction obtained after column chromatography with the new system. The technique permitted migration of cholesterol 1/2 the distance from the origin to the solvent front (rf = 0.5) and utilized Chromarsheets (Mallinckrodt Co.). For separation, a solvent system of petroleum ether, ethyl ether, and methanol (75:24:1) and a development time of 35 minutes at 25°C was used. The free sterol fractions from several samples were pooled and analyzed.

The origin, solvent front, a diglyceride spot, and the cholesterol spot were analyzed for C^{14} activity and for the possible presence of phosphorus.

Study III - per se. Both parts of this study utilized the most refined techniques which permitted quantitation of lipids as well as C^{14} incorporation.

From the TL fraction (25 ml), 1 and 1/2 ml aliquots were taken for C^{14} analysis and twenty ml were removed for lipid analysis.[1] The remaining portion was saved for confirmative C^{14} analysis if the first two samples did not yield comparable results.

The twenty ml which were taken from the TL fraction were evaporated three times in acetone by the procedure described earlier for chloroform (see page 25). One half ml of $MgCl_2$ in ethanol was then added to the fraction to cause precipitation of the phosphate containing lipids. To insure precipitation, the "solution" was kept at 4°C overnight. Precipitated material was then separated from soluble material

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[1] Recall that carbon activities in the first studies were expressed as counts per minute. In Study III, activity is expressed as disintegrations per minute (DPM).

by filtration. The acetone soluble material was collected in a 10 ml flask and was designated as the TLC fraction. The TLC solvent (acetone) was then slipped over to 5% ethyl ether in hexane by repeated evaporation. The final volume was raised to 10 ml.

The precipitated phospholipids were redissolved from the filter and collected in a 10 ml flask and labeled. One and 1/2 ml samples were taken from both the TLC and PL fractions for C^{14} analysis.

The TLC fractions were now ready for chromatographic analysis. Analysis utilized the newly developed semi-automatic system as described in APPENDICES I and II. Some minor modifications were made after the calibration values in APPENDIX II were obtained.[1] One such modification was a change from a gradient elution system to a graded one (Table 1). Recalibration was again performed and data from the two systems were found to be comparable. The following graded elution system was used to effect lipid separation of the TLC fractions taken from post-copulatory rats.

The methanol eluent, which removed any remaining material from the column, was not run onto the tape. It was, instead, collected in a scintillation vial directly from the base of the column. Following the methanol strip, the column was regenerated (as described in APPENDIX I) and prepared for the next run.

After having been run through the R-4 analyzer, individual

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[1]Any time a change in the system was made, new calibration values were obtained. These values were then applied to correction of data which were collected in the time period covered by that calibration. Consequently, concentration data, as it is presented in the results, have all been calculated using the appropriate calibration values. All data are, therefore, comparable.

classes of lipids were recollected and adjusted to volume in 10 ml volumetric flasks. These samples were designated as sterol esters SE, triglycerides TG_3, free sterols FS, and free fatty acids FFA. From each fraction, 2 and 4 ml aliquots were taken for C^{14} analysis. The remaining 4 ml portions were saved for further study.

TABLE 1

GRADED ELUTION SYSTEM

Eluate Number	Solvent System	ml used	Flow Rate (ml/min)
1	5% ethyl ether 95% hexane	13	.815
2	20% ethyl ether 80% hexane	13	.815
3	50% ethyl ether 50% hexane	15	.815
4	50% ethyl ether 50% methanol	8:3	.815:.612[1]
5	100% methanol	20	4.35

One class of lipids from this study was subjected to further analysis. The pooled samples of free sterol fractions from each 24 hour period from both the in vivo and in vitro studies were analyzed for free cholesterol content. Cholesterol analysis was performed using a Technicon Autoanalyzer (method $N\text{-}24_a$).

[1] As the eluate containing methanol emerged from the column base, the flow rate was decreased to facilitate evaporation of the solvent.

CHAPTER III

RESULTS

Introduction

Before the role of lipid metabolism in uterine function could be investigated, two tissue capabilities were essential: synthesis of fatty acids from acetate and esterification of these acids to yield major lipids. The inability of the investigated tissues to synthesize or esterify these would have prevented further studies by the methods described. No studies have been reported which demonstrate that either human endometrium or rat uterus possess these properties without external hormonal stimulation. The ability of both human endometrial and rat uterine tissues to synthesize lipids is reported in the following data.

Fatty Acid Synthesis and Esterification

As will be discussed in detail later, incorporation of C^{14} into the total lipid extract was observed in 15 incubations of human endometrium (Page 47). For the purposes of this preliminary discussion, during the proliferative phase about 25,000 CPM (per mg defatted and dried tissue) were obtained and in the secretory phase about 6,000 CPM. Thus, endometrial C^{14} incorporation into lipids compared favorably with human liver which incorporated 225 CPM of C^{14} per mg wet weight.(47) Study II included comparisons of C^{14} incorporation by rat uterine and

liver tissues (Table 2). In one rat, the liver was 7 times more active than uterine tissue; in another rat, the liver was only 1.4 times as active.

Human and rat tissues distributed fatty acids containing C^{14} to several major classes of lipids. The distribution is illustrated by the examples presented in Table 3 (from Tables 6 and 10).

TABLE 2

COMPARISON OF ACETATE INCORPORATION INTO LIPIDS BY RAT LIVER AND UTERUS

	Total Lipid Carbon 14 Activity		
Rat No.	Liver	Uterus	Liver/Uterus
88	7325	1058	6.9
87	12350	6925	1.8
89	5400	1662	3.2
66	5600	3888	1.4
69	3800	1088	3.5

The activities are presented as CPM/mgm d-d

TABLE 3

CARBON 14 DISTRIBUTION IN VARIOUS LIPID CLASSES

	Lipid Classes						
	TL	PL	SE	TG_3	FS	FFA	Other
Rat Uterus	338	23	20	93	82	58	47
Human Endometrium	29280	1670	266	699		26650	0

TL=Total Lipid; PL=Phospholipid; SE=Sterol Esters; TG_3=Triglycerides; FS=Free Sterols; and FFA=Free Fatty Acids

Activities are expressed as CPM/mgm d-d

Fatty Acid Population Distribution and Relative Carbon 14 Incorporation

During Study III, the population distribution of fatty acids from rat uterine tissue was determined with the help of Dr. B. Ahluwalia. Gas liquid chromatographic analysis was performed on each of four classes taken from six rats (from day 0 through 5 postcopulation, Tables 4 and 5). No relationship existed between time and fatty acid distribution in any class (Tables 4 and 5). However, fatty acids 16:0 and 16:1 together equaled 30 to 56%, and fatty acid 18:1 represented from 10 to 35% of the total population.

Distribution ratios of C^{14} from acetate into the various free fatty acids for two postcopulation days were determined (Figure 1). The small quantities remaining from Study III necessitated pooling specimens from four rats (two on day 1 and two on day 4). The C^{14} activity of one fatty acid, not identified but eluted between 12:0 and 14:0, was low on day 1 and high on day 4. Conversely the activity of fatty acid 14:0 was high on day 1 and low on day 4. In addition to these acids, 16:1 and 18:1 also had interesting differences between days 1 and 4.

Cholesterol Synthesis

Presumably, these tissues could have incorporated acetate into cholesterol since they synthesized fatty acids. However, neither rat uterine nor human endometrial tissue appeared to synthesize significant amounts of cholesterol from acetate.

Following digitonin precipitation and dibromination, measurable C^{14} was present in only 8 of 19 purified cholesterol samples obtained from human endometrium.(14) Similarly, when free sterol fractions from

TABLE 4

POPULATION DISTRIBUTIONS OF SHORT CHAIN FATTY ACIDS
PRESENT IN FOUR LIPID CLASSES

RAT #	DAY POSTCOPULATION	CARBON CHAIN LENGTH 10	12	12:1	X	X	X	14:0	14:1
				STEROL ESTERS					
52	0	2.3	5.1					7.0	
36	2	7.6	7.4		4.5			7.6	
43	2	2.9	3.3					8.1	
42	3	2.2	4.2					8.2	
37	5	15.9	10.6		6.6			4.8	3.5
40	5	6.1	5.8		3.3	.8	.8	9.6	
				TG3					
52	0	2.9	2.9		2.5	2.7		8.3	
36	2	1.9	3.6		2.6	1.4		5.6	0.9
43	2	2.3	2.2		2.8			7.4	
42	3	4.9	1.9					3.8	
37	5	2.1	4.2		2.2	1.4	1.8	5.1	4.0
40	5	2.6	2.7		1.5			5.3	
				FREE STEROL					
52	0	2.6	2.6					4.4	
36	2	1.9	2.4		2.9			7.7	
43	2	1.6	2.5		1.9	1.0		7.3	0.6
42	3	4.2	3.1		3.7	1.1		6.3	
37	5	2.3	0.9					2.9	
40	5	4.2	6.9					4.5	
				FREE FATTY ACIDS					
52	0		3.6					2.4	
36	2	3.8	5.2		3.3	2.8		10.5	4.8
43	2	2.3	2.5					6.8	
42	3	4.1	3.1					6.3	
40	5	4.8	3.4					8.9	5.1

This table presents the distribution ratios of fatty acids present in four lipid classes as functions of time postcopulation in the rat. The total ratios of the short chain plus the long chain ratios for any given class on any given day equals 1.0.

TABLE 5

POPULATION DISTRIBUTION OF LONG CHAIN FATTY ACIDS
PRESENT IN FOUR LIPID CLASSES

RAT		CARBON CHAIN LENGTH									
#	DAY	15	15:1	15:2	16	16:1	16:2	17	18	18:1	18:2
					STEROL ESTERS						
52	0	4.8			36.4	17.4			12.4	13.3	
36	2	4.0			33.1	10.8			7.2	13.3	4.2
42	3	6.4			28.6	20.4		3.1	7.1	19.9	3.4
43	2	5.8	3.4		33.5	19.5		3.0	8.3	11.8	
37	5	10.6			38.5	9.3					
40	5	5.9	4.3		37.2	15.5			6.2	8.9	
					TG3						
52	0	4.5	6.0	5.6	35.3	18.2			13.2	23.8	
36	2	3.7	2.9		28.2	13.9			6.6	20.7	7.6
43	2	3.1	1.8		32.7	17.0			7.8	22.7	7.9
42	3	3.5	2.2		32.6	14.1			6.0	26.7	8.8
37	5	4.0			28.8	15.1	2.7	3.9	6.8	19.6	2.2
40	5	2.8			23.2	9.8			23.3	30.9	
					FREE STEROL						
52	0	4.3			32.2	16.5			20.2	18.5	
36	2	5.7	3.7		34.0	11.6	2.8		11.2	13.3	2.6
43	2	5.2	4.2		32.3	17.3	2.4		8.9	18.3	
42	3	5.7	4.7		33.8	11.5	2.1		5.8	13.1	13.5
37	5				26.5	4.7			10.6	34.2	17.7
40	5	6.8			40.6	15.3			11.7	13.1	
					FREE FATTY ACIDS						
52	0	4.5			39.3	15.8			14.4	18.7	
36	2	3.7			29.3	14.4			7.0	13.2	
43	2	5.2	3.6		32.5	19.9		3.8	8.3	18.2	
42	3	4.0			38.3	15.5			8.8	18.7	
40	5	5.1			35.2	15.5			14.2	12.6	

This table presents distribution ratios of fatty acids present in four lipid classes. These data complement the data presented in Table 4.

Relative Carbon 14 Activities of Free Fatty Acids
from Rat Uteri. Day 1 Postcopulation vs Day 4.

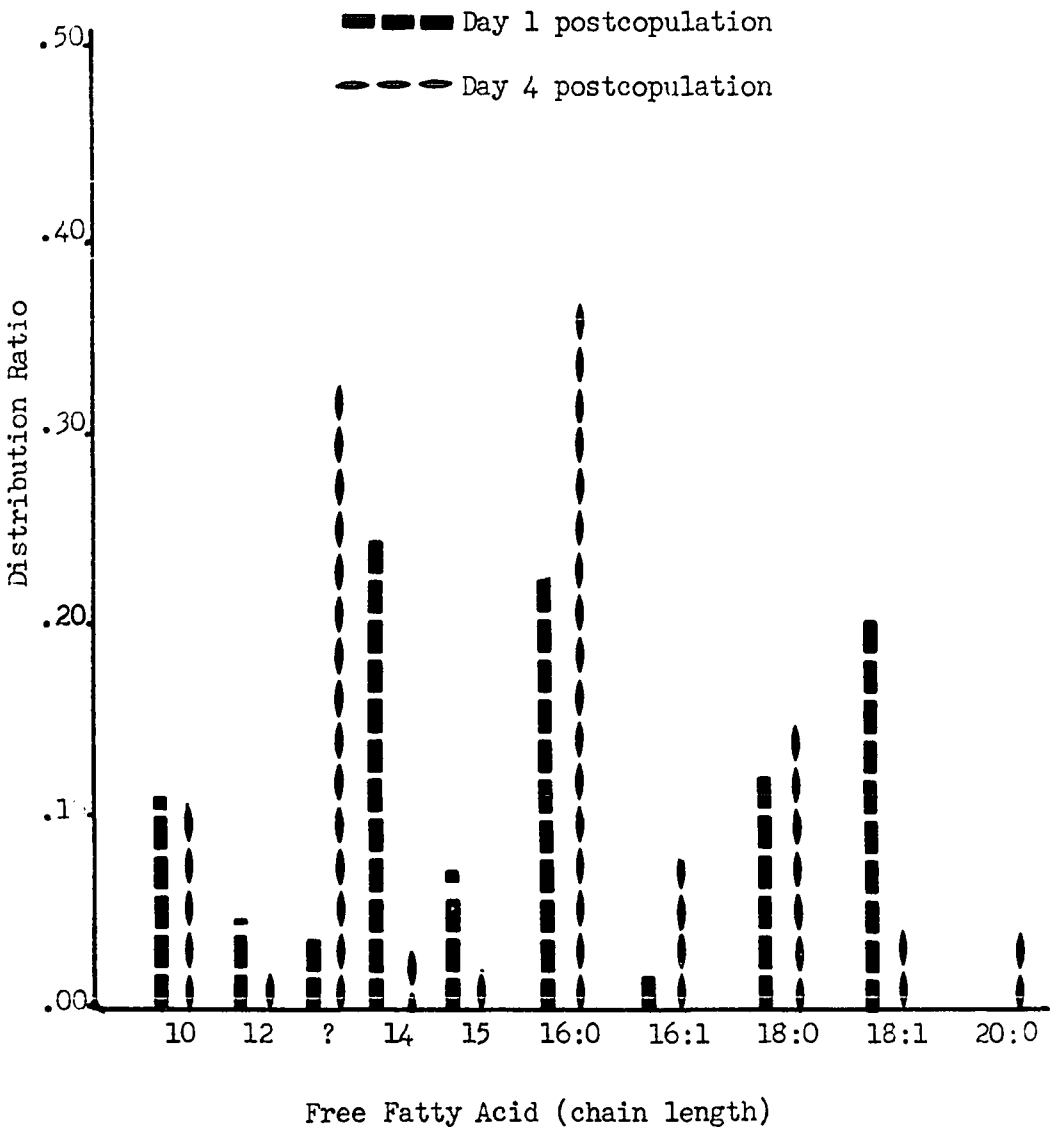


Figure 1 - The relative C^{14} activities were derived from pooled Fatty Acids taken from TG_3, FFA, and TLC fractions. The ratio equals the C^{14} into individual fatty acids divided by the total C^{14} in all the fatty acids.

rat uteri were pooled and subjected to digitonin precipitation, the purified cholesterol contained little C^{14}. Initially, the pooled samples contained an activity of 1705 CPM. Of the 1125 CPM recovered, the soluble portion contained 1100 and the cholesterol contained only 25.

Pooled free sterol fractions from rats were also subjected to thin-layer analysis (Figure 2). Of the four lipid spots developed, cholesterol comprised the greatest concentration: small quantities of diglycerides were also present. Carbon 14 analysis of the four spots demonstrated that the diglyceride area contained 39% of the activity on the plate. The plate origin and solvent front areas contained significant activity and left only 25% of the total C^{14} in the cholesterol area.

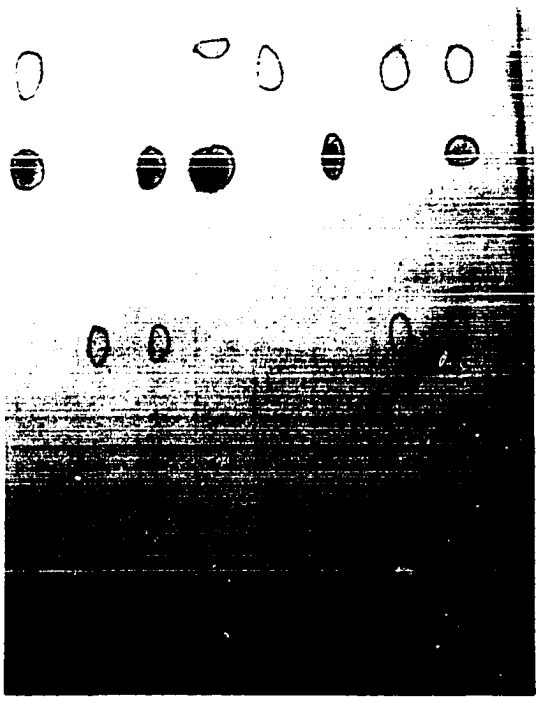
Because the cholesterol spot contained 25% of the C^{14}, and the digitonin purification left less than 2% of the C^{14} with the cholesterol fraction, a phosphorus assay was run on each thin-layer area. A positive result was obtained only for the cholesterol area: The higher counting diglyceride area contained no phosphorus.

Presumably, some newly synthesized fatty acids entered the acetone soluble phospholipids and thus were not removed by the precipitation processes. It is equally possible that the C^{14} was present in compounds other than cholesterol which do not form a digitonin complex but did have similar "relative fronts" in the chromatographic system employed.

Considerations such as these, plus the high C^{14} activity in the diglyceride area, point to interesting future studies.

Results of the studies in the sections above demonstrated

Illustration of the Thin-Layer System for
Analysis of the Free Sterol Fractions



1 2 3 4 5 6 7 8

Sample Numbers

Figure 2 - The following samples were used in this analysis. Concentrations are expressed as micrograms of material.

1) free cholesterol, 31.6; diolein, 75.
2) monolein, 75.
3) free cholesterol, 31.6; monolein, 75.
4) UNKNOWN (free sterol fraction from semi-automatic analysis).
5) diolein, 75.
6) free cholesterol, 16.
7) diolein, 75; monolein, 75.
8) free cholesterol, 16; diolein, 75; monolein, 75.

The solvent system was benzene, ethyl ether, ethanol, and acetic acid (50:40:2.0:0.2). Development time was 40 minutes. Development solvent was phosphomolybdic acid. The chromatographic plate was a Chromarsheet 7 x 9 inches (Mallinckrodt Co.).

unequivocally that rat uterine and human endometrial tissues synthesize fatty acids and esterify these acids to yield major lipids. In addition, these tissues produced little cholesterol from acetate.

Based on the knowledge that these essential biosynthetic functions occurred in human endometrium and rat uterine tissue, esterification patterns of fatty acids could be investigated. Data from each study served as the foundation for each additional study. Results from each study will be presented and will be followed by a discussion, which includes the rationale for the succeeding study.

Study I (Normal Human Endometrial Lipids)

Noyes, Hertig and Rock classified proliferative endometrium from humans as either early (days 4 to 7), mid (days 8 to 10), or late (days 11 to 14); onset of menses was day 1.(53) Secretory endometrium was classified by days. Human data which follow utilized both the menstrual history of the patient and the above classification system for accurate dating of the endometrium. Both dating criteria had to agree before a tissue was considered normal.

Table 6 and Figures 3 through 6 show C^{14} activities of four lipid classes from 15 normal endometrial tissues on which defatted and dried tissues were obtained. Statistical analysis demonstrated that in three fractions greater C^{14} incorporation occurred during the proliferative phase than during the secretory phase (TL, $p < .001$; TG_3 $p < .01$; PL $p < .02$). While the SE fractions appeared to contain more C^{14} activity in the secretory phase, this could not be demonstrated statistically ($p < .20$). Statistical analysis of linearities of regression between time and incorporation were not performed on these data.

TABLE 6
CARBON 14 INCORPORATION BY NORMAL ENDOMETRIUM

	c.p.m. x 10^{-2}/mg.				
Histology	TL	PL	SE	TG_3	NEL
Day 1	292.8	16.7	2.66	6.99	266.5
Day 7	349.	81.1	21.1	56.1	190.8
Day 7	321.7	72.9	1.7	56.9	190.2
Day 9	308.3	107.6	23.4	5.0	72.2
Day 11	103.6	36.2	19.1	16.9	31.4
Day 13	222.3	68.6	8.14	15.2	130.4
Prolif. (mean)	249.6	63.9	12.7	26.2	146.9
Day 17	61.5	11.8	21.1	8.5	20.0
Day 17	35.0	2.9	10.7	0.37	21.1
Day 18	80.9	0.37	33.7	0.64	46.1
Day 21	74.2	8.6	24.7	3.3	37.6
Day 21	45.4	0.91	31.0	0.46	13.0
Day 22	38.8	19.3	10.8	3.2	5.5
Day 22	76.0	28.6	4.4	1.3	41.7
Day 22	90.6	-	-	-	-
Day 26	49.1	0.30	37.3	0.56	10.9
Secretory (mean)	61.3	9.1	21.7	2.3	24.5

The C^{14} titre expressed as counts per minute per milligram defatted and dried weight for total lipid extract (TL), phospholipids (PL), sterol esters (SE), triglycerides (TG_3), and the remainder of the total lipid extract (NEL) of various endometrial specimens.

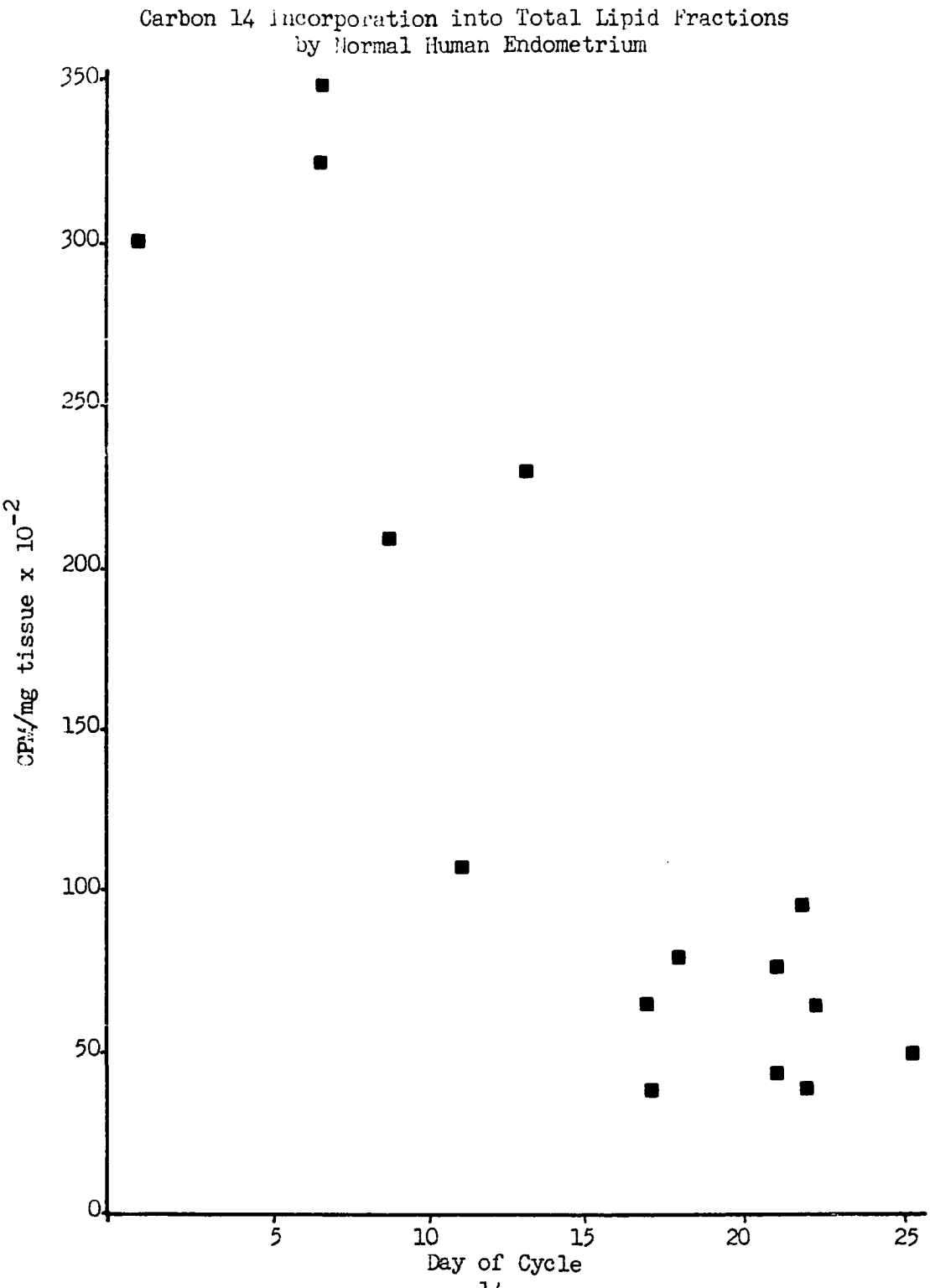


Figure 3 - The acetate-1-C^{14} incorporation into total lipid fractions is illustrated as a function of time in a normal menstrual cycle. Activity is expressed as CPM per mg of defatted and dried tissue from which the lipids were extracted.

Carbon 14 Incorporation into Triglyceride Fractions
by Normal Human Endometrium

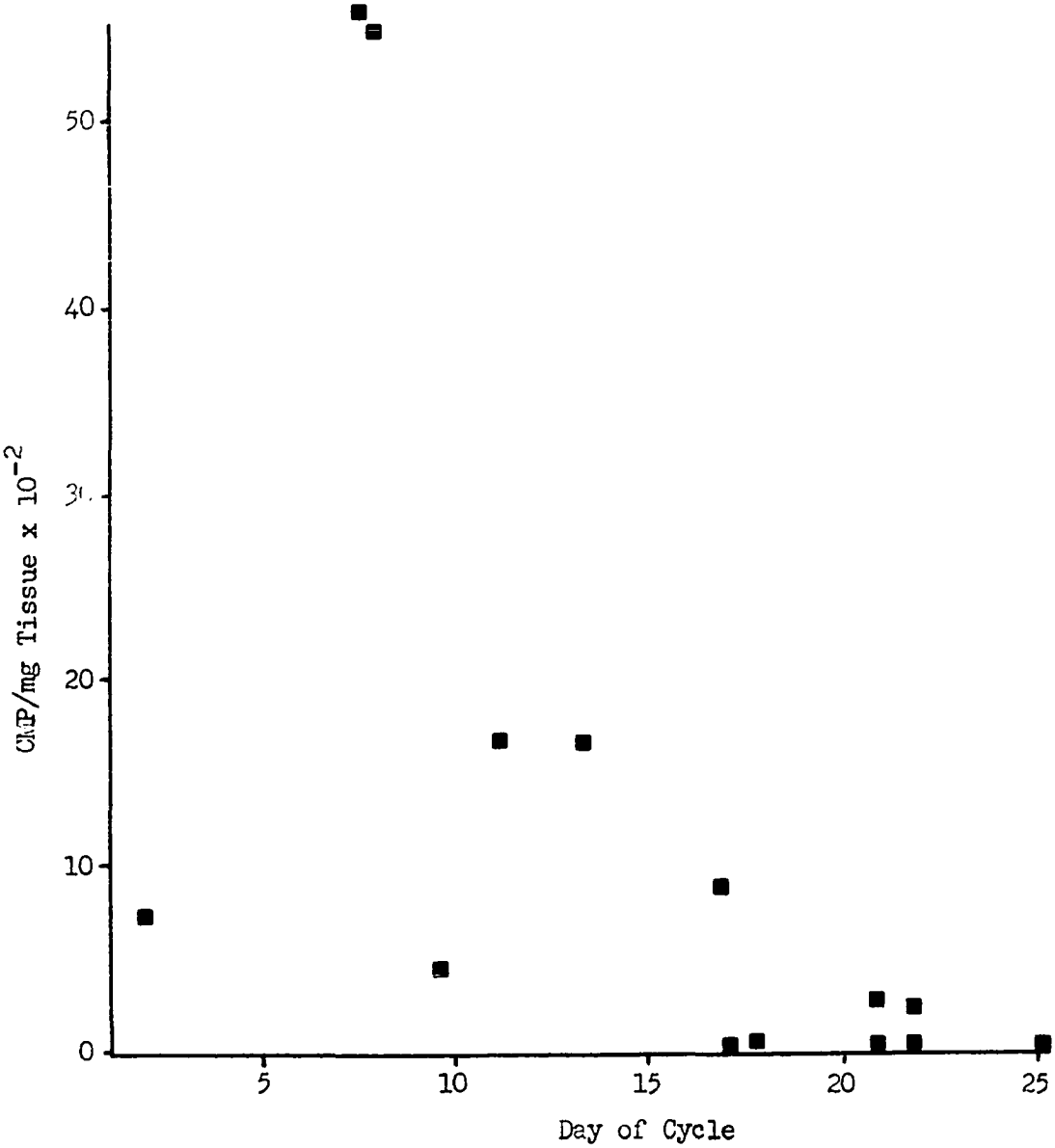


Figure 4 - The acetate-1-C^{14} incorporation into triglyceride fractions is illustrated as a function of time in a normal menstrual cycle. Carbon 14 activity is expressed as counts per minute per mg of defatted and dried tissue from which the lipids were extracted.

Carbon 14 Incorporation into Phospholipid Fractions
by Normal Human Endometrium

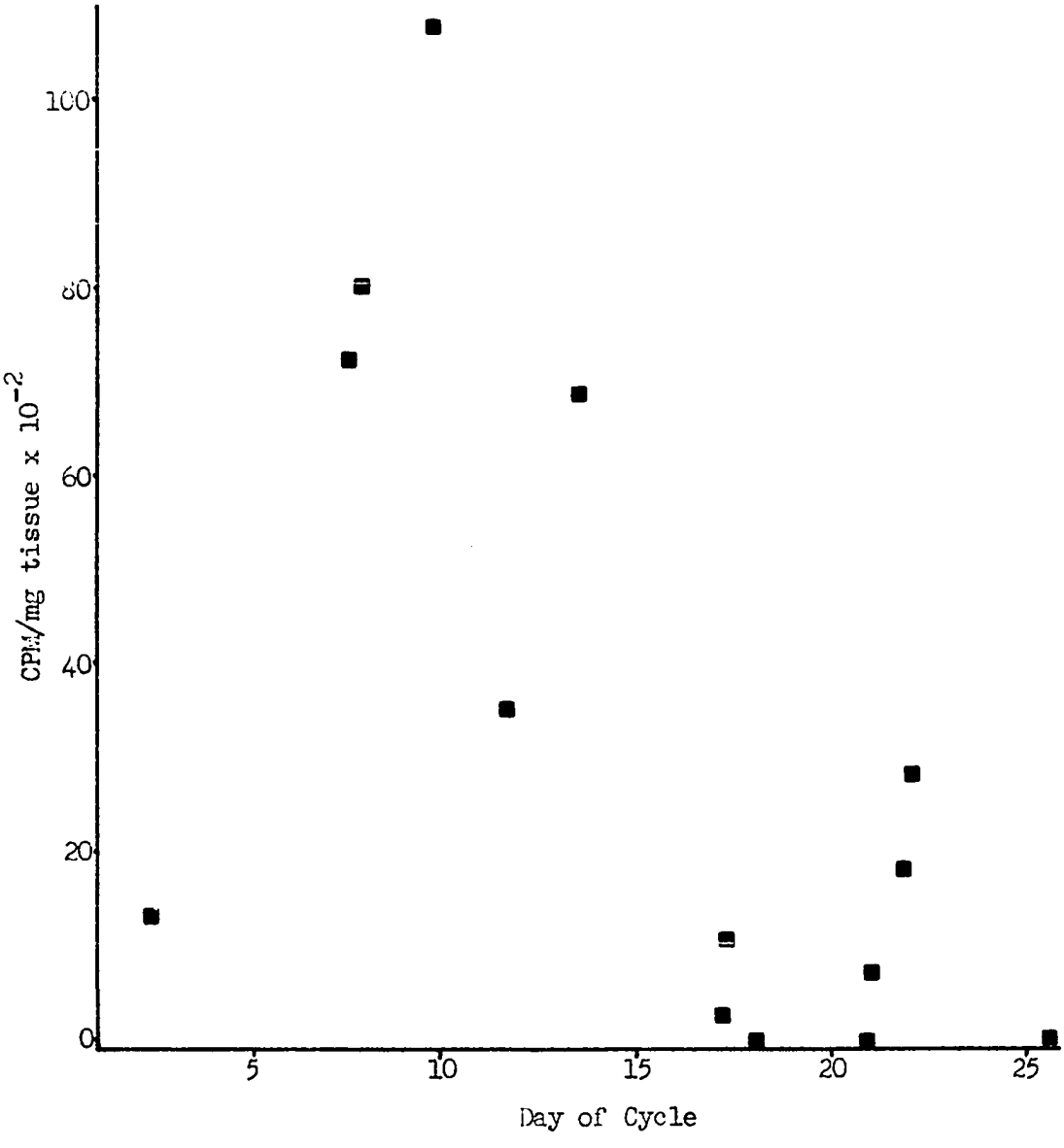


Figure 5 - The acetate-1-C^{14} incorporation into phospholipid fractions is illustrated as a function of time in a normal menstrual cycle. Carbon 14 activity is expressed as counts per minute per mg of defatted and dried tissue from which the lipids were extracted.

Carbon 14 Incorporation into Sterol Ester Fractions
by Normal Human Endometrium

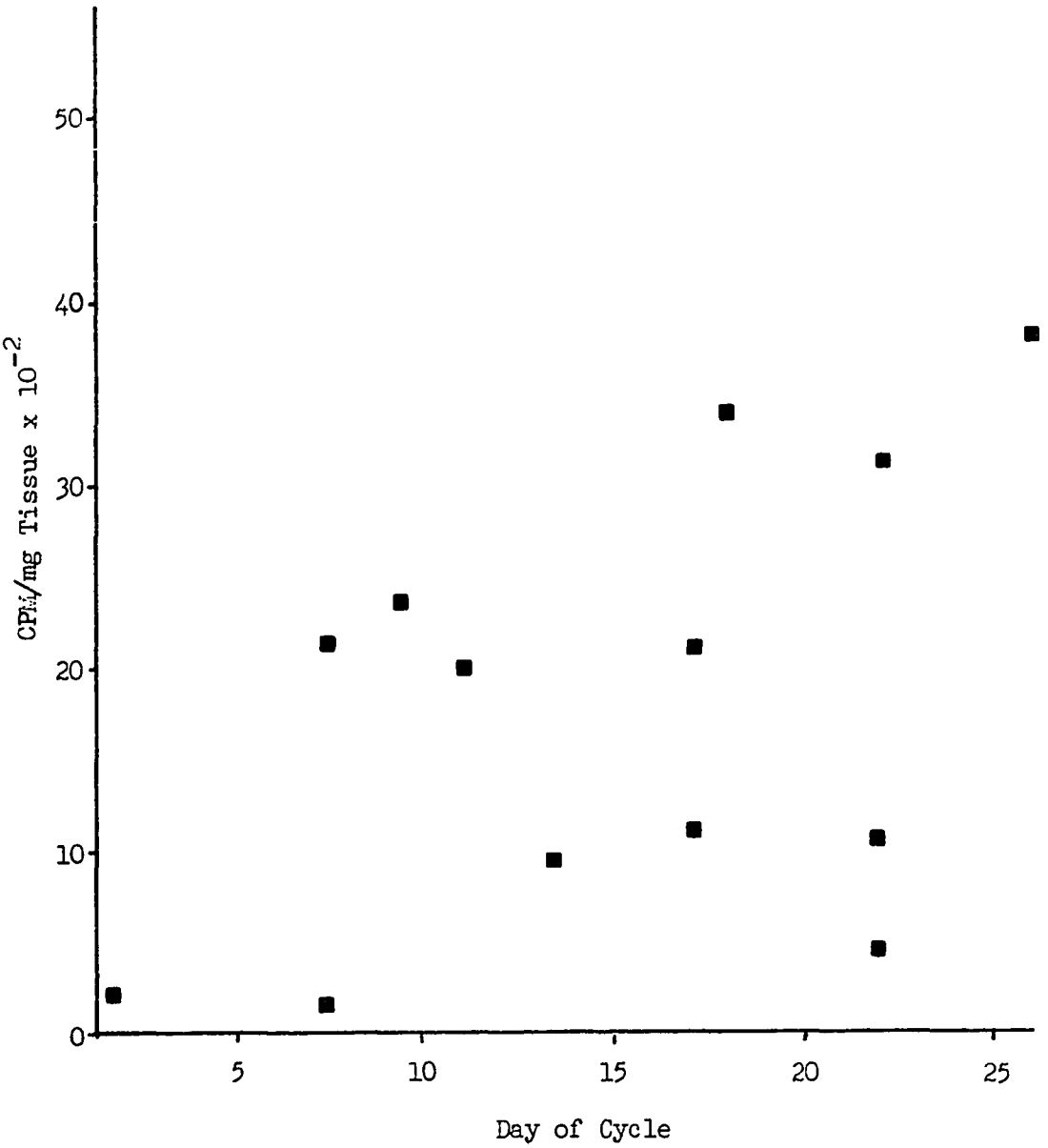


Figure 6 - The acetate-1-C^{14} incorporation into sterol ester fractions is illustrated as a function of time in a normal menstrual cycle. Carbon 14 activity is expressed as counts per minute per mg of defatted and dried tissue from which the lipids were extracted.

Twenty-two additional tissues were incubated and lyophilized with the sera, following incubation. Consequently, tissue and sera dried together and prevented determination of defatted and dried tissue weights; thus only count ratio analyses were possible.[1]

Table 7 presents two series of count ratio data; each was calculated differently. One series utilized three lipid classes ($PL+SE+TG_3$) for computation; the other series utilized only TG_3 and SE fractions.[2] Of the TG_3 and SE classes, most C^{14} entered the TG_3 class during the proliferative phase of the cycle, when estrogen secretion is greatest. The SE fractions contained the most C^{14} during the secretory phase when progesterone is secreted. Figures 7 and 8 present these data in graphic form. A sudden shift in the esterification pattern of newly synthesized fatty acids occurred during the periovulatory period. Day 16 marked the onset of the changes; this was the second day in the secretory phase. Furthermore, day 16 is reported as being the first day when the histological changes are rapid enough to permit accurate endometrial dating.(56) The histological changes reflect the outpouring of progesterone by the ovary.(56) Hence, the change in esterification probably results from the effects of progesterone on the

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[1] The term "count ratio," used frequently in this dissertation, describes a value obtained in C^{14} analysis. The C^{14} activity from any two or more lipid classes is totaled; the sum is then divided into the activity of each class which contributed to the total. Fatty acids are esterified to yield sterol esters, triglycerides or phospholipids: Thus a ratio analysis permits detection of qualitative changes in esterification patterns. Each Table and Figure will present the specific ratios which were determined.

[2] When the PL ratios were included, greater statistical variances occurred (as expected). In addition, count ratios of the SE+PL and TG_3+PL fractions demonstrated no significant patterns of incorporation.

TABLE 7

THE DISTRIBUTION RATIOS OF CARBON 14 INCORPORATION INTO LIPID FRACTIONS BY ENDOMETRIUM

	Distribution Ratios				
		PL, SE, TG_3 / Sum PL+SE+TG_3		SE, TG_3 / Sum SE+TG_3	
Day	PL	SE	TG_3	SE	TG_3
1	0.63	0.10	0.27	0.28	0.72
1	0.52	0.11	0.38	0.22	0.78
1	0.09	0.23	0.68	0.25	0.75
3	0.65	0.17	0.18	0.48	0.52
5	0.24	0.27	0.49	0.35	0.65
6	0.07	0.55	0.38	0.59	0.41
6	0.16	0.14	0.70	0.16	0.84
7	0.03	0.45	0.53	0.46	0.54
7	0.21	0.40	0.40	0.50	0.50
8	0.40	0.31	0.29	0.52	0.48
8	0.30	0.34	0.36	0.48	0.52
9	0.44	0.19	0.37	0.33	0.67
11	0.51	0.26	0.23	0.53	0.47
11	0.03	0.36	0.61	0.37	0.63
11	0.02	0.08	0.90	0.08	0.92
12	0.08	0.45	0.47	0.49	0.51
13	0.75	0.09	0.17	0.35	0.65
13	0.27	0.13	0.61	0.17	0.83
13	0.29	0.26	0.45	0.37	0.63
14	0.26	0.43	0.31	0.58	0.42
15	0.04	0.32	0.64	0.33	0.67
15	0.42	0.25	0.33	0.44	0.56
15	0.10	0.40	0.50	0.44	0.56
15	0.37	0.28	0.35	0.44	0.56
16	(0.003)	0.98	0.02	0.98	0.02
16	0.20	0.72	0.08	0.90	0.10
17	0.29	0.51	0.20	0.71	0.29
17	0.21	0.77	0.03	0.96	0.04
18	0.01	0.97	0.02	0.98	0.02
19	0.37	0.37	0.25	0.60	0.40
21	0.24	0.67	0.09	0.88	0.12
21	0.03	0.96	0.01	0.99	0.01
22	0.58	0.32	0.10	0.77	0.23
22	0.36	0.38	0.26	0.59	0.41
22	0.46	0.21	0.33	0.38	0.62
22	0.02	0.30	0.68	0.30	0.70
26	0.01	0.98	0.01	0.99	0.01

Ratios are obtained by dividing the C^{14} titre expressed in counts per minute by the sum of the fractions considered.
PL = phospholipids; SE = sterol esters; TG_3 = triglycerides.

TABLE 7

THE DISTRIBUTION RATIOS OF CARBON 14 INCORPORATION INTO LIPID FRACTIONS BY ENDOMETRIUM

	Distribution Ratios				
	$\frac{PL, SE, TG_3}{Sum\ PL+SE+TG_3}$			$\frac{SE, TG_3}{Sum\ SE+TG_3}$	
Day	PL	SE	TG_3	SE	TG_3
1	0.63	0.10	0.27	0.28	0.72
1	0.52	0.11	0.38	0.22	0.78
1	0.09	0.23	0.68	0.25	0.75
3	0.65	0.17	0.18	0.48	0.52
5	0.24	0.27	0.49	0.35	0.65
6	0.07	0.55	0.38	0.59	0.41
6	0.16	0.14	0.70	0.16	0.84
7	0.03	0.45	0.53	0.46	0.54
7	0.21	0.40	0.40	0.50	0.50
8	0.40	0.31	0.29	0.52	0.48
8	0.30	0.34	0.36	0.48	0.52
9	0.44	0.19	0.37	0.33	0.67
11	0.51	0.26	0.23	0.53	0.47
11	0.03	0.36	0.61	0.37	0.63
11	0.02	0.08	0.90	0.08	0.92
12	0.08	0.45	0.47	0.49	0.51
13	0.75	0.09	0.17	0.35	0.65
13	0.27	0.13	0.61	0.17	0.83
13	0.29	0.26	0.45	0.37	0.63
14	0.26	0.43	0.31	0.58	0.42
15	0.04	0.32	0.64	0.33	0.67
15	0.42	0.25	0.33	0.44	0.56
15	0.10	0.40	0.50	0.44	0.56
15	0.37	0.28	0.35	0.44	0.56
16	(0.003)	0.98	0.02	0.98	0.02
16	0.20	0.72	0.08	0.90	0.10
17	0.29	0.51	0.20	0.71	0.29
17	0.21	0.77	0.03	0.96	0.04
18	0.01	0.97	0.02	0.98	0.02
19	0.37	0.37	0.25	0.60	0.40
21	0.24	0.67	0.09	0.88	0.12
21	0.03	0.96	0.01	0.99	0.01
22	0.58	0.32	0.10	0.77	0.23
22	0.36	0.38	0.26	0.59	0.41
22	0.46	0.21	0.33	0.38	0.62
22	0.02	0.30	0.68	0.30	0.70
26	0.01	0.98	0.01	0.99	0.01

Ratios are obtained by dividing the C^{14} titre expressed in counts per minute by the sum of the fractions considered.
PL = phospholipids; SE = sterol esters; TG_3 = triglycerides.

Ratios of Carbon 14 Activities in Triglyceride Fractions from Endometrium Versus Time

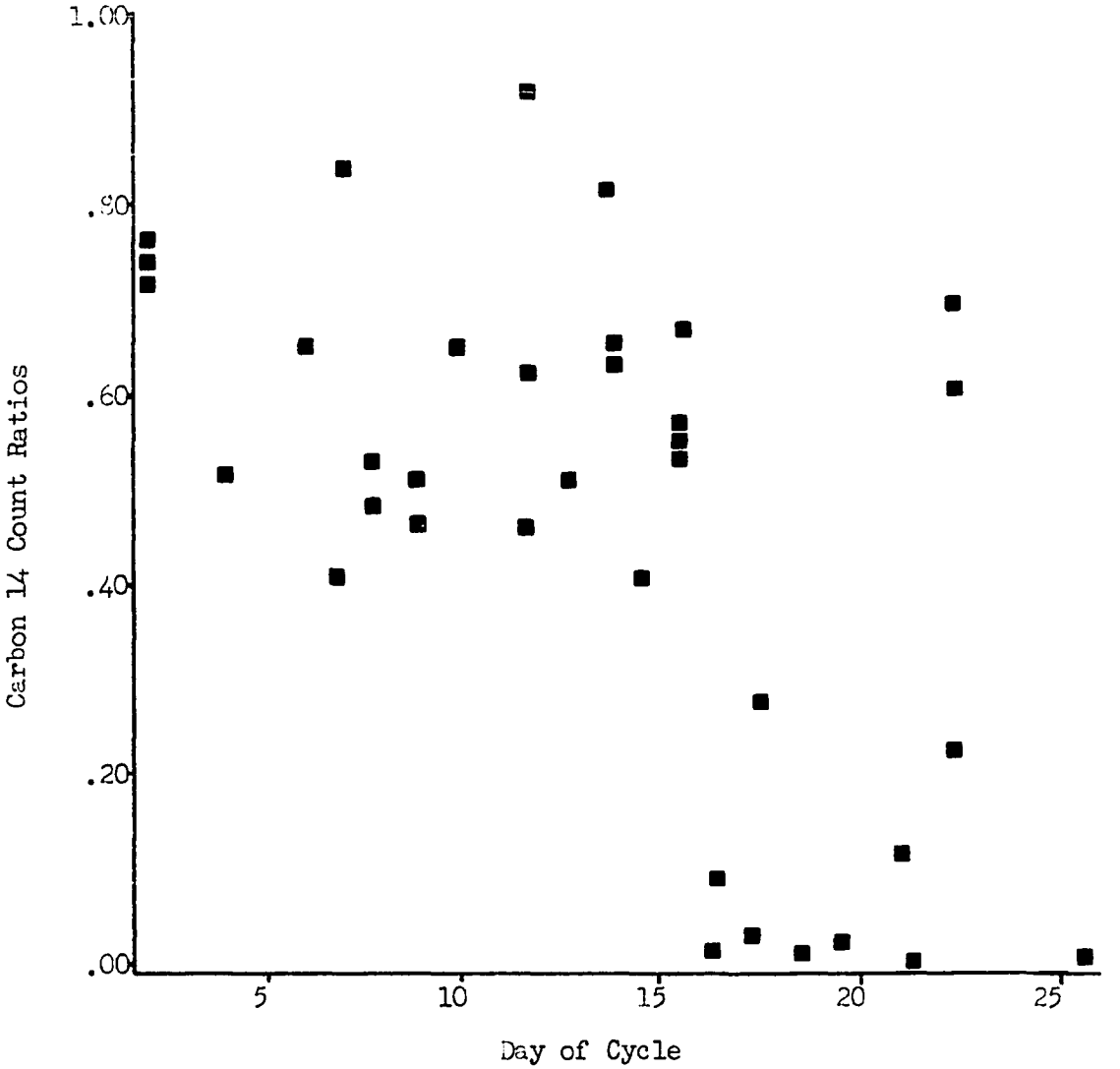

Figure 7 - This figure illustrates the ratios of C^{14} incorporated into triglyceride fractions as determined by dividing individual activities in the triglyceride fractions by the sum of sterol ester and triglyceride activities from the same tissue specimen.

Ratios of Carbon 14 Activities in Sterol Ester Fractions from Endometrium Versus Time

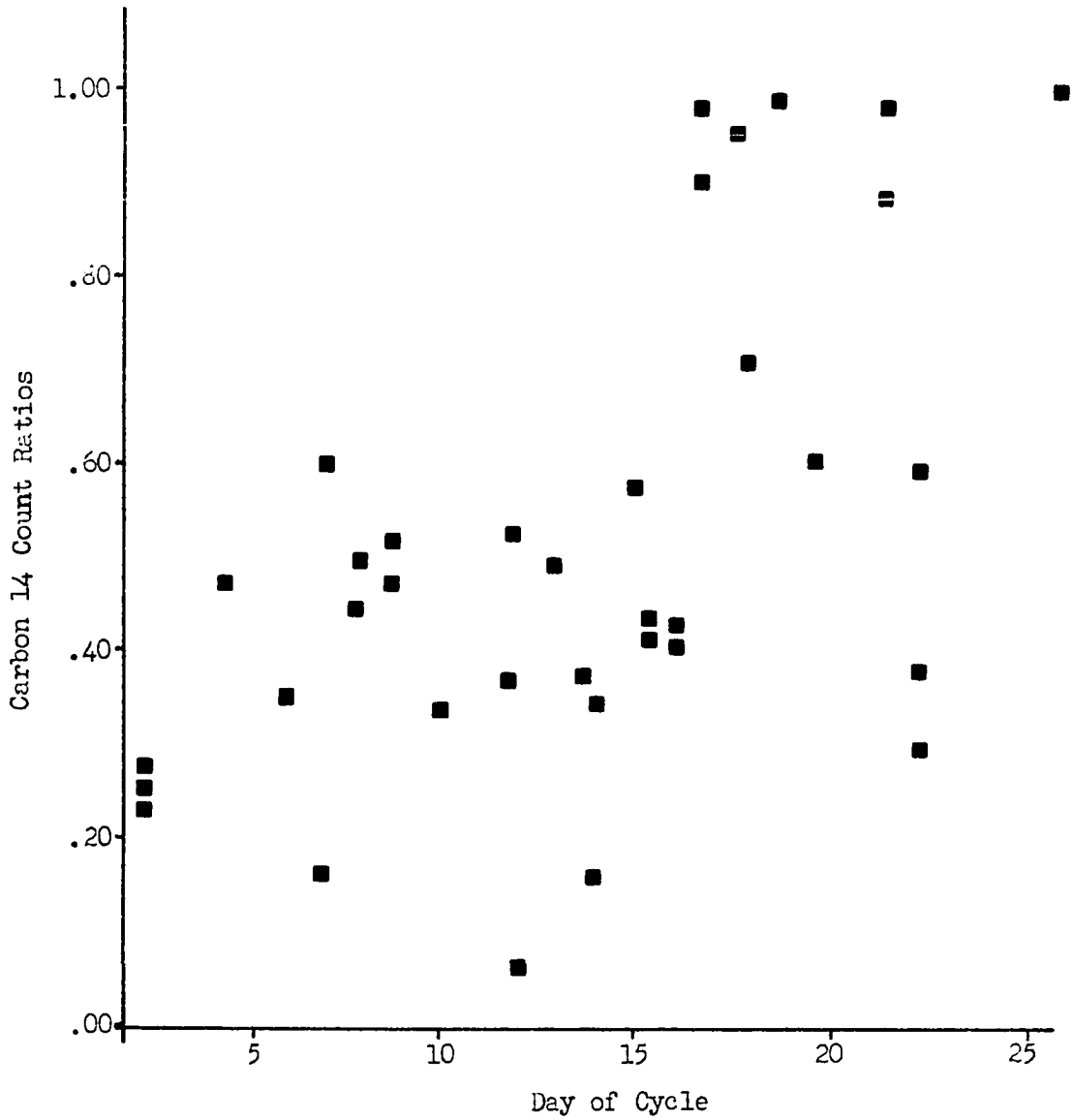

Figure 8 - This figure illustrates the ratios of C^{14} incorporated into sterol ester fractions as determined by dividing individual activities by the sum of the sterol ester plus triglyceride activities from the same tissue.

endometrium. These changes were independent of changes in C^{14} activities of the total lipid fractions as illustrated in Figure 3. Aside from this independence, the SE class appeared important because their C^{14} titres did not mimic the pattern of the TL fractions (Figures 3 and 6). Furthermore, the C^{14} in the SE fractions increased during the secretory phase. Thus two changes were evident prior to and during the period of the nidation of the blastocyst.

The results of Study I supported the original premise that uterine lipid metabolism is important to pregnancy. The biological variance of humans, coupled with the inability to control the donor's status in the cycle, encouraged the use of laboratory animals. Of several available species, rats were chosen because the literature cited earlier indicated their uteri contained sufficient material for analysis. In addition, their vaginal cytology reflects changing ovarian hormones and permits timing of the estrous cycle. However, Long and Evans demonstrated that ovarian changes in the rat are not always "in phase" with changing vaginal cytology.(38) Still, the rat appeared to be the ideal animal to use simply because it had been reported upon extensively.

Study II (Rat Uterine Lipids During the Estrous Cycle)

Analysis and presentation of the results utilized a "normal" estrous cycle as established by the criteria of Long and Evans.(38) The cycle is expressed in these results by stages. While all data were recorded at specific times in the cycle, the times could not be estimated more accurately than ± 2 hours. Elongated dots represent

this inaccuracy in Figures 10 and 11.

Gross and histological examinations of the ovaries were made to determine, as precisely as possible, the maturity of ripening follicles and presence of corpora haemorrhagicae. Precise timing of ovulation was feasible by use of these ovarian indices.

The average C^{14} incorporation was greater during Stages I and II of the cycle than during Stages IV and V (Figure 9).

Since estrogen titres increase during Stages I and II, the increased C^{14} activities were probably related to the effects of estrogen on uterine metabolism. Furthermore, estrogen administration to ovariectomized rats stimulates lipid biosynthesis. In the Study II rats, the rising estrogen titres, culminating at estrus, had been acting on a uterus previously released from maximal estrogen stimulation. Thus a simulation of the effects noted in the castrated rat upon parenteral estrogen administration was to be expected.

Counts per minute of TL fractions from liver tissues ranged from 810 to 15150/mg d-d tissue (average 7161); no time related changes in C^{14} activities occurred (Table 8). Thus the observations made in uterine tissues were due to independent changes in that tissue.

A change in esterification near the time of ovulation was sought since the human data have revealed such a change. The count ratio data of these rats had demonstrated similar changes in both the PL and TG_3 fractions (Figures 10 and 11). However, in contradistinction to the human study, SE count ratios from rat uteri did not change (Table 9).

As Figure 10 illustrates, the TG_3 ratios decreased during the

Carbon 14 Activities of TL Fractions from Rat Uterine Tissues during the Estrous Cycle

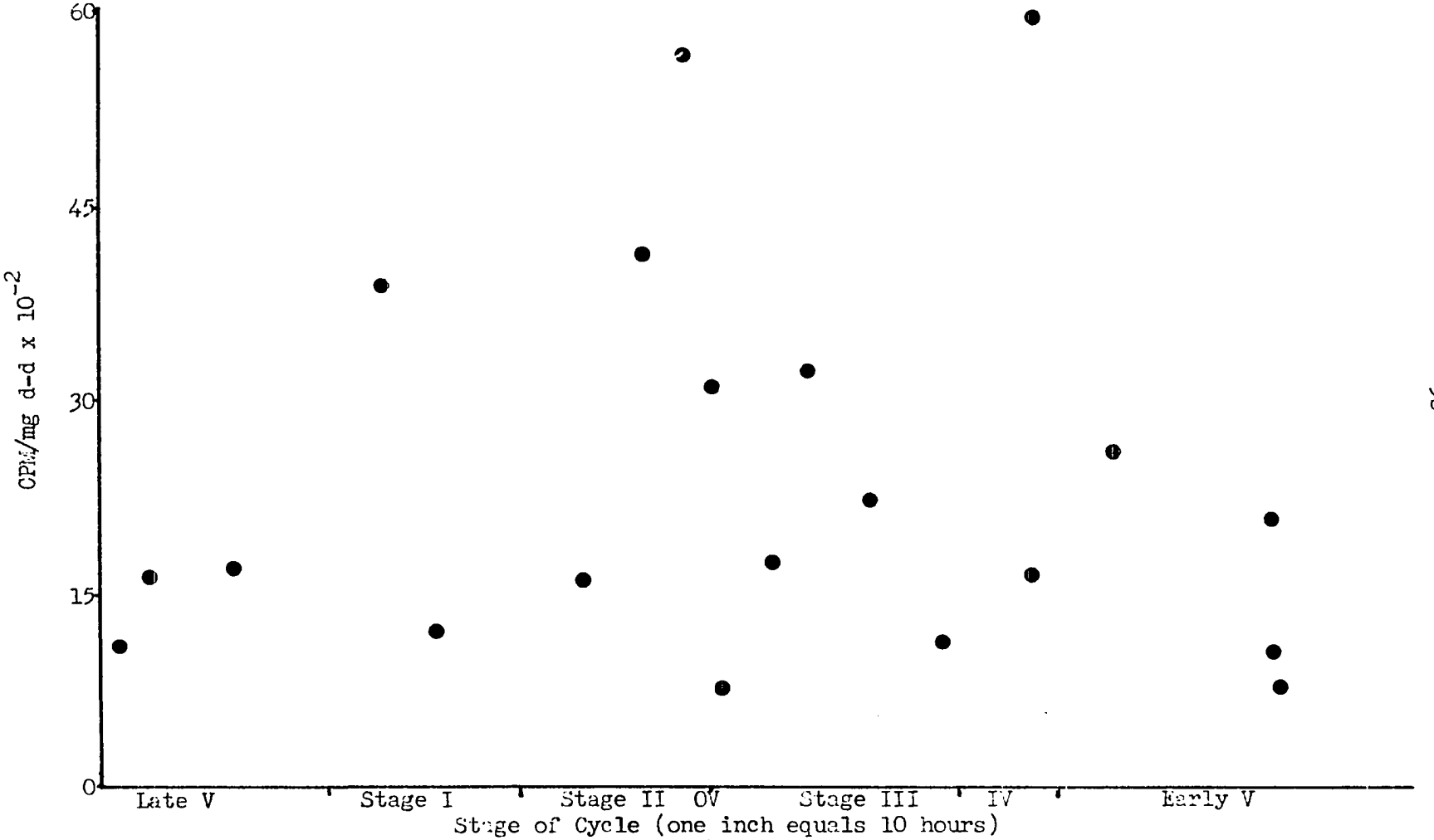

Figure 9 - Activities are expressed as CPM/mg d-d tissue. Time is presented as stages in the cycle.

Ratios of Carbon 14 Incorporated into the Triglyceride Fractions by the Rat Uterus During the Estrous Cycle

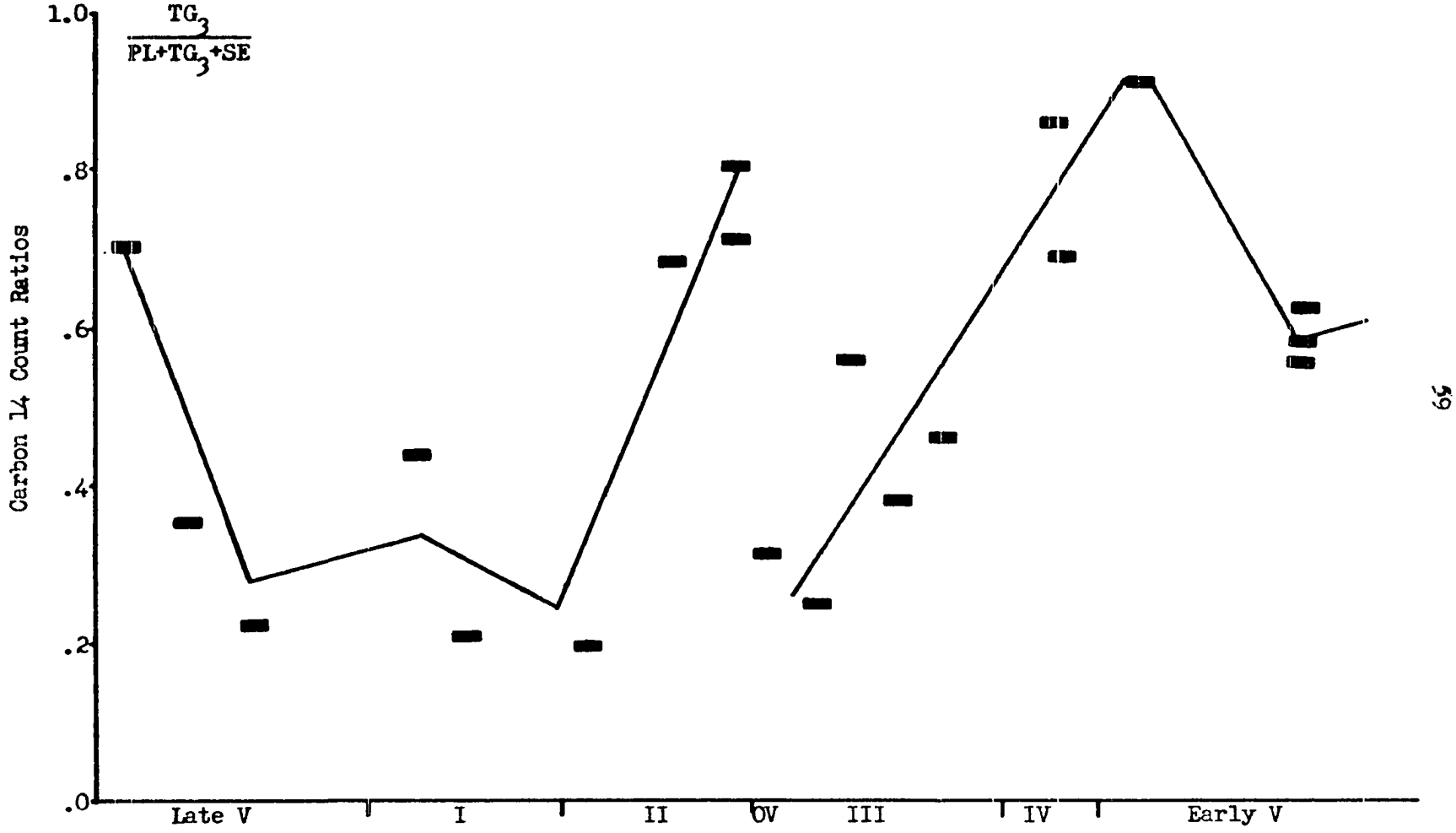

Figure 10 - Ratio formation and timing of the estrous cycle are discussed on pages 26 and 46.

Ratios of Carbon 14 Incorporated into the Phospholipid Fractions by the Rat Uterus During the Estrous Cycle

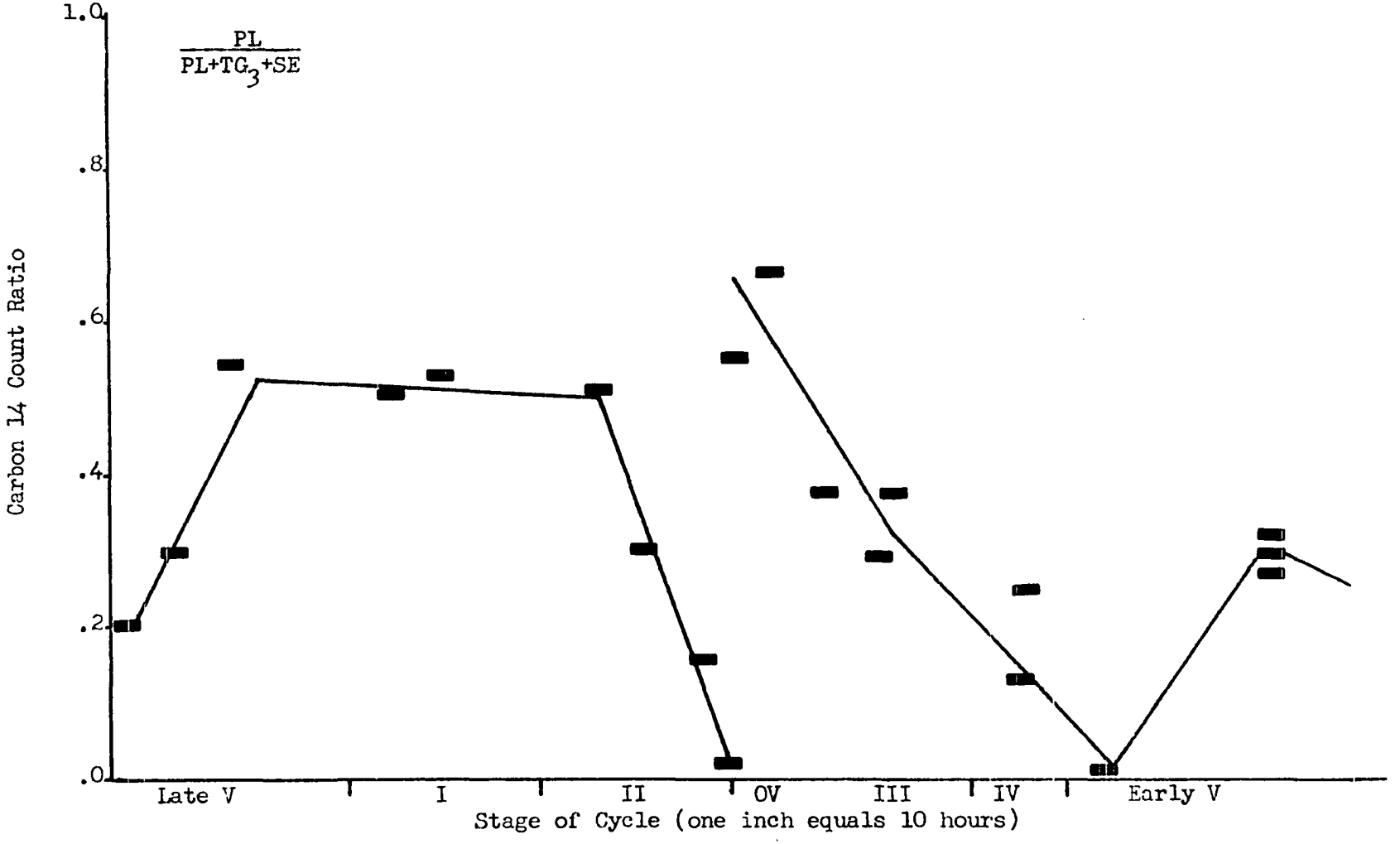

Figure 11 - Ratio formation and timing of the estrous cycle are discussed on pages 25 and 46.

TABLE 8

CARBON 14 INCORPORATION INTO MAJOR LIPID FRACTIONS BY LIVER TISSUE AS FUNCTIONS OF TIME IN A NORMAL ESTROUS CYCLE

STAGE OF CYCLE	RAT NUMBER	LIPID FRACTION		
		TL	TLC	PL
Late V	88	7325	4650	1360
	87	12350	10900	608
	89	5400	500	613
I	66	5600	4700	1430
	69	3800	2875	393
II	78	6375	261	138
	85	11362	421	178
	90	5700	218	773
III	81	1245	943	73
	82	15150	11950	1208
IV	79	15100	10750	273
Early V	70	810	670	128
	77	2875	5000	313

This table illustrates the C^{14} incorporation into major lipid fractions by rat liver tissue (as CPM/mg of defatted and dried tissue) as a function of time in a normal estrous cycle. All data which were obtained are presented without regard for completeness of recovery following separation of TLC and PL fractions. Stages of the cycle were determined by the methods discussed on page 26.

TABLE 9

RATIOS OF CARBON 14 INCORPORATED INTO THREE LIPID CLASSES BY RAT UTERINE TISSUE AS FUNCTIONS OF TIME

STAGE	RAT NUMBER	PHOSPHO-LIPIDS	STEROL ESTERS	TRI-GLYCERIDES
LATE V	88	.70	.10	.20
	87	.35	.32	.33
	89	.22	.24	.54
I	66	.43	.07	.48
	69	.20	.27	.53
II	65	.19	.32	.49
	78	.68	.08	.24
	85	.70	.14	.16
	68	.80	.19	.01
	90	.38	.05	.57
III	71	.24	.08	.68
	81	.55	.09	.36
	82	.38	.24	.38
	67	.47	.27	.26
IV	79			
	80	.85	.01	.14
EARLY V	84	.95	.04	.01
	70	.62	.09	.29
	72	.57	.11	.32
	77	.59	.10	.31

The ratios were calculated by dividing the C^{14} activity in each individual fraction by the total activity fractions. Determination of the stages of the estrous cycles is discussed on page 25.

preovulatory phases from late in Stage V. Within 4 hours of ovulation the ratios decreased dramatically. Then during the postovulatory phases, TG_3 count ratios again increased until late in Stage V when they returned to a low level.

Conversely, the count ratios of PL fractions decreased during the preovulatory period. At ovulation, the ratios increased from near 0.0 to 0.6. Following ovulation, the ratios decreased again to a low point early in Stage V. During the last half of Stage V, and through Stage I, the count ratios increased again to the preovulatory levels (Figure 11).[1]

Hormonal titres during the cycle are related to these ratio changes. As luteinizing hormone and progesterone increase during the postovulatory period, TG_3 ratios increase and PL ratios decrease. Similarly, as follicle stimulating hormone and estrogen increase during late diestrus and early estrus, TG_3 ratios decrease and PL ratios increase.

In addition to changes in TG_3 and PL ratios, differences in C^{14} titres existed between the classes. Individual "T" tests performed on ratio data ($SE+TG_3+PL+FFA$) demonstrated that the average SE and TG_3 ratios were significantly smaller ($p < .05$) than the average FFA ratio (Table 10).

In summary, in both humans and rats, the PL fractions received the newly synthesized fatty acids when estrogen stimulated the uterus. When progesterone influenced the uterus in rats, the TG_3 fractions

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[1]Linear regression lines were calculated for the TG_3 and PL ratios during the preovulatory and postovulatory phases: Each of the four lines was different from zero ($p < .05$).

TABLE 10

RATIOS OF CARBON 14 INCORPORATED INTO THE PL, SE, TG_3 AND FFA FRACTIONS BY RAT UTERINE TISSUE AS FUNCTIONS OF TIME

STAGE	RAT	PL	SE	TG	FFA
Late V	88	.47	.06	.13	.34
	87	.12	.07	.14	.67
	89	.08	.08	.19	.65
I	66	.26	.05	.29	.40
	69	.02	.03	.05	.90
II	65	.03	.05	.08	.83
	78	.40	.04	.14	.42
	85	.31	.10	.11	.48
	68	.22	.05	0	.73
	90	.03	.01	.05	.91
III	71	.02	.01	.07	.90
	81	.10	.02	.06	.82
	82	.12	.08	.12	.68
	67	.13	.07	.07	.73
IV	79				
	80	.10	.00	.02	.88
Early V	84	.27	.01	.01	.71
	70	.19	.03	.09	.70
	72	.17	.03	.10	.70
	77	.23	.04	.12	.60
Average		.16	.04	.09	.65

In this table, the ratios were calculated by dividing the C^{14} activity in each individual fraction by the sum of C^{14} activity in all four fractions. The average TG_3 and SE ratios were less than the average FFA ratio ($p < .05$). Determination of the stages of the cycle was discussed on page 26.

received the newly synthesized fatty acids. In humans the SE fractions received the fatty acids.

While the results to date had answered several questions, their significance was speculative since lipid concentrations were unknown. Without concomitant concentration data, the incorporation studies only pointed to lipid classes of possible importance. While definite biosynthetic patterns of lipids had obviously reflected changing hormonal titres, uterine lipid concentrations could have been functions of dietary changes. Consequently, future work was designed to include concentration measurements.

There was at this time a hiatus in actual lipid studies as the semi-automatic system of lipid analysis was developed. After the system was functional, Study III was started.

Study III (Rat Uterine Lipids Following Copulation)

The analytical system used in Study III permitted measurement of the parameters listed in Table 11.

Pilot experiments for Study III revealed that mincing of the uterus prior to incubation reduced between rat variation in C^{14} incorporation without affecting lipid concentration. Consequently, the incubation methods were modified for this study (METHODS, page 27).

Incubation time and substrate concentration influenced incorporation.(83) The following control incubations measured the influence of the variables on C^{14} incorporation.

Uterine horns from six rats were cooled, minced and incubated (200 mg wet weight/flask). Individual incubations utilized various times and concentrations. Carbon 14 activity in the TL fractions

TABLE 11

VALUES WHICH WERE MEASURED BY THE NEW ANALYTICAL SYSTEM

Lipid Sample	Concentration	Carbon 14
Total Lipid Extract (TL)	no	yes
Total Lipid Extract less the Phospholipids (TLC)	yes	yes
Phospholipids (PL)	no	yes
Sterol Esters (SE)	yes	yes
Triglycerides (TG_3)	yes	yes
Free Sterols (FS)	yes	yes
Free Fatty Acids (FFA)	yes	yes

increased during the first two hours of incubation, then leveled off. Two hours were retained as the incubation time (Figure 12). Over the range investigated, C^{14} activity increased as a linear function of acetate concentration (Figure 13). Incubations in this study utilized the median concentration of 1 mc/deciliter.

Several methodological changes were instituted in this study. In addition, the study consisted of two parts. One part utilized uteri which were incubated (in a medium containing acetate-1-2-C^{14}) under carefully controlled *in vitro* techniques. The second part utilized uteri from animals previously injected with acetate-1-2-C^{14} (*in vivo* incubations). Both parts of the study included measurements of lipid concentrations and C^{14} titres.

Uterine Lipid Concentrations Following Copulation

There were no time related changes in concentrations of the SE,

Incubation Standard; Time vs Incorporation

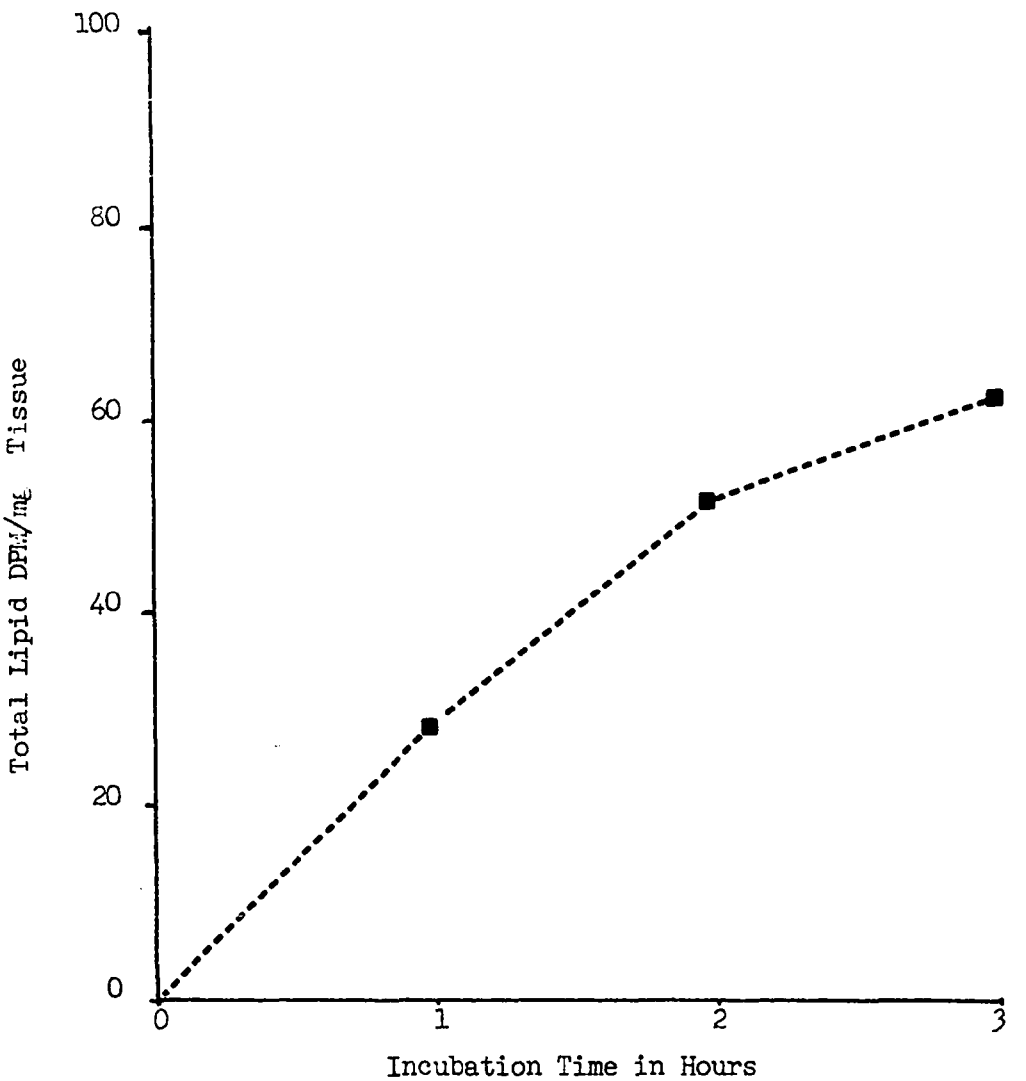

Figure 12 - This figure illustrates the relation between Time and acetate-1-2-C^{14} concentration of 0.1 mc per liter was used.

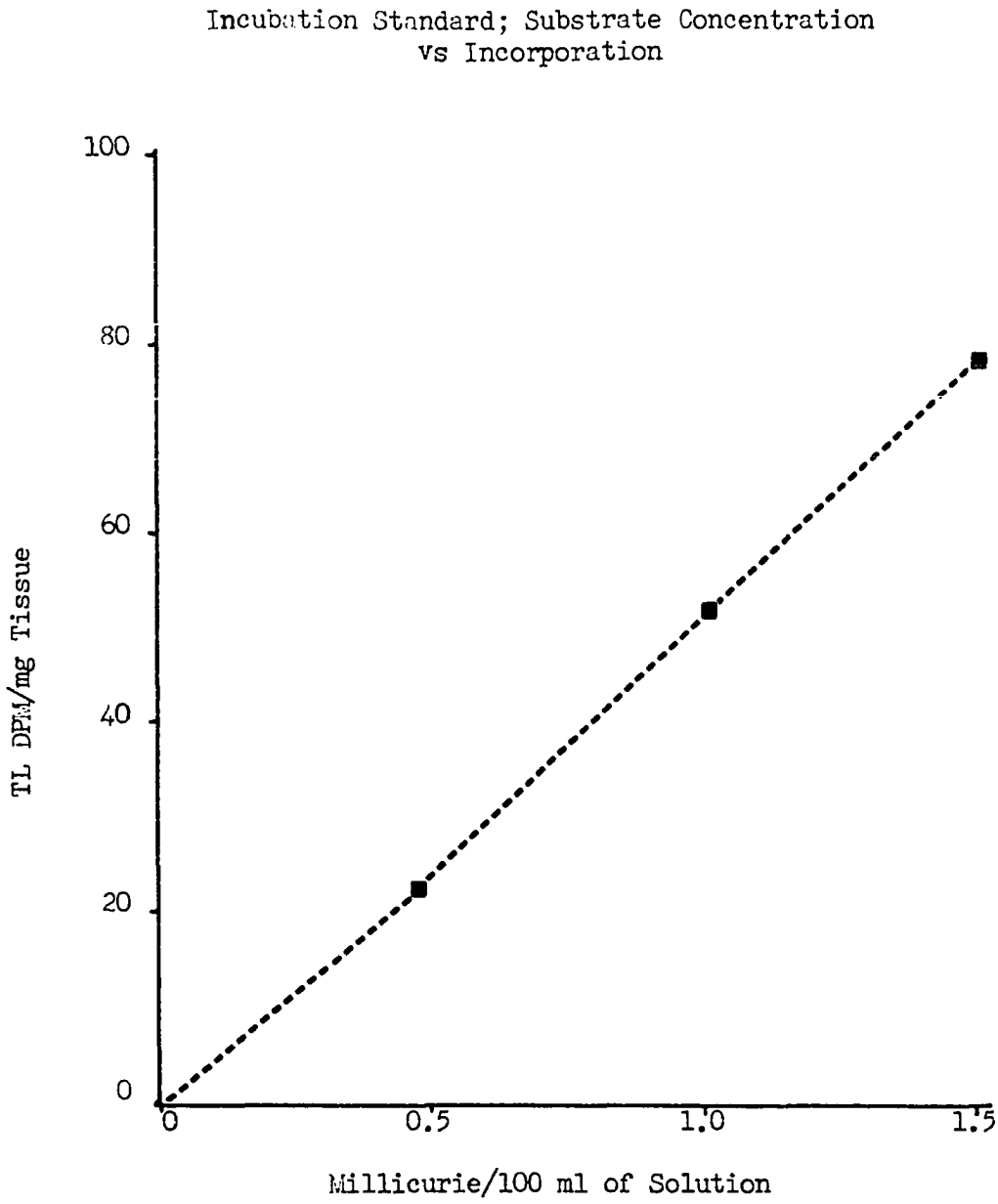

Figure 13 - This figure illustrates the relation between acetate-1-2-C^{14} incorporation into the Total Lipid fractions and substrate concentration. Incorporation is expressed as DPM per milligram of tissue net weight. An incubation time of two hours was used.

FS and FFA classes (all "p" values exceeded 0.05; Table 12). Since these concentrations were unrelated to time, a statistical "T" test was applied to evaluate concentration differences between the in vitro and in vivo incubated uteri. None of the three classes had significant concentration differences between tissues from the different systems (all "p" values exceeded 0.25).

As will be shown, the TG_3 concentrations of tissue from both incubation systems changed with time postcopulation. Consequently, uterine TG_3 concentrations could not be compared by the simple "T" test.

Concentrations of the SE fractions ranged from 1.2 to 10.8 (average 5.2) micrograms/mg d-d tissue. Concentrations of the FS fractions ranged from 6.5 to 38.0 (average 15.1) mcg/mg d-d tissue. Free fatty acid concentrations from 3.1 to 34.5 (average 13.2) mcg/mg d-d tissue.

From these data, the average FS concentration exceeded the average SE concentration by a factor of about 3 ($p < .01$). Thus, the postcopulatory rat uterus contained about 75% of the steroid moiety in the unesterified form. Recall that phosphorous, diglycerides, and cholesterol had contaminated the FS fractions (thin-layer analysis, page 45).

An auto-analyzer (available through the courtesy of Dr. Merl Steinberg, Schering Corporation) permitted the following cholesterol concentration analyses. Known portions of the FS fractions from rats were pooled. The pooling process kept incubation systems (i.e., in vitro and in vivo treated tissues) and time postcopulation separated. Thus, cholesterol concentrations from both analytical systems were

TABLE 12
CONCENTRATIONS OF RAT UTERINE LIPIDS
POSTCOPULATION (I)

STEROL ESTER FRACTIONS
Days Postcopulation

Incubation	0	1	2	3	4	5	6
In Vitro	1.6	1.9	5.4	9.0	4.7	3.0	2.7
	7.6	8.6	6.7	4.9	2.2	4.4	10.8
	1.3	5.1	2.9	12.0	5.8	2.8	2.9
In Vivo	1.4	5.1	X	6.2	9.1	2.3	3.6
	2.6	5.5	8.1	2.4	9.5	4.1	8.7
Average	2.9	5.2	5.8	6.9	6.3	3.3	5.9

FREE STEROL FRACTIONS
Days Postcopulation

Incubation	0	1	2	3	4	5	6
In Vitro	14.7	12.3	24.1	17.8	11.7	22.8	17.1
	14.9	10.8	16.0	12.6	20.3	10.4	20.0
	10.3	13.2	18.9	12.0	18.3	14.4	22.1
In Vivo	13.1	11.8	X	14.4	15.9	11.5	13.3
	9.4	6.5	13.3	12.8	14.3	15.1	20.7
Average	12.5	10.9	18.1	13.9	16.1	14.8	18.6

FREE FATTY ACID FRACTIONS
Days Postcopulation

Incubation	0	1	2	3	4	5	6
In Vitro	9.3	16.3	23.1	7.0	9.0	13.8	24.8
	11.6	23.7	20.8	9.0	21.0	4.0	34.5
	7.8	14.0	22.0	7.8	24.0	32.5	8.5
In Vivo	4.9	3.3	X	9.0	20.8	5.1	9.9
	4.4	7.7	5.5	3.2	13.5	8.7	3.1
Average	7.6	13.0	17.9	7.2	17.7	12.8	16.2

This table shows the concentrations of sterol ester, free sterol, and free fatty acid fractions as functions of time. Concentrations are expressed as micrograms of lipid per milligram of defatted and dried tissue. As there were no concentration differences between the *in vitro* and *in vivo* treated tissues, the data were pooled in calculation of the average values.

compared with time postcopulation. As the semi-automatic system measured all material eluted with a given fraction, a ratio analysis (i.e., total material concentration divided by cholesterol concentration) permitted evaluation of the contaminating material. If the FS fractions contained a constant concentration of contaminating material, then the ratio would have remained unchanged with time after copulation. However, concentrations of the FS contaminants changed remarkably in tissues from both systems (Figures 14 and 15).

A significant time related increase in their concentration occurred in the FS fractions from *in vivo* treated tissues ($p < .001$, Figure 14). In the *in vitro* treated tissues, a transient increase in contaminants occurred near day 2 postcopulation: The significance of this "bump" is not known. If diglycerides caused the increases (recall the thin-layer analysis, page 45), one would expect the TG_3 concentrations to increase similarly.

Table 13 and Figures 16 and 17 show that the TG_3 concentrations from *in vitro* incubated tissues increased from days 0 through 4. They then decreased dramatically on days 5 and 6 postcopulation. With the exception of the two low aberrant points, TG_3 concentrations from *in vivo* treated tissues increased lineally from day 1 through 6 postcopulation (Figure 17). Recall, the SE, FS and FFA concentrations were similar in tissues incubated differently. Consequently, the TG_3 data from *in vitro* and *in vivo* incubated tissues were also combined to yield more accurate information of concentration changes. When combined, the TG_3 concentrations increased lineally from day 1 through 4. On days 5 and 6 only the variance increased.

Ratios of Free Sterol Concentrations Divided by
Free Cholesterol Concentrations as Functions
of Time Postcopulation (In Vivo)

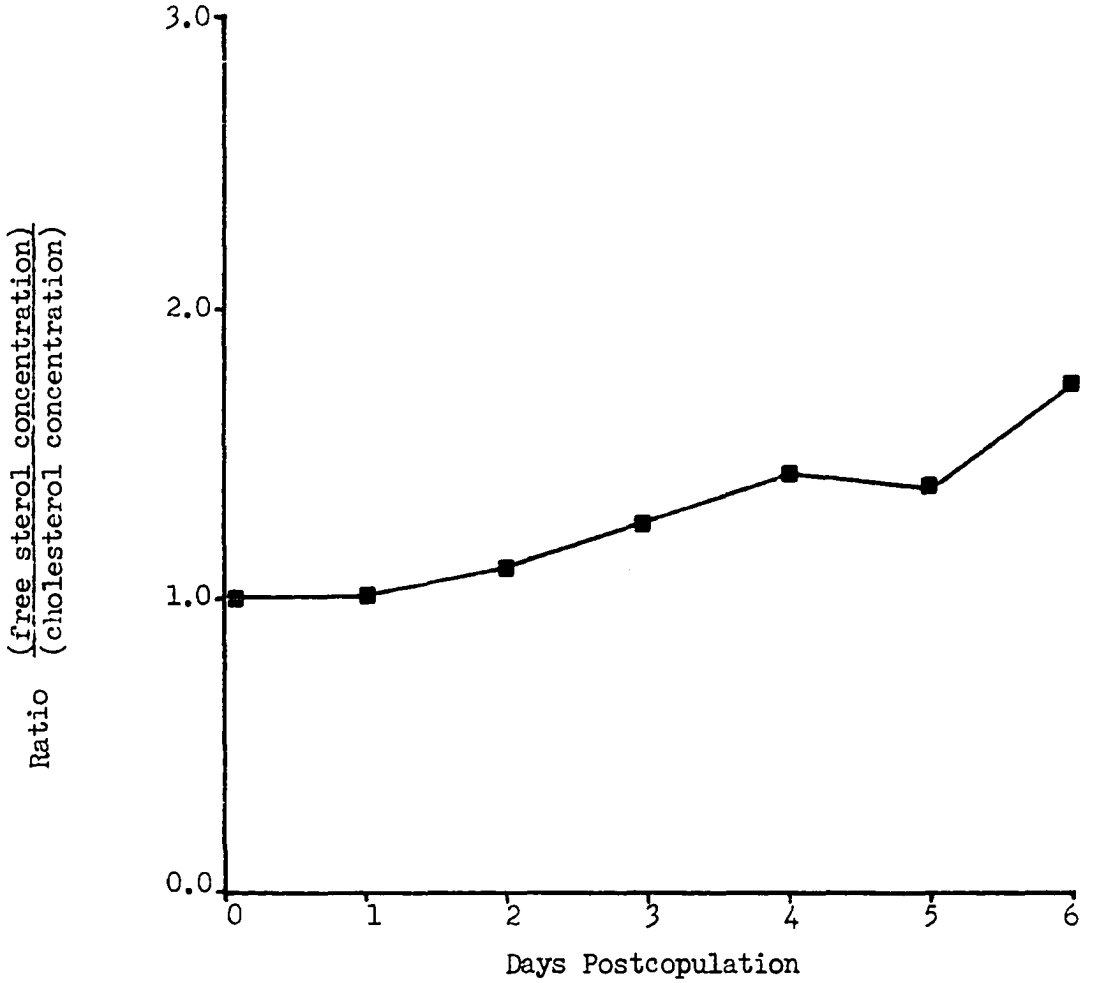

Figure 14 - This figure presents ratios of lipid concentrations as functions of time. The ratios were derived by dividing concentrations of pooled free sterol fractions by the cholesterol concentrations in those fractions as determined by Auto-Analyzer.

Ratios of Free Sterol Concentrations Divided by Free Cholesterol Concentrations as Functions of Time Postcopulation (In Vitro)

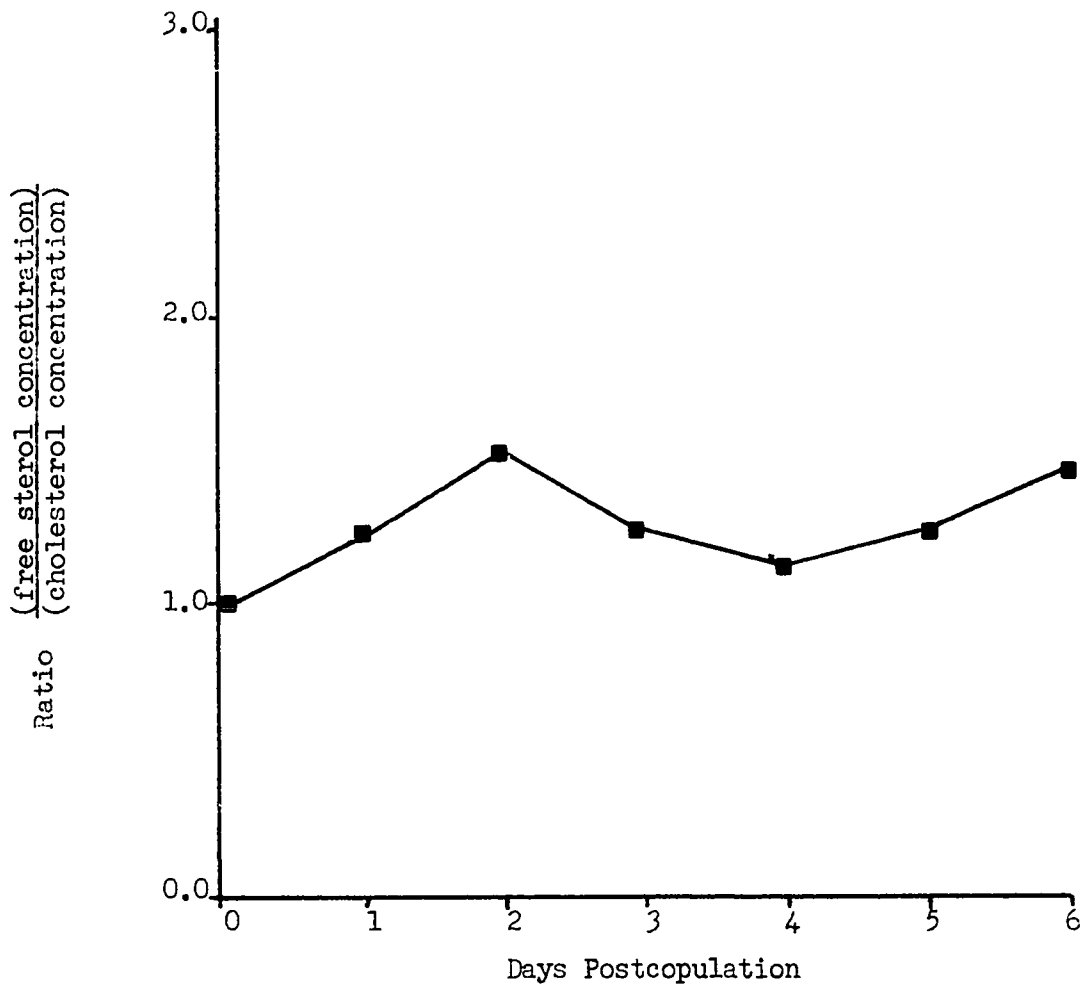

Figure 15 - This figure presents ratios of lipid concentrations as functions of time. The ratios were derived by dividing concentrations of free sterol fractions by the cholesterol concentrations in those fractions as measured by Auto-Analyzer.

TABLE 13

CONCENTRATIONS OF RAT UTERINE LIPIDS
POSTCOPULATION (II)

TRIGLYCERIDE FRACTIONS
Days Postcopulation

Incubation	0	1	2	3	4	5	6
In Vitro	13.8	3.2	7.5	16.3	37.0	9.4	17.8
	16.9	13.7	31.1	13.8	42.5	6.0	19.4
	6.1	9.4	19.0	35.1	49.1	10.4	26.4
In Vivo	1.4	11.1	X	24.9	29.1	37.1	9.8
	6.4	6.0	16.6	15.8	11.1	46.9	45.4
Average	11.5	8.7	18.6	21.2	33.8	22.0	23.8

TOTAL LIPID FRACTIONS
Days Postcopulation

Incubation	0	1	2	3	4	5	6
In Vitro	41.5	33.7	60.1	50.0	62.5	49.0	62.4
	51.0	56.8	74.7	40.3	85.0	24.8	84.7
	25.5	41.7	81.0	56.1	97.3	60.1	59.9
In Vivo	25.0	31.3	X	54.5	74.9	56.0	36.7
	22.6	25.6	43.5	34.1	48.4	74.8	77.9
Average	33.1	37.8	64.8	47.0	73.6	52.9	64.3

This table shows the concentrations of triglycerides and total lipid fractions as functions of time postcopulation. Concentrations are expressed as micrograms of lipid per milligram of defatted and dried tissue. Both in vitro and in vivo treated tissues are presented.

Rat Uterine Triglyceride Concentrations
as Functions of Time Postcopulation
(In Vitro)

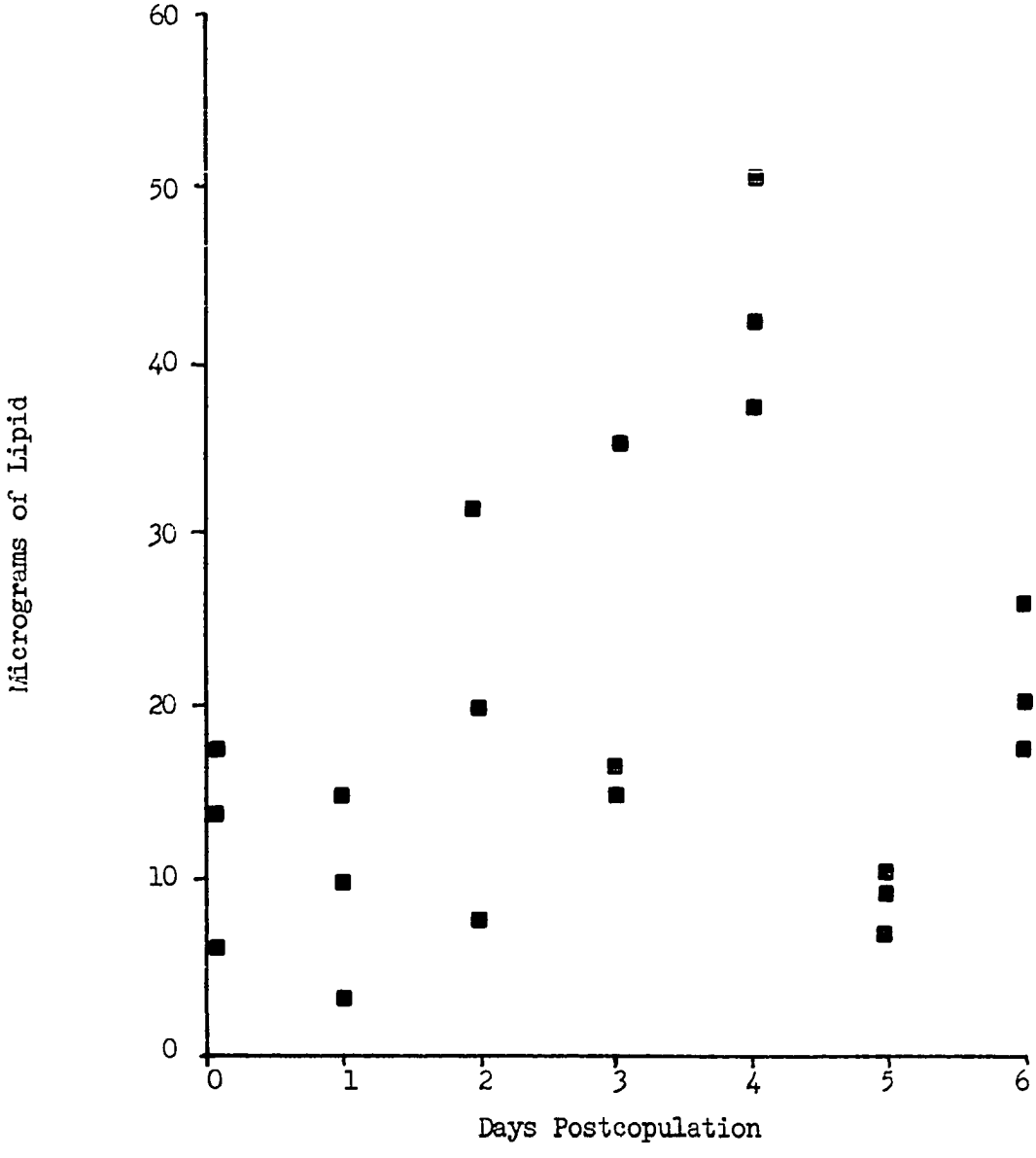

Figure 16 - This figure presents concentrations of Triglycerides in uterine tissues incubated by in vitro techniques as functions of time postcopulation. There is a significant linear relationship between concentration and time from day 1 through day 4 postcopulation. Concentrations are expressed as micrograms of lipid per milligram of defatted and dried tissue ($p < .01$).

Rat Uterine Triglyceride Concentrations
as Functions of Time Postcopulation
(In Vivo)

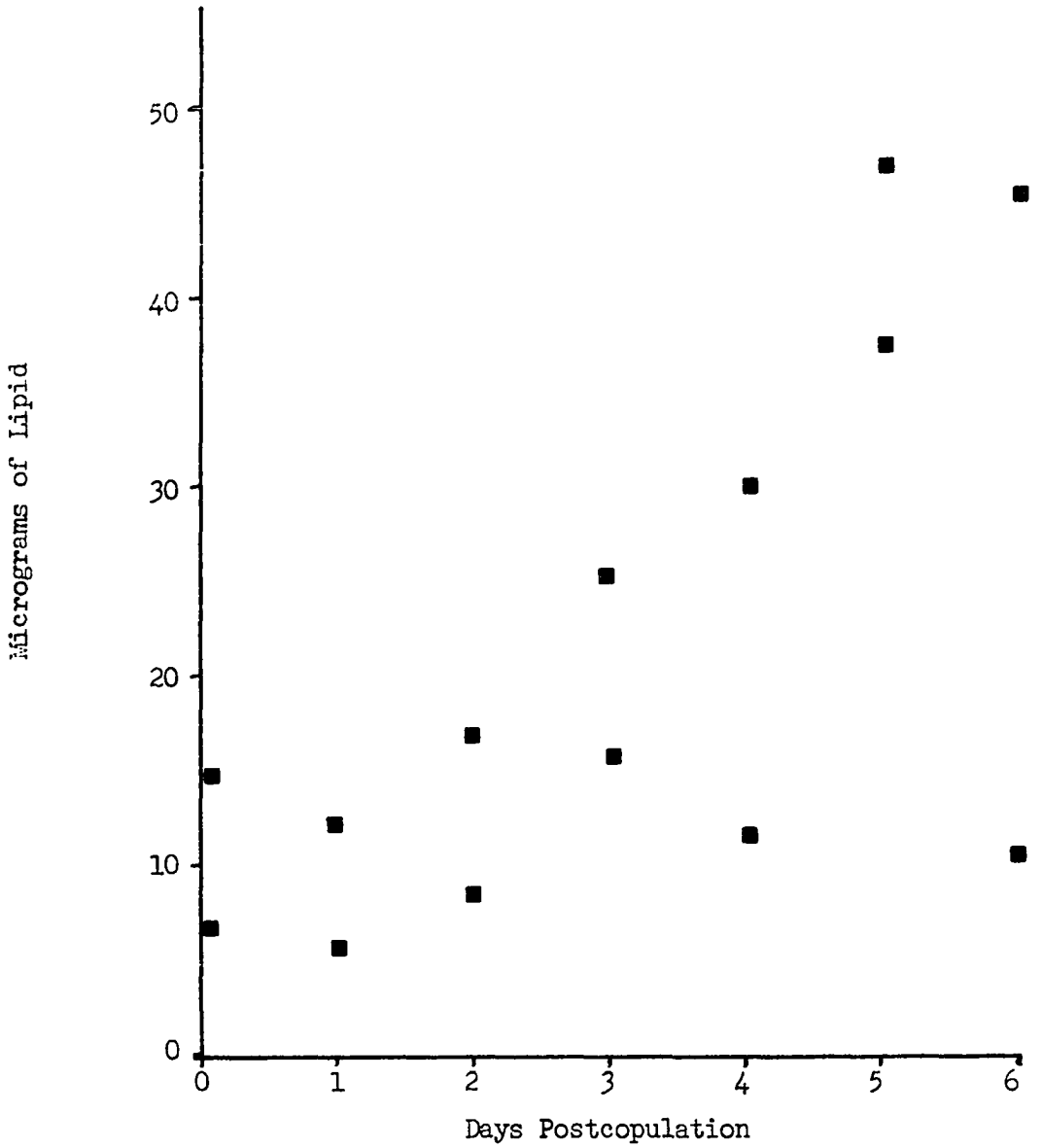

Figure 17 - This figure presents triglyceride concentrations in uterine tissues incubated by in vivo techiques as functions of time postcopulation. Concentrations are expressed as micrograms of lipid per milligram of defatted and dried tissue. A significant linear relationship exists from day 1 through day 4 postcopulation ($p < .05$).

Interestingly, classical studies have segregated days 1 through 4 as a time when no histological changes in uterine tissue occurred. (13,71) These same studies demonstrated histological changes on day 0 and from day 5 through partus. Since the uterus was histologically constant from days 1 through 4, this period was selected for a linear regression analysis of the TG_3 data. TG_3 concentrations increased significantly with time in tissues from both incubation systems ($p < .05$). The obvious increase in variance on days 5 and 6 was demonstrated statistically as follows: Linear regression statistics were determined for TG_3 concentrations from days 1 through 6 for both sets of tissues. While the concentrations from *in vitro* treated tissues were not related to time during this period ($p > .10$), *in vivo* treated tissues were time related ($p < .01$).

In an effort to determine the cause of variance, uteri were removed and incubated on day 7 postcopulation. At this time, embryonic growth was sufficient to permit the implantation areas to be separated by gross dissection from normal uterine tissue. Consequently, lipid analyses with comparisons of these two tissues, within each rat, were possible (Table 14). When the lipid concentrations between similar classes (from different tissues) were compared, only the TG_3 class had a consistent tissue distribution. In all five rats, the implantation areas (decidua) contained less TG_3 material than the comparable uterine (endometrium) tissue from the same rat. Therefore, the variance in TG_3 concentrations on days 5 and 6 had a logical explanation. The implantation processes (which commence on day 5 and extend over a protracted period) apparently utilized the triglycerides which the

TABLE 14

LIPID CONCENTRATIONS OF ENDOMETRIAL AND DECIDUAL TISSUE ON DAY 7, POSTCOPULATION

RAT	TG DEC	TG ENDO	TG RATIO	SE DEC	SE ENDO	SE RATIO
VITRO						
72	6	74	0.08	4	4	1.0
73	4	10	0.40	2	5	0.4
71	125	360	0.35	20	36	0.5
VIVO						
74	14	82	0.17	9	5	1.8
75	21	38	0.55	4	.75	5.3

RAT	F.S. DEC	F.S. ENDO	F.S. RATIO	F.F.A. DEC	F.F.A. ENDO	F.F.A. RATIO	TNL DEC	TNL ENDO	TNL RATIO
VITRO									
72	13	12	1.1	11	13	0.9	34	103	0.2
73	13	3	4.3	12	9	1.3	31	27	1.1
71	36	59	0.6	16	31	0.5	197	486	0.3
VIVO									
74	26	21	1.2	21	16	1.3	70	124	0.6
75	13	11	1.2	24	4	6.0	62	54	1.1

This table presents lipid concentrations of five lipid functions in both decidual and endometrial tissue from rats seven days after copulation. The ratios were derived by dividing the decidual lipid concentration by the endometrial lipid concentration.

uterus previously accumulated.

Thus these data provide: (1) a second change in lipid titre with time after copulation, and (2) evidence of a specific utilization of the TG_3 class, resulting in an unequivocal change in their concentration.

Uterine Lipid Carbon 14 Titres Following Copulation

Introductory comments. Similar studies of other tissues indicated the most logical means of presenting C^{14} data was by calculating specific activities. However, the data presented in Table 15 illustrate that the variance was excessive when this analytical tool was applied.

TABLE 15

SPECIFIC ACTIVITIES OF TG_3 FRACTIONS FROM RAT UTERI POSTCOPULATION (IN VITRO)

Rat #	Day PC	DPM/mg d-d	Mg lipid/mg d-d	DPM/mg lipid (Specific Activity)
97	1	64	3.2	19.75
98	1	48	13.7	3.51
95	2	37	7.5	4.95
99	2	93	31	3.00
92	3	226	13.7	16.41
92	3	306	35.1	8.71

As this table indicates, the TG_3 concentrations varied considerably (Figure 16 illustrates the variance for all TG_3 concentration data). In addition, C^{14} incorporation into the TG_3 fractions also varied. Consequently, when these two sets of data were combined, the resultant variance was excessive (as was expected). With ideal circumstances in

similar problems, change in C^{14} titres should be measured as functions of the tissue DNA concentration. However, a literature survey demonstrated that uterine cellular growth is virtually nonexistent from copulation to implantation.(13,60,71) Thus, the number of uterine cells (hence tissue DNA concentrations) do not change significantly during this period.(42) Rat uterine DNA data by Saldarini and Yochim demonstrated this point.(60) However, the same publication contained wet weight data which could be interpreted to indicate cellular proliferation. Since this was the only report which indicated significant mitotic activity might occur in rat uteri during the postcopulation period, I contacted Dr. Saldarini. By personal correspondence, he confirmed that the total number of cells in the rat uterus does not change appreciably during the pre-implantation period. Thus, a defatted and dried tissue weight was a good (if not the best) base criterion for determining changes in cellular activities in this study.

As would be expected from the substrate concentrations (METHODS, page 28) in vivo and in vitro treated tissues incorporated measurably different quantities of C^{14}. Consequently, activity data from the two parts of the study are presented separately.

Carbon 14 titres on days 1 through 6 postcopulation. Figure 18 shows the TL C^{14} titres (computed as per mg d-d tissue) as a function of time postcopulation. The C^{14} titres increased from days 1 through 6. Statistical tests showed the increase was linear and significant from days 1 through 4 and 1 through 6 ($p < .05$). However, TL C^{14} activities of in vivo treated tissues did not increase comparably (Table 16).

When the TL fractions were separated into TLC and PL portions,

TL Carbon 14 Activities
Versus Time Postcopulation (In Vitro)

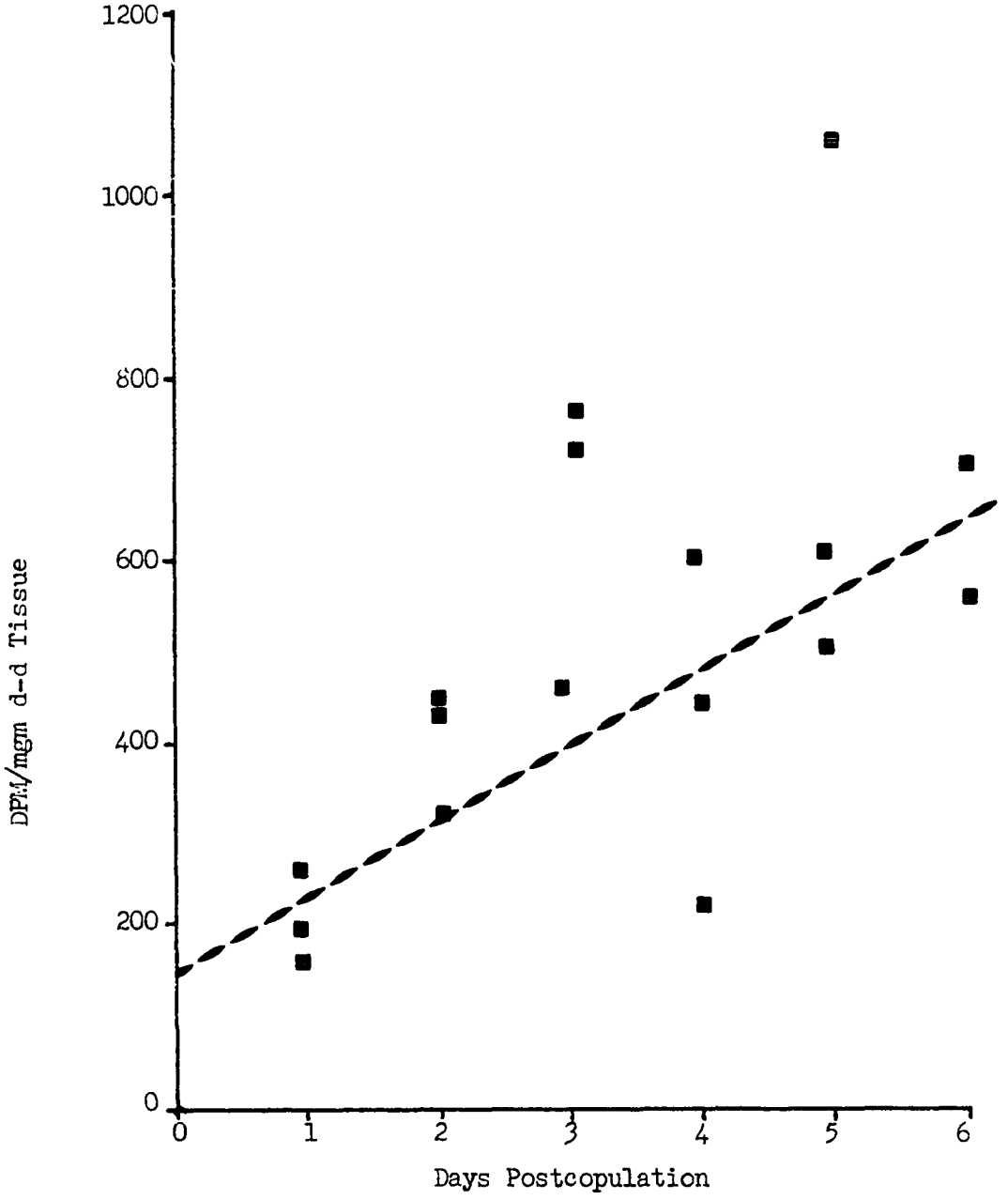

Figure 18 - This figure presents the C^{14} activity in Total Lipid fractions from rat uteri incubated by in vitro techniques as functions of time. Numerical values are presented in Table 16.

the TLC portions contained the most C^{14} (Table 16). The C^{14} titres in these fractions were not related to time postcopulation (Table 16). The C^{14} titres of PL fractions from in vitro treated tissues decreased within 24 hours after copulation (Table 16). However, PL C^{14} activities from in vivo treated tissues did not change similarly.

Analyses of the TLC fractions permitted determination of C^{14} activities in the SE, FS, FFA and TG_3 portions. Briefly, C^{14} incorporation into the SE, FS and FFA fractions (from tissues of both parts of the study) did not change with time after copulation (all "p" values exceeded 0.5; Table 20). SE C^{14} titres of in vivo treated tissues averaged 5 DPM/mg d-d (range 1 to 20): C^{14} titres from in vitro incubated tissues averaged 24 DPM/mg d-d (range 10 to 52). FS C^{14} titres averaged 22 and 98 for in vivo and in vitro treated tissues, respectively (ranges were 4 to 52 and 13 to 303). The FFA fractions contained C^{14} titres of 11 and 78 DPM/mg d-d for in vivo and in vitro treated tissues, respectively (ranges were 2 to 28 and 26 to 124).

As the data in Table 17 shows, C^{14} titres in the TG_3 fractions from in vitro incubated tissues increased from days 1 through 6 ($p < .05$). Carbon 14 activities peaked on day 3 in these tissues (statistical significance not determined). In the in vivo data, comparisons of days 0, 1 and 5, 6 with days 2 and 3 also demonstrated a peak of C^{14} activity on days 2 and 3. Because these peaks existed, a linear relationship failed to accurately reflect the TG_3 C^{14} activities. Consequently, a method was sought to reduce the variance and permit "true" changes in C^{14} activities to become visible. Patterns of C^{14} incorporation into these fractions might have been visible had all tissues incorporated

TABLE 16

CARBON 14 INCORPORATION INTO LIPID FRACTIONS AS A FUNCTION OF TIME (I)

TOTAL LIPID FRACTIONS

	DAYS POSTCOPULATION						
	0	1	2	3	4	5	6
VITRO	406	248	164	459	432	611	55
	493	145	234	666	597	555	703
	764	184	240	683	214	1077	x
AVERAGE	554	192	213	603	414	748	253
VIVO	44	15	177	107	101	38	62
	51	x	55	98	40	41	189
AVERAGE	48	15	116	103	71	40	126

TOTAL LIPIDS FOR CHROMATOGRAPHY

	0	1	2	3	4	5	6
VITRO	361	253	166	440	398	638	288
	454	151	212	589	601	468	699
	707	167	235	633	198	949	x
AVERAGE	508	190	204	554	399	685	329
VIVO	46	73	54	83	56	34	99
	37	14	140	85	65	38	48
AVERAGE	42	44	97	84	61	36	74

PHOSPHOLIPID FRACTIONS

	0	1	2	3	4	5	6
VITRO	28	5	10	4	6	17	145
	35	5	1	24	5	9	8
	29	2	5	4	2	6	3
AVERAGE	31	4	5	11	4	11	52
VIVO	6	2	37	9	31	1	10
	5	53	1	2	x	9	76
AVERAGE	6	28	19	6	31	5	43

This table presents the C^{14} activities of the various lipid fractions as separated by the semi-automatic techniques described in the APPENDICES. Activities are expressed as DPM/mg of defatted and dried tissue.

TABLE 17

CARBON 14 INCORPORATION INTO LIPID FRACTIONS AS A FUNCTION OF TIME (II)

STEROL ESTER FRACTIONS

	DAYS POSTCOPULATION						
	0	1	2	3	4	5	6
VITRO	24	16	14	31	46	23	16
	30	13	23	38	14	28	17
	16	17	28	52	10	38	X
AVERAGE	23	15	22	41	23	30	17
VIVO	2	3	4	6	5	2	1
	4	20	7	3	2	2	2
AVERAGE	3	12	6	5	4	2	1

FREE STEROL FRACTIONS

	0	1	2	3	4	5	6
VITRO	98	65	60	78	59	231	109
	110	13	39	115	188	127	203
	223	26	58	110	41	220	X
AVERAGE	144	35	52	101	96	193	156
VIVO	24	4	50	34	15	16	24
	12	12	16	15	22	15	52
AVERAGE	18	8	33	25	19	16	38

FREE FATTY ACIDS

	0	1	2	3	4	5	6
VITRO	69	70	26	63	93	80	62
	80	45	37	88	64	85	124
	183	42	51	53	31	213	X
AVERAGE	111	52	38	68	63	126	93
VIVO	7	2	28	13	12	5	9
	10	15	9	17	11	5	10
AVERAGE	9	9	19	15	12	5	10

TRIGLYCERIDE FRACTIONS

	0	1	2	3	4	5	6
VITRO	111	64	37	226	183	199	101
	137	48	93	306	259	196	223
	158	56	77	365	98	326	X
AVERAGE	135	56	69	299	180	240	162
VIVO	12	2	48	29	23	11	11
	13	15	14	30	11	8	18
AVERAGE	13	9	31	30	17	10	15

This table presents the C^{14} activities of the lipid classes which comprise the TLC fractions. Activities are presented as DPM/mg of defatted and dried tissue.

exactly the same quantity of acetate. The data in Figure 19, which compares the relationships of the TL and TG_3 concentrations, gave some credence to this assumption. As is obvious, the TG_3 concentrations increased with the TL concentrations. It seemed possible that the degree of lipid storage in each rat could influence its lipid metabolism. However, the very nature of these studies precluded the use of basal or constant metabolic conditions within the animals. For example, the animals had to have normal estrous cycles, normal functioning adrenal glands, etc. Consequently, a correction factor was derived from the TL C^{14} activities which reduced the variance of the TG_3 activity data.[1]

The "corrected" data produced the C^{14} activity patterns presented in Figure 20. In these data, C^{14} activities peaked on day 3 in tissues from both incubation systems. A Dunnett's multiple range test applied to the *in vitro* data showed that C^{14} activities were significantly larger on days 3 ($p < .01$) and 4 ($p < .05$) than on other days. The same test, when applied to the *in vivo* data, indicated that C^{14} activities on day 1 were less than on days 2, 3 ($p < .01$), 4, and 5 ($p < .05$). In addition, C^{14} activities on days 2, 3 and 4 exceeded C^{14} activity on day 6 ($p < .05$). Thus, both sets of data demonstrated decreased TG_3 synthesis on days 5 and 6 postcopulation. The increase in C^{14} activities coincided with the estrogen surge (which initiates implantation on day 5) on days 3 to 4.(78) Thus, rising estrogen titres most likely effected the preimplantation changes in C^{14} incorporation into TG_3 fractions.

---

[1]The correction factor was derived by dividing the average TL activity into each TL fraction activity which contributed to the average. This ratio (i.e., average TL activity/individual TL activity) was then divided into the individual TG_3 activities to yield the "corrected" data.

Rat Uterine Triglyceride Concentrations vs
Total Neutral Lipid Concentrations

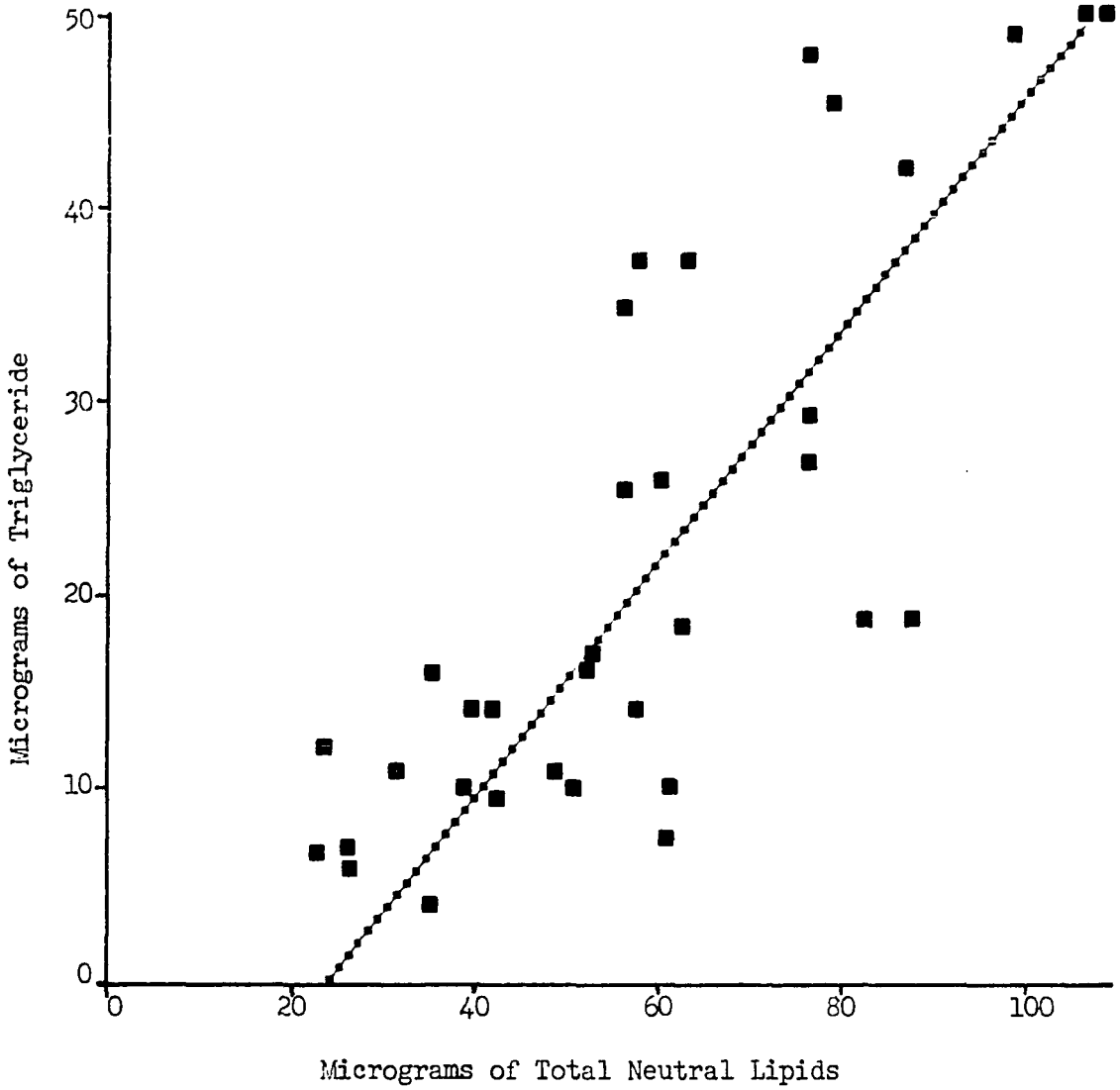

Figure 19 - This figure illustrates the relation between triglycerides and total neutral lipid concentrations extracted from rat uteri. Time postcopulation was not considered in this illustration.

Carbon 14 Activities of Triglyceride Fractions after a Mathematical correction for Tissue Variability

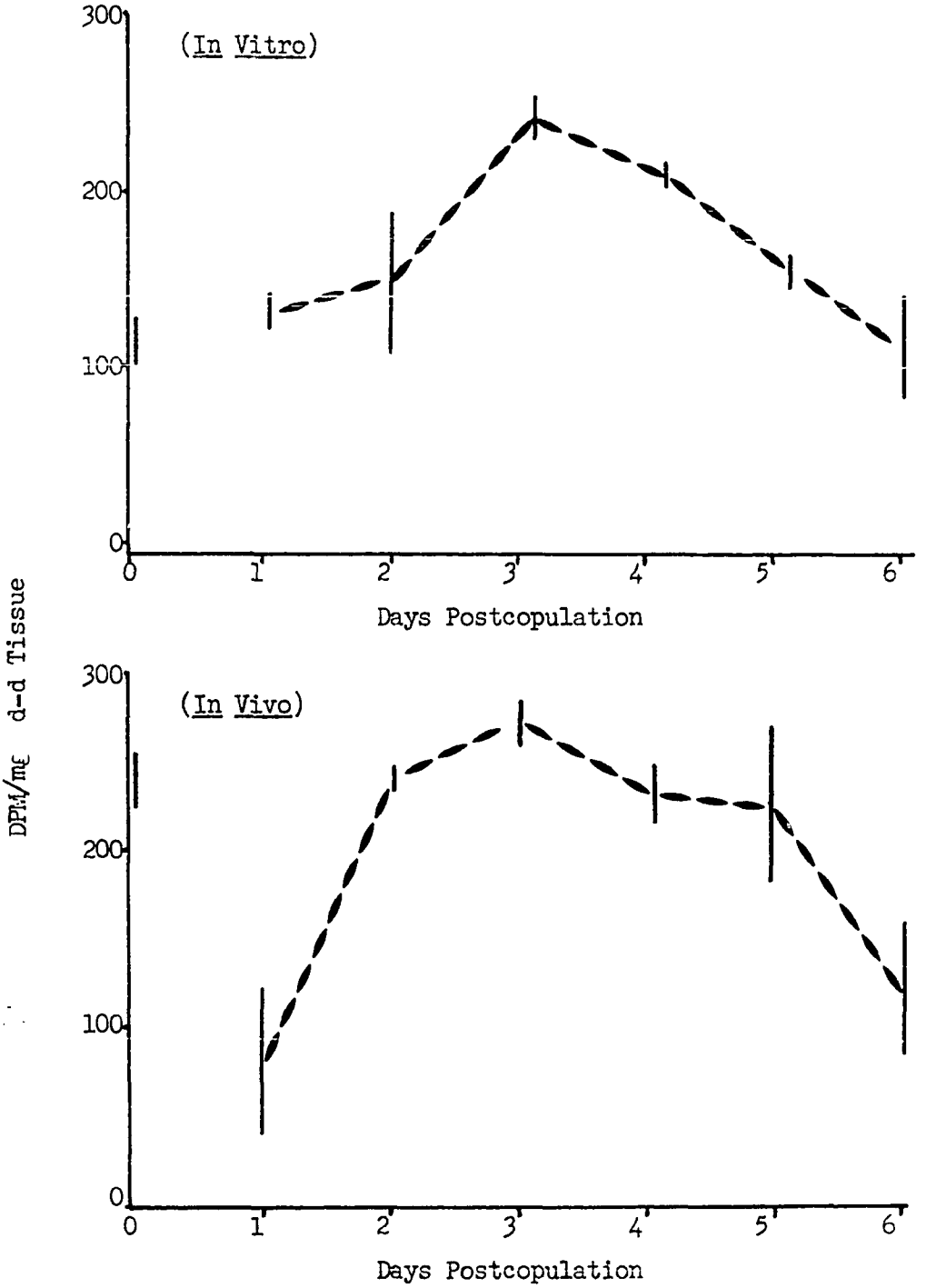

Figure 20 - These graphs illustrate the C^{14} activities of triglyceride fractions when the tissues were "forced" mathematically to have incorporated equal amounts of C^{14} into the TL fractions.

Carbon 14 titres on day 7 postcopulation. As stated earlier, tissues from day 7 rats were separated by gross dissection into implantation areas (decidual) and uterine tissue (endometrial) (page 77). Histological examination of implantation areas from day 7 postcopulation uteri revealed that less than 10% of the total tissue in the areas was embryonic tissue. Thus, the changes in TG_3 concentrations discussed earlier (page 77) reflected changes in uterine lipids. However, embryonic tissue during the entire period of gestation has a tremendous growth rate and, therefore, a capacity for rapid lipid synthesis.[1] Consequently, the incorporation data presented in this section may reflect the metabolism of embryonic tissues more than decidual tissues.

The analyses of C^{14} utilized tissue ratios (i.e., Dec/Endo from each rat) to reduce the variance resulting from inadequate separation of these tissues by gross dissection.

Decidual tissues incorporated about three times more C^{14} than endometrial tissue (Table 18). Recall, endometrial tissues contained more lipids per unit weight than decidual tissues (Table 18). These two facts demonstrated that decidual tissues had the higher rate of lipid metabolism; a relationship which was expected.

After the TL fractions were divided into TLC and PL portions, two endometrial PL fractions were spoiled. This prevented both C^{14} analysis and determination of tissue ratios for PL's.

Decidual tissues consistently incorporated more C^{14} into the TLC

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[1]Additional studies, not presented in this dissertation, substantiate this point. Twelve day old rabbit embryos incubated for 1 hour under the same conditions, incorporated over 8,000 DPM/mg d-d into the TL fractions.

TABLE 18

CARBON 14 ACTIVITY OF ENDOMETRIAL AND DECIDUAL TISSUE LIPID FRACTIONS ON DAY 7 POSTCOPULATION

	TOTAL LIPID			TOTAL LIPID FOR CHROM.			STEROL ESTER		
RAT	DEC	ENDO	RATIO	DEC	ENDO	RATIO	DEC	ENDO	RATIO
VIVO									
72	99	55	1.8	103	54	1.9	3	2	1.5
73	373	50	7.5	242	41	5.9	2	5	0.4
VITRO									
71	1763	1231	1.4	1262	1184	1.1	120	28	4.3
74	1327	629	2.1	1349	619	2.2	142	20	7.1
75	1182	657	1.8	1032	653	1.6	29	11	2.6

	TRIGLYCERIDE			FREE STEROL			FREE FATTY ACID		
RAT	DEC	ENDO	RATIO	DEC	ENDO	RATIO	DEC	ENDO	RATIO
VIVO									
72	15	12	1.3	50	26	1.9	19	9	2.1
73	14	12	1.2	70	22	3.2	23	9	2.6
VITRO									
71	296	526	0.6	202	311	2.3	58	174	0.3
74	360	161	2.2	691	158	4.4	161	111	1.5
75	206	295	0.7	524	361	1.5	232	84	2.8

This table presents the C^{14} activities in major lipid fractions of: (1) embryonic and decidual tissues (DEC) and (2) normal uterine tissues, grossly unaffected by nidation (ENDO). Activities are expressed as DPM/mg d-d tissue. The ratios were obtained by dividing DEC C^{14} activities by ENDO C^{14} activities.

fractions than endometrial tissues.

Of the four lipid classes comprising the TLC fractions, only FS fractions contained a consistent tissue distribution pattern: Decidual FS fractions contained more activity than endometrial FS fractions. However, decidual and endometrial tissues had similar distributions of C^{14} activities in the SE, TG_3 and FFA fractions.

plays a significant role in uterine function. Thus, the original premise of these studies has been supported by the experiments performed. Only the delineation remains of how these findings relate to the earlier observations of other workers.

Goswami, Kar and Chowdhury (1963) by conventional techniques, analyzed concentrations of unsaturated fatty acids from mice uteri.(28) They concluded, "It is unlikely that (estrogen) exerts any significant influence on metabolism of unsaturated fatty acids per se." The study of postcopulation, free fatty acid distribution in the rat uterus yielded data which: (1) confirmed their results, (2) included saturated fatty acids, and (3) included fatty acids of sterol esters and glycerides. According to the population distribution studies, no specific fatty acid is necessary, in uterine tissue, for growth of the blastocyst or implantation. However, incorporation patterns of C^{14} in fatty acids changed interestingly between days 1 and 4 postcopulation. Possibly specific fatty acids were synthesized and utilized at rates which prevented their accumulation. These specific conditions would have prevented detection of important fatty acids.

The limited C^{14} incorporation into sterols indicated either synthesis was slow, with conditions employed, or acetate was not the primary substrate. However, uterine tissue from immature and/or ovariectomized rats does incorporate acetate into cholesterol following hormonal administration.(74)

Changes in C^{14} activities of the TL fractions from all studies were understandable. In humans, increased C^{14} activities of TL fractions reflected the high metabolic activity of proliferating endometrial tissue. Rat uterine tissue incorporated the most acetate into lipids

during estrogen stimulation. An increased C^{14} incorporation during estrus was expected because: (1) estrogens stimulate uterine lipid synthesis, and (2) estrogen titres increase during estrus.(1,25,26,38, 51,85)

The postcopulatory rat uteri also incorporated C^{14} into TL fractions rapidly on day 0, i.e., estrus. In these studies, activity decreased temporarily within 24 hours after mating and then again increased after day 1. This increase coincided with increased estrogen titres on day 3 and with beginning mitosis on days 4 and 5.[1] Decidual tissues containing fetal tissues also incorporated C^{14} rapidly into TL fractions: Their incorporation rates consistently exceeded those of endometrium. This was predictable since postimplantation embryonic tissue is metabolically more active than endometrial tissue per se.

Elftman demonstrated (by histochemical methods) that the phospholipid rich Golgi apparatus increased in the rat uterus during estrus and decreased in other stages of the cycle.(20) The esterification data support his studies. For example, phospholipid fractions contained more C^{14} during preovulatory periods than during postovulatory periods. Specifically, this relationship existed during the human menstrual cycle, and after copulation in the rat (in vivo incubated tissues only). Count ratio data obtained during the normal estrous cycle in the rat substantiated these periovulatory changes in phospholipid synthesis by decreasing sharply after ovulation.

These phospholipid data also support chemical analyses of uterine tissue by other workers. According to Davis and Alden, rat

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[1] This activity is not sufficient to alter statements concerning the use of defatted and dried tissue weight as a base number (page 79).

uterine phospholipid concentrations increased from proestrus to estrus during the cycle.(19) Goswami et al., found similar changes in mice uterine phospholipids during the estrous cycle.(28) They concluded, "Under the influence of progesterone, the synthesized fatty acids are perhaps channelled towards triglycerides for incorporation because the phospholipid bases are not available in adequate amounts."

Apparently, from the total information available, the uterus synthesizes required phospholipids during the proestrus periods (i.e., preovulatory) of the cycle when estrogen stimulation is greatest. After rapid cell proliferation has subsided following ovulation, uterine phospholipid synthesis and concentration decrease. Thus, proper cellular development (during preparatory periods preceding fertilization) requires phospholipid synthesis. However, phospholipids do not likely serve any nutritive function of the blastocyst since both concentration and synthesis decrease after ovulation when nurture is required of the uterus.

The synthesis and accumulation of triglycerides after copulation in the rat compared to reported studies of changes in these lipids during the estrous cycle. For example, Davis and Alden demonstrated that neutral fats (which included triglycerides) increased in concentration during the rat estrous cycle from estrus to metestrus.(19) During diestrus of the cycle (with no active corpora lutea), the triglycerides decreased. After implantation the decidual tissues contained less triglycerides than the endometrial tissues. Thus, the triglycerides which were accumulated during the preimplantation period apparently became depleted during implantation or in early embryonic development.

Possibly they were utilized as biochemical energy sources for developmental processes. The literature contains histochemical data which support this concept. Williams (1948) and Krehbiel (1937) reported that the stainable lipids present in the decidualizing areas of rat uteri decreased during the early days following implantation.(34,71)

In the animal studies, synthesis, count ratios, and/or concentrations of the sterol esters, free sterols, and free fatty acids, varied tremendously during the reproductive cycles. Consequently, normal uterine function does not require specific concentrations or distributions of these compounds. However, a minimal quantity of them may be requisite since all classes were present in every tissue examined.

In 1969, Malinowska and Fotherby wrote, "There is relatively little information regarding biochemical, as opposed to histochemical and histological, changes in the uterus during early pregnancy."(42) With the completion of this dissertation, their statement is no longer valid.

CHAPTER V

SUMMARY AND CONCLUSIONS

Methods

Human endometrial and rat uterine tissues were incubated with acetate containing C^{14}. Following incubation lipids were extracted from the tissues and analyzed. Methods of analyses utilized column, thin-layer, and gas liquid chromatography. All studies included C^{14} analyses of the lipid classes. A semi-automatic technique for performing lipid analyses was developed. Measurements of lipid concentrations, by the semi-automatic system, were obtained in one study of rat uterine lipid metabolism following copulation.

Results

The studies reported in this dissertation demonstrated that human endometrium and rat uterine tissues synthesized fatty acids rapidly from acetate. The tissues then esterified the fatty acids to yield major lipid classes. However, sterols were not synthesized from acetate in any significant quantities.

A study of free fatty acid distribution within each lipid class indicated no specific fatty acid was present in a concentration related to lipid class or time postcopulation. However, C^{14} distribution ratios of some fatty acids did change from day 1 to day 4 postcopulation.

Evaluation of patterns of synthesis and esterification then demonstrated that C^{14} was rapidly incorporated into phospholipids during the preovulatory phases of reproductive cycles. Incorporation then decreased greatly during the postovulatory periods. Following copulation in the rat, phospholipid synthesis remained diminished through day 6. Both these data and the literature indicate that phospholipids are produced by the uterus during preovulatory uterine development when cell proliferation is greatest. In addition, they indicate that uterine phospholipids are not an energy source for developing blastocysts or for implantation processes.

These studies also demonstrated that uterine triglyceride synthesis and concentration increased following copulation. Similarly, TG_3 count ratios increased after ovulation in the rat estrous cycle. When these findings were correlated with reported studies, the uterus appeared to synthesize and accumulate triglycerides after ovulation. The stored triglycerides were then utilized during implantation and early embryonic development.

Carbon 14 and lipid concentrations of the other classes had considerable variance which appeared to be normal for uterine tissue. With the possible exception of sterol esters in human endometrium, the sterol esters, free sterols, and free fatty acids *per se* are probably not requisite in any specific concentration for normal fertilization and implantation.

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APPENDIX I

APPENDIX I

INTRODUCTION AND DEVELOPMENT OF A SEMI-AUTOMATIC METHOD OF LIPID ANALYSIS

General Introduction

Early in these lipid studies, attention was also directed toward development of a semi-automatic system for lipid analysis of the TLC fractions. The system utilized standard procedures of chromatography for lipid separation. In addition, it permitted measurement of lipid concentration plus C^{14} activity.

Development of the system took some 4 years and was started soon after the human endometrial studies were underway. Because development and proof of reliability of the methods were a necessity before detailed investigation of uterine lipids could be pursued, a detailed description of the instrument calibration and operation will be presented. Since the system developed has independent and significant value in areas other than uterine lipid investigation, a general description of the procedure will precede the specific methodology.

Rationale of the System

It is desirable to be able to observe a process as it separates a mixture of compounds. Since chromatography came into general use, means to achieve continuous observation of colorless compounds have been

sought. Chromatography plays a central role in lipid assay and analysis. Its significance is well documented in Marinetti's monograph on lipid chromatography.(81)

All the uterine studies which are presented in this dissertation were concerned with understanding lipid metabolism, particularly biosynthetic mechanisms. As an example, the first study, lipid biosynthesis by human endometrium, can be cited.(47) As mentioned earlier, standard techniques of column and thin-layer chromatography were employed to perform this study. The small quantity of material available (a few milligrams of biopsy tissue in many instances) limited the information that could be gained. For example, before the lipid concentrations in a tissue could be measured, it was necessary to decide which lipids would be measured. Measurement of one lipid class concentration could preclude the accurate evaluation of another. Separation of classes for determining the distribution of radioactivity, as was done in the early studies, prevented quantitative determinations of lipid concentration: All available material was needed for the C^{14} assay.

Within these analytical limitations, potentially significant changes were shown to occur in lipid metabolism near the time of ovulation. In addition, normal human endometrium did not characteristically synthesize cholesterol but malignant tissue did. These findings provided a stimulus to devise additional analytical methods with a greater information yield than those initially employed.

The refractometer, described below, was developed in 1964 and presented the possibility of a new analytical method. About the same time, James published a method for continuous examination of the lipid

content of a column eluate.(32) During the subsequent four year period the refractometer and principles of continuous solvent evaporation (as presented by James) were combined into a working unit which proved to be satisfactory for uterine lipid analysis.

However, the usefulness of the system is not limited to uterine lipid problems. Its general application is demonstrated by at least three points.

1) The instrument can detect and quantitatively determine as few as 10 micrograms of various lipids at 1/2 its full sensitivity under routine working conditions.

2) If silica gel chromatography and solvent evaporation with an inert gas (N_2 or CO_2) are not capable of altering lipids under study, then the apparatus will not change compounds whose concentrations it determines.

3) After measurement of the lipid concentration, all the material is available for further study if desired. Consequently, other desirable procedures like C^{14} analysis are permitted.

In summary, the problems of uterine lipid measurement were solved by a system capable of general application. In theory, the system (with appropriate adjustments) should be applicable to the analysis of any set of compounds which are soluble in volatile solvents and can be separated by column chromatography.

The sensitivity as delineated later is not the limit of the system. A tenfold sensitivity increase should be relatively easy to achieve. Perhaps equally important, the system also has the capacity to separate and measure larger quantities of lipids.

The analytical system as described below will include only the range of concentrations found in uterine lipids from rats.

General Considerations of System Operations

The total eluate from the chromatographic column is evaporated on a moving inert plastic tape (Mylar or Teflon, 1.375 in. wide x 0.005 in. thick). The residue is then redissolved in a suitable solvent which passes continuously through a recording refractometer (type R-4, manufactured by Waters Associates). The change in refractive index of the solvent is shown on a potentiometric recorder, the magnitude of change being a function of the concentration of the lipid. Successful operation requires that:

1) the tape be clean
2) there is controlled speed of tape movement
3) there is control of column flow rate
4) rapid and complete evaporation of eluate from the column occurs
5) continuous solubilization of eluated material by successive volumes of eluate is prevented
6) the redissolving solvent is maintained at a constant level of purity (which specifically includes constant concentrations of dissolved gases)
7) flow rate of the redissolving solvent is constant, and
8) the redissolving solvent is completely removed by the pick-up process.

Specific operations of the apparatus are described below. These operations are illustrated in Figures 21 and 22.

Mechanical Operations

The plastic tape moves through a continuously changing solvent bath in Chamber A (Figure 21) to be washed and then over rollers through the first air lock (1) to Chamber B. In this chamber, the tape is guided below a horizontal plane through a drying oven (3). The tape, now carrying eluate residues, emerges from the oven and is returned to the horizontal plane by a roller.

Diagrammatic Sketch of Eluate Collection, Evaporation and Resolution System

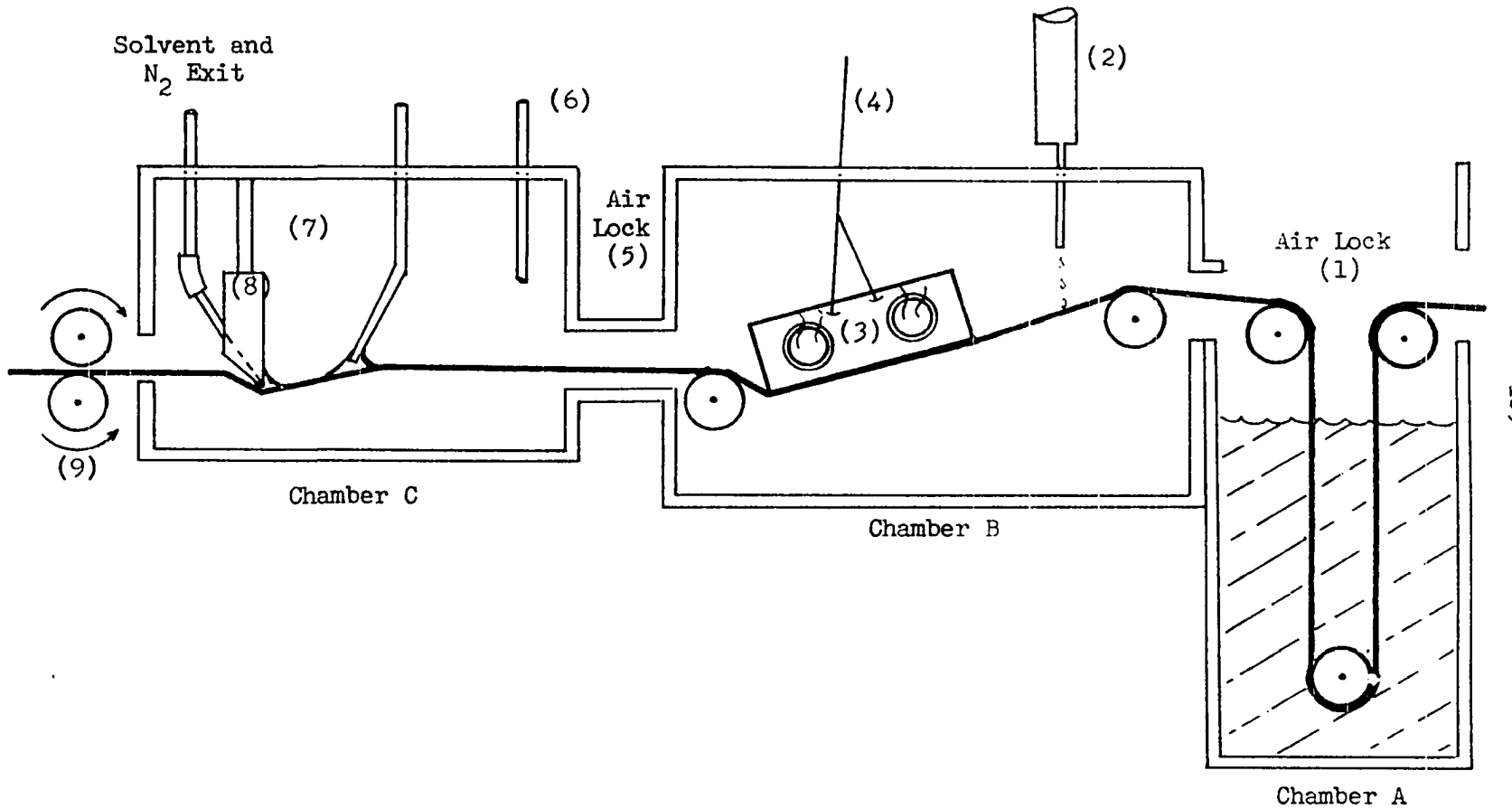

Figure 21 - This figure illustrates the mechanical system for collecting the fractions eluted from the chromatographic column. The mechanics of this system are discussed in detail in the text.

Immediately after entry into Chamber B, the tape receives the eluate from the chromatographic column (2). The tape is crimped into a trough by guides on the outside edges of the drying oven. The eluate is thereby kept in the center of the tape. Nitrogen or other inert gas is forced (via 4) over 50 watt heaters in the oven so that the heated gas strikes the tape and is deflected towards the central pool of eluate. Two heating elements and gas streams at different temperatures are necessary for complete control of solvent evaporation.

Column eluate flows onto the tape in pulses. (The method of producing the pulses will be described later.) The pulsation coupled with the slope at which the tape is held prevents the following solvent from redissolving the residue from earlier portions of eluate. The solvent pool "buds off" under these conditions, producing discrete separate large drops which remain separate as they evaporate. Eluates which form crystalline residues show the residue as small circles after evaporation is complete.

An exhaust vacuum is applied to the drying Chamber B to remove gases of the evaporated solvents.

For both collection of the material by the redissolving solvent and observation of changes in refractive index, the tape moves into the redissolving chamber (Chamber C) through a second air lock (5). Nitrogen gas saturated with the solvent used to redissolve the residue (ethanol in these studies) is continuously passed through a port (6) into Chamber C to completely exclude air. Flushing this chamber with saturated gas also reduces evaporation of the redissolving solvent during the pick-up phase and during solvent removal from the chamber.

The redissolving solvent flows to the tape via a Teflon applicator (7), 1" wide. This is located at a height above the tape that allows the solvent to form a bridge between the applicator and the tape.

After passing under the applicator, the tape is inclined downwards to the solvent pick-up which presses the tape into a gap between two guide plates. The upper surfaces of the guide plates match the convex base of the pick-up unit. The pick-up unit is a heavy metal section whose base is convex and polished to a fine edge. At the lowest point of the base is an orifice (0.04") that connects to the negative pressure produced by the pseudo-aspirator (Figure 25) at the base of the condenser (to be described later).

The negative pressure "sucks" the solvent, plus nitrogen, from the chamber to a liquid-gas separator mounted above the refractometer. The separator unit is a glass Y-shaped tube, which allows the solvent to flow from the bottom to the refractometer while the N_2 passes to the aspirator. If the refractometer is not used, an appropriate vertical length of tubing is needed to permit the pick-up and Y-tube combination to function and deliver samples to collection vials. The liquid separation unit cannot function unless some negative liquid pressure is available at its base. Flow rate of liquid from the Y-tube is easily adjusted by maintaining the exit port at an appropriate level below the separator.

When the refractometer is employed, the ambient temperature in the Y-tube must be greater than the internal temperature of the refractometer. Careful adjustment of the negative pressure determined by the level of the exit port is also necessary. Because the solvent

contains N_2, these adjustments must be properly made or small gas bubbles will form in the refractometer and render the instrument useless. Fortunately, gas bubbles are easily recognizable as pulses in the recording and can be differentiated from meaningful peaks.

Tape Drive Mechanism

The tape is drawn through the apparatus by a capstan (9, Figure 21) having one metal and one rubber roller, driven by a synchronous motor via gearing to provide a range of speeds (minimum movement is 1"/min). The pressure exerted by the rollers of the capstan is sufficient to ensure that changes in tape resistance to movement will not cause changes in tape speed.

Cog tape drives, normally used for ensuring constant tape speed, were avoided because of the higher tape cost and increased difficulty in tape manipulation within the instrument. By using the cheaper unperforated tape, it is practical to use the tape only once.[1]

Solvent Flow System

The system utilizes a still located at floor level. Vapor from the still rises through a partially insulated column to ceiling height and enters a condenser. The condensed liquid flows into a reservoir with two exits (1, Figure 22).

From the upper exit (an overflow), the solvent flows to a constant level reservoir (2) which feeds the applicator (7, Figure 21).

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[1] When isotopic labeling was used, failure to completely remove material containing label from the tape might have caused an error in subsequent usage of the same tape. Consequently, a tape was used only once.

Diagram of the Solvent Flow System

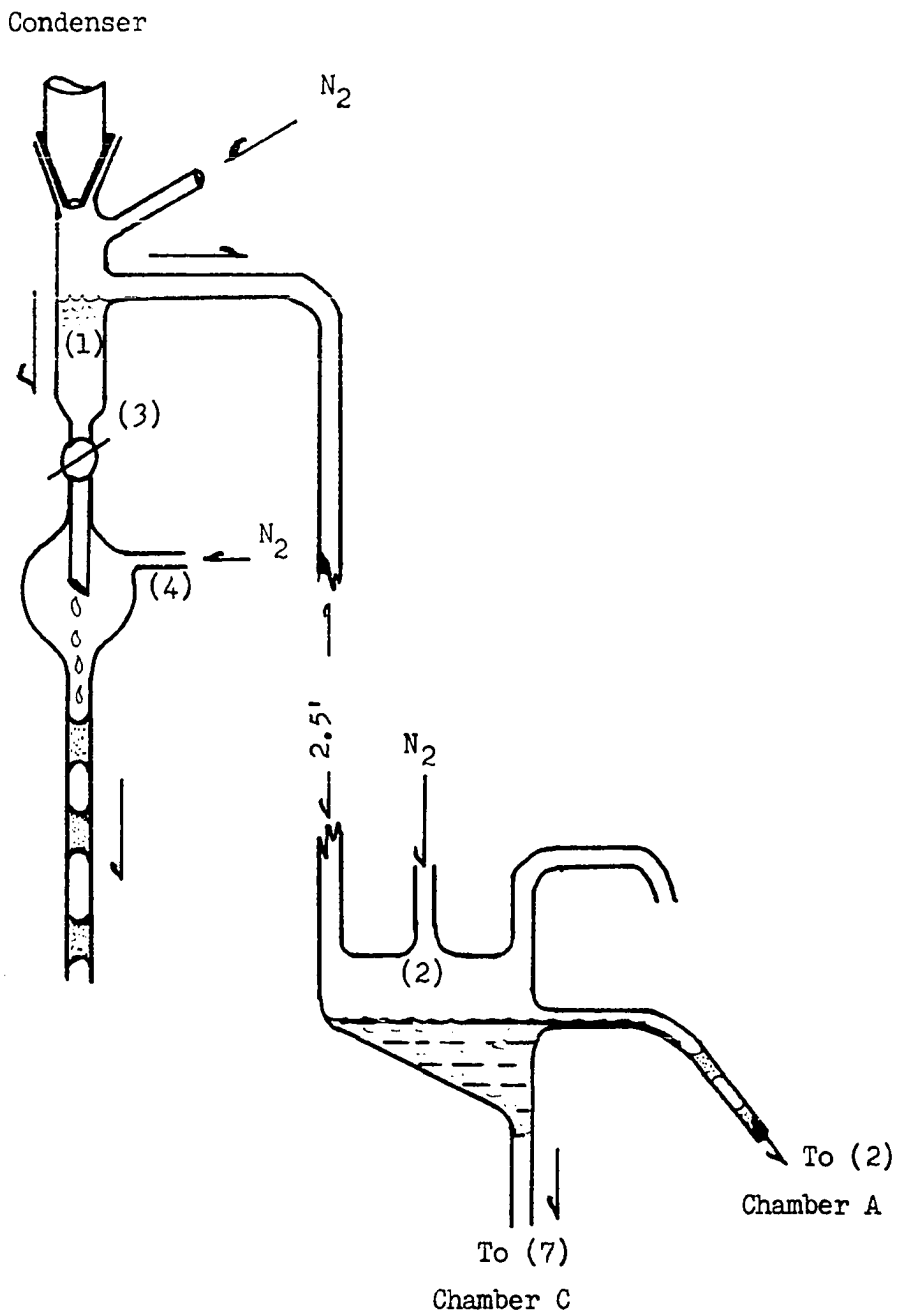

Figure 22 - This figure illustrates the solvent flow system which creates the negative pressure used to remove materials from the Mylar tape. Details are discussed in the text.

Adjustment of the height of this reservoir provides the desired flow rate to the tape. Overflow from the reservoir (2) runs into the tape washing unit (Chamber A, Figure 21). Overflow from here is returned to the still.

The second exit from the reservoir (1) is fitted with a fine Teflon stop cock to control flow (3, Figure 22), the condensate then passes into the aspirator (4). The flow from the stop cock is adjusted so that discrete "slugs" of liquid form in the exit tube and draw N_2 in through the side tube. The negative pressure so obtained is used to "lift" the redissolving solvent from the tape.

This simple system, which was found satisfactory for producing the absolute constancy of flow and negative pressure needed, has additional advantages which result from the use of a still as the source of driving power. First, constant distillation of the redissolving solvent assures a minimum of change in that solvent. Secondly, the adjustments that control flow rates are working against gravity. Therefore, once set and locked, they can be expected to remain constant (only changes in flow resistance can then alter flow rates). By adjusting the distillation rate to exceed flow through the aspirator and the redissolving unit, only the amount of fluid entering the washing chamber is subject to change with factors which may affect distillation rate (for example, temperature and/or voltage changes).

Temperature changes of the redissolving solvent affect its viscosity. Thus, a solvent flow rate through a fixed resistance at a constant pressure gradient can change. Ambient temperature changes must, therefore, either be controlled or flow rates must be considered in the

calibration procedure.

The flow rate of the ethanol was from 0.6 to 0.8 ml/min. Since the refractometer measures concentration, fluctuations in rate of more than 1% or 0.006 to 0.008 ml/min. were undesirable during the analysis. Any change in flow rate could have been reflected as a significant change in calibration value. Equally important, changing flow rates caused "noise" and poor baselines in the recordings. No inexpensive mechanical pumps were found capable of maintaining this degree of constancy at such low flow rates.

The instrument is best described as a differential refractometer because it measures the difference in refractive index (at a sensitivity of 10^{-6}) between the fluids in the reference cell and the sample cell. If the solvents in the two chambers are maintained constant, a zero reading (arbitrarily set on the recorder scale) results. However, because of the nature of the instrument, factors other than rate of flow will affect refractive index as it is measured.

The refractive index of the solvent can be altered under conditions of constant flow and temperature by impurities (i.e., dissolved gases, liquids, or solids). The degree of alteration from the true refractive index is a function of the concentration and refractive index of the dissolved impurity.

In these studies, where a measurement of the amount of an "impurity" was sought, the degree of change from zero obtained when all other sources of change were held constant was a direct function of the lipid concentration. A change in flow rate of the ethanol (either onto or off the tape) during the period in which a specific amount of lipid

was available for redissolving would have changed the calibration value.

This area (i.e., relation of solvent volume to sensitivity of the device) is where future developments may provide radical advances. The smaller the volume of solvent employed to redissolve a deposited lipid, the higher the recording sensitivity. Pragmatically, the relationship is linear so that a doubling of concentration is reflected in a doubling of the area (see calibration curves, Figure 24).

Operation of the Column and Solvent Pumps

While any of several chromatographic column systems could have been used, the column used in these studies consisted of a glass tube 46 cm long, 6 mm internal diameter, containing 4 gms of silica gel. The base of the column was fitted with an ultrafine Teflon stop cock (the volume between the sintered glass filter at the column base and the stop cock was 0.02 ml). The column was kept at 19°C by passing water through a concentric jacket. The top of the column terminated in a standard taper (10/30) male joint, below which was fitted a Teflon collar. The solvent line was connected to the column top via a Teflon cylinder into whose base was cut a standard female tapered joint. This cylinder was secured to the column top by an adjustable connection to the Teflon collar. The cylinder contained two ports each with a diameter of 0.04 inches. The stainless steel solvent line from the column pump was fitted to one of these ports and a small needle valve (for gas extrusion at the column top) to the other.

Into the solvent line either a manifold (fed by solvent pumps) or a mixing chamber was connected, permitting use of either a gradient elution system or a graduated solvent elution system.

The glass tubing leading from the base of the Teflon stop cock at the bottom of the column was drawn to a fine point. Eluate dropped into a narrow short funnel (9 mm internal diameter at top, capillary at base). The funnel fitted into a 2 cm length of Teflon tubing which in turn was connected to the stainless steel (internal diameter 0.04 inches, external diameter 0.0655 inches) tubing which directed the eluate to the tape (Figure 21). The base of the tubing was held about 5 mm from the tape. This multidiameter line in which a portion was capillary tubing retained most fluid in it until the fluid seeping into the larger diameter tubing below the capillary exerted sufficient negative pressure to pull air through the capillary base of the funnel. This was true only if the flow rate into the funnel was not too great to prevent a flow break. By this simple means, a pulsating flow (discussed earlier on page 110) can be developed from the constant flow issuing from the base of the column.

Since development of the system started, solvent pumps of various types have been employed, the most satisfactory of which have been the constant infusion pumps (glass syringes driven by constant speed drives) manufactured by the Harvard Apparatus Company. They served well for all solvents except ethyl ether when high resistance columns were used. For low resistance columns, they were satisfactory for all solvents.[1]

Only Teflon, glass, stainless steel or comparable materials

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[1]When the higher pressures encountered in high resistance columns must be overcome, the use of special tight syringes with Teflon plungers must be considered. Low viscosity solvents will leak between the piston and cylinder walls of a glass syringe.

were used in these pumping systems.[1] Plastics other than Teflon were found to leach plasticizers into the solvents in appreciable quantities for months.

The standard squeeze bottle (unless made of Teflon) was not even used for solvent storage of lipid solvents in these studies and should not be used with this system.

Special Problems Posed by the Elution Solvents

Because the detection system eployed was sensitive to any compound soluble in the redissolving solvent, a minimum of non-volatile residue in the eluting solvents was critical. Even hexane required purification by passage through large silicic acid columns followed by distillation.

As a solvent purity test in the initial studies, 10 ml of hexane which were evaporated on to a small area of the tape had to yield no signal indicating residue before the hexane was considered sufficiently pure for use. Ether and methanol of analytical reagent grade usually passed this test without further purification. In later studies, special methanol (Nanograde provided by Mallinckrodt Co.) proved more satisfactory but still had some limitations (see below).

There are several "escapes" from the dilemma of working in the microgram range where the solvents employed can, by the limits of purity they possess, interfere with the observations the analyst is attempting. These are use of (1) blank runs, (2) elaborate purification techniques,

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[1] Stainless steel fittings were also used exclusively because metals other than stainless steel appeared to leak oil or otherwise contaminate the solvents. (The oil apparently penetrated the metal during machining operations and leached out slowly into the solvents.)

and (3) working in a restricted concentration range. The lipid studies utilized a combination of all three "escapes."

The blank run approach is relatively simple but required precision of operation and careful preplanning to minimize non-productive runs. Blank runs were conducted frequently during both the calibration and experimental phases of these studies. Figure 23 is a recording from such a blank run. There were no significant peaks recorded until methanol was used in the eluant sequence. In this run, had the tape been stopped as each eluant came from the column, small peaks (0.04 square inches or less) would have likely been produced by each solvent front. However, they became invisible within the baseline variations when stretched over several minutes of elution and evaporation. The methanol containing eluate, however, appeared to extract from the column a portion of impurity which was absorbed by the column from prior eluates.

Therefore, in general, successful application of the blank run principle requires a precision operation of a suitable column in a fashion which (1) minimizes the volume of elution solvent needed, and (2) removes desired fractions from the column in as sharply defined peaks as possible. Such chromatographic technique and artful column operation could easily form a separate subject of discussion. They are mentioned here only to emphasize the point that the better the chromatographic technique, the more accurate will be the signals which are provided by the observational portion of this system.

Solvent purification to reduce the impurity level below significance at all concentrations employed is the ideal solution to the dilemma. As mentioned earlier, hexane purification was necessary in the

LEGEND FOR FIGURE 23

Presented in this figure are tracings of two column calibrating experiments performed at maximal instrument sensitivity. Tracing Number I represents a blank run. It illustrates the peak seen when methanol strips the column of residue deposited by the earlier eluants.

The vertical lines at the base of each tracing represent 10 minute intervals. The vertical numbered lines represent the onset of pumping the following eluants through the column:

#1 - 12.0 ml of 5% Ethyl Ether in Hexane

#2 - 12.0 ml of 20% Ethyl Ether in Hexane

#3 - 20.0 ml of 50% Ethyl Ether in Hexane

#4 - 12.0 ml of 50% Ethyl Ether in Methanol

#5 - 12.0 ml of Methanol

All eluants, except the methanol, were pumped at 0.84 ml per minute. The methanol was pumped at 0.4 ml per minute.

Tracing Number II represents the separation and calibration of 97.2 micrograms of cholesterol and 114 micrograms of cholesterol linoleate which were placed on the column. The expected areas (based on complete calibration data) were 1.13 and 0.98 square inches for the free cholesterol and cholesterol ester, respectively. The areas found for this particular experimental run were 1.02 and 1.04 square inches, respectively.

The column was regenerated by passing through it 30 ml of methanol, 30 ml of ethyl ether and 15 ml of 5% ether in hexane. Column volume was 9 ml.

Representative Machine Tracings of Lipid Samples
used in Calibration of the Semi-automatic System

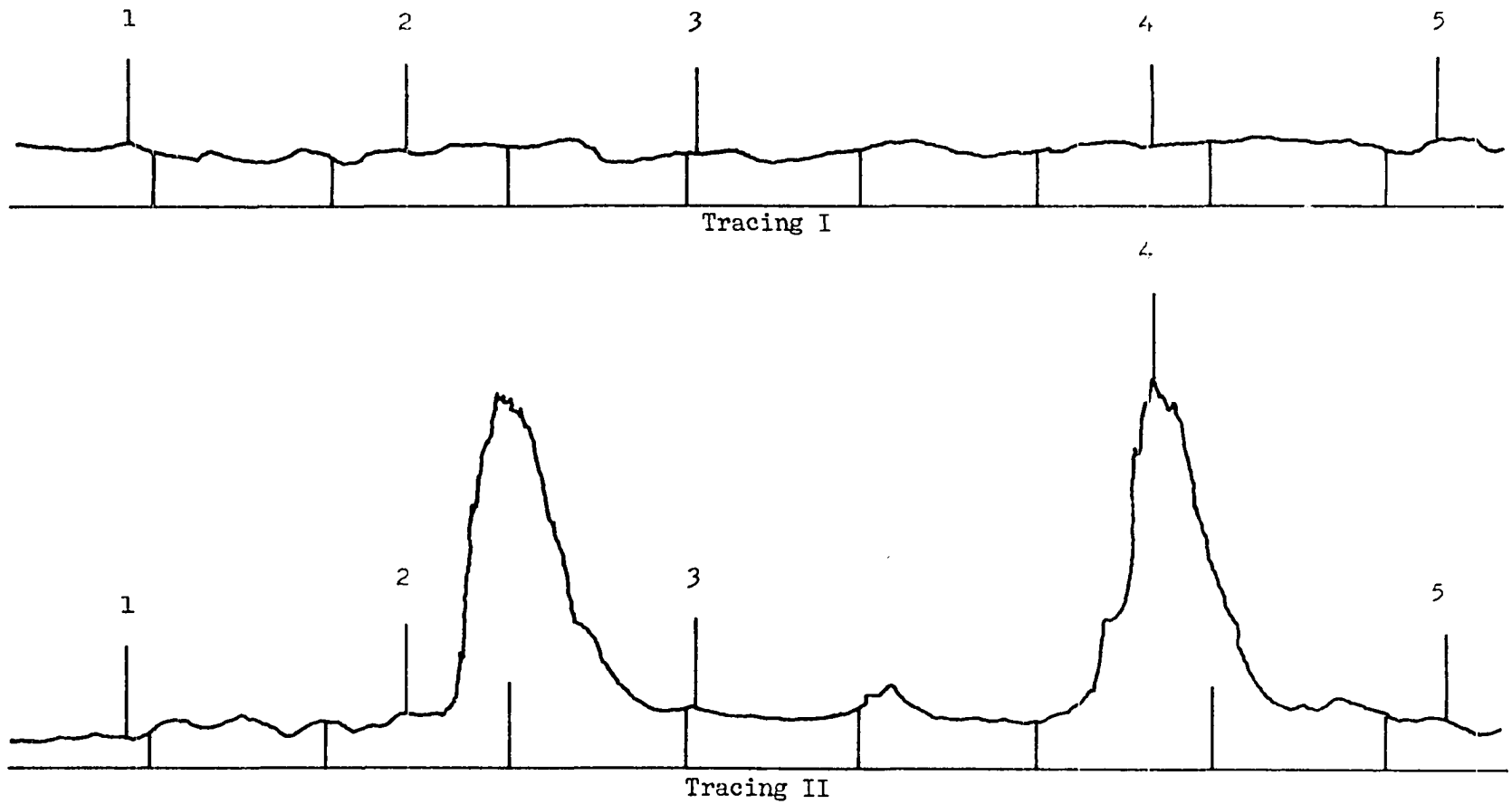

Figure 23 - Details concerning these tracings are discussed in text.

lipid studies. The difficulty in achieving solvent purification is easily seen by recognizing that (as will be discussed later) over 10 ml of solvent was used as an eluate and the instrument can detect as little as 5 micrograms of lipid material. Such levels of purity (0.5 micrograms/10 ml) are not always present in commercially available solvents. If a solvent of the desired purity is not available, then the labor of purification is a necessary part of the effort involved in the total analysis. At this point the advantage of a restricted working range becomes self-evident.

Usually it is not necessary to employ a system capable of separating 5 milligram components of assorted lipids when observations at the 10 to 50 microgram levels are to be made. This was the case in the lipid analysis studies on which this description is based. A reduced column size required reduced volumes of solvents. At the levels of column and solvent used, the degree of solvent impurity (hexane excepted) in these studies did not interfere with the accuracy of determining 10 to 50 micrograms of lipid.

Column Composition and Use

With some grades of silica gel, regeneration of the column was possible. A suitable grade was silica gel "Ultra-pure" (0.05 to 0.20 micrometers) by Brinkmann Instrument Co. Column regeneration was performed by passing three column volumes of methanol, followed by a similar volume of ethyl ether through the column. Refilling the column with hexane or 5% ethyl ether in hexane completed the procedure.

With continued use of the entire system, it was found necessary to develop criteria for (a) properly constructed columns, and (b) a

column's continued ability to function. The criteria which were used are as follows. (1) When a column was constructed, several blank runs were made. If the column did not meet expected blank run standards (see Figure 23), it was discarded. Columns which did meet these standards were subjected to frequent confirmative runs to assure that the blank values were maintained. (2) An artificial mixture of known compounds (usually cholesterol-esters and cholesterol) were separated by the column. If the results of this test conformed to expected values, the column was ready for separation of unknown lipid mixtures. (3) A column history was maintained. Failure of the column to retain proper performance either during test runs or at any time thereafter terminated its use. Several columns, once found acceptable, functioned well through over fifty regenerations and runs.

Choice of Redissolving Solvent

In general, the sensitivity of the refractometer assay system is at a maximum when the solvent used for redissolving the lipids has a refractive index far removed from that of the lipids. The most suitable solvent, from this aspect, for most blood and tissue lipids is methanol. Unfortunately, however, triglycerides are poorly soluble in methanol.

Since a still is used for solvent distribution and reuse, a composite solvent such as chloroform-methanol cannot be employed. Such "constant boiling" mixtures are difficult to use because changes in barometric pressure will alter the still's output. There are other considerations which play a role in the choice of the redissolving solvent (for example toxicity, volatility, and stability). Consequently for these lipid studies, a compromise of refractive index, solubility,

and other conditions was reached by using ethanol as the redissolving solvent.

APPENDIX II

APPENDIX II

CALIBRATION OF THE SYSTEM

In accordance with the studies undertaken, development of the overall system was limited to accurate separation and measurement of sterol esters, triglycerides, free sterols, and free fatty acids as discrete classes.

The elution sequence employed in calibration runs (as contra-distinguished from experimental and blank runs) of the analytical system was as follows:

1) 5% ethyl ether in hexane was used to fill the column after regeneration and to dissolve the lipid samples applied to the column.

2) After application of the lipids to the column, 5% ether in hexane was passed through the column for 10 minutes at the rate of 1 ml/min.

3) Continuous gradient elution was then begun by adding (at a constant rate) 100% ether to the 90 ml volume of 5% ether in hexane in the mixing chamber.

4) When the peak corresponding to the free sterols was seen, gradient elution was stopped and 100% methanol was put through the column at a reduced rate of flow. The resulting peak would be expected to contain free fatty acids, residual phospholipids, and other very polar lipids. This fraction in the first stage of development was used only to prove recovery of lipids by tracer analysis.

The partial content of the various peaks observed was proven by adding known quantities of specific substances to a reference standard

extract (described below). Known quantities of each material were first deposited directly onto the tape, permitting an expected increase of area in a given peak to be predicted. By the procedure of finding a specific peak area predictably increased, it was demonstrated that the material was totally eluted from the column and the specific peak contained the substance.

Sterol containing peaks were also proved and/or identified by use of the Liebermann-Burchard reaction.(16)

When all solvent volumes and flow rates related to the gradient elution system were held constant, prediction of peak appearance to within 2 to 3 minutes was possible.

For a reference standard of a biological mixture of lipids (instead of a synthetic mixture) 50 ml of deep frozen human plasma were thawed and extracted with CM as described earlier for the uterine tissue lipids. The extract was evaporated to an oil which was then re-extracted with chloroform. The phospholipids were then removed by acetone precipitation and a serum TLC fraction dissolved in 5% ether in hexane was obtained. Although this procedure can be quantitative (as well it was in the uterine lipid studies), no effort was made in this preparation to recover all available lipids. The prime concern was simply the exclusion of phospholipids from the TLC fraction because of their well-known capacity to interfere with neutral lipid chromatography.

TLC aliquots ranging from an equivalent of 0.015 ml of plasma to 0.125 ml were assayed with the elution sequence described above. The resulting calibration curves for the sterol esters, triglycerides and free sterol fractions are presented in Figure 24. The free fatty acid

Calibration of Human Serum versus Peak Area

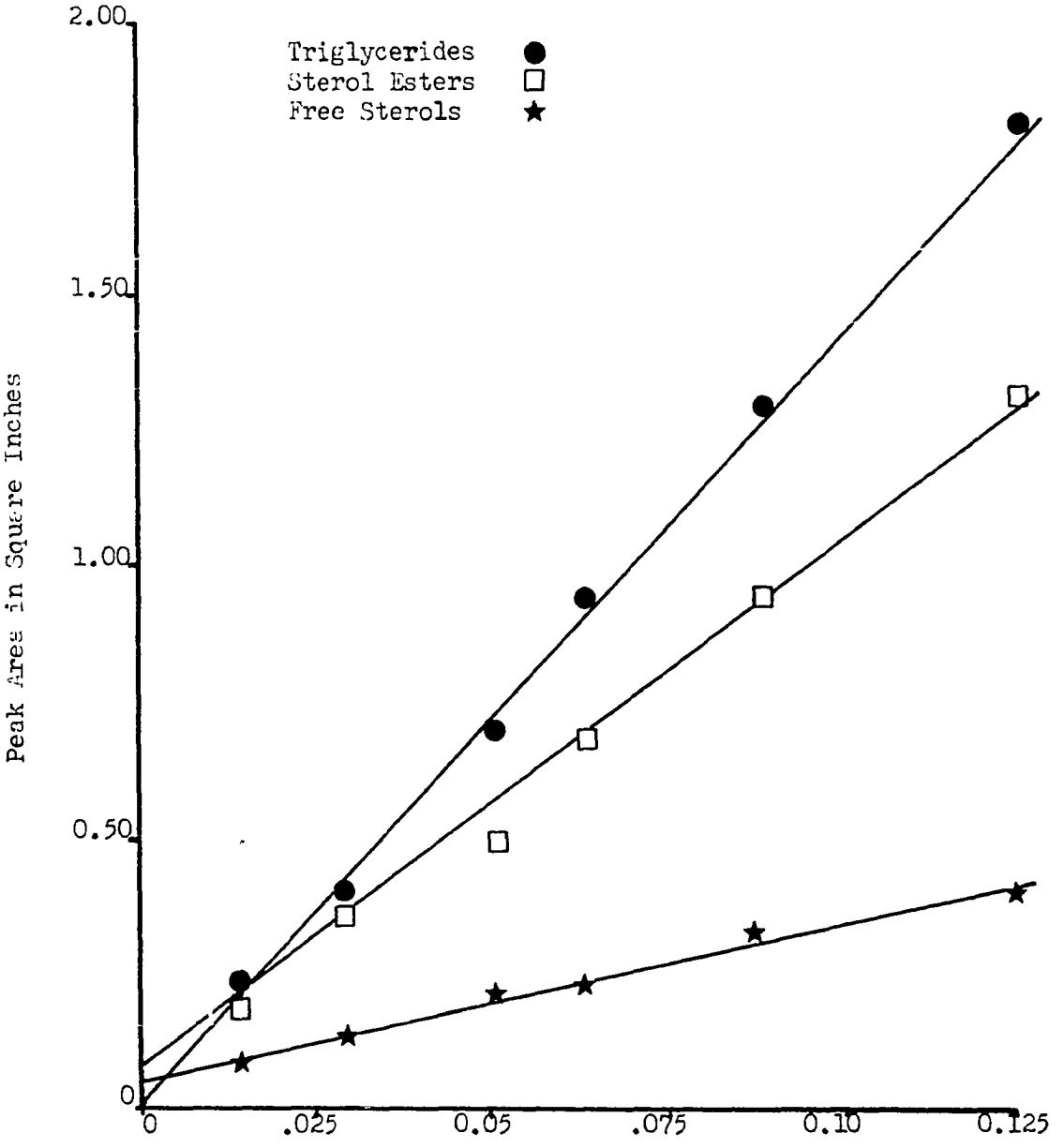

Figure 24 - This figure illustrates the relation between ml equivalents of human serum lipids and peak areas as recorded by the new system.

levels were not observed because at this early stage of procedural development, the blank methanol fraction (which contained low amounts of free fatty acids) showed erratic peaks due to contaminants. Further development of the system after these calibration values were obtained permitted accurate measurement of the free fatty acid fractions from uterine lipid samples.

As Figure 24 shows, the device gave a linear response to concentration of each of the three classes. However, only the triglyceride line passed through the origin. Computation of linearity by the methods of least squares gave intercepts of 0.038, 0.050, and 0.010 square inches for the free sterols, sterol esters, and triglycerides respectively. Theoretically, each of these lines should pass through zero since a blank run similar to Figure 23 produced no peaks.

Promising explanations of the observed discrepancy at that time were (a) that the solvents were not sufficiently free of contaminants and (b) that when a lipid class moved down the column, it carried with it an accumulation of residue that was not moved in a similar fashion by the solvents alone.

Another possibility could have been the incomplete evaporation of eluate solvents from the lipids. Retention of that solvent, if it was constant and directly related to the amount of lipid deposited on the tape, would fit the observations. This seemed a remote possibility in view of the efficient performance of the evaporation oven. In addition, neither rate of solvent evaporation nor nature of the solvent employed to deposit a particular sample on the tape had been found to influence the resulting signal. Had retained solvent contributed to the

size of the signal, then deposition of a sterol ester sample on the tape dissolved in methanol should have produced a markedly different peak from the same amount of ester deposited in a chloroform solution. Such checks were applied early in development of the system and were found to yield a constant area per unit of lipid irrespective of the solvent employed.

It was not until the refractometer was improved (recall the entire system was in a state of improvement during all studies) and a different system of elution was employed that it became clear that solvent impurity was the primary source of the difficulty. This is discussed below.

As part of the original calibration of the system, sterol esters, triglycerides (as olive oil) and cholesterol were added to serum aliquots. The area obtained for each peak was corrected by subtraction of the area expected for the serum aliquot alone. In the initial phases of development, with an ethanol flow of 0.78 ml/min and with the original refractometer at 1/2 full sensitivity, 0.02 square inch peaks were easily seen. The final calibration values for that stage of development are presented in Table 19.

TABLE 19

MICROGRAMS PER SQUARE INCH OF PEAK
OF THREE LIPID CLASSES

Sterol ester	268 micrograms/sq.in.
Triglyceride	390 micrograms/sq.in.
Cholesterol	206 micrograms/sq.in.

Through the kindness of Dr. David Kritchevsky, an independent

analysis of the three major classes of lipids in the serum extract was obtained. The sterol ester and free sterol areas obtained by the use of the new system were corrected, for this comparison, by the value of the area the intercept. The triglyceride value was not corrected. Comparative results are shown below:

	Source of Analysis	
	Dr. Kritchevsky	New system
Sterol Esters	156 mg/100 ml	161 mg/100 ml
Triglycerides	595	579
Free Cholesterol	51	58

The values for the sterol esters and triglycerides lie within the probable error of both techniques. The free cholesterol values are significantly different and illustrate the inherent problem of any assay system of the type being described. The higher free cholesterol value obtained by using the refractive index method is undoubtedly due to the well-known fact that other substances besides cholesterol are eluted by the solvent mixture which elutes cholesterol from a column of this type. A further analysis of free sterol fractions taken from rat uteri will be presented later.

This series of calibrations demonstrated the usefulness of the overall system. A more sensitive refractometer was then obtained which, fortunately, also provided a more stable baseline. Representative calibration data for this instrument are presented below.

Representative calibration values, determined on new instrument, employed a planimeter accurate to 0.01 square inches. The varying concentrations of purified cholesterol (via dibromination) and cholesterol linoleate (synthetic) were placed on the tape through the same funnel system used for column eluates. A wash of 0.3 ml of 5% ether in hexane

was used to ensure complete deposit of standard on the tape. Redissolving solvent flow rate was 0.714 ml per minute. Instrument sensitivity was maximal.

CHOLESTEROL DETERMINATIONS

Micrograms	Square Inches	Micrograms per Square Inch
97.2	1.163	83.6
121.5	1.463	83.0
145.8	1.673	87.1
48.6	0.550	88.4
24.3	0.293	82.9
97.2	1.093	88.9

Calibration value = 85 ± 2.8 micrograms per square inch.

CHOLESTEROL LINOLEATE DETERMINATION

Micrograms	Square Inches	Micrograms per Square Inch
28.5	0.250	114.0
57.0	0.500	114.0
85.5	0.763	112.1
114.0	0.890	128.1
142.5	1.190	119.7
171.0	1.520	112.5

Calibration value 116.7 ± 6.2 micrograms per square inch.

With successful production of a usable system, full attention was once again directed toward the studies of lipid biosynthesis by the rat uterus.