THE EFFECT OF HORMONES ON THE
INTERMEDIARY METABOLISM OF MAMMARY GLANDS

DISSERTATION
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by
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Advisor
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This Dissertation is dedicated to my wife

JUNE FABER READ

whose unfailing support made this project possible
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I Introduction

During the last several decades endocrinology has grown to the stature of a major branch of science. In the course of this period dozens of hormones have been discovered, their physiological role elucidated, and in several cases the chemical properties have been determined. Nevertheless, the mode of interaction between a specific hormone and normal body metabolism has in no case been discovered.

The mammary gland is well suited to the study of hormone metabolism. It may be easily removed from the body because it is located outside the main body of the animal, its development is dramatically controlled by hormones, and the rate of mammary metabolism is sufficiently high that the results of altered conditions may be easily observed. In addition, the mammary gland has synthesizing capacities equalled by no other single organ of the body, possibly excepting the liver. For these reasons it has been recently used by many investigators for experimentation.
II. Hormonal Effects in Mammary Development and Lactation

A. Gross Anatomy

The mammary gland is a complex organ consisting of a tree-like structure of branching ducts, which originate in alveoli lined with the actual secretory cells and terminate at the teat. In non-pregnant females the ducts are rudimentary prior to puberty, when they grow by apical proliferation to form a complex tubular structure. The ducts and alveoli develop during pregnancy under the influence of the female sex hormones, the estrogens and progesterone. Lactation begins following parturition due to the secretion of prolactin from the anterior pituitary. It is maintained by other "galactopoietic" substances from the anterior pituitary, which apparently differ from prolactin.

B. Estrogen and Progesterone

The role of the female sex hormones in the development of the mammary gland during pregnancy has been elucidated only with great difficulty. Even after over twenty years of intensive investigation, there is no unanimity on the exact role of these substances. The primary cause of dissension arises from species differences in the hormones arising from outside the ovary, i.e. from the adrenals, the placenta, etc.
That the pituitary also plays an important role is apparent from the fact that mammary development stops following hypophysectomy.

The most widely accepted explanation of mammary growth may be attributed to the classic work of W.O. Nelson and his co-workers. It was early found by this group that progesterone, as well as estrogen, was involved in mammary hyperplasia (213). They also recognized that the growth and the secretion of the mammary gland were separate phenomena (211). Optimal development in the rat could be brought about by injections of estrone followed by injections of progesterone, whereas lactation depended upon an additional injection of anterior pituitary extract. Histological studies of developing rat mammary tissue, reported by Reece and Bivins (248) in 1942, clearly demonstrated that estrogens caused duct proliferation, whereas progesterone stimulated the development of the alveoli. This series of events has been confirmed for the rabbit (256). As a partial explanation, it is interesting that Jeener (145) reports that the injection of estradiol into castrated female rats caused an increase in the ribose nucleic acid (RNA) content and the phosphomonoesterase activity of the uterus. The phosphomonoesterase activity was associated with the synthesis of fibrous structural proteins.

It has become apparent that in some animals estrogens
alone are capable of initiating lactation, notably in ruminants. Folley and his co-workers have been the leaders in this field. Imunion of the udder with diethylstibestrol, and artificial estrogen, has resulted in initiation of lactation in virgin goats (103) and nulliparous cattle (104).

Similar results have been reported following the subcutaneous implantation of estrogens in cattle (193). In the latter case anterior pituitary extracts enhanced lactation. The milk produced by these methods closely resembled colostrum in its chemical composition (99,104), containing increased amounts of globulins and less fat than normal milk. The production of milk decreased after the initial increase, but the lactation level was again increased to even higher levels following the removal of the implanted tablet. Whether this lactation was a direct result of the administered hormone or was mediated through the pituitary is disputed. It has been amply shown that injected estrogen increases the prolactin content of the pituitaries of rats (169,194,246,248), mice (194), and rabbits (193). However, the dosage level is vital inasmuch as "low" levels achieve the above-mentioned results whereas "high" concentrations are inhibitory to prolactin production (87,193). Progesterone also depends on concentration for its effect (81,248).
Mammary growth and lactation depend on the ratio of progesterone to estrogen (182), 1:20 (expressed as units) being optimal in the rat, and 1:240 to 1:960 in the rabbit. A rapid increase in this ratio due to increases in progesterone is apparently instrumental in the onset of lactation post-partum. That this occurs in vivo has been shown by Atkinson and his co-workers (5, 246). This will explain the frequent reports that estrogens inhibit lactation once it has been initiated (84, 98, 153).

Turner and his co-workers expanded the concept of sex hormone-pituitary interaction into a theory of Mammogens which, at its height, embodied two separate products, Mammogens I & II. Mammogen I (168) was supposed to be produced by the pituitary under the influence of estrogen whereas Mammogen II was produced under the influence of progesterone (203). This theory has now been reduced to only one Mammogen which acts on both the ducts and the alveoli (284). This theory has been discredited by most current investigators as a result of the inunction and implantation experiments previously mentioned, as well as by the demonstration that intraduct injection of prolactin alone can initiate lactation in suitably prepared rabbit mammary glands (178). Nevertheless, this theory is accepted insofar as the concept of pituitary interaction in mammary development is concerned. Numerous experiments have
been reported in which hypophysectomy has inhibited estrone-stimulated mammary growth (214) although pregnancy and mammary development may be maintained in similar animals by the implantation of placental tissue (162). Since placental tissue is an active source of gonadotrophin (8) the effects of hypophysectomy are probably due in part to a decrease in the amount of circulating gonadotrophin. Further mammary development may be achieved by the administration of progesterone (120).

Recent work has also implicated prolactin as functional in the development of lactation. Lyons (quoted in 63) has recently demonstrated that prolactin, as well as estrone and progesterone, was necessary for the full development of rat mammary tissue. In particular, prolactin was found essential for alveolar growth. Daane and Lyons (66) have also reported a synergistic effect between prolactin and estrone-progesterone combinations injected into castrated male mice. Perhaps one of the most satisfactory explanations of the many conflicting reports concerning mammary development is that proposed by Cowie and Folley (63). Briefly, they suggest that low levels of estrogen in the circulating blood are lactogenic due to stimulation of prolactin secretion from the pituitary. The high levels of estrogen found during early pregnancy inhibit lactogenesis, however, by indirect action
on the mammary gland. Later in pregnancy lactogenic activity is inhibited by the presence of progesterone while the two hormones act synergistically with prolactin for full mammary development. At parturition the blood progesterone level once again drops thus permitting lactogenesis to occur under the influence of prolactin secretion, stimulated by the low levels of estrogen in the blood. This theory is supported by the observations by Meites and Sgouris (198) that an antagonism exists between prolactin and combinations of the two steroid hormones. Lactation in ovariectomized rabbits depended upon the relative levels of the three hormones.

C. The Lactation Hormone Complex

The stimulation of lactation may be divided into two distinct phases (108). The initiation of lactation is referred to as lactogenic activity, whereas the maintenance of established lactation is called galactopoietic activity. There is only one lactogenic hormone, prolactin. Galactopoiesis, on the other hand, is the result of a complex of hormones arising from the pituitary, adrenals, thyroids, parathyroids, etc. The role of the female sex hormones has not, as yet, been determined except for their influence on the production of adrenocorticosteroids.

Prolactin was first reported by Stricker and Grueter in 1928 (272). Although the existence of a lactation-initiating
hormone was disputed for a long time, it was one of the first pituitary hormones to be isolated in a pure form.

All prolactin preparations appear to have the same properties, regardless of the source, except for slight differences in solubility in salt solutions and in tyrosine content. Prolactin is a protein containing no carbohydrate, thiol groups or phosphorus (293). The molecular weight has been variously reported as 22,000 to 33,000. The latter is considered the most probable value. Li has made a thorough study of the chemical properties of prolactin (170). He reports that the amino acids so far determined account for 99.9% of the nitrogen and all of the sulfur. He concludes that of the eighteen amino groups, six are terminal. Recent work by Cole and Li (36) has demonstrated the terminal amino groups to be part of the threonine components of the molecule. The presence of free amino groups is essential for prolactin action inasmuch as covering only 35% of them decreases prolactin activity 95% (32,170,174). Although the tyrosine hydroxyl groups are not essential, iodination of the tyrosine component inactivates the molecule (173). Esterification of only 10% of the free carboxyl groups with methanol decreases the activity by 50-65% (171). However, the activity cannot be restored by hydrolysis of the ester, suggesting that the loss of activity may be due to the rupture of hydrogen or
ionic bonds resulting in crucial structural alterations. It is also interesting to note that prolactin is inactivated by thiol compounds (111). Whether this is correlated with the four cystine residues per molecule (170) is not known. It is of interest that of the non-skeletal tissues reported, mammary slices inactivate prolactin more rapidly than any other (258). That this is probably an enzymatic process is indicated by the loss of inactivating ability upon boiling the tissue.

The role of prolactin in mammary function has been fairly definitely established. Following the discovery of a lactogenic factor in pituitary extracts (272) there has been repeated verification that prolactin is the initiator of milk production. As noted previously, prolactin activity requires the prior preparation of the mammary gland by estrogen and progesterone action. This explains why pregnant mammary tissue cannot utilize prolactin to any marked extent.

In laboratory animals, hypophysectomy prior to parturition will prevent the onset of lactation (210,211,212,257) as well as stop lactation once it has been started (210,211,257). Subsequent injection of impure anterior pituitary extracts has been successful in bringing about lactation in these cases (119,170,210). Use of purified prolactin, however, would not replace the pituitary in hypophysectomized guinea pigs (119,211). If, in addition to pure prolactin,
ACTH (adrenocorticotrophic hormone) (120,211) or adrenal cortex extracts (119,212,247) were injected immediately following parturition then lactation was initiated. In spite of continued injections, the mammary tissue subsequently underwent gradual involution even though continuing to secrete milk (120). Further experiments have shown that the administration of growth hormone (GH) as well as ACTH and prolactin would completely maintain lactating mammary tissue in hypophysectomized rats (180). Additional studies in hypophysectomized castrated male rats (181) have shown that complete mammary development may be achieved by injecting estrone, progesterone, prolactin, and growth hormone. Milk secretion was achieved by the further injection of prolactin, growth hormone and ACTH. Of this lactogenic trio, prolactin was the only mandatory requirement, the others only augmented prolactin-stimulated lactation. Similar results have been obtained with suitably prepared virgin female rats (180,181). Additional evidence that the role of ACTH in lactation is only supportive comes from the fact that lactation will be naturally initiated in adrenalectomized rats but the adrenal hormone requirements for lactating females are greater than for non-lactating females (96,116,117). It is not surprising that salt solutions also aid lactation in adrenalectomized rats (82,117). Further evidence that prolactin itself is the
primary agent for initiating milk secretion was afforded by Lyons (178), who stimulated rabbit mammary glands to secrete milk by the intraduct injection of prolactin. Milk secretion only occurred in the immediate area of the injection. The secretion was accompanied by a rapid proliferation of the alveolar epithelial cells with the supra-nuclear cytoplasm being sloughed to form the secretion (see also 196).

The pituitary is the most important endocrine gland in galactopoiesis by virtue of its control over many of the other endocrines. Prolactin itself has no uniquely galactopoietic ability (101,106,108). Rather, galactopoiesis appears to be related to glycotrophic or "anti-insulin" effect (106). All pituitary extracts which are galactopoietic are also diabetogenic (89). It thus follows that since purified growth hormone has been shown to be diabetogenic in the cat (60), most of the galactopoietic properties of anterior pituitary extracts may be accounted for on the basis of growth hormone (59). The galactopoietic properties once assigned to prolactin (106) have been found to be quantitatively equivalent to the growth hormone contamination of the samples (59).

The importance of the adrenals has already been mentioned with the concomitant importance of ACTH. Further supporting this concept is the fact that the pituitary and adrenals hypertrophy during gestation and particularly during
lactation (116). Beneficial effects of thyrotrophin have occasionally been reported (107) but it is believed by many investigators that the decrease in lactation following thyroidectomy (85) is due to the simultaneous removal of the parathyroids (86). The administration of parathormone (86, 102) or implantation of parathyroid tissue (102) will counteract the drop in lactation. Further evidence has been obtained from parathyroidectomy, which causes a decrease in lactation (62). On the other hand, thiouracil-stimulated-thyroidectomy, which leaves the parathyroids functional, causes a decrease in lactation simultaneously with a decrease in pituitary prolactin content (86). The administration of thyroxine will stimulate lactation in thyroidectomized rats (69,105,195) or thiouracil treated rats (52). In either case, thyroxin or parathormone are required only to provide milk precursors, and do not directly stimulate the mammary gland in any unusual way.

It can thus be seen that lactation is truly the result of a complex of hormones. It is initiated by the action of prolactin and maintained through the activity of other hormones. Prolactin itself may also be implemented in maintenance of lactation inasmuch as the pituitary content remains high throughout lactation. Although it does not have "anti-insulin" properties, injections of prolactin will augment
milk production post partum (107,109). But, like the other hormones discussed, its role is supportive rather than uniquely galactopoietic. The latter property would appear to be primarily that of growth hormone. Both prolactin and growth hormone are aided by other hormones from the thyroid, parathyroid, pancreas, and adrenals which control precursor levels. The secretion of these hormones, in turn, is controlled by the pituitary and possibly the female sex hormones.
III. Mammary Metabolism

Although many observations have been made of the gross effects of hormones on mammary development and secretion, it is impossible to assign chemical roles to the hormones until the metabolic processes peculiar to mammary tissue are thoroughly understood. Presumably, of course, mammary metabolism is similar to that found in other tissues possessing synthesizing abilities, such as liver, suitably modified to permit the production of the various milk constituents. It has been only recently, however, that detailed investigations into these phenomena have been undertaken. The resulting voluminous data are often times contradictory and unclear so that explanations must be sought through comparable studies made in other reproductive organs. In this way coherent metabolic pathways are slowly being elucidated.

A. Substrate origins

Inasmuch as protein synthesis is an extremely important function of the lactating mammary gland, the source of the constituent amino acids has been extensively studied. Acetate has been reported to be the principle source of the non-essential amino acids of casein (30, 282). Only about 5% of the blood glucose can serve as a source
for this acetate (157). On the other hand, the essential amino acids have been found to arise directly from the free amino acids of the blood in the rabbit (47) and the goat (4). In the goat, at least, hydrolysis of the blood proteins does not measurably contribute to the synthesis of casein (4).

Most of the blood glucose (80%) absorbed by the mammary gland is utilized for the synthesis of lactose rather than lipids or energy (29,157). Both the glucose and the galactose moieties of the lactose molecule arise from blood glucose (250). The enzymes necessary for this conversion have been identified (156,264).

The source of lipid precursors has been extensively studied in many laboratories. In ruminants the acetate produced in the stomach is apparently transported to the udder where it serves as the primary precursor of the fatty acids (94,113,233,234,235). In non-ruminants (e.g. the rat) blood acetate also serves as a fatty acid precursor although glucose can also serve in this respect (295). Recently, Shaw (260) has proposed a modified theory of fatty acid sources which appears to be noteworthy. It has been found in his laboratory that the shorter chain fatty acids (10 carbons or less) disappear from the milk following fasting whereas
the longer chain fatty acids do not. The latter are normal blood constituents. The decrease in short-chain fatty acids was reversed by introducing \( \beta \)-hydroxybutyric acid into the blood supply and also by administering acetate in carbon dioxide-rich plasma. Similar results were obtained with perfused udders in vitro. These results have led to the proposal that synthesis of fatty acids is restricted to the short chain acids originating from blood acetate or \( \beta \)-hydroxybutyrate and that the milk fatty acids of greater than 12-carbon chain length come directly from the blood stream. The concept of \( \beta \)-hydroxybutyrate as a fatty acid precursor in mammary tissue is in agreement with the recent data of Popjak, et al, (231,235). Further verification of this theory is required.

One group of hormones, in particular, appears to be associated with the transfer of precursors across mammary cell walls. These are the hormones of the adrenal gland. It was very early found that adrenalectomy caused a decrease in lactation in experimental animals. In 1936, Gaunt and Tobin (117) reported that this decrease could be partially reversed with salt solutions and completely reversed by injections of salt solutions plus adrenal extracts. Cowie and Folley (62) reported the role of the
adrenal hormones to be the maintenance of a proper Na/K ratio in the cell. Pursuing this, Folley and Greenbaum (96) demonstrated an increase in intracellular water resulted from the administration of deoxycortisone to adrenalectomized rats. It is also interesting that a hormone-reversible reduction in mammary respiration occurred following adrenalectomy (92) which closely resembled similar changes resulting from inanation. Thus the adrenal hormones are associated with water and salt transfer across mammary membranes and also possibly the transfer of other precursors and nutrients.

B. Energy yielding reactions.

It is readily apparent that the extensive synthetic reactions and secretory processes characteristic of mammary tissue require large amounts of energy. Oxygen consumption studies on mammary slices, reported repeatedly by the Folley group since 1949 (92), have provided indirect evidence of the occurrence of the Krebs cycle in mammary tissue. It was not until 1951 that the aerobic enzymes were definitely demonstrated by Moore (204,205) and Terner (277), showing the enzymes to be essentially identical to those found in other tissues. Aerobic oxidation is considerably lower than anaerobic oxidation (92,139) suggesting that oxidation is
deflected in the general area of "active-acetate" (Acetyl-CoA) metabolism, a concept supported by Terner's results using dinitrophenol (DNP) (277). Here only a fraction of the total pyruvate metabolized by rat mammary slices went for energy production. The larger portion entered synthetic reactions.

Folley and French (95) have suggested that the increased energy requirements necessary for lactation come from an increase in "respiration" rather than in aerobic glycolysis. Subsequent reports from their laboratory, notably by Terner, have clearly shown that the term "respiration" must be expanded to include the formation of acetyl-CoA in that this reactive compound is apparently pivotal in mammary metabolism. Terner has reported a series of exhaustive experiments designed to fully elucidate the role of acetyl-CoA. His experiments employ the uncoupling of oxidative phosphorylations with DNP and/or inhibiting, with fluoride ions, the utilization of high energy phosphate (\(^{\sim}P\text{O}_4\)) compounds for the activation of ionic acetate. Terner reported that, as in most tissues, the rate of mammary glycolysis is regulated by the capacity of the \(^{\sim}P\text{O}_4\) acceptor systems. Oxygen consumption by a pyruvate-fumarate substrate was greatly increased by uncoupling these reactions.
with DNP (276), particularly if tissue slices depleted of endogenously substrate were employed (277). In the absence of DNP citric acid accumulated in the medium whereas no citrate accumulated in the presence of DNP inasmuch as the Kreb cycle proceeds in an uncontrolled fashion under these conditions. The accumulation of citrate in the absence of DNP confirmed the results of Moore and Nelson (20). The latter investigators reported no further oxidation of citric acid occurred in mammary homogenates, presumably due to the lack of the required nucleotide cofactors. However, Terner claims citrate accumulation to be the product of excess citric acid synthesis over its oxidation (280).

Total pyruvate utilization by rat mammary tissue treated with DNP increased during lactation to a peak, several days after parturition, then remained high until weaning (276). In the absence of DNP, only one-third of the pyruvate oxidized was converted to energy; the remainder entered various synthetic reactions. The products in both cases indicated that acetyl-CoA was a transitory intermediate which did not collect appreciably in the medium.

As was discussed previously, rat mammary homogenates can utilize acetate for citrate and fatty acid synthesis in the presence of glucose or Kreb cycle intermediates (e.g. fumarate). Fluoride ions inhibit the utilization of $\sim$PO$_4^-$.
compounds produced during Krebs cycle oxidations. Since the activation of ionic acetate depends on such compounds as energy sources, it is not surprising that fluoride inhibited rat mammary homogenate utilization of acetate. Fluoride did not inhibit the incorporation of pyruvate into citric acid. Similarly, DNP inhibited acetate utilization by uncoupling oxidative phosphorylations (279).

It is thus apparent that the area of acetyl-CoA metabolism is particularly important in mammary metabolism both for energy production and for the synthesis of milk precursors. In the rat, pyruvate is converted to acetyl-CoA and hence to citric acid, etc., by a scheme relatively independent of simultaneous Kreb cycle oxidations. Presumably in the ruminant, however, the activation of blood acetate requires the simultaneous production of ~PO₄ compounds which are produced by the oxidative phosphorylations associated with Kreb cycle metabolism.

A correlation between respiratory quotient and prolactin has been noted in in vitro experiments. At parturition, the respiratory activity of mammary homogenates rises to several times that observed in pregnancy (93,95), this increase being associated with an increase in the utilization of glucose for synthetic reactions (17,19). In vitro
studies have shown that the addition of prolactin to the system raised the R.Q. (glucose or acetate) of homogenates from rats 1-5 days post-partum but did not in homogenates on the twentieth day of pregnancy (14,33). Desoxycorticosterone gave the reverse results. Subsequent investigations have shown that at least part of this stimulatory action of prolactin was due to contamination with Intermedin B (63,90) although prolactin apparently does have some intrinsic ability to stimulate respiration. Other metabolic effects of prolactin have not been reported. However, it is obvious that prolactin must be involved in the biological pathways of carbohydrate oxidation and fat or protein synthesis as seen from the rapid increase in these properties post-partum. Due to the convergence of the many synthetic reactions at the point of acetyl-CoA metabolism, it is an attractive theory that prolactin may be an activator of CoA-dependent reactions. Unfortunately, however, Balmain and Folley (14) have shown that prolactin does not affect the in vitro synthesis of fatty acids in rat mammary tissue.

Of the body tissues, the lactating mammary gland has the highest citric acid concentration (282). Citric acid is formed by the condensation of acetyl-CoA and oxalacetic
Deturi and Gurin (70) found that citric acid increased fatty acid synthesis twenty-fold. In conjunction with this, it has been proposed by Moore (204) that the high concentration of citrate keeps the concentration of nucleotides (DPN, TPN) low thus favoring synthetic reactions originating from acetate metabolism. Conversely, Villee and co-workers have reported a role for estradiol, and to a lesser extent the other estrogens, in the oxidation of citric acid to provide energy. Although estrogens do not stimulate respiration in liver tissue (75), they do increase oxygen consumption in rat uterus (50,154) and human placenta (286,287,288). Villee concludes that estradiol stimulates a DPN-linked isocitric dehydrogenase system (287), probably facilitating the hydrogen transfer by a reversible oxidation of estradiol to estrone. An enzyme for this reaction has been isolated in numerous tissues, including mammary tissue, by Ledogar and Jones (164). It is interesting to note that one other reaction reported to be responsive to estrogens (163) also is associated with a DPN-linked dehydrogenase.

Although only a few studies have been made in mammary tissue, the activity of several dehydrogenases of the Krebs cycle have been found to change during the various phases of estrus, pregnancy, and lactation in the other
reproductive organs. In the corpus luteum there are increases in succinic dehydrogenase (200,201) throughout pregnancy and lactation. Malic dehydrogenase and cytochrome-c (expressed as units per mg) increase in this tissue until midpregnancy, then fall until parturition (190); the increase in the size of the corpus luteum, however, maintains these two factors at high levels until parturition. Cytochrome oxidase activity parallels succinic dehydrogenase activity in rat corpus luteum (201). These changes are apparently correlated with the production of steroids, possibly serving as an "energy-siphon". In guinea pig mammary tissue a similar increase in succinic dehydrogenase activity also occurs late in pregnancy (204) followed by comparable increases in the cytochrome oxidase activity several days later. Both enzymes remain high during lactation. These results appear to correlate well with the levels of circulating progesterone reported for the rat (7).

The Krebs cycle is, of course, not the only source of energy in the mammary gland. Energy also may be obtained from the enzymes of anaerobic glycolysis. The respiration of pregnancy mammary tissue is largely by the anaerobic scheme (139). Judging from the results obtained with other
reproductive tissues, the activity of the anaerobic enzymes probably is controlled by the steroid female sex hormones. Estrogen mobilizes in vivo glycogen in the guinea pig liver (132), but it decreases the utilization of uterus glycogen in vitro. Kun reports (163) that ovariectomy activates the formation of phosphopyruvate from 3-phosphoglyceric acid, a series of reactions involving a DPN-linked dehydrogenase. The activity of this enzyme system in vitro is increased or decreased according to the concentration of diethylstilbestrol. The in vitro oxygen consumption of rat mammary homogenates increases slowly during pregnancy with a marked increase at parturition (93, 95, 139). As previously noted, the increase at parturition may be associated with the hormone prolactin.

C. Synthetic Reactions

1. Lipids

The glycolytic scheme of carbohydrate metabolism is important for more than just energy production in mammary metabolism. It serves as the direct source of citric acid (204, 280) which is present in high concentration in milk and mammary tissue (282). It also serves as a major source of the glycerol portion of lipids and of the acetyl-CoA necessary for the synthesis of fatty acids, etc.
Popják, et al, (113,236) have reported that in rabbit mammary tissue, glucose could be utilized as a glycerol precursor in vivo. It has further been reported by these investigators (19) that glycerol arises from glucose at 65-90% of the rate that lactose is formed from glucose. It is thus apparent that glycerol formation can not be a rate-limiting step in lipogenesis, as originally proposed by this same group (113). In vivo experiments have shown that glycerol can be metabolized by rabbit mammary tissue to yield acetate and hence fatty acids (236). This has been confirmed in vitro with rat and sheep homogenates (17). In neither case could the conversion of acetate to glycerol be achieved. Thus the glycerol portion of milk triglycerides probably arises directly from anaerobic glycolysis rather than by synthesis from blood acetate.

Another possible source of glycerol also occurs in mammary tissue, however. This route is by the hexose-monophosphate shunt, the method of so-called direct glucose oxidation. As outlined by Dickens and Glock (71) glucose-6-phosphate is oxidized to 6-phosphogluconate and hence oxidatively decarboxylated to yield a pentose-phosphoric ester. The enzymes responsible for these transformations have been isolated in rat and rabbit tissues (238).
Abraham, Hirsch, and Chaikoff (1) have studied this oxidative scheme in rat mammary tissue. It is their conclusion that nearly 60% of the glucose is metabolized by this method in the rat. As proposed by Horecker (140) the product is a pentose phosphate which is further metabolized to a triose phosphate and an active glycolaldehyde. The triose portion is more readily incorporated into triglycerides than is the glycolaldehyde, but even the latter is appreciably utilized during lipogenesis. Another interesting observation made by Glock and McLean (118) was that glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase activities increase many fold in mammary tissue during lactation. Thus it is entirely possible that much of the glycerol required for lipid synthesis during lactation may arise from the direct oxidative scheme.

It has been suggested (17) that glycerol production is regulated by insulin. This interpretation arose from experiments showing that glycerol added to acetate-glucose substrates caused an increase in respiration equivalent to that caused by insulin in identical homogenates. However, the action of insulin is now believed to be correlated with the direct utilization of glucose rather than with glycerol or fatty acid synthesis.
Probably the most important single product of the glycolytic pathway in mammary tissue is acetyl-CoA. Although metabolic differences in precursors occur between ruminants and non-ruminants, it is at the point of acetyl-CoA utilization that milk synthesis by the two groups of animals becomes identical.

The first indication that mammary fat synthesis occurred from an acetate substrate was obtained by Folley and co-workers (91,93,95). In experiments with tissue slices they found that non-ruminants required glucose as well as acetate to produce a R.Q. greater than 1.0. This high R.Q. was taken to indicate the synthesis of fats. Somewhat lower values could be obtained with glucose alone. On the other hand, increased R.Q. values with ruminant tissue could be obtained with acetate alone; glucose alone was without effect. Thus both types of animals utilize acetate as the immediate precursor, with slight modifications as to the requirements according to the precursors provided by the blood. Direct confirmation of fat synthesis came shortly from experiments on goats injected with carboxy-labelled sodium acetate (234). The resultant fatty acids contained the tagged carbon from the acetate. Furthermore, the mammary lipogenesis was considerably more rapid than the accumulation of tagged lipids in the blood, thus indicating that synthesis was indeed occurring in
in the mammary gland itself. Similar fat synthesis has been demonstrated in the lactating rabbit (113) and the perfused cow udder (61).

It has been repeatedly demonstrated that mammary fatty acid synthesis involves a condensation of 2-carbon units (acetyl-CoA) (94, 113, 233, 234, 235, 295) in both ruminants and non-ruminants. Using carboxy-labelled acetate the resultant fatty acids are marked on the alternate carbon atoms. It has been fairly well established that the acetate addition occurs at the carboxyl end of the fatty acid being lengthened (235). For the shorter chain fatty acids it is probable that the synthesis occurs analogously to that outlined for butyric acid (268):

\[
\begin{align*}
\text{Acetyl-CoA} \\
\text{Acetoacetyl-CoA} \\
\beta\text{-hydroxybutyryl-CoA} \\
\text{Crotenyl-CoA} \\
\text{Butyryl-CoA}
\end{align*}
\]

The necessary enzymes have been isolated from other tissues and the conditions for their optimal operation determined (see 28, 216 for reviews). In mammary gland there is some information that a 4-carbon compound other than butyric acid also serves as the precursor for the C₆ to C₁₀ fatty acids.
It is probable that this compound is \( \beta \)-hydroxybutyric acid (231, 260).

Although the synthesis of long-chain fatty acids--\( C_{12} \) and longer--from acetate has been demonstrated \textit{in vivo} in the goat (234) and \textit{in vitro} with rat mammary homogenates (238), the quantities have been quite small compared to the yield of the short-chain acids. This would seem to confirm Shaw's hypothesis (231) that only short-chain fatty acids are synthesized by mammary tissue, the remainder coming directly from the blood. This also is a possible interpretation of the comparative studies on goat milk and blood fatty acids following the injection of carboxy-labelled acetate (234).

It is interesting to observe that citrate serves to increase fatty acid synthesis in crude tissue preparations (35, 70). A partial explanation for this might be found in the recent work by Popják and Tietz. Using both slices and cell-free suspensions of rat mammary tissue, these investigators demonstrated that fatty acid synthesis was activated by the simultaneous oxidation of \( \alpha \)-ketoglutarate or oxalacetate (238, 239). The synthesis was apparently dependent on DPN\(^+\) and ATP, which can substitute for the Kreb cycle intermediates (134, 240). It is possible that the simultaneous oxidation of citrate, \( \alpha \)-ketoglutarate, or oxalacetate served to maintain the ATP levels necessary for fatty acid synthesis. Popják and
Tietz (241) further explain the results as providing DPN⁺H necessary for reducing the double bonds created during lipogenesis. However, malonate, which inhibits Krebs oxidations (45,242), also activates fatty acid synthesis. This contradictory situation confirms the proposal of Moore and Nelson (204) that interruption of the Kreb cycle (e.g. at the point of citrate oxidation in their experiments) conserves the nucleotides for synthetic reactions. One possible interpretation compatible with all of these observations is that the proposed inhibition of citrate oxidation causes the accumulation of citrate in the system which, in turn, directly activates the synthesis of fatty acids. Such an interpretation would explain the beneficial effects of succinate, α-keto-glutarate, pyruvate, and oxalacetate as due to the production of citric acid. It would also explain the observation that oxalacetate, while activating fatty acid production, also inhibits the further oxidation of acetate (238,242). Finally, the beneficial effect of malonate might be explained as an inhibition of whatever acetate oxidation normally occurs in spite of the presence of citrate; thus all the available acetate would be diverted to synthetic reactions. It is obvious, however, that additional investigations into the interrelationships between lipogenesis and glycolysis are required.
It is certainly not unexpected that in purified mammary systems, CoA is required for fatty acid synthesis (134).

Sterol synthesis also is dependent upon the acetyl-CoA in mammary tissue, just as in other cells. Marked acetate has been shown to be a precursor for cholesterol in mammary tissue, in vivo in rabbits (113, 232) and in goats (234) as well as in perfused cow udder (61). It has been stated, in fact, that lactating mammary tissue has the greatest cholesterol-synthesizing ability of any organ yet studied (232). The sterol synthesizing ability of yeast cells (158) and rat liver (159) parallels the CoA content of the cell.

Of the hormones studied from the viewpoint of influence on acetate metabolism, insulin has probably been the most intensely investigated. Yet the results of these investigations are still sufficiently unclear to prevent an unequivocal interpretation. Insulin has been repeatedly shown to accelerate the in vitro synthesis of fatty acids from acetate in the rat (16, 34, 131, 192) but it has no effect on ruminant fatty acid synthesis (18, 76). The presence of glucose in the system was found to be essential for rat lipogenesis (13, 15, 16). Inasmuch as diabetes results in a decrease in lipogenesis in the liver (see 271 for a review), Drury (74) has suggested that insulin has an activating role in fatty acid synthesis. This concept has received support from the
work of others (31,131,135,192). However, Baker, Chaikoff, and Schusdek (11) report that the decreased fat synthesis found in alloxinlzed rat livers could be overcome by feeding a diet high in fructose prior to sacrifice. This indicates that the primary result of insulin insufficiency was a block in the phosphorylation of glucose, thus preventing its further oxidation to yield acetate for lipogenesis. This concept is not inconsistent with studies on the insulin effect on mammary fat synthesis. In studies with rat homogenates, the presence of glucose was essential for the demonstration of a beneficial effect of insulin (13,15,16). Furthermore, in experiments with labelled acetate (13,16) the resultant fatty acids were not investigated as to the positions of the marked carbon atoms so it is possible that the fatty acid synthesis was stimulated by a general increase in precursor concentration rather than by a direct stimulation of the synthetic enzymes. Further confirmation of this hypothesis comes from the observation that the introduction of glycerol could simulate the beneficial effects of glucose plus insulin (15). Therefore, although a direct role for insulin in lipogenesis can not yet be excluded, it would appear probable that insulin serves to increase the oxidation of glucose to yield the acetyl-CoA necessary for fatty acid synthesis.
It is thus apparent that mammary tissue is the site of extensive synthesis of lipids and sterols. Unlike the liver, mammary lipogenesis probably is limited to the shorter chain fatty acids (C<sub>10</sub> or less); however, in both tissues the synthesis involves the condensation of acetyl-CoA segments to yield even-numbered fatty acids. The hormone insulin is probably not directly involved in lipogenesis by mammary tissue but the lactogenic hormone, prolactin, may be.

2. Amino acids and proteins:

Protein synthesis is a major function of mammary tissue. Nevertheless, considerably less has been done towards elucidating this phase of mammary metabolism than either the energy reactions or lipogenesis. This is not surprising however, inasmuch as the enzymes instrumental in protein synthesis have not been isolated from any tissue.

The essential amino acids used for protein synthesis arise directly from the blood supply to the mammary gland (4, 47) Askovas, et al, (4), using goat mammary glands, further reported that blood proteins were not degraded to provide amino acids for casein synthesis; only the free amino acids of the blood were utilized. On the other hand, the non-essential amino acids have been reported to be derived from blood acetate (30) and glucose (29). Both are presumably
utilized through the transaminations originating from the Kreb cycle intermediates, as is true for other tissues.

The reactions involved in amino acid synthesis are too numerous to be discussed in detail here. They have been summarized in a recent review by Ehrensvard (77). A summary of his review includes the following salient points.

The three primary sources of amino acids are the transaminases schematically presented below:

<table>
<thead>
<tr>
<th>Alanine</th>
<th>( \alpha )-Ketoglutaric Acid</th>
<th>Aspartic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glutamic-Pyruvic Transaminase</td>
<td></td>
</tr>
<tr>
<td>Pyruvic Acid</td>
<td>Glutamic Acid</td>
<td>Oxalacetic Acid</td>
</tr>
<tr>
<td></td>
<td>Glutamic-Oxalacetic Transaminase</td>
<td></td>
</tr>
</tbody>
</table>

Thus the production of glutamic acid yields either pyruvic acid or oxalacetic acid, depending on the enzyme system. Glutamic acid can also be obtained from \( \alpha \)-ketoglutaric acid by the action of glutamic dehydrogenase. All of these reactions are reversible depending upon the relative concentrations of the reactants and products. From alanine two other amino acids are synthesized: serine and cysteine. The glutamic acid resulting from \( \alpha \)-ketoglutaric transformations has been found to be the precursor for ornithine and proline by a rather complex series of reactions. Tyrosine is
synthesized from an initial acetate and glucose condensation, as is tryptophane. It is thus apparent that the synthesis of some of these acids is directly dependent upon acetate metabolism. The rest are indirectly dependent on acetate metabolism through the requirement for a properly functioning Kreb cycle. Although not all of these reactions have been identified in mammalian tissue, enough have that it may be assumed that the others probably occur.

Greenbaum and Greenwood (124) have studied extensively several of the enzymes involved in the above transformations using rat mammary tissue. They reported that glutamic dehydrogenase activity was low until late in pregnancy and then rose sharply at parturition. It remained considerably above the pregnancy levels until weaning. Glutamic-oxalacetic transaminase activity also rose late in pregnancy and remained high throughout lactation until after weaning. A correlation between these enzymes and protein metabolism was first demonstrated by Braunstein and Azarkh (37) who observed that amino acids could be oxidatively deaminated by a two-step method; (1) transamination with α-ketoglutaric acid to yield glutamic acid and (2) resynthesis of α-ketoglutaric acid by the increased activity of glutamic dehydrogenase. Conversely, it has been suggested (124) that a low level of glutamic dehydrogenase would stimulate amino acid synthesis by a reversal of
this transamination scheme. This inverse relationship was not observed in mammary tissue by Greenbaum and Greenwood (124). These investigators explain the inconsistency between their data and the theory by suggesting that mammary mitochondria, which contain the dehydrogenase, continually change their enzyme content due to the continual destruction and resynthesis of the mitochondria. Such changes are a reflection of the different metabolic requirements of any given time. The transaminases, on the other hand, remain constant in the non-mitochondrial portion of the cell. This interpretation would appear overdrawn were it not for the observation of changes in mitochondrial form (146) and number (68) which have been reported during pregnancy and lactation. It also receives some support from the report by Glock and McLean (118) that the levels of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase in rat mammary tissue rose steadily during lactation. These enzymes are members of the glucose-shunt, a scheme which has been proposed as the source of the ribose moiety of ribonucleic acid.

The steady rise in mammary cathepsin found by Greenbaum and Greenwood (124) parallels the milk production. Mammary arginase rises to a peak at mid-lactation thus facilitating gluconeogenesis during lactation.
There are indications that the pituitary growth hormone is implicated in the maintenance of lactation. As has been previously noted, the galactopoietic qualities of the growth hormone are intimately related to its "anti-insulin" property. In addition to this hyperglycemic action, the injection of growth hormone into experimental animals lowers the level of amino nitrogen in plasma and liver (172, 202), apparently due to an increase in protein deposition (255). It also causes ketosis (78). The metabolic explanations offered for these results are extremely varied. The "anti-insulin" effect is explained on the basis of an inhibition of hexokinase activity (57). There is also a proposed stimulation of the conversion of amino acids to protein or possibly an inhibition of protein catabolism (137). Mobilization of lipid fat has been frequently observed (125, 165, 273) accompanied by an increase in fatty acid catabolism (126). Also in the area of fat metabolism are observations that growth hormone inhibits the conversion of carbohydrate or acetate to fat (130, 292).

It has been proposed frequently that the changes in fat and carbohydrate metabolism elicited by growth hormone administration are reflections of a general change in metabolism aimed at increasing protein deposition. Occasional observations indicating a decrease in activity of in vitro amino acid metabolism resulting from growth hormone addition to
homogenates from hypophsectomized animals have been reported, notably with liver arginase (112), glutamic-oxalacetic transaminase (24, 27, 114), glutamic-pyruvic transaminase (114), and glutamic dehydrogenase (114, 115). Gaebler (294) concludes that those changes, although important for protein deposition, are not critical for the metabolic results of growth hormone. Certainly they do not correlate well with the observed changes in these enzymes in mammary tissue.

Coenzyme A metabolism is implicated in some way with the action of growth hormone. Pantothenic acid deficient rats are unable to respond to growth hormone stimulation (26). Furthermore, hypophysectomized rats stimulated with growth hormone developed acute pantothenic acid deficiencies. Hypophysectomy has also been found to decrease significantly the CoA content of rat liver (25, 253), this decrease being reversible by the injection of growth hormone or of pantothenate solution. Whether these effects are to be interpreted as a primary reaction for growth hormone activity or as a reflection of the increased demand for amino acid synthesis via transaminations of the Kreb cycle intermediates is not clear. It is of interest to mention here that Ringler and Nelson (254) have found that the injection of growth hormone into ovariectomized female guinea pigs did not alter the CoA content of hormonally produced mammary tissue. The significance of these
results is uncertain, however, as the authors noted that the glands were not maximally developed by the hormone treatment. Ringler, et al, (252) have noted a marked increase in CoA content of mammary tissue following the normal initiation of lactation.

A correlation has also been proposed between the female steroid hormones and protein metabolism. In guinea pig uterus (185), estradiol and progesterone act synergistically to increase peptidase activity. In rat uterus, these sex hormones have also been reported to increase nucleic acid content (3, 53, 275), the increase in RNA being proportional to the total amount of fibrous or structural protein. In contrast, prolactin injections into pigeon gizzard cause an increase in DNA which is proportional to non-structural protein synthesized (comparable to casein?).

Many investigators have taken the frequently close correlation between alkaline phosphatase activity and protein accumulation as indicative of a fundamental role for the phosphatase in protein synthesis. In spite of diligent investigation, however, no proof for this role has been obtained. Nevertheless, the circumstantial evidence in support of this theory can not be ignored, and in particular, the suggestion that the level of phosphatase activity may be influenced by the female sex hormones. It is the concensus of
many workers that estradiol, when injected into the intact animal, causes an increase in the alkaline phosphatase of many organs, e.g. mouse uterus (2), pigeon uropygial gland and breast muscle (150), guinea pig uterus (185), and rat genitalia (203). Progesterone decreases the activity of this enzyme in pigeon uterus (151) and guinea pig uterus (185). Similar interactions of these hormones with mammary alkaline phosphatase have been reported for cows (48), guinea pigs (97), and rats (96). Furthermore, mammary alkaline phosphatase increases during pregnancy (96) when structural proteins are being synthesized and remains constant during lactation. There are reports that changes in alkaline phosphatase activity are associated with the sex hormone-induced changes in nucleic acids discussed above. There is no change in alkaline phosphatase under the influence of prolactin. It is the opinion of this author that the changes in alkaline phosphatase activity of the various sex glands are correlated better with the changes in the secretory activity of the cell, as proposed by Stafford, et al, (267), than with protein synthesis.

Other correlations of the estrogens with protein metabolism are found in the increase in histamine found in human blood (148,230) when progesterone concentration is increased and an increase in histaminase activity when estrogens are
injected (148, 244). This, correlated with reports that estrogens cause the breakdown of acetylcholine (20, 51, 224), and that progesterone effects in the uterus may be simulated by injected histamine, has prompted European investigators to propose that these hormones influence protein metabolism by stimulation of the sympathetic nervous system. This view has not been widely accepted in England and the United States.

It is apparent that the role of the sex hormones in protein synthesis has not been resolved. It is possible that part of their effect may be indirect through an hydrogen-transport system such as has been discussed in a previous section. Further investigations into the area of hormonal influences on amino acid and protein syntheses are clearly required.

3. Lactose:

Observations have been made that in vivo lactose synthesis in the mammary gland occurs from blood glucose in the cow (29, 157) and goat (23, 250). Furthermore, the synthesis of lactose has also been reported using mammary slices from guinea pigs (184, 251) and rats (49, 135).

Studies with glucose tagged on carbon-1 have shown that both the glucose and the galactose portions of the lactose molecule arise equally from a glucose precursor (23, 72, 156, 250)
without the rearrangement of the carbon chain (72,250). Caputto and Trucco (49) have demonstrated the presence of unidentified lactose-containing compounds in lactating rat mammary tissue which they believe to be intermediates in lactose synthesis. It is probable that these compounds are related to the phosphorylated lactose identified in rat mammary homogenates (187).

Recent work by Smith and Mills (264) using rat mammary tissue has permitted them to propose the following scheme for lactose synthesis:

\[
\begin{align*}
\text{Uridyl-triphosphate} + \text{Glucose} - 1\text{-phosphate} & \xrightarrow{\text{Uridyl Transferase}} \text{Uridyl-diphosphogluucose} + \text{pyrophosphate} \\
\text{Uridyl-diphosphogluucose} & \xrightarrow{\text{Galactowaldenase}} \text{Uridyl-diphosphogalactose} \\
\text{Uridyl-diphosphogalactose} & \xrightarrow{\text{Glucose}} \text{Lactose} + \text{Uridyl-diphosphate}
\end{align*}
\]

These authors isolated uridyl transferase from rat homogenates. Further confirmation comes from the high uridyl-diphosphate content of mammary tissue (187,265). It would thus appear that the synthesis of lactose has been fairly well established for lactating mammary tissue.
IV. Coenzyme A Metabolism

From this review of the literature it is obvious that the point of acetyl-CoA metabolism is vitally important to the metabolism of the lactating mammary gland. At parturition, when prolactin secretion into the blood stream is greatly increased, a marked increase in the respiration of mammary homogenates occurs. This increase, which may be stimulated in vitro by the addition of prolactin to the reaction medium, is associated with an increase in the synthesis of lipids and proteins through acetyl-CoA metabolism rather than with an increase in Kreb cycle oxidations. At the time of parturition, mammary CoA content also markedly increases, paralleling the prolactin concentration in the blood. These observations lead to the conclusion that the role of prolactin may be related to CoA action in mammary tissue. Three alternatives for such a role are possible: (1) increasing of CoA synthesis, (2) inhibiting CoA destruction, or (3) directly activating CoA linked reactions. A review of the literature concerning the methods of study of these alternatives will be presented in this section.
A. The Structure of Coenzyme A:

Coenzyme A was first reported by Lipmann and Kaplan (175) as a coenzyme for the acetylation of compounds such as sulfanilamide or choline. Subsequently the acetylation of sulfanilamide, using pigeon liver enzymes, was refined (149) to provide the most commonly used method for the assay of CoA in biological samples.

Shortly after the discovery of CoA, the presence of a new growth factor (LBF) for Lactobacillus bulgaricus was reported (294). The alkaline hydrolysis of LBF yielded pantothenic acid and \( \beta \)-alanine (188), accompanied by an odor typical of sulfur-containing compounds. Snell, et al., (265) determined the sulfur-containing moiety to be \( \beta \)-mercaptoethylamine thus permitting the identification of LBF as N-(pantothenyl) -\( \beta \)-aminoethanethiol or its disulfide, named pantetheine and pantethine, respectively. A similar LBF-active fraction was obtained from CoA treated with intestinal alkaline phosphatase (38,219). The hydrolysis of CoA by a mixture of enzymes, intestinal alkaline phosphatase plus a crude preparation from pigeon liver, liberated pantothenic acid (176). Gregory and Lipmann (129) confirmed \( \beta \)-mercaptoethylamine as the only S-containing portion of the CoA molecule. This information prompted the suggestion (189)
that LBF was a normal intermediate in the synthesis of Coenzyme A; this was subsequently verified in other laboratories (121,155).

The presence of a pyrophosphate bridge was anticipated by the degradative action of snake venom pyrophosphatase on CoA. Proof of this structure was not obtained, however, until the products were separated chromatographically and identified as diphosphoadenosine and phosphopantetheine (215). The latter was identified by chromatographic comparison to pantothenate and pantetheine derivatives synthesized by Baddiley and Thain (9). Confirmation that the pyrophosphate was attached to the 4' hydroxyl of the pantothenate moiety was obtained by synthesis (10).

The third phosphate group (217) in the CoA molecule was found to be on the adenosine portion of the molecule (215). Wang, Shuster, and Kaplan (290) found the exact position to be on carbon-3 of the ribose moiety, thus differing from the configuration found in TPN or ATP.

Putting these facts together the structure of CoA has been determined to be as in Figure I (215,216). Biologically it may exist either as the sulfhydryl shown, as a disulfide of two CoA molecules (142), or as a mixed disulfide with another sulfhydryl compound (e.g. pantetheine) (40,41). However, CoA must be in the reduced form to function in biological reactions (41).
FIG. 1. THE STRUCTURE OF COENZYME A, SHOWING POINTS OF ENZYMATIC ATTACK
BASED ON NOVELLI (216)
B. The Synthesis of Coenzyme A:

All of the pantothenic acid found in biological materials is apparently present as part of the Coenzyme A molecule (219). Inasmuch as CoA and its Kaplan-Lipmann active precursors are not absorbed from the digestive tract of animals (122), absorption must occur as pantothenic acid or a non-active derivative. Resynthesis to CoA appears to occur primarily in the liver since this organ has the highest CoA content of any tissue yet reported (220). It is believed that transport of the liver-stored CoA to other body organs is not in the form of the intact CoA molecule, however. Intravenous administration of CoA results in the rapid accumulation in the blood of split products active as CoA with the Kaplan-Lipmann complex of enzymes (122). Therefore it is probable that most, if not all, body tissues contain enzyme systems capable of synthesizing CoA from these split products as well as pantetheine and/or pantothenic acid.

An early theory of CoA synthesis was proposed by Craig and Snell (129). This theory envisioned two phases to the synthesis: (a) the formation of pantetheine from pantothenic acid by unidentified tissue enzymes followed by (b) the synthesis of the CoA molecule by steps including the reversal of the degradative alkaline phosphatase reaction (38, 219). Although the second step(s) in this scheme have been
disproven, the first phase has been expanded through the
identification of the enzymes involved.

Extracts of the bacterium *Proteus morganii* indicate that
in the presence of cysteine and ATP, pantothenic acid is con­
verted to N-pantothenylcysteine (42). In the presence of any
of several sulfhydryl-reducing compounds N-pantothenylcysteine
is decarboxylated to form pantetheine by *Acetobacter suboxy­
dans*. Recently Hoagland and Novelli (136) have demonstrated
that rat liver is also capable of synthesizing pantetheine
from either pantothenic acid or N-pantothenylcysteine. The
synthesis from pantothenic acid has obligatory requirements
for cysteine, Mg++, and ATP. The decarboxylation of N-
pantothenylcysteine does not involve a phosphorylation step
as proposed by Brown and Snell (42). It is interesting to
note that pigeon liver preparations do not exhibit these
reactions (136).

The synthesis of Coenzyme A from pantetheine was first
reported by Levintow and Novelli in 1952 (166). They re­
ported the synthesis to be a three-step process (see Figure
II) involving (a) phosphorylation of pantetheine, (b) con­
densation of phosphopantetheine with ATP to yield dephospho
CoA, and (c) the phosphorylation of the ribose moiety of
the dephospho CoA molecule to yield CoA. The enzymes have
been found to be heat-labile, particularly for step (a).
In fact, the customary aging of pigeon liver acetone powder extracts inactivates (a) thus explaining why CoA synthesis was not previously observed. The properties of these enzymes have been reported in detail by members of Novelli's laboratory.

The enzyme which phosphorylates pantetheine to produce phosphopantetheine has been named pantetheine kinase (167), usually abbreviated $K_\text{T}$. In the absence of substrate, the enzyme is inactivated in four hours at 25°C or in ten minutes at 50°C. It slowly loses activity even when stored frozen. It requires 0.01 M Mn++ or Mg++ for full activity, with Mn++ serving as a slightly better activator. The pH range is broad with an optimum at 7.2 in the presence of Mg++. The addition of phosphate ions is also beneficial, doubling the reaction when present in only small quantities. The obligatory source of phosphate for phosphorylation is ATP, with ADP as the product.

The condensation of phosphopantetheine with ATP to yield dephospho CoA and pyrophosphate is achieved with the enzyme dephospho CoA pyrophosphorylase (136), usually abbreviated "C". The pH optimum is 7.5 with Mg++ and cysteine serving as activators. The latter maintains the substrate in the reduced form.

Dephospho CoA kinase, abbreviated as $K_\text{II}$, is the
PANTOTHENIC ACID + ATP → CYSTEINE + PANTOTHENYL-CYSTEINE

N-PANTOTHENYL-CYSTEINE + CO₂ → PANTETHENE

PANTETHENE + ATP, Mg²⁺ or Mn²⁺, PQQ → PANTETHENE KINASE (Kₐ)

PANTETHENE KINASE (Kₐ) + ATP, Mg²⁺, CYSTEINE, ATP → "CONDENSING ENZYME" (C')

PANTOTHENIC ACID + ATP, Mg²⁺, CYSTEINE, ATP, PQQ → "CONDENSING ENZYME" (C')

PANTETHENE + ATP → "CONDENSING ENZYME" (C')

COENZYME A + ATP, Mg²⁺ → DEPHOSPHOCOENZYME A

DEPHOSPHOCOENZYME A + ATP, Mg²⁺, CYSTEINE → DEPHOSPHOCOENZYME A KINASE (Kₐ)

DEPHOSPHOCOENZYME A KINASE (Kₐ) + ATP, Mg²⁺, CYSTEINE → COENZYME A
Fig. II, Coenzyme A - Linked Reactions
enzyme responsible for the formation of CoA from desphospho CoA (136). The products include, in addition to CoA, another molecule of ADP. The requirements of this enzyme are reported to be the same as for "C" except that the broad pH range has an optimum at 9.0 rather than 7.5. It is recommended by the authors that when studying the three enzymes \(-K_I, "C", \text{ and } K_{II}\) simultaneously that the broad pH range of each enzyme makes the use of a pH 8.0 buffer the most satisfactory.

A summary of the synthesizing system from pantothenic acid to CoA is presented in FIGURE II.

C. The Degradation of Coenzyme A:

The degradation of Coenzyme A has not been as fully studied as has the synthesis of the coenzyme, probably because at first glance degradation would appear to be of less physiological importance. A survey of the literature shows, however, that there are probably several pathways by which this coenzyme might be degraded. Not all of these pathways have been identified in mammalian tissues.

As discussed in the preceding section, CoA is synthesized from pantetheine by a three-step series of condensations summarized as follows:

\[
Pantetheine + 3 \text{ ATP} \rightarrow CoA + PP + 2 \text{ ADP}
\]
The reactions are reported to be completely reversible under appropriate conditions (136, 166, 167, 218) so that physiological CoA concentrations in animal tissues may well depend on the ratio of synthesis to degradation in one or more of these reactions.

Other reactions have also been reported to degrade CoA. Brown, et al. (38) have reported that alkaline phosphatase will degrade the coenzyme to yield a product active as LBF. The site of the reaction is at 3 on FIGURE I. This reaction is inhibited by sodium fluoride (219). Prostatic phosphatase yields dephospho CoA by an attack at position 1. Position 2 is cleaved to yield phosphopantetheine through the action of a nucleotide pyrophosphatase found in potatoes and snake venom. Those three reactions are reversible. Another point of enzymatic attack is at 4. Enzymes prepared from hog or avian liver and kidney irreversibly split β-mercaptoethyamine from either CoA or pantetheine (38, 129, 215). This reaction is not inhibited by NaF.

That the degradation of Coenzyme A does occur as a normal physiological reaction has been demonstrated by Govier and Gibbons (122). They report that CoA injected intravenously into dogs is degraded to CoA precursors which are transported to the "target organs" where resynthesis
occurs. However, the identification of any of the synthesizing or degrading enzymes in mammary tissue has not yet been reported. Ringler, et al, (252,254) have reported that mammary homogenate prepared with phosphate buffer does not exhibit CoA-degrading activity.

D. The Synthesis of Citric Acid:

As has been previously mentioned, mammary tissue has the highest citric acid content of any non-skeleton tissue (282). Studies with mammary homogenates from rats (276,277) and guinea pigs (204) have further shown that citric acid accumulates in the medium in the absence of DNP (276). Terner (280) explains this accumulation as due to an excess of synthesis over oxidation whereas Moore (204) interprets the accumulation as due to an inhibition of citric acid oxidation resulting in an increased concentration of the nucleotides (TPN,DFN) necessary for synthetic reactions. Citric acid has been reported to increase twenty-fold the synthesis of fatty acids in tissue homogenates (70).

For these reasons it is believed that a study of citric acid production in mammary tissue might yield interesting information concerning mammary metabolism. Furthermore, the Kaplan-Lipmann condensing enzyme is responsible for the production of citric acid by the condensation of
acetyl-CoA and oxalacetate (270). Total citric acid production in mammary tissue might therefore be expected to serve as a measure of CoA-linked reactions, as has been recently suggested by Terner (281).

Potter's laboratory has developed a method of measuring *in vivo* citrate synthesis. It was first reported in 1949 that the intraperitoneal injection of sodium monofluoracetate (NFA) caused the accumulation of citric acid (44). It has been proven that the inhibition occurs specifically in the oxidation of citric acid (46), apparently by acting on aconitase. None of the other Kreb cycle oxidations are affected. The inhibiting compound produced is monofluorocitric acid (226,227). The amount of citrate formed is dependent on NFA dosage and the time interval between injection and sacrifice (239).
V. Experimental Techniques

A. Source of Special Reagents

The prolactin used in these experiments was a gift from Dr. S.L. Steelman of the Armour Research Laboratories (Chicago, Illinois). The two samples were from lots #R-491153 (cow) and #759-39BB (sheep). The pantethine (Lot #1918x66) was a gift from Dr. O.D. Bird of the Parke-Davis and Company (Detroit, Michigan). Purified Coenzyme A and adenosine-triphosphate (ATP) as well as the cysteine-HCl and the sulfanilamide were purchased from Nutritional Biochemicals, Inc., Cleveland, Ohio. The tris-hydroxymethylaminomethane used for the preparation of the "Tris" buffers was purchased from Sigma Chemical Company, St. Louis, Missouri. Coenzyme A determinations required the coupling of sulphanilamide with N-(1-naphthyl)ethylenediamine dihydrochloride which was purchased from Eastman Kodak Company, Rochester, New York. The sodium monofluoroacetate was obtained from Monsanto Chemical Company, St. Louis, Missouri.

B. Care and Selection of Experimental Animals

The guinea pig was employed as the experimental animal in these investigations. All animals raised at Ohio State University were maintained on the standard vitamin C-enriched Rockland Guinea Pig Pellet supplemented occasionally with...
lettuce. Water was offered ad libitum from a drinking bottle. In the later experiments at the Medical Nutrition Laboratory, the guinea pigs were raised on Purina Rabbit Chow plus sufficient lettuce to provide all of their liquid requirements. In general, the number of deaths from respiratory infections on the latter regimen were greatly reduced compared to the deaths on the vitamin C-enriched guinea pig ration. These differences are not believed to have altered the results, however, since only healthy animals were used for experimentation.

For breeding purposes, five females and one male were housed together in galvanized iron cages. No animal was bred until it had achieved a weight of 350 grams or more. All females used in these experiments were undergoing their first pregnancy, following which the time of parturition was noted and the experiments begun twelve, twenty-four, or thirty-six hours later. All litters were reduced to two nursing young as soon as possible after birth. The mother and litter were left in the "home" cage throughout the course of the experiment in order to reduce environmental disturbances to a minimum.

C. In vivo Experiments

In undertaking these investigations, it was felt that the point of departure should be in the in vivo changes caused
by hormone administration. However, most methods reported so far in the literature require large numbers of animals, each yielding a small segment of information, and requiring extensive statistical analysis for accurate interpretation. Consequently, a new method for studying in vivo metabolism has been developed which partially obviates the difficulties normally inherent in animal investigations.

The mammary gland of the guinea pig is divided into two well separated pads of tissue lying just beneath the skin of the abdomen. Surgical removal of one or both glands may be easily effected without affecting the normal metabolic processes outside the gland. Using this principle it was possible to surgically remove one gland of a lactating female. The metabolic state of the excised tissue was determined by appropriate methods. Following hormonal injections into the remaining gland it was removed and again the biochemical determinations repeated. By the application of this procedure, it has been possible to determine the changes associated with hormone action within a given animal. It has also been possible to determine in the usual way those changes which occurred naturally within the same group of animals. Thus each animal served as its own control and also yielded twice as much information as was heretofore obtainable.
1. The surgical procedure.

Two surgical methods have been developed which differ primarily in the post-operative handling of the animal and in the time-sequence of the various injections. In both cases the animal was anaesthetized with aqueous nembutal at the appropriate time post-partum. The optimal intraperitoneal dose was found to be 3mg. per 100 gm. body weight. If this proved insufficient, additional nembutal or ethyl ether was administered until surgical anaesthesia was achieved.

The usual precautions were observed during surgery. All equipment was sterilized with 80% ethanol or an autoclave. The surgery table and immediate work area were sterilized with a disinfectant of the quaternary ammonium variety. The animal was prepared for surgery by shaving the hair from the immediate vicinity of the operation with an electric razor. This area was then disinfected. The animal was strapped to the operating table, covered with sterile cloths, and the surgery performed. The gland was removed as quickly as possible and chilled in ice while the wound was stitched shut. The teat was removed prior to stitching since it was found that, in those experiments involving subsequent nursing, this discouraged the young from worrying the wound unnecessarily. The animal was kept warm under blankets and a heater
until she was recovered from the anaesthesia (about five hours) or the experiment was completed, whichever came sooner.

2. Injection and post-operative handling

All injections of prolactin or saline were made directly into the gland under investigation. The solutions used for these injections were as nearly isotonic as possible in order to avoid upsetting the mineral metabolism of the lactating gland. The prolactin solutions contained 100 IU of prolactin per ml. of isotonic KCL. The volume injected provided 10 IU of prolactin per 100 gm. body weight. The control animals were injected with proportional volumes of isotonic KCL solution.

As was noted previously, two series of surgical studies were developed which differed primarily in the time of injections and in the post-operative handling of the experimental female. The first of these series, hereafter referred to as the "24-hour surgery" series, employed two separate injections of prolactin: one four hours after the removal of the first gland and the second twenty hours after the removal of the gland. Following the recovery of the animal from anaesthesia, the animal was allowed to nurse the young with the remaining teat until time for sacrifice. Twenty-two hours
after the removal of the first gland, an injection of sodium monofluoroacetate (NFA) was made intraperitoneally and the time carefully noted. Exactly two hours later (twenty-four hours after the first gland was surgically removed) the animal was sacrificed by cervical fracture and exsanguination. The remaining gland was quickly removed and chilled in ice for subsequent analysis.

In the second of these series, henceforth referred to as the "1-hour surgery" series, the animal was not permitted to recover from surgical anaesthesia during the experiment. At the time of the removal of the first gland, a prolactin injection was immediately made into the remaining gland. One hour later the animal was sacrificed by cervical fracture and exsanguination. The remaining mammary gland was quickly excised and chilled in ice for subsequent analysis. The brief interval between hormone administration and sacrifice did not permit the injection of NFA in this series of investigations.

3. Preparation of the samples for analysis

The chilled, excised mammary glands, trimmed of excess body fat, were rapidly weighed on an analytical balance and homogenized in a Potter-Elvehjem glass homogenizer with an equal volume of cold isotonic KCL. The resultant homogenate was made to 25 ml. with cold KCL, maintaining the flask in an
ice bath until all experiments were completed. Duplicate 1 ml. aliquots of this homogenate were used for dry weight determinations (24 hours, 105° C.). The dry weight of a 1 ml. aliquot of KCL was determined simultaneously to correct for the KCL content of the homogenate.

Five ml. of the homogenate were precipitated with an equal volume of 20% trichloroacetic acid (TCA), homogenized, and filtered. The filtrate was diluted to 25 ml. with water and stored in the refrigerator until citric acid determinations were performed. Another 10 ml. aliquot was boiled for two minutes with constant stirring. The product was then homogenized, frozen for twenty-four hours, thawed, and centrifuged. The resultant clear, pale-yellow supernatant was used for CoA assay.

4. The determination of citric acid

Potter's laboratory has recently developed a method of measuring in vivo citrate synthesis. It was first reported in 1949 that the intraperitoneal injection of 5 mg. of sodium monofluoroacetate (NFA) into a rat caused the accumulation of citric acid (44). It has been proven that the inhibition occurs specifically in the oxidation of citric acid (46), apparently by acting on aconitase. None of the other Krebs cycle oxidations are affected. The inhibiting
compound produced is monofluorocitric acid (226,227). The
amount of citrate formed is dependent on dosage and the time
interval between injection and sacrifice (243).

In view of the dependence of citrate accumulation on
dosage and time, an experiment was run to determine the con­
ditions for optimum citrate accumulation in the guinea pig.
Injection of animals with varying amounts of NFA demonstrated
that, in the guinea pig, the optimal dosage lay in the region
of 1.0-1.5 mg. NFA per kilogram body weight. Greater dosages
killed the animal prematurely. The optimal time between in­
jection and sacrifice was determined by injecting a series
of animals with 1.5 mg. NFA per kilogram and varying the time
of sacrifice. The kidney and liver were removed, chilled in
ice, homogenized in KCl, and the proteins precipitated with
20% TCA. Citric acid was determined as described subse­
quently. The results of this experiment are presented in FIGURE
IV. It may be seen that citrate accumulation was negligible
during the first sixty minutes following injection, then rose
linearly during the interval of 90-150 minutes. As a result
of this experiment it was felt that two hours would be the
most satisfactory time interval between injection and sacri­
fice. Occasional animals have died within this time limit
but, as a rule, it has proven very satisfactory.
FIG. 4 CITRATE ACCUMULATION IN THE KIDNEY OF SODIUM MONOFUOROACETATE POISONED GUINEA PIGS

DOSE: 1.5 Mg. NFA/100 Gm. BODY WEIGHT
The citric acid content of the excised mammary gland was determined by the Ettinger, Goldbaum, and Smith modification (80) of the pentabromacetone method of Natelson, Lugovy, and Pincus (206). This modified method has been reported to be specific for citric acid in the presence of other Kreb cycle intermediates. A standard curve was run covering the range of 1 µg.m. to 10 µg.m. citric acid per ml. A suitable dilution for the assay of citric acid was found to be 1 ml. of TCA filtrate (see section V:C:3.) diluted to 25 ml. with water. In applying this technique to the surgical method, the citric acid content of both glands from a given animal were determined simultaneously and expressed as µg.m. per mg. dry weight.

5. Coenzyme A content

The method used for CoA estimation in these samples was the sulfanilamide acetylation method proposed by Kaplan and Lipmann in 1948 (149). The enzyme was prepared from pigeon liver as described in the original article. The enzyme isolation was performed in a cold-room. It was not found necessary to use in vacuo P₂O₅ dessication of the acetone precipitate; "Drierite" dried samples had equal activity and the dessicant was easier to handle. A standard curve was prepared for each batch of pigeon liver using a
crude CoA preparation from rat liver (149) or a commercial purified CoA product. The Kaplan-Lipmann method of CoA estimation was chosen in preference to the arsenolysis methods because it is responsive to the intermediates of CoA synthesis. It thus covers a wide range of reactions which might be influenced by prolactin.

The crude CoA extract of mammary tissue was prepared as previously described (see section V:C:3.). The resultant CoA-containing supernatant was stable for about four days when stored frozen or for one day when maintained at room temperature. It was also found advisable to assay the supernatant undiluted.

The substrate used for the Kaplan-Lipmann CoA assay method contained 10 ml. of 0.004 M sulfanilamide, 2.5 ml. of 1 M sodium acetate, 8.0 ml. of 0.05 M ATP, and 10.0 ml. of sodium citrate. This was neutralized to pH 7.0 and stored frozen until used. A typical system for CoA estimation was as follows:

0.3 ml. substrate
0.3 ml. CoA extract
0.25 ml. of enzyme
0.08 ml. of freshly prepared 1 M NaHCO₃
0.1 ml. of cysteine-HCl
0.48 ml. of water
The assay was run in duplicate simultaneously with two blanks, one leaving out the substrate and the other adding 4.0 ml. 5% TCA before introducing the enzyme. At the end of two hours the reaction was stopped with 4 ml. of 5% TCA. Centrifugation of this produced a water-white solution. Two ml. of this solution plus seven ml. of water and one ml. of 2N HCl were assayed for sulfanilamide by the method of Bratton and Marshall (36). CoA content was then expressed in terms of units per mg. dry weight.

D. In vitro Experiments

The mammary tissue used in the in vitro experiments was obtained from animals as close to the designated time post-partum as possible. All homogenates used in these studies were prepared using 2 gm. of chilled fresh tissue and 8 ml. of ice-cold glass-distilled water. Following homogenization in a cold Potter-Elvehjem glass homogenizer, the homogenate was strained through four layers of cheese cloth in order to remove the larger pieces of unmacerated tissue. The resultant homogenate was stored in an ice-bath until used. Where necessary, dry weights of 1 ml. aliquots of the homogenate were determined (24 hours, 105°C.). In the experiments on phosphate inhibition of CoA metabolism the homogenates were prepared in a similar way using 0.067 M phosphate buffer pH 7.4.
The acetone-powders employed for the extraction of the various enzyme fractions were prepared by blenderizing fresh tissue with twenty volumes of cold acetone for two minutes in a Waring Blendor. The resultant slurry was filtered under suction and washed with five volumes each of cold acetone and cold ethyl ether. The powder obtained was dried over "Drierite" in a dessicator stored at 4°C. Crude extracts of the dried, sifted powder were prepared using ten volumes of freshly prepared cold 0.02 M KHCO₃ followed by centrifugation in the cold. The supernatant will henceforth be referred to as AP extract.

For use in the in vitro studies the prolactin was dissolved in 0.05 M "Tris" buffer pH 8.0 ("Tris" is the commonly used abbreviation for tris-hydroxymethylaminomethane). The hormone solution was added to the reaction mixture after the introduction of the homogenate unless otherwise stated.

1. Coenzyme A synthesis

CoA synthesizing activity was measured with the following system:

\[
\begin{align*}
2.5 \times 10^{-5} \text{ M } & \text{MgCl}_2 \\
2.0 \times 10^{-5} \text{ M } & \text{K}_2\text{ATP} \\
0.67 \times 10^{-5} \text{ M } & \text{KH}_2\text{PO}_4 \\
5 \times 10^{-5} \text{ M } & \text{Cysteine-HCl (neutralized with KOH)}
\end{align*}
\]
1 x 10^-7 M Pantethine
5 x 10^-5 M "Tris" Buffer pH 8.0
Water to a total volume of 4.5 ml.

When liver or mammary AP extracts were employed as the enzyme source, a volume of 0.8 ml. was used in place of an equal volume of water. One ml. of fresh homogenate was used in the experiments on synthesis by fresh homogenates as well as in those experiments concerned with the inhibition of AP extract synthesis by fresh homogenate. These reactions were incubated for two hours at 37° C. Samples were taken at T0 and T120 and boiled for two minutes for subsequent analysis for CoA or pantetheine content. Mg++ was found to be a slightly better activator than Mn++ for mammary CoA synthesizing enzymes. Also, increasing the concentration of phosphate ions did not further increase the measurable CoA synthesis.

Care was taken in all the studies on CoA synthesis to maintain a prolactin/cysteine ratio sufficiently low to prevent the inactivation of the hormone (111).

2. Pantetheine and coenzyme A degradation

In the experiments concerned with potential degradation of pantetheine by mammary homogenates the reaction mixture contained:
1 x 10^{-8} \text{ M pantethine} \\
5 x 10^{-5} \text{ M cysteine-HCl (neutralized with KOH)} \\
1 x 10^{-3} \text{ M "Tris" buffer pH 8.0} \\
1.0 \text{ ml. fresh homogenate} \\

Water to a total volume of 3.6 ml.

The incubation time was one hour at 37^\circ \text{ C}. Samples were taken at T_0 and T_{60}, boiled, and assayed by the appropriate technique.

Contrary to the reports by Ringler and his co-workers (252,254), mammary tissue has been found to have potent CoA degrading enzymes. Investigations showed that CoA degradation could be measured with a system containing 40-50 units of CoA, 5 x 10^{-4} \text{ M "Tris" buffer pH 8.0, 0.6 ml. fresh homogenate, and water to a volume of 4.5 ml. The presence of phosphate ions was scrupulously avoided. Incubation time was one hour at 37^\circ \text{ C}. The T_0 and T_{60} samples were boiled for two minutes prior to assay for CoA or CoA precursors. Neither the addition of Mg^{++} nor cysteine aided the degradative system. For studies on the effect of phosphate ions on CoA degradation, phosphate homogenate was employed in place of the aqueous homogenate; additional phosphate was also used in place of the water. The studies on the effect of pH on degradative activity utilized "Tris" buffers of the appropriate pH substituted for the pH 8.0 buffer ordinarily
3. Differential coenzyme A determinations

Estimations of CoA content have been conducted by two different methods. The method most frequently employed was the Kaplan-Lipmann (KL) enzyme complex. The details of this assay were presented in a previous section (V:C:5). The only modification necessary for the assay of the samples from the in vitro studies was the addition of 0.1 ml. of 1.0 M "Tris" buffer pH 7.0 to the reaction system. For a more detailed estimation of the products of degradative activity, the enzymes involved in CoA synthesis have been partially purified from pigeon liver according to the method of Levintow and Novelli (167). Through a series of fractionations utilizing protamine sulfate precipitation and absorption with calcium phosphate gel, two separate fractions have been obtained from pigeon liver AP extracts. One of these, PK, contained nearly all the pantetheine kinase activity whereas the second, SA, contained the phosphopantetheine condensing enzyme ("C"), dephospho-CoA kinase (KII), and sulfanilamide acetylation enzyme (see Figure II).

Fraction SA was found to be responsive to CoA in a manner analogous to the KL system using the following assay system:
0.3 ml. KL substrate
5 x 10^{-5} M cysteine
3 x 10^{-6} M MgCl_2
1 x 10^{-4} M "Tris" buffer pH 8.0
0.25 ml. fraction SA
Unknown containing 3 units CoA or less
Water to a total volume of 1.45 ml.

This system was incubated for two hours at 37° C. at which time the reaction was stopped with 4.0 ml. 5% TCA. The quantity of sulfanilamide acetylated was determined as previously outlined for the KL system. In order to maintain uniform units throughout all experiments, the SA standard curve was prepared using CoA solutions previously standardized against the KL system. Qualitatively the SA curve obtained with these Pure CoA solutions resembled the standard curves obtained with the KL system.

In investigating mixed reaction products, the SA system was employed to determine the total CoA and CoA precursors above phosphopantetheine (see FIGURE II). The concentration of pantetheine could also be determined on a similar sample of the same unknown by substituting 0.4 ml. of PK for an equal volume of water in the SA mixture. By subtracting the amount of CoA determined using the SA system alone from the
CoA determined with the combination of SA plus PK, the CoA equivalents of the pantetheine present were obtained.
VI. Results and Discussion

A summary of the naturally occurring in vivo changes in citric acid and CoA concentration as determined from the mammary glands removed by surgery prior to any hormone treatment is presented in TABLE I. A study of the averages obtained suggests that the citric acid content of mammary tissue undergoes a rapid increase immediately after parturition followed by a period of "leveling-off" whereas the CoA content undergoes a somewhat more gradual increase. In order to verify these observations, statistical analysis of the data was performed employing the t-test for testing the differences between the means obtained. In comparing the 24-hour and the 12-hour concentrations of citric acid in mammary tissue, it was found that the t-value obtained was slightly less than significant at the 5% probability level. The differences between the 12-hour and the 36-hour samples were significant at the 5% probability level but the comparison of the 24-hour and the 36-hour concentrations showed them to be not significantly different. It therefore appears that the citric acid content of mammary tissue undergoes a rapid increase following parturition such as might be expected from the direct or indirect intervention of circulating prolactin in citric acid synthesis. Similarly, the
TABLE I

THE IN VIVO ACCUMULATION OF CITRIC ACID AND COENZYM E A IN GUINEA PIG MAMMARY TISSUE
DURING EARLY LACTATION

<table>
<thead>
<tr>
<th>Citric Acid</th>
<th>Coenzyme A</th>
</tr>
</thead>
<tbody>
<tr>
<td>(µgm./mg. dry weight)</td>
<td>(Units/mg. dry weight)</td>
</tr>
<tr>
<td>Hours Post-partum</td>
<td>12</td>
</tr>
<tr>
<td>--------------------</td>
<td>----</td>
</tr>
<tr>
<td>8.37</td>
<td>13.62</td>
</tr>
<tr>
<td>8.50</td>
<td>10.93</td>
</tr>
<tr>
<td>7.90</td>
<td>10.75</td>
</tr>
<tr>
<td>4.98</td>
<td>7.10</td>
</tr>
<tr>
<td>5.47</td>
<td>7.23</td>
</tr>
<tr>
<td>5.89</td>
<td>9.15</td>
</tr>
<tr>
<td>4.73</td>
<td>3.61</td>
</tr>
<tr>
<td>--</td>
<td>10.84</td>
</tr>
<tr>
<td>--</td>
<td>5.46</td>
</tr>
<tr>
<td>--</td>
<td>10.14</td>
</tr>
<tr>
<td>--</td>
<td>11.45</td>
</tr>
<tr>
<td>Average</td>
<td>6.75±1.65</td>
</tr>
</tbody>
</table>

NOTE: Values obtained from mammary glands surgically removed at the indicated time post-partum. See section V:C for experimental details.
### TABLE I (Continued)

Results of Statistical Analysis by the t-Test

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Citric Acid</th>
<th>Coenzyme A</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 vs 24 hours</td>
<td>2.092#</td>
<td>2.786*</td>
</tr>
<tr>
<td>12 vs 36 hours</td>
<td>2.457*</td>
<td>2.930**</td>
</tr>
<tr>
<td>24 vs 36 hours</td>
<td>0.0321</td>
<td>0.931</td>
</tr>
</tbody>
</table>

# Significant at 6% probability level
* Significant at 5% probability level
** Significant at 1% probability level
CoA concentration exhibited a rapid increase during the 12- to 24-hour interval which was significant at the 5% level of probability. By thirty-six hours post partum the increase in CoA concentration was significant at the 1% level when compared to the 12-hour tissue. However, the comparison between the 24-hour and 36-hour tissue suggests that a leveling off of the CoA increase subsequently occurs although the increase continues to be apparent. The rapid increase in CoA concentration confirms a recent report by Ringler, et al (252).

From the biochemistry of citric acid formation it would appear that the increase in CoA concentration was instrumental in causing the observed increase in citric acid content of lactating mammary tissue. A close correlation between these two compounds was demonstrated by determining the correlation coefficient between the CoA and citric acid concentrations in those glands where both determinations were made. Taking all of these animals as a group, the coefficient obtained was significant at the 5% level. However, it would appear that the continued accumulation of CoA thirty-six hours post-partum indicates a diversion of at least part of the CoA to other non-oxidative reactions in the lactating mammary gland.

Holst and Turner (138) have investigated the prolactin
content of guinea pig pituitaries. They report that the prolactin content remains low (1.5 units per 100 gm. body weight) during pregnancy then rises sharply at parturition to a value of 3.5 units per 100 gm. body weight at forty-eight hours post-partum and does not fall greatly until the tenth day. Comparison of the data obtained here with that of Holst and Turner suggests that the rapid increase in CoA and citric acid concentration is intimately associated with prolactin action. Additional information about this possibility has been obtained from the surgical studies. The results of these investigations are presented in TABLES II-IV.

The first of these studies, summarized in TABLES II and III, was planned to investigate the effects of prolactin when injected into the lactating mammary gland of a female continuing to nurse her young. Injections of saline were made into other animals to serve as controls. TABLE III summarizes the relative changes in citric acid and CoA contents which occurred, comparing the non-injected and the injected glands from single females. In general, the injection of prolactin did not cause marked increases in the citric acid content of the treated glands. Coenzyme A content, on the other hand, was appreciably increased as a result of prolactin injection, particularly during the first 24 hours post-partum.
### TABLE II

**IN VIVO EFFECTS OF PROLACTIN INJECTIONS ON CITRIC ACID AND COENZYME A CONCENTRATION IN LACTATING GUINEA PIG MAMMARY TISSUE AS DETERMINED BY THE "24-HOUR" SURGICAL TECHNIQUE**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Citric Acid</th>
<th>Coenzyme A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td><strong>12 hours post-partum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control:</td>
<td>8.50</td>
<td>10.10</td>
</tr>
<tr>
<td></td>
<td>7.90</td>
<td>8.32</td>
</tr>
<tr>
<td></td>
<td>4.98</td>
<td>6.15</td>
</tr>
<tr>
<td>Prolactin:</td>
<td>5.47</td>
<td>6.29</td>
</tr>
<tr>
<td></td>
<td>5.89</td>
<td>6.81</td>
</tr>
<tr>
<td></td>
<td>4.73</td>
<td>5.63</td>
</tr>
<tr>
<td><strong>24 hours post-partum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control:</td>
<td>10.75</td>
<td>11.97</td>
</tr>
<tr>
<td></td>
<td>7.10</td>
<td>10.51</td>
</tr>
<tr>
<td></td>
<td>7.23</td>
<td>8.13</td>
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<tr>
<td></td>
<td>9.15</td>
<td>9.87</td>
</tr>
<tr>
<td>Prolactin:</td>
<td>3.61</td>
<td>6.44</td>
</tr>
<tr>
<td></td>
<td>5.46</td>
<td>8.76</td>
</tr>
<tr>
<td></td>
<td>10.14</td>
<td>12.48</td>
</tr>
</tbody>
</table>

**NOTE:** Values in columns A obtained from mammary gland surgically removed at indicated time post-partum. Values in columns B obtained from mammary gland removed twenty-four hours later following two injections of prolactin or saline. Values in columns B/A represent relative change comparing the B value to the A value. See Section V: C for experimental details.
TABLE II (Continued)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Citric Acid</th>
<th>Coenzyme A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td><strong>36 hours post-partum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control:</td>
<td>7.21</td>
<td>7.59</td>
</tr>
<tr>
<td></td>
<td>7.28</td>
<td>7.07</td>
</tr>
<tr>
<td></td>
<td>11.15</td>
<td>10.47</td>
</tr>
<tr>
<td>Prolactin:</td>
<td>7.16</td>
<td>7.17</td>
</tr>
<tr>
<td></td>
<td>10.50</td>
<td>9.55</td>
</tr>
<tr>
<td></td>
<td>7.67</td>
<td>6.84</td>
</tr>
</tbody>
</table>
TABLE III

SUMMARY OF AVERAGE B/A VALUES FROM TABLE II

<table>
<thead>
<tr>
<th>Time post-partum</th>
<th>Treatment</th>
<th>Citric Acid</th>
<th>Coenzyme A</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 hours</td>
<td>Control</td>
<td>1.15</td>
<td>0.784</td>
</tr>
<tr>
<td>12 hours</td>
<td>Prolactin</td>
<td>1.17</td>
<td>0.941</td>
</tr>
<tr>
<td>24 hours</td>
<td>Control</td>
<td>1.20</td>
<td>0.774</td>
</tr>
<tr>
<td>24 hours</td>
<td>Prolactin</td>
<td>1.54</td>
<td>1.14</td>
</tr>
<tr>
<td>36 hours</td>
<td>Control</td>
<td>0.987</td>
<td>0.853</td>
</tr>
<tr>
<td>36 hours</td>
<td>Prolactin</td>
<td>1.053</td>
<td>0.921</td>
</tr>
</tbody>
</table>
However, the results obtained would not appear to be sufficient to explain the dramatic changes in post-partum mammary metabolism which may be attributed to prolactin action. It is possible, of course, that the post-operative stress during the twenty-four hour interval following surgery may have overshadowed the changes resulting from hormone injections. Furthermore, the four hour interval between the final prolactin injection and sacrifice may have been too long to adequately demonstrate prolactin action. Thus, instead of having the cumulative effect anticipated, the prolactin activity may have been dissipated prior to sacrifice. Support for this concept comes from the report by Sgouris and Meites (258) that mammary tissue inactivated prolactin in vitro as measured by pigeon crop stimulation. Cox (64) also demonstrated rapid inactivation of I\(^{131}\)-tagged prolactin by mammary tissues.

In view of these possibilities, a second series of surgical experiments were designed in which the prolactin injection was administered immediately following the removal of the first gland. The animal was sacrificed one hour later. The results of these experiments on animals twelve hours post-partum is presented in TABLE IV. A clear correlation between prolactin injection and mammary tissue CoA content was obtained. The smaller standard deviation also shows that
### TABLE IV

**IN VIVO EFFECTS OF PROLACTIN INJECTION ON COENZYME A CONCENTRATION IN GUINEA PIG MAMMARY TISSUE AS DETERMINED BY THE "1-HOUR" SURGICAL TECHNIQUE**

<table>
<thead>
<tr>
<th>Units Coenzyme A per mg. Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>0.068</td>
</tr>
<tr>
<td>0.057</td>
</tr>
<tr>
<td>0.048</td>
</tr>
<tr>
<td>--</td>
</tr>
<tr>
<td>Prolactin</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>0.053</td>
</tr>
<tr>
<td>0.029</td>
</tr>
<tr>
<td>0.045</td>
</tr>
<tr>
<td>0.058</td>
</tr>
</tbody>
</table>

**NOTE:** Values in columns A obtained from mammary gland surgically removed 12 hours post-partum. Values in columns B obtained from mammary gland removed one hour later following one injection of prolactin or saline immediately following the removal of the first gland. Values in columns B/A represent relative change comparing the B value to the A value. See Section V:C for experimental details.
fewer complications arising from post-operative stress were elicited. Unfortunately, the short time between removing the two glands did not permit the determination of citric acid production by the HFA method.

The demonstration that prolactin and CoA metabolism were correlated in the metabolism of lactating mammary tissue could be explained by several different mechanisms. It was possible that prolactin increased the level of CoA or CoA precursors in the blood. However, this seemed improbable inasmuch as the significant correlation was obtained by the intramammary injection of the hormone. Furthermore, the beneficial effects of prolactin on in vitro respiration which have been reported (14, 33) could not be explained on the basis of changes in blood constituents. Another possibility was that prolactin may have inhibited degradation of CoA in mammary tissue thus conserving it for synthetic reactions. Since Ringler, et al, (252, 254) had reported that phosphate buffer homogenates did not degrade CoA, this possibility was considered unlikely. The third possibility was that prolactin activity increased CoA synthesis in mammary tissue. Experiments were undertaken to test this hypothesis.

As the first step in this investigation, attempts were made to demonstrate CoA synthesis in fresh mammary homogenates. The method outlined by Hoagland and Novelli (136)
employed leaching the aqueous homogenate for thirty minutes at \(0^\circ\) C. This technique was applied to mammary homogenates. Following centrifugation the supernatant was tested for synthetic activity using a system similar to that in Section V:D:i but devoid of phosphate ions. No synthesis could be demonstrated. Neither the direct addition of 5 IU prolactin per ml. to the reaction mixture nor the preliminary incubation of the supernatant enzymes with prolactin permitted the demonstration of CoA synthesis. Subsequent investigation indicated that the addition of phosphate ions would increase the demonstrable CoA synthesis when acetone powder extracts were used as the enzyme source. By applying this principle to fresh homogenates the synthesis of 5 IU of CoA per ml. of 20% homogenate in two hours was obtained. Repetition of the prolactin addition experiments did not indicate any beneficial effect of prolactin on the CoA synthesizing enzymes. Data from typical experiments on the effect of prolactin on \textit{in vitro} CoA synthesis by mammary homogenates are presented in TABLE V. The data in this table also shows that increasing the phosphate concentration above \(0.67 \times 10^{-5}\) M did not further increase the demonstrable CoA synthesis.

Although prolactin activation of CoA synthesis could not be shown with fresh mammary homogenates, the possibility remained that the low levels of synthesis obtained prevented
TABLE V

EFFECTS OF PHOSPHATE AND PROLACTIN ON COENZYME A SYNTHESIS
BY MAMMARY HOMOGENATES

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Hours Post-partum</th>
<th>Phosphate Concentration</th>
<th>Units CoA Synthesized by 1 ml. Homogenate in 2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$0.67 \times 10^{-5}$ M</td>
<td>$3 \times 10^{-4}$ M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>12</td>
<td>+6.8</td>
<td>+6.6</td>
</tr>
<tr>
<td>93</td>
<td>12</td>
<td>-1.08</td>
<td>-0.54</td>
</tr>
<tr>
<td>88</td>
<td>36</td>
<td>+3.4</td>
<td>+3.4</td>
</tr>
<tr>
<td>101</td>
<td>36</td>
<td>+5.0</td>
<td>+6.0</td>
</tr>
</tbody>
</table>

* Prolactin concentration expressed as units per ml. of reaction mixture.

NOTE: See Section V:D:1 for experimental details.
the determination of a beneficial effect. Preliminary studies indicated that the phosphate-containing system (Section V:D:1) gave optimal determinations of synthetic activity with both rat and guinea pig liver acetone powder extracts. One ml. of extract from either of these sources synthesized 9-10 units of CoA in two hours (see TABLE VI). Both systems were activated by magnesium. For the rat extract system, manganese was an even better activator than magnesium whereas manganese completely inhibited CoA synthesis in guinea pig liver extracts. Application of the magnesium activated system indicated the mammary extract to have three to five times the synthetic activity of the liver extracts.

Several experiments were set up to determine the influence, if any, that prolactin might have on the mammary acetone powder extract system. The concentration of hormone used was 10 IU per ml. of reaction mixture. Neither incubation of the hormone with the complete system nor preincubation of the hormone with the extract before adding the other components had any effect on total synthesis.

Frankel-Conrat (110) has reported that thiol compounds cause the reduction of the cystine moiety of prolactin to cysteine and also that thiol compounds probably form addition compounds with the hormone protein. Pantetheine and CoA are both sulfhydryl compounds. Pantetheine has been shown
### TABLE VI

**CATION ACTIVATION OF COENZYME A SYNTHESIS BY ACETONE POWDER EXTRACTS FROM VARIOUS SOURCES**

<table>
<thead>
<tr>
<th>Source of Extract</th>
<th>Cation Activator</th>
<th>Cation Concentration</th>
<th>Units CoA Synthesized by 1 ml. Extract in 2 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Liver</td>
<td>Mg</td>
<td>$2.5 \times 10^{-5} \text{M}$</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>$2.5 \times 10^{-5} \text{M}$</td>
<td>10.5</td>
</tr>
<tr>
<td>Guinea pig liver</td>
<td>Mg</td>
<td>$2.5 \times 10^{-5} \text{M}$</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>$2.5 \times 10^{-5} \text{M}$</td>
<td>3.4</td>
</tr>
<tr>
<td>Guinea pig mammary tissue</td>
<td>Mg</td>
<td>$2.5 \times 10^{-5} \text{M}$</td>
<td>27.9</td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>$2.5 \times 10^{-5} \text{M}$</td>
<td>11.4</td>
</tr>
</tbody>
</table>

**NOTE:** Synthesizing system same as in Section VId:1 except for indicated changes in cation concentration.
to form mixed disulfides (40,41) which are active as precur-
sors for CoA synthesis. One of these is the now well-known
LBF. In view of these facts it was believed possible that
prolactin and pantetheine might form such a mixed disulfide
having increased activity towards the synthesizing enzymes.
This possibility was investigated by incubating the hormone
with the other reaction ingredients before introducing the
enzyme. The results were negative under the experimental con-
ditions employed.

During the course of the experiments on potential pro-
lactin activation of CoA synthesis an experiment was devised
to test the hypothesis that acetone-fractionation of the mam-
mary tissue eliminated a cofactor necessary for prolactin
action. Although the results did not contribute to the
studies on CoA synthesis, they were nevertheless significant
for the progress of this investigation. In these experiments
the method for determining CoA synthesis was the same as that
used in the other experiments (Section V:D:1). The volume of
homogenate added was 1.0 ml. in each reaction tube. The re-
sults of these experiments are presented in TABLE VII. Sur-
prisingly, the addition of homogenate prepared from animals
twelve hours post-partum to the semi-purified system inhibited
the synthesis of CoA. In four out of five experiments, 10 IU
prolactin per ml. of reaction mixture reversed this inhibition,
restoring nearly all of the original synthetic activity. In three of the experiments the introduction of 5 IU prolactin partially reversed the inhibition. One of the latter which showed low levels of inhibition by the homogenate was completely reactivated by both levels of prolactin. The only reasonable explanation for these results appeared to be that fresh mammary homogenate had pantetheine or CoA degrading activity sufficient to prevent accumulation of the coenzyme synthesized by the powder extract. Furthermore, the inhibitory action of fresh homogenate was reversible, in part at least, by the hormone prolactin. Since it had been shown that prolactin had no effect on mammary acetone powder synthesis of CoA, it was apparent that prolactin must have reversed the inhibitory effect of the whole homogenate by reversing the homogenate degradation of either CoA or pantetheine.

Extending these studies of extract synthesis to include homogenates from animals thirty-six hours post-partum (TABLE VII) gave results which were somewhat more erratic than those from the 12-hour homogenate experiments. In the first animal an increase in CoA synthesis resulted from the introduction of homogenate to the system. No studies on the effects of prolactin were made with this animal. The second animal duplicated the results of the 12-hour studies in that homogenate introduction inhibited CoA synthesis by the acetone powder
extract; this inhibition was reversed by prolactin. The third animal again exhibited an increase in synthesis in the combined extract and homogenate tube. Prolactin addition gave no beneficial effect. In the case of this animal, homogenate synthesis studies were run simultaneously (second 36-hour animal, TABLE V). A demonstrable synthesis was observed approximately equaling the increment between the synthesis by the extract and the extract plus homogenate. Prolactin did not further increase this synthesis. These results may partially explain the observed results with extract synthesis.

In order to explain fully the inhibitory effects of fresh homogenate on extract synthesis, however, it was necessary to determine the effects of incubating fresh homogenate with pantetheine. It was possible that the homogenate might have degraded the pantetheine so as to remove it from CoA synthesis by the acetone powder extracts.

Several experiments were performed to test this hypothesis using CoA synthesis by mammary powder extracts as an index of pantetheine content both before and after incubation of the pantetheine with fresh homogenate. The results are summarized in TABLE VIII. As can be seen, pantetheine was not degraded by incubating it with the fresh homogenate. Later, after the SA-PK system for pantetheine assay had been perfected, these experiments were repeated and extended to include
### TABLE VII

**THE EFFECT OF HOMOGENATE PLUS PROLACTIN ON COENZYME A SYNTHESIS BY MAMMARY ACETONE POWDER EXTRACTS**

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Hours Post-Partum</th>
<th>Extract Alone</th>
<th>Extract + Homogenate</th>
<th>Extract + Homogenate + 5 IU Prolactin*</th>
<th>Extract + Homogenate + 10 IU Prolactin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>12</td>
<td>22.1</td>
<td>13.2</td>
<td>--</td>
<td>21.2</td>
</tr>
<tr>
<td>54</td>
<td>12</td>
<td>15.3</td>
<td>6.0</td>
<td>10.5</td>
<td>--</td>
</tr>
<tr>
<td>63</td>
<td>12</td>
<td>14.1</td>
<td>9.9</td>
<td>--</td>
<td>13.5</td>
</tr>
<tr>
<td>65</td>
<td>12</td>
<td>13.1</td>
<td>7.9</td>
<td>8.3</td>
<td>12.0</td>
</tr>
<tr>
<td>83</td>
<td>12</td>
<td>6.9</td>
<td>3.6</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>60</td>
<td>24</td>
<td>4.4</td>
<td>6.8</td>
<td>8.3</td>
<td>10.4</td>
</tr>
<tr>
<td>79</td>
<td>36</td>
<td>6.0</td>
<td>15.6</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>88</td>
<td>36</td>
<td>16.8</td>
<td>13.6</td>
<td>17.4</td>
<td>16.8</td>
</tr>
<tr>
<td>101</td>
<td>36</td>
<td>7.7</td>
<td>14.5</td>
<td>14.0</td>
<td>14.1</td>
</tr>
</tbody>
</table>

*Prolactin concentration expressed as units per ml. of reaction mixture.

**NOTE:** See Section V:D:1 for experimental details.
the effect of prolactin on the reaction. Typical results from these experiments are also included in TABLE VIII. Once again it was apparent that pantetheine was not destroyed by fresh mammary homogenate, either with or without prolactin present.

In view of these results, it was decided that further investigations into the possibility of CoA degradation by fresh mammary homogenate were warranted. This phase of the problem had been neglected due to the reports by Ringler, et al, (252, 254) that CoA was not degraded by mammary homogenates containing phosphate buffer. Using aqueous homogenates this investigator quickly found that fresh mammary homogenates have a very high level of CoA degrading activity (see TABLES IX to XII). These high levels of activity required the use of 40-50 units of CoA as substrate as well as an incubation period of one hour or less for assay. This suggested that Ringler's group had inadvertently chosen conditions inhibitory to CoA degradation in preparing their homogenates. Therefore investigations of the influence of phosphate ions on CoA degradation were undertaken. As may be seen in TABLE IX, $3 \times 10^{-4}$ M phosphate ion almost completely inhibited the degradative activity of fresh homogenate. The addition of sodium or potassium ions at the same concentration as was used in Ringler's buffer had no effect on degradation in the absence of phosphate. Additional studies were undertaken to determine the effect of
### TABLE VIII

EFFECTS OF MAMMARY HOMOGENATE ON PANTETHINE

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Conditions</th>
<th>CoA Equivalents of Pantetheine Before Incubation with Homogenate</th>
<th>CoA Equivalents of Pantetheine After Incubation with Homogenate</th>
<th>Pantetheine Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>104</td>
<td>No prolactin</td>
<td>7.0</td>
<td>6.8</td>
<td>-0.2</td>
</tr>
<tr>
<td>99</td>
<td>No prolactin</td>
<td>4.5</td>
<td>6.0</td>
<td>+1.5</td>
</tr>
<tr>
<td>95</td>
<td>No prolactin</td>
<td>7.0</td>
<td>7.3</td>
<td>+0.3</td>
</tr>
</tbody>
</table>

a) Assay by synthesis of CoA by mammary acetone powder; CoA assay with KL:

b) Direct assay with SA-PK system:

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Conditions</th>
<th>CoA Equivalents of Pantetheine Before Incubation with Homogenate</th>
<th>CoA Equivalents of Pantetheine After Incubation with Homogenate</th>
<th>Pantetheine Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>No prolactin</td>
<td>11.7</td>
<td>12.2</td>
<td>-0.5</td>
</tr>
<tr>
<td>10 IU prolactin*</td>
<td>11.5</td>
<td>12.2</td>
<td></td>
<td>-0.7</td>
</tr>
</tbody>
</table>

*Prolactin concentration expressed as units per ml. reaction mixture.

NOTE: See Section V:D:2 for experimental details.
pH on CoA degradation. Typical results are presented in TABLE X. It was found that at pH 7.4 CoA degradation was reduced by about 55%, which was not so great as the effect of phosphate at pH 8.0. However, the combination of phosphate plus a pH of 7.4 completely inhibited CoA degradation. Thus the results by Ringler and his co-workers are confirmed and explained. Fluoride ion has also been found to be an inhibitor of CoA degradation (see TABLE XI).

The inhibition studies yield clues as to the mechanism of the degradative reaction. Phosphate inhibition suggests that degradation might occur by one of two methods. The first might be by the action of an enzyme in mammary tissue which would hydrolyze the ribose-phosphate linkage. On FIGURE I much as has been demonstrated with prostatic phosphatase. However, the product of such an hydrolysis would be dephospho-coenzyme A, a compound which behaves as CoA in the impure KL assay system. Inasmuch as the formation of this product would not register as CoA destruction with the KL system, this possibility appears unlikely. The second degradative scheme might be the reversal of the synthetic pathway to yield phosphopantetheine. Phosphopantetheine may be dephosphorylated to pantetheine by the reversal of pantetheine kinase or by the phosphorylytic action of alkaline phosphatase. The synthesis of phosphopantetheine by pantetheine kinase is activated by
### Table IX

**EFFECT OF PHOSPHATE ON COENZYME A DEGRADATION BY MAMMARY HOMOGENATE**

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Without phosphate</th>
<th>In presence of $3 \times 10^{-4}$ M phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>18.9</td>
<td>1.2</td>
</tr>
<tr>
<td>77</td>
<td>18.9</td>
<td>1.4</td>
</tr>
<tr>
<td>79</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>88</td>
<td>24.5</td>
<td>2.7</td>
</tr>
<tr>
<td>93</td>
<td>15.5</td>
<td>2.3</td>
</tr>
</tbody>
</table>

**NOTE:** See Section V:D:2 for experimental details.
TABLE X

**pH EFFECTS ON COENZYME A DEGRADATION BY MAMMARY HOMOGENATES**

<table>
<thead>
<tr>
<th>pH</th>
<th>Concentration of Phosphate Ion</th>
<th>Units of CoA Degraded per 0.6 ml. Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>0</td>
<td>15.5</td>
</tr>
<tr>
<td>8.0</td>
<td>3 x 10^{-4} M</td>
<td>2.3</td>
</tr>
<tr>
<td>7.4</td>
<td>0</td>
<td>6.8</td>
</tr>
<tr>
<td>7.4</td>
<td>3 x 10^{-4} M</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**NOTE:** See Section V:D:2 for experimental details.
TABLE XI
EFFECT OF FLUORIDE ON COENZYME A DEGRADATION
BY MAMMARY HOMOGENATES

<table>
<thead>
<tr>
<th>Concentration of Sodium Fluoride</th>
<th>Assay Method</th>
<th>Units CoA/ml of product</th>
<th>Units * pantetheine/ml Product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Zero</td>
<td>After</td>
</tr>
<tr>
<td>0 M</td>
<td>KL</td>
<td>11.7</td>
<td>4.4</td>
</tr>
<tr>
<td>5x10⁻³ M</td>
<td>KL</td>
<td>13.0</td>
<td>9.3</td>
</tr>
<tr>
<td>0 M</td>
<td>SA-PK</td>
<td>11.0</td>
<td>3.5</td>
</tr>
<tr>
<td>5x10⁻³ M</td>
<td>SA-PK</td>
<td>12.5</td>
<td>8.8</td>
</tr>
</tbody>
</table>

*Units of pantetheine expressed as CoA equivalents
NOTE: See Section V:D:2 for experimental details.
# TABLE XII

**EFFECT OF PROLACTIN ON COENZYME A DEGRADATION BY MAMMARY HOMOGENATE**

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Hours Post-Partum</th>
<th>Units CoA Degraded per Hour per ml. Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Homogenate</td>
</tr>
<tr>
<td>63</td>
<td>12</td>
<td>19.5</td>
</tr>
<tr>
<td>65</td>
<td>12</td>
<td>17.0</td>
</tr>
<tr>
<td>93</td>
<td>12</td>
<td>21.1</td>
</tr>
<tr>
<td>77</td>
<td>36</td>
<td>31.4</td>
</tr>
<tr>
<td>79</td>
<td>36</td>
<td>13.3</td>
</tr>
<tr>
<td>88</td>
<td>36</td>
<td>40.7</td>
</tr>
</tbody>
</table>

*Prolactin expressed as units per ml. reaction mixture.

NOTE: See Section V:D:2 for experimental details.
phosphate ions (167) and therefore it may be presumed that the degradative activity of this enzyme would be inhibited by phosphate. Furthermore, the dephosphorylation of phosphopantetheine by intestinal alkaline phosphatase is inhibited by NaF (38). This evidence suggests that the degradation of coenzyme A is probably by the reversal of the synthetic reaction with the pivotal reaction being the formation of phosphopantetheine.

If the reversal of pantetheine kinase should be the key step in mammary CoA degradation then pantetheine would be anticipated as a degradation product. However, mammary acetone powder extracts, which synthesize CoA from pantetheine, could not resynthesize CoA from the degradation products. Therefore the degradation products apparently do not include pantetheine. This was further verified in subsequent experiments using the SA-PK assay system. Inspection of TABLE IV shows that CoA degradation resulted in a net loss of pantetheine in the three animals investigated. In conjunction with this it is significant to note that fluoride inhibition of CoA degradation did not affect the disappearance of the endogenous pantetheine from that system (TABLE XI). It is therefore probable that the disappearance of the large amounts of pantetheine normally present in mammary tissue is unrelated to the process of CoA degradation. That it occurs at all is noteworthy, however, because the direct action of homogenate on pantetheine was
not demonstrable (TABLE VIII).

Having established the conditions under which mammary homogenate degrades CoA, studies were undertaken of the effect of prolactin on this degradation. The results of these investigations are presented in TABLE IV. Using tissue from animals twelve hours post-partum the introduction of 10 IU prolactin per ml. of reaction mixture decreased CoA degradation approximately 50%. In only one case was inhibition achieved with 5 IU prolactin. Similarly, with tissue from animals thirty-six hours post-partum, 10 IU prolactin inhibited CoA degradation, although not so dramatically as with the 12-hour homogenates. Two of the three homogenates also exhibited response to 5 IU prolactin. It would thus appear that prolactin inhibits the degradation of CoA. It is interesting that, insofar as comparison is possible, the effects of prolactin on homogenate degradation parallel the results obtained with prolactin plus homogenate in the acetone powder extract synthesis experiments.

As a corollary to the other studies, quantitative estimates of CoA degradation were made on several animals. These values have been tabulated in TABLE XIII. There is apparently wide variability in degrading activity in mammary tissues selected at either twelve or thirty-six hours post-partum. However, the averages at both twelve and thirty-six hours are
nearly equal so that it would appear that the inhibitory effects by prolactin on CoA degradation are not the result of the permanent binding of prolactin to the degradative enzymes. If it were, then a decrease in measurable degradation would be expected in the 36-hour tissue.

Finally, investigations were undertaken to determine in greater detail the products of CoA degradation by mammary homogenate using the SA-PK system of analysis. The results of these studies are listed in TABLE XIV. The most illuminating result of these investigations was the lack of agreement between the CoA assays with the KL and the SA systems. At zero time, the two methods yielded fairly comparable values when it is remembered that the SA system contains $K_{11}$, $C$, and the acetylation enzyme in high concentrations whereas the KL system contains a relatively large concentration of the acetylation enzyme plus the other enzymes in lesser concentrations. Thus the KL system intrinsically assays for a smaller percentage of the CoA precursors than does the SA system. A comparison of the products of degradation in the absence of prolactin also shows a similar relationship between the two methods of assay. However, following the incubation of prolactin with homogenate and CoA, the results of the two systems differ significantly. Using SA analysis, no inhibition of CoA degradation was found. Nevertheless, assay of
# TABLE XIII

**QUANTITATIVE ESTIMATE OF MAMMARY TISSUE COENZYME A DEGRADING ACTIVITY AT VARIOUS TIMES POST-PARTUM**

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>12 Hours Post-partum</th>
<th>36 Hours Post-partum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units CoA Degraded per mg. Dry Weight</td>
<td>Animal Number</td>
</tr>
<tr>
<td>72</td>
<td>0.35</td>
<td>77</td>
</tr>
<tr>
<td>75</td>
<td>0.83</td>
<td>79</td>
</tr>
<tr>
<td>83</td>
<td>0.92</td>
<td>88</td>
</tr>
<tr>
<td>93</td>
<td>0.59</td>
<td>101</td>
</tr>
<tr>
<td>Average</td>
<td>0.67</td>
<td></td>
</tr>
</tbody>
</table>

---

**NOTE:** See Section V:D:2 for experimental details.
these same samples with the KL system once again demonstrated the inhibition of CoA degradation by prolactin. Only one explanation of these divergent results appears possible. The SA complex of enzymes is a relatively restricted group associated with the assay of CoA and several of its precursors. The crude KL System also assays for the precursors above pantetheine as well as for CoA. However, the KL system also contains many enzymes which have not yet been identified. One or more of these must have liberated CoA from a bound complex, presumably including prolactin, so that acetylation of sulfanilamide could progress in the normal manner. That the binding of CoA to prolactin by mammary homogenates must be an enzymatic reaction may be inferred from the similarity of the similarity of the zero time assays with or without the addition of prolactin. No routine decrease in CoA content was apparent from the SA or KL assays merely because prolactin was present. It was only after the incubation that the systematic difference manifested itself.

Whether the prolactin-CoA complex contains CoA per se or one of the CoA precursors can not be definitely determined from this data. It is probable that the CoA-active portion of the complex is not pantetheine, however, inasmuch as pantetheine is not a product of mammary CoA degradation. Furthermore, prolactin does not influence the rate of
TABLE XIV

PRODUCTS OF COENZYME A DEGRADATION BY MAMMARY HOMOGENATE

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Prolactin Concentration(^*)</th>
<th>Assay Method</th>
<th>Units CoA per ml. Product Zero Time</th>
<th>Units CoA per ml. Product After Incubation</th>
<th>Change</th>
<th>Units Pantetheine per ml. Product Zero Time</th>
<th>Units Pantetheine per ml. Product After Incubation</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>118:</td>
<td>0</td>
<td>KL</td>
<td>8.3</td>
<td>2.6</td>
<td>-5.7</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10 IU</td>
<td>KL</td>
<td>8.3</td>
<td>4.6</td>
<td>-3.7</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>SA-PK</td>
<td>10.0</td>
<td>2.6</td>
<td>-6.8</td>
<td>38.0</td>
<td>13.3</td>
<td>-24.7</td>
</tr>
<tr>
<td></td>
<td>10 IU</td>
<td>SA-PK</td>
<td>10.5</td>
<td>3.5</td>
<td>-7.0</td>
<td>35.5</td>
<td>12.5</td>
<td>-23.0</td>
</tr>
<tr>
<td>120:</td>
<td>0</td>
<td>KL</td>
<td>11.7</td>
<td>4.4</td>
<td>-7.3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10 IU</td>
<td>KL</td>
<td>8.7</td>
<td>5.6</td>
<td>-3.1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>SA-PK</td>
<td>11.0</td>
<td>3.5</td>
<td>-7.5</td>
<td>30.0</td>
<td>12.0</td>
<td>-18.0</td>
</tr>
<tr>
<td></td>
<td>10 IU</td>
<td>SA-PK</td>
<td>8.0</td>
<td>3.5</td>
<td>-5.5</td>
<td>29.0</td>
<td>11.0</td>
<td>-17.0</td>
</tr>
<tr>
<td>116:</td>
<td>0</td>
<td>KL</td>
<td>7.8</td>
<td>2.1</td>
<td>-5.7</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10 IU</td>
<td>KL</td>
<td>8.7</td>
<td>5.1</td>
<td>-3.6</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>SA-PK</td>
<td>9.5</td>
<td>2.0</td>
<td>-7.5</td>
<td>24.0</td>
<td>16.0</td>
<td>-8.0</td>
</tr>
<tr>
<td></td>
<td>10 IU</td>
<td>SA-PK</td>
<td>10.7</td>
<td>1.7</td>
<td>-9.0</td>
<td>26.3</td>
<td>10.0</td>
<td>-16.3</td>
</tr>
</tbody>
</table>

\(^*\)Prolactin concentration expressed as units per ml. reaction mixture.

NOTE: See Section V:D:3 for experimental details.
disappearance of pantetheine from a system simultaneously degrading CoA (TABLE IV) nor does it alter the measurable pantetheine using the SA-PK system of assay (TABLE VIII).

Several other proposals concerning the nature of this complex can be made from the data presented and from the chemistry of the prolactin protein. The most apparent conclusion is that the complex involves the sulfhydryl end of the CoA molecule. Prolactin has been shown to react with thiol compounds to form addition compounds (110). These products are not active as prolactin until properly reconstituted as a disulfide structure (111). The stronger the binding agent the more difficult the addition product is to reactivate by chemical means, e.g. thioglycolic acid inactivates fifty times as much prolactin as cysteine. Thiol compounds have been shown to act primarily by cleaving the cystine disulfide structures which are believed to serve as linkages between the folds of the protein chain in the prolactin molecule. Coenzyme A also has been shown to form mixed disulfides in biological systems (40,41) which must be cleaved before CoA activity may be demonstrated. Therefore CoA would be anticipated to form a disulfide structure through cystine cleavage. The resultant complex probably would not be cleaved by cysteine without enzymatic intervention if the hormone-CoA mixed disulfide linkage were
semi-internal, that is between amino acid chains as has been suggested as the position of the cystine residues. From the experiments reported here it would appear that enzymatic cleavage is necessary for disrupting the prolactin-CoA complex. Were this the only point of attachment for the CoA molecule, however, then an effect of prolactin on pantetheine disappearance would also be anticipated. Such was shown not to be the case. Another site of potential reactivity would be the phosphate on carbon-3 of the ribose moiety. Such a bonding would logically be through an hydroxyl on the surface of the prolactin molecule. Three hydroxyl-containing amino acids have been reported in the prolactin structure (170): tyrosine, serine, and threonine. Apparently the tyrosine hydroxyl is so situated in the molecule that it may be more easily acetylated with ketene than the amino groups (173), contrary to the situation in other hormones. Therefore a phosphate bridge might be anticipated through this structure. However, acetylation of 20% of the tyrosine residues has no adverse effect on prolactin activity as measured by the pigeon crop test. Further acetylation results in the simultaneous acetylation of the free amino groups with the concomitant inactivation of the hormone. Reconstitution of the tyrosine hydroxyl does not reactivate the protein. Since the amino groups have been shown to be essential by other
techniques (32, 170, 174), it has been concluded that the tyrosyl hydroxyl is not necessary for prolactin action even though the entire phenolic structure of tyrosine apparently is (171). Of the other two amino acids, serine may be presumed to be internal in its position in the prolactin structure although the nature of its hydroxyl group has not been studied. Threonine, on the other hand, has been reported to be terminal in the prolactin chain (170) as determined by the phenylisocyanate and the dinitrofluorobenzene methods of analysis. This, then, would appear to be a possible site for combining the ribose phosphate group of CoA to prolactin during the formation of the proposed prolactin-CoA complex. In any case, the attachment of this end of the CoA molecule would explain the inhibition of CoA degradation inasmuch as degradation apparently involves the reversal of the CoA synthesizing scheme. The necessary first step for this is cleavage of the ribose-phosphate linkage.

The concept of a degradation-inhibiting prolactin-CoA complex is entirely in keeping with the available information on mammary metabolism. The rapid rise in glucose utilization by post-partum mammary homogenate (93, 95) indicates an increased need for CoA, particularly since this increase primarily reflects changes in anaerobic glycolysis. Approximately two-thirds of the resultant pyruvate is diverted to
synthetic reactions (277). Without a rapid post-partum increase in CoA concentration for use in synthetic reactions the increased pyruvate and acetyl-CoA would quickly flood the Kreb cycle, which is restricted by a limited oxidative-phosphorylation capacity (276). Such flooding would, in turn, back up the entire oxidative process so as to inhibit mammary syntheses. In vitro studies also support the concept of prolactin inhibition of CoA degradation. It has been observed (14,33) that prolactin stimulates the oxidation of glucose or acetate by lactating mammary homogenates. The concept of prolactin inhibition of CoA degradation explains these observations since mammary tissue quickly degrades the available CoA in phosphate-free systems. In the experiments cited, CoA concentration was dependent on the endogenous CoA level of the tissue; no further CoA was added to the medium. Thus the prolactin activity probably was not directly stimulatory but rather permitted the full expression of enzyme activity under the conditions employed.

The reasons for the decreased Kreb cycle oxidative-phosphorylations post-partum are not entirely clear. Whether there is a direct intervention of a hormone in phosphate transfer or is an indirect effect arising from decreased DH~H oxidation has not been resolved. The latter explanation coincides with a post-partum decrease in circulating estrogen. Villee (287)
110.

has shown that estrone is an activator of DPNH oxidation in uterine preparation. Also of interest in this respect is a report (123) that progesterone inhibits the oxidation of malate plus pyruvate in vitro. Thus the post-partum decrease in estrogen and increase in progesterone reported by Atkinson and Leathem (7) would appear to explain the low level of Kreb cycle activity in lactating mammary tissue. If this is true then the sex hormones and prolactin work synergistically to increase the utilization of acetate for synthetic reactions rather than excessive energy production.

The refractoriness of pregnancy mammary homogenates to prolactin (14) might be explained by a similar mechanism. The high level of estrogen in mammary tissue during pregnancy would allow the maximal development of the oxidative-phosphorylation systems necessary for full Kreb cycle activity. The Kreb enzymes do not require the CoA concentrations necessary for synthetic reactions and hence inhibition of CoA degradation would merely increase CoA concentration beyond the level needed for the maximally functioning Kreb cycle. Paraphrasing Moore and Nelson's suggestion (203), the estrogen stimulated oxidation of DPNH would maintain conditions antagonistic to synthetic reactions. This would further decrease the need for high levels of CoA.
A role for the prolactin-CoA complex as a CoA carrier may also be inferred. Several CoA-dependent reactions are dependent on a carrier intermediate. In purified fatty acid oxidase systems Green and Milii (126) have found that succinyl-CoA will serve as the necessary CoA carrier. Similarly, Stern, et al, (269) have found that succinyl-CoA or other Kreb intermediates were required for citrate synthesis by purified systems. In the absence of succinyl-CoA or other Kreb cycle carriers, prolactin might perform this function. Certainly the positive correlation between CoA and citric acid concentration in post-partum mammary tissue as well as the correlation between CoA content and prolactin injection within a given animal indirectly suggests such a possibility. On the other hand, citrate might function in this respect, as suggested by citrate stimulation of fatty acid synthesis (35, 70).

Without a doubt the results obtained in these experiments are definitely correlated with prolactin activity. Growth hormone has been variously reported as associated with CoA levels. However, Dr. Steelman, from whom the prolactin samples used in these studies were obtained, stated that the prolactin preparations were free from growth hormone contamination, as well as ACTH and other pituitary hormone activities.
The observation by Folley's group (63,90) that a part of their respiration-stimulation activity was due to contamination with Intermedin-B would also not appear to apply here since the prolactin samples employed were stated to be better than 97% electrophoretically pure. The fraction of impurity was reported to be free from pituitary hormone activities. Thus the observed complex formation must have been due to prolactin itself rather than some other pituitary hormone activity.
VII. Summary and Conclusions

Guinea pig mammary tissue has been demonstrated to have rapidly increasing citric acid and coenzyme A concentrations during the first thirty-six hours post-partum. These two compounds are closely correlated with each other as shown by the highly significant correlation coefficients determined by comparing the two values obtained from the same mammary glands. The increases in CoA and citric acid content of mammary tissue appear to parallel the pituitary prolactin content reported by other investigators.

The development of a surgical technique in which an animal served as its own control has been described. One mammary gland was removed at a specified time post-partum and the young permitted to nurse the remaining gland for twenty-four hours. During this interval hormone injections were made into the remaining gland. The animal was then sacrificed and the second mammary gland removed. Employing this technique, demonstrable increases in CoA and citric acid contents were obtained in the hormone treated gland. Lower values were obtained in tissues from similar saline-injected control animals. Shortening the period between surgery plus prolactin injection and sacrifice showed an even greater increase in CoA concentration resulting from
hormone treatment.

Fresh mammary homogenates have been shown to have low levels of CoA synthesizing activity using pantetheine as the substrate. The presence of small amounts of phosphate ion was mandatory for this demonstration. Guinea pig mammary acetone powder extracts have been found to contain high concentrations of the CoA synthesizing enzymes, approximately three to five times the concentration observed in comparable guinea pig or rat liver preparations. Extracts from both guinea pig liver and mammary acetone powder were inhibited by manganese ions and activated by magnesium ions. Prolactin had no effect on CoA synthesis by mammary acetone powder extracts. Prolactin also did not react with pantetheine to increase CoA synthesis by mammary acetone powder extracts.

The addition of fresh homogenate to mammary acetone powder extracts decreased the synthesis of CoA by the extracts. This decrease was reversed by the addition of prolactin. Using mammary powder synthesis as an index of pantetheine concentration, no destruction of pantetheine following its incubation with fresh homogenates was observed. Additional studies using a purified pigeon liver enzyme system for assay also indicated no destruction of pantetheine by fresh homogenates.

Aqueous homogenates of mammary tissue have been shown
to degrade CoA at a rapid rate at pH 8.0. The presence of fluoride ions and low concentrations of phosphate ions inhibited the degradative activity. A combination of pH 7.4 and phosphate ions completely inhibited mammary tissue degradation. Prolactin also inhibited CoA degradation in a manner analogous to the beneficial effects of prolactin upon homogenate inhibition of mammary acetone powder extract synthesis of CoA. Mammary homogenates did not degrade pantotheine when the latter was used as the sole substrate.

Although inhibition of CoA degradation by mammary homogenates was demonstrated by employing the Kaplan-Lipmann method of CoA assay, the application of a purified pigeon liver assay method did not indicate the inhibition in the same samples. The results have been interpreted as indicating the formation of a bound form of CoA which is not active in the purified system but is active in the Kaplan-Lipmann assay system. Presumably the complex contains prolactin. Formation of the complex is probably enzymatic inasmuch as the mere mixing of prolactin, CoA, and fresh homogenate did not lead to the differential results noted above.

Speculation suggests the prolactin-CoA complex to contain doubly-bound CoA. The sulfhydryl end of the CoA molecule is probably bound as a disulfide by the reduction of the cystine residues of the prolactin molecule. The ribose-
3-phosphate of the CoA may also be bound as a phosphate bridge to the terminal threonin moiety of the prolactin amino acid chain. These conclusions would explain the observed data concerning the behavior of the proposed prolactin-CoA complex towards the CoA assay systems as well as explain prolactin inhibition of the indicated method of CoA degradation in mammary tissue. It is possible that the prolactin-CoA complex also serves as a CoA carrier for synthetic reactions.

These results explain, in part, the *in vivo* and *in vitro* results of prolactin administration observed by others.
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AUTObIOGRAPHY

I, Merrill Stafford Read, was born in Baltimore, Maryland, June 3, 1928. I received my secondary school education in the public schools of Kirkwood, Missouri. My undergraduate training was obtained at Northwestern University, from which I received the degree Bachelor of Science in 1949. I received the degree Master of Science from The Ohio State University in 1951. While in residence at The Ohio State University I acted in the capacity of Graduate Assistant in the Department of Agricultural Biochemistry. From September, 1951, through June, 1952, I served as Head Assistant in the Department of Agricultural Biochemistry. From June until August, 1952, I held the Dry Milk Institute Fellowship under the direction of Dr. Fred E. Deatherage. Commencing in September, 1952, I received the appointment of Research Fellow at The Ohio State University, where I specialized in the Department of Agricultural Biochemistry under the supervision of Dr. Richard O. Moore. This appointment was retained throughout 1953. In 1954 I was inducted into the United States Army. While serving in the Army I registered for off-campus research at The United States Army Medical Nutrition Laboratory, Denver, Colorado, beginning in January, 1955. I continued this off-campus research.
under the direction of Dr. Richard O. Moore for fifteen months while completing the requirements for the degree Doctor of Philosophy.