HORMONAL REGULATION OF LIPID SYNTHESIS BY MOUSE MAMMARY GLAND IN VITRO

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ABSTRACT

HORMONAL REGULATION OF LIPID SYNTHESIS BY MOUSE MAMMARY GLAND IN VITRO

By

Joseph A. Cameron

Hormone-supplemented organ cultures of mammary gland explants from midpregnant mice show that a combination of insulin, prolactin, and corticosterone induce an active secretory appearance characterized by increased synthesis of specific milk proteins and carbohydrates. The effects of these hormones on the uptake of precursors and synthesis of milk fat were examined in this study.

Mammary explants from midpregnant mice were cultured for various times in medium 199 with each of the following hormone supplements: no hormone, insulin, insulin + prolactin, insulin + corticosterone, and insulin + prolactin + corticosterone. Labeled glucose was added to cultures at 4 hours prior to termination, and explant viability, endogenous lipid, glucose uptake, and lipid synthesis were studied as a function of time. The results show that without hormones, explants take up glucose and synthesize lipid at minimal rates. After 48 hours these activities appear to be primarily those of adipose tissues since the epithelial and connective tissue degenerate. Triglyceride synthesis is primarily the result of turnover of preformed glycerides, since glycerol accounts for 89% of ^{14}C radioactivity in triglycerides. Also, the

percentage of short chain fatty acids in triglycerides declines over time.

Insulin stimulates cellular proliferation for 24 hours and maintains survival over 96 hours. Its actions on lipogenesis and glucose transport are temporally distinguishable with an earlier effect on the former parameter. Insulin stimulates the incorporation of newly synthesized medium and some short chain length fatty acids into triglycerides.

The addition of prolactin to insulin-containing cultures has little effect on glucose uptake and lipid synthesis; however, it does produce minimal secretions in alveolar lumina, suggesting synthesis and secretion of milk products. The absence of intracellular vacuoles indicates that these products probably contain little lipid. In contrast to the action of prolactin, corticosterone intensifies the effects of insulin on lipogenesis, although it inhibits glucose transport and has little apparent effect on the morphology of explants. These observations suggest that the action of corticosterone on lipid synthesis occurs primarily in adipose tissue. Although corticosterone increases the percentage of short chain fatty acids in triglycerides, its effect is no greater than that found with insulin + prolactin.

The three hormone combination produces a highly secretory condition by 48 hours with little change thereafter. Although this combination of hormones has no effect on glucose uptake above that obtained with insulin alone, it induces maximal quantitative and qualitative changes in lipid synthesis that are temporally correlated with milk secretion.

The results of this study indicate, therefore, that lipid synthesis and secretion during induced lactogenesis *in vitro* require the same triple complement of hormones necessary for the synthesis of milk proteins and carbohydrates.

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INTRODUCTION

It is well known that hormones affect the development of the mammary gland. However, interpretations of hormonal effects on the morphology, physiology, and biochemistry of the mammary gland require specific knowledge of the hormone-tissue interaction. It is important to recognize species specificity and tissue sensitivity to hormones, as well as the time during development when the tissue becomes capable of response. For example, the normal sequence of tissue and cellular development might be altered by experimental hormonal manipulations, since in some cases the rate of development of a tissue may require greater amounts of hormones than available endogenously. In addition, the subcellular site of action, the precursor-product nature of the response, and the mechanism by which the hormone initiates the response are problems of equal importance in hormonal investigations of mammary growth and differentiation.

Milk fat has long been known to be of a dual origin, i.e. from the blood and from de novo synthesis by the mammary gland. Although the relative contributions of these two sources in the non-ruminant have not been determined, it has been reported that ruminant blood lipids represent approximately 25% of milk fat (Glascock et al., 1956, 1958). The evidence is generally conclusive that long chain fatty acids (C_{18} and above) are absorbed directly from the blood, while short and medium chain fatty acids are synthesized by the mammary gland

from small precursors, i.e. glucose and acetate (see reviews by Folley and McNaught, 1961; Jones, 1969). The glycerol moiety of milk fat is primarily derived from glucose via α-glycerophosphate and to a lesser extent from hydrolysis of plasma and mammary gland triglycerides (Dils and Clark, 1962; see also Jones, 1969). In addition, the phospholipids of milk fat are synthesized within the mammary gland and are thus independent of blood phospholipids (Garten, 1955). The latter author showed that ^{32}P-phospholipids injected into the lactating rabbit had no effect on the specific activity of milk phospholipids, whereas ^{32}P-orthophosphate produced a significant rise in radioactivity of milk phospholipids.

The de novo synthesis of milk fat has been studied extensively in ruminants both in vivo and in vitro. However, in the non-ruminant, studies have been done primarily in the rat and only recently in the rabbit and mouse. The techniques utilized include R.Q. determinations, radioactive isotopes, incubation of homogenates, soluble enzyme systems and tissue slices and, more recently, organ culture of mammary gland explants.

The classical studies of Folley and French (1948a,b; 1949a,b,c; 1950) provided early evidence that lipogenesis in the mammary gland varied with the physiological state of the animal. Using mammary tissue slices from pregnant, lactating, and weaned rats, these workers showed that the respiratory quotient (R.Q.) was above unity only in slices from lactating rats. This suggested the synthesis of oxygen-poor compounds (lipids) from oxygen-rich compounds (carbohydrates). In addition, they reported that mammary gland slices from non-ruminants

utilized glucose for fatty acid synthesis, but failed to use acetate. However, Balmain et al. (1954) showed that rat mammary gland slices formed fatty acids from glucose and acetate when both were present in the incubation media.

The role of glucose as the major lipogenic substrate prompted many investigators to examine the various metabolic pathways involved in its utilization during the lactation cycle, i.e. pregnancy, lactation, and involution. In this regard, enzymes of the Embden-Myerhof and hexose monophosphate oxidative pathways have been found to increase during pregnancy, to rise rapidly during lactation, and to decrease to low levels during involution. However, the latter pathway changes more drastically than the former (Glock and McLean, 1958; McLean, 1958; Baldwin and Milligan, 1966; Bartley et al., 1966; Karlsson and Carlsson, 1968). In addition, the first two enzymes of the hexose monophosphate pathway, i.e. glucose-6-phosphate dehydrogenase (G-6PDH) and 6-phosphogluconate dehydrogenase (6-PGDH) are particularly important as they serve as the major source of reduced nucleotides ($NADP \cdot H_2$) for fatty acid synthesis. Isocitrate dehydrogenase and malic enzyme provide additional reduced $NADP \cdot H_2$ for this purpose (Katz and Wals, 1972).

The original work of French and Popjak (1951) and Popjak et al. (1949) demonstrated that ^{14}C-glucose injected intravenously into a lactating rabbit produced radioactivity in the following products over time: glucose, pyruvate, acetate, and fatty acids. These workers provided the basic working hypothesis for lipogenesis in the mammary gland, following which subsequent investigations have almost completely elucidated the intermediate steps. In brief, the available evidence

suggests that glucose is oxidized to pyruvate via the Embden-Myerhof and pentose phosphate pathways. Pyruvate is oxidized within the mitochondria to acetyl CoA which reacts with oxaloacetate to form citrate. Citrate passes through the mitochondrial membrane and is hydrolyzed to acetyl CoA and oxaloacetate by ATP-dependent citrate ligase. This enzyme increases rapidly during lactation and declines during involution (Spencer and Lowenstein, 1966; Jones, 1967). The results of these investigations have been reviewed by Folley and McNaught (1961) and Jones (1969).

In the mammary gland of non-ruminants there are three routes for the biosynthesis of fatty acids. The mitochondrial and microsomal pathways are essentially elongation pathways and serve only to lengthen the carbon chain of preformed fatty acids. The major route for de novo fatty acid synthesis is the malonyl-coenzyme A pathway, which is cytoplasmic in nature (Dils and Popjak, 1962). Jones (1969), in his review article, describes fatty acid synthesis as a 2-step reaction:

$$\text{Acetyl CoA} + CO_2 + \text{ATP} \rightarrow \text{malonyl-CoA} + \text{ADP} + P_i, \qquad (1)$$

$$\text{n-malonyl-CoA} + \text{acetyl-CoA} + 2n\text{-NADPH}_2 \twoheadrightarrow$$

$$CH_3\text{-}CH_2\text{-}(CH_2)_{n-1}\text{-}CH_2\text{-COCoA} + n\text{-NADP} + n\text{-}CO_2 \qquad (2)$$

Reaction (1) is the rate-limiting reaction and is catalyzed by a single enzyme, acetyl CoA carboxylase, which is Mg^{++} and biotin-dependent. This reaction is effectively inhibited and stimulated by avidin (biotin inhibitor) and malonate, respectively. The second reaction describes a complex cyclic sequence of steps involving a multicomplex enzyme system, i.e. fatty acid synthetase. The steps involved have not been studied in detail in the mammary gland. However, studies on homogenate fractions

from the lactating rat (Abraham et al., 1961; Dils and Popjak, 1962) and rabbit (Smith and Dils, 1963, 1966) have shown that these tissues synthesize both short and long chain fatty acids via the malonyl-CoA pathway. This pathway involves the successive condensation of malonyl-CoA with acetyl-CoA, with the free carboxyl group of malonyl-CoA, which originates from carbon dioxide, being eliminated in the process. Recent studies have been centered around the possible mechanism for termination of growth of the carbon chain in fatty acid synthesis. Smith and Abraham (1971) reported that, in contrast to the variety of fatty acids produced by homogenates of rat mammary gland, the isolated fatty acid synthetase produced almost exclusively palmitic acid. They suggest that a separate fatty acid synthetase may be present in the mammary gland for the synthesis of the shorter chain fatty acids. In addition, it has been shown that mouse milk contains primarily medium and some shorter chain varieties (Dils and Popjak, 1962; Smith et al., 1969). These authors found that whereas the milk fat contained approximately 38% fatty acids of 18 carbons and above, the mammary gland possessed approximately 60% fatty acid of 18 carbons or above. This difference was primarily due to the decrease in stearic and oleic acids in milk.

The specific intracellular site for triglyceride synthesis has not been determined, although Stein and Stein (1967), using autoradiography and electron microscopy (EM), claim that triglyceride synthesis occurs in the rough endoplasmic reticulum of lactating mouse mammary glands. This point of view is supported by perfusion and EM studies of mammary glands of lactating rats and hamsters (Bargmann and Welsch, 1969). However, Kurosumi et al. (1968) suggested that the

smooth endoplasmic reticulum is "the site of elaboration of secretory fat droplet in the mammary gland." The fat droplets accumulate in the cytoplasm and are released from the cells surrounded by cellular membrane (Hollmann, 1969).

The endocrine control of normal growth, differentiation, and function of the mammary gland has been abundantly studied both *in vivo* and *in vitro*. From the *in vivo* standpoint, the classical experiments of Lyons *et al*. (1958) and Nandi (1969) show that normal mammary development and function can be produced in hypophysectomized-ovariectomized-adrenalectomized virgin rats and mice, respectively, by sequential injections of ovarian (estrogen plus progesterone), pituitary (prolactin plus somatotrophin), and adrenal corticosteroid hormones.

The above *in vivo* technique of gland removal, followed by hormone replacement therapy, is particularly useful for determination of hormonal requirements for growth and development; however, it is inadequate for the distinction of direct vs. indirect effects. For this reason many investigators have isolated the tissue in buffered solutions of various types. One of the frequently utilized *in vitro* techniques is that of organ culture which allows maintenance of tissue in chemically defined media for relatively longer periods of time than possible by slice methods.

In a series of papers Elias, Rivera, and coworkers (Elias, 1957, 1959; Elias and Rivera, 1959; Rivera and Bern, 1961; Rivera, 1964) provided *in vitro* verification of the direct effects of ovarian, pituitary and adrenal hormones on normal development of the mammary gland. In addition, these workers found that insulin, either individually

or in combination with other hormones, was required for maintenance of viable mammary gland explants (Elias, 1959; Rivera and Bern, 1961).

Explants from mid- and late pregnant mice required prolactin and/or somatotrophin in addition to insulin and an adrenal corticosteroid to induce secretion in the former and to maintain it in the latter (Bern and Rivera, 1960; Rivera and Bern, 1961; Rivera, 1964). In general, these workers provided the basic histological evidence that insulin, prolactin and a glucocorticoid represent the minimum hormonal complement required to induce and maintain secretion in explants of mid- and late pregnant mice.

The question of hormone and species specificity was examined by Rivera (1964a,b; 1966). She found that mammary tissues of C3H mice responded equally well to cortisol, corticosterone, or aldosterone in combination with insulin, prolactin and growth hormone. Similar results were reported by Turkington et al. (1967b), who compared the effects of 16 steroid compounds on histological changes in casein synthesis. He noted that prednisolone and 21-deoxycortisol fell in the same group of most effective compounds. In addition, Rivera (1964b) found that mammary tissues of C3H mice were 4 times more sensitive to prolactin than tissues of strain A mice.

Several studies have attempted to determine the earliest time of development when the mammary tissue becomes competent to respond to hormones in vitro. Ichinose and Nandi (1964, 1966) noted that mammary glands of 3 to 4 week old BALB/c strain mice given estrogen and progesterone in vivo were later responsive by full lobulo-alveolar development to a combination of insulin, estrogen, progesterone, aldosterone,

prolactin and growth hormone *in vitro*. However, glands not primed with ovarian hormones were only maintained *in vitro*, and glands cultured without priming and without hormones *in vitro* showed extensive degeneration. Prop (1966) reported that mammary tissues from 7 week old CBA mice responded better to hormones in culture than those 5 weeks old. Rivera (1964a) observed that the actively growing terminal ducts and end buds of mammary tissue from young mice were highly sensitive to the lack of insulin. These studies suggest that differentiation in the mammary gland requires sufficient prior exposure to growth-promoting hormones.

The influence of hormones on biochemical parameters, such as nucleic acid and protein synthesis and enzyme activities during mammary gland differentiation *in vitro*, has been reviewed by Denamur (1971) and Forsyth (1971). Stockdale and Topper (1966) reported that insulin alone induced maximum DNA synthesis from ^{3}H-thymidine by 24 hours in mammary explants of midpregnant mice. Similar results were found by Lockwood *et al*. (1967) and Turkington (1968). Mills and Topper (1970) claimed that insulin induced a doubling of the average number of cells per alveolar cross section. Insulin had similar effects on adult virgin (Stockdale and Topper, 1966) and immature 3-week old C3H mice (Voytovich and Topper, 1967; see also Forsyth, 1971; Denamur, 1969, 1971). El-Darwish and Rivera (1970) demonstrated that insulin induced DNA synthesis by one day of culture. However, insulin + prolactin and insulin + prolactin + corticosterone was required for maintenance of this effect over two and five days, respectively. Likewise, Mayne and

Barry (1970) reported that insulin + prolactin + corticosterone inhibited the decline in DNA synthesis which occurred with insulin alone.

Since RNA synthesis is indicative of protein synthesis and subsequent function of secretory tissues, several workers have examined hormone-induced changes in RNA. Mayne et al. (1966) found that insulin increased the incorporation of ^{14}C-adenine into RNA by 60% over controls in the first 3 hours of culture. This effect was maintained over 12 hours (Mayne and Barry, 1967) without significant changes in total tissue RNA, which suggested rapid turnover of RNA. Stockdale et al. (1966) reported that the rate of RNA synthesis doubled by 24 hours in insulin-containing cultures. After 48 hours the rate increased maximally with insulin, prolactin and hydrocortisone, while insulin and insulin + hydrocortisone cultures showed no change in rate. Green and Topper (1970) compared RNA synthesis in mammary fat pads free of epithelium and those containing epithelium. They reported that prolactin, added in combination with insulin and cortisol stimulated RNA synthesis by epithelial cells but not fat cells after 4 days of culture.

The function of the mammary gland involves the synthesis and secretion of milk. Some of the components of milk are absorbed directly from the blood while others (caseins, whey proteins, lactose, and shorter chain fatty acids and glycerol of glycerides as well as phospholipids of milk fat) are synthesized within the mammary gland. Organ culture has been utilized to study the hormonal control of these parameters. In this regard, Juergens et al. (1965) reported that a combination of insulin, prolactin and hydrocortisone induced the synthesis of a "casein-like" phosphoprotein by midpregnant C3H mouse mammary gland

explants. Other hormone combinations were ineffective. The maximum synthesis of casein occurred after 48 hours of culture. This observation provided biochemical confirmation of the morphological responses observed by Elias and Rivera (1959), Rivera and Bern (1961), Rivera (1964), and Stockdale et al. (1966). Electrophoretic analysis of the "casein-like" phosphoprotein showed 4 major components with the same mobility pattern as that found for authentic mouse milk casein (Turkington et al., 1965). Further support of this observation was provided by Topper (1968), who showed that the maximal rate of induced casein synthesis, which occurred by 48 hours, was approximately 50% of that seen in tissues from 10 day post-partum mice.

Lockwood et al. (1966) and Turkington et al. (1967) reported that the whey proteins (α-lactalbumin and β-lactoglobulin) responded in an identical manner to the same complement of hormones, while increased synthesis of non-milk proteins required only insulin (Lockwood et al., 1966). Analysis of mouse milk showed that the ratio of casein to whey proteins was similar to that found in hormone-induced secretion.

The hormonal control of lactose synthesis, which is mediated by lactose synthetase, an enzyme composed of a nonspecific galactosyltransferase (A protein) and a specifier protein (B protein, α-lactalbumin) (Brodbeck and Ebner, 1966), has also been studied in organ culture of mammary gland explants. In this regard, Turkington et al. (1968) reported that the same 3 hormone combination required for casein synthesis, i.e. insulin + prolactin + hydrocortisone, induced maximum activity of lactose synthetase. They noted also that, in contrast to the asychronous development of the A and B proteins in vivo, the 3 hormones

induced maximum synthesis of these proteins synchronously by 48 hours of culture. Similar requirements for the 3 hormone combination for increased activity of lactose synthetase were found by Palmiter (1969). In addition, Delouis and Denamur (1971) observed that insulin and prolactin induced synthesis of lactose from ^{14}C-glucose in mammary explants from pseudopregnant rabbits. Although corticosterone was not required for increased lactose synthesis, it enhanced the effects of insulin and prolactin (see also Forsyth, 1971).

The first _in vitro_ evidence of hormonal control of lipid biosynthesis in the non-ruminant mammary gland was shown by Balmain and co-workers (Balmain _et al._, 1952, 1954; Balmain and Folley, 1951, 1952). These workers incubated mammary gland slices of pregnant, lactating and weaned rats in a buffered solution containing insulin and ^{14}C-glucose plus ^{14}C-acetate. They determined the respiratory quotient (R.Q.) and measured the ^{14}C-fatty acids produced and found that insulin was highly effective in stimulating lipogenesis in slices from lactating rats, but was totally ineffective in slices from pregnant and weaned rats. Abraham _et al._ (1957) and Abraham and Chaikoff (1957), using differentially labeled glucose, showed the same distinction with respect to increased oxidation of glucose via the hexosemonophosphate pathway. In addition to their studies on the rat, Balmain and Folley (1951) reported that insulin increased the respiration of slices from lactating rabbits and mice, but to a lesser extent in the latter species. No analogous studies were done on pregnant and weaned animals.

With regard to prolactin and corticosteroids, Balmain and Folley (1952) showed that cortisone enhanced respiration considerably in mammary slices from late pregnant rats, whereas prolactin was without effect. The results were exactly the reverse in slices of early lactating rats. These results were interpreted to indicate the necessity of corticosteroids for the induction of milk secretion and of prolactin for its maintenance. In contrast, McLean (1960) found that prolactin markedly stimulated ^{14}C-glucose oxidation and incorporation into lipids of mammary slices from pregnant rats, but had no consistent effect on slices from lactating rats. Although Balmain and Folley (1951) observed no additive effects of these hormones, Abraham et al. (1960) demonstrated that both prolactin and hydrocortisone acetate were required to increase lipogenic parameters in mammary slices of rats hypophysectomized at midpregnancy and injected post-partum with various hormone combinations.

Analysis of glucose oxidation and fatty acid synthesis in mammary slices of the adrenalectomized lactating rat has shown that both the Embden-Myerhof and pentose shunt oxidative pathways are decreased, while the citric acid cycle and fatty acid synthesis from ^{14}C-acetate are unaffected (Willmer, 1960; Greenbaum and Darby, 1964). These results led Greenbaum and Darby (1964) to suggest that adrenalectomy results in a metabolic block at the level of the decarboxylation of pyruvate, thereby preventing formation of acetyl CoA and subsequent fatty acid synthesis.

The above studies in the rat suggest that insulin and prolactin may have similar effects on lipogenesis. As stated by McLean

(1960), "the parallel increase in the oxidation of glucose and synthesis of lipid is apparent in both cases." However, she also suggested different mechanisms of action, since prolactin acted during pregnancy while insulin was effective only during lactation. All of the aforementioned studies involved slices of mammary tissues and, as stated by Jones (1969), "the results have not been easy to interpret and have sometimes been contradictory." The development of the organ culture method (Elias, 1957; Elias and Rivera, 1959; Rivera, 1964) has provided investigators with a hormonally-responsive system in which long term biochemical effects can be observed in vitro. In this regard, the organ culture system has recently been used to study lipogenic parameters in the rabbit and mouse.

Organ cultures of mid-pregnant and pseudopregnant rabbits show that prolactin alone can stimulate an increased rate of synthesis as well as a shift in the pattern of fatty acid produced, i.e. from long chain (C_{18}) to shorter chain (C_8) and (C_{10}) fatty acids (Bolton and Bolton, 1970; Dils et al., 1971; Forsyth et al., 1972; Strong et al., 1972). This shift in the pattern of fatty acids was similar to that seen during normal differentiation of the mammary gland and to that found in rabbit milk. The prolactin-induced effects were increased by 40-fold in cultures containing insulin, prolactin and corticosterone.

Since glucose is the major lipogenic substrate in non-ruminant mammary glands and since most of the enzymes involved in its metabolism show significant changes during normal and induced mammary differentiation, some recent studies have attempted to evaluate glucose uptake and to separate the enzymatic reactions responsive to metabolic hormones

from those responsive to differentiative hormones. Moretti and DeOme (1962) and Moretti and Abraham (1966) demonstrated that after the first day, insulin stimulated glucose uptake over 3 to 4 days of culture in organ culture of midpregnant mouse mammary explants. Mayne and Barry (1970) reported that prolactin and corticosterone added to insulin cultures had no additional effect.

With regard to the enzymatic reactions involved in glucose oxidation, Rivera (1972) reported that in freshly-dissected explants of midpregnant mice, the enzymes glucose-6-phosphate dehydrogenase (G-6-PDH), 6-phosphogluconate dehydrogenase (6-PGDH), phosphoglucose isomerase (PGI), and lactate dehydrogenase (LDH) show low activity with respect to post-partum increases. Insulin increased the activity of G-6-PDH and 6-PGDH by 1 day of culture (Leader and Barry, 1969; Rivera and Cummins, 1971a,b), while insulin plus prolactin produced maximum responses within two days (Leader and Barry, 1969; Rivera and Cummins, 1971a,c). Maintenance of the maximum response over 3 days of culture required insulin, prolactin and corticosterone (Rivera and Cummins, 1971a,c).

In contrast to the pentose shunt enzymes, the glycolytic enzymes phosphoglucose isomerase (PGI) and lactate dehydrogenase (LDH), responded maximally to insulin alone, while prolactin and corticosterone had no additional effect (Rivera and Cummins, 1971a,b,c; Rivera, 1972). These results led to the suggestion (Rivera, 1972) that increases in the enzymes of the pentose phosphate pathway represent prolactin-induced differentiative responses, whereas insulin-induced increases in glycolytic enzymes are secondary metabolic responses. Inhibitor

studies (Leader and Barry, 1969; Rivera and Cummins, 1971b) have shown that hormone-induced increases in G-6PDH and 6-PGDH are independent of DNA synthesis, but require RNA and protein synthesis.

The utilization of glucose for fatty acid synthesis has also been studied in the mouse mammary explant system. Moretti and Abraham (1966) found that insulin stimulated fatty acid synthesis from ^{14}C-glucose in explants from pregnant and lactating mice, but to a much greater extent in the latter. Mayne and Barry (1970) reported that prolactin and corticosterone had no additional effect above that of insulin. However, Wang et al. (1972), using ^{14}C-acetate, found that prolactin and corticosterone produced synergistic effects with insulin, both individually and in combination. The latter authors also reported that the combination of insulin, prolactin and corticosterone produced a fatty acid pattern resembling that of mouse milk fat, while the pattern produced by other hormone combinations resembled that found in adipose tissue.

The relative lack of information on the hormonal regulation of lipogenesis in the mouse mammary organ culture system, as compared to other biochemical parameters (e.g. protein and lactose synthesis), suggests that further study would be valuable with regard to other interpretations of hormone-induced lactogenesis. The overall objective of this investigation was to provide further insight into the role of differentiative and metabolic hormones in lactational studies in vitro. More specifically, questions with regard to temporal and synergistic actions of insulin, prolactin and corticosterone on glucose uptake and triglyceride and total lipid synthesis are considered. In addition,

the effects of these hormones on the fatty acid patterns of the triglycerides are examined.

MATERIALS AND METHODS

Tissues

Adult nulliparous Swiss albino mice in their 13th day of pregnancy were killed by cervical dislocation. Explants were aseptically removed with fine iridectomy scissors and placed in small amounts of sterile hormone-free media. The small explants ranged in size from 0.4 to 0.8 mg.

Culture Methods, Hormones, and Isotope Studies

The culture methods have been previously described by Rivera (1971). Medium 199 (BBL) containing Penicillin G (50 i.u./ml) was used as the basal culture medium. Bovine insulin (Calbiochemical, activity 26 USP units/mg) and ovine prolactin (NIH-P-S-8) were dissolved in small amounts of 0.005 N HCL and 0.0001 N NaOH, respectively. Medium 199 was added in sufficient volume to obtain stock solutions of 100 µg/ml, which were then filtered through millipore filters with an average porosity of 0.45 µ. Corticosterone (Upjohn, Lot number 62931) was dissolved in absolute ethanol to obtain a stock concentration of 100 µg/ml. Aliquots of the stock hormone solutions were diluted with medium 199 to a final concentration of 5 µg/ml (insulin and prolactin), and 1 µg/ml (corticosterone).

The following 5 treatments were used in each experiment: no hormones, insulin, insulin + prolactin, insulin + corticosterone, and

insulin + prolactin + corticosterone. Five large Falcon petri dishes were set up, each containing 3 circles of Whatman filter paper saturated with 5 ml of sterile distilled water. Three halves of smaller petri-dishes (35 x 10 mm) were placed in each large petri dish, and 3 ml of medium were pipetted into each of the former. Seventy-five explants from each of 2 mice were randomly and equally distributed among the 5 large petri-dishes giving a total of 15 explants from each animal in each treatment. The large petri dishes were covered, placed in a plastic box, which was closed tightly, and incubated at 37°C with a 95% O_2-5% CO_2 gas flow. The pH of the medium was maintained at 7.4 by adjusting the rate of gas flow.

Cultures were terminated at 4, 24, 48, 72, and 96 hours. The medium was changed at 48 hours in the 72 and 96 hour experiments. Experiments were repeated four times with the exception of the 4 hour cultures, which were done only twice.

^{14}C-glucose, 0.25 μc/ml (Amersham/Searle, specific activity 230 mc/mM) was added to cultures 4 hours prior to termination. For specific activity determinations during longer culture times, it was necessary to know the amount of unlabeled glucose present in the medium at the time of pulse labeling. This was determined by measuring the amount of glucose remaining in the medium at termination, assuming linearity of uptake, and computing the hourly rate. Corrections for the amount of unlabeled glucose loss from the medium during the 4 hour pulse labeling period which involved the assumption of linear uptake were minor and specific activity corrections had no significant effects

on treatment differences. At termination, explants were rinsed, blotted on filter paper and weighed.

Lipid Extraction

Tissue

Lipids were extracted according to the method of Folch, Lees, and Sloane-Stanley (1957). Weighed tissue explants were homogenized in 20 volumes of chloroform-methanol (2:1, v/v) in a Potter-Elvehjem glass homogenizer at 2-4°C. The homogenate was mixed for 15 minutes and filtered through tarred Whatman #1 filter paper into an appropriate-sized separatory funnel. An additional 21 ml of rinse solvent was filtered through the precipitate. Twice distilled water (0.2 percent of total solvent volume) was added to the filtrate, which was shaken vigorously and allowed to sit in the refrigerator at 4°C overnight.

Phase separation was determined by the appearance of a distinct phase boundary when the mixture was allowed to return to room temperature. The lower chloroform phase and upper phase wash were collected in a round bottom Erlemeyer flask and evaporated to dryness at approximately 40°C with a Buchi rotary evaporator. Lipid extracts were quantitatively transferred to preweighed 5 dram vials, taken to dryness under a fine stream of nitrogen at 60°C (N-evap, Organomation Association) and weighed on a Mettler balance. Vials were weighed upside down as a precautionary measure to inhibit autoxidation. Dried lipid samples were dissolved in 0.5 ml of chloroform and stored under nitrogen at -20°C. Teflon cap liners were inserted in each vial cap to prevent evaporation.

Media

All media samples were extracted in the same manner as the tissue but without homogenization and with a 20 ml volume of chloroform-methanol (2:1, v/v). All methanol-water upper phases were saved for analysis of glucose concentration and specific activity.

Qualitative Analyses

Thin Layer Chromatography (TLC)

Lipids were separated into various components using silica gel thin layer chromatography. Thin (0.25 mm) precoated silica gel G plates (Analtech, Inc.) were activated in an oven at 110°C. for 1-2 hours and stored in an air tight dessicator until used.

To insure optimum recovery of ^{14}C-lipids, all TLC plates were marked off in equal columns. This procedure was not necessary for preliminary experiments involving unlabeled samples. The chloroform lipid samples were applied in triplicates with Hamilton microliter syringes in 10 µl aliquots. Known standards of neutral lipid mixtures (Applied Science, Inc.) were cochromatographed as controls.

Several developing solvents with varying concentrations were used in preliminary experiments. Of these solvents, petroleum ether: diethyl ether:acetic acid (90:10:1, v/v/v) (Randerath, 1966) and (80:20:2, v/v/v), (Stahl, 1965) were used to separate lipid classes, as well as neutral lipid components. Triglycerides were (1) scraped directly into scintillation vials and (2) scraped, eluted with chloroform-methanol (2:1, v/v), and converted to fatty acid methyl esters (FAME) for gas chromatographic analysis (GC). At least eight

triglyceride spots of samples were scraped for gas chromatographic analyses. In addition, 10 μl aliquots of total lipid samples were (1) placed directly into scintillation vials and (2) spotted on silica gel and scraped into scintillation vials.

Lipids were detected primarily by exposure to iodine vapors. However, in some initial cases, sulfuric acid charring (conc. H_2SO_4:70% ethanol, 1:1, v/v), ultraviolet detection, phosphomolybdic acid, rhodamine B and other detection agents were used. The efficacy of these procedures for rapid detection and subsequent analyses were deemed less than that of iodine detection.

Since iodine inhibits the accuracy of subsequent GLC analysis (Nichaman et al., 1963), plates were covered with clean glass plates, cut specifically to leave only outer columns uncovered. This prevents absorption of iodine by spots to be scraped, while allowing localization of spots.

Preparation of Fatty Acid Methyl Esters

Triglyceride samples were scraped from TLC plates, eluted with chloroform:methanol (2:1, v/v) through fritted disc filters into round bottom flasks, and evaporated to dryness with a Buchi rotary evaporator. Samples were redissolved in chloroform:methanol (2:1, v/v) and quantitatively transferred to 15 ml screw cap culture tubes. Teflon inserts were used in all caps to prevent leakage. Samples were taken to dryness under nitrogen and 1 ml of methanol: 1 N HCl was added. The methanolic HCl samples were then heated in an oven at 75°C for 18 to 24 hours. To insure a tight seal, a sealing tape was used in addition to

the teflon liners. All tubes were checked for leakage after 15 minutes in the oven.

After allowing time for tubes to return to room temperature, a few drops of distilled water and 3 ml of hexane (b.p. 4°C) were added and mixed well. After layer formation, the upper hexane layer was removed with a Pasteur pipette and filtered through anhydrous sodium sulfate. The extraction procedure was repeated 3 times, and the combined extracts were concentrated to dryness under a stream of nitrogen. The dried fatty acid methyl ester (FAME) extracts were dissolved in 0.5 ml of hexane and aliquots were analyzed by gas-liquid chromatography (GLC).

Gas-Liquid Chromatographic Analysis

All gas-liquid chromatographic analyses were performed using the Hewlett Packard Model F and M 402 apparatus equipped with a hydrogen flame ionization detector. Chromatography of fatty acid methyl esters (FAME) was done on glass columns with dimensions 1.8 m x 2 mm (length and internal diameter) packed with chromosorb W (80-100 mesh) coated with 3% S.E. 30, or 5% diethylline glycol succinate (DEGS). Nitrogen was used as the carrier gas at 30 psi., or at an approximate flow rate of 30 to 40 cc per minute. Chromatography was carried out both isothermally (150-180°C oven temperature) and with programmed temperature increases of 5°C per minute from an initial 100°C. The injector (inlet) and detector temperatures were routinely kept at 30-50°C above oven temperature.

Fatty acids were identified by comparing retention time versus chain length of known fatty acid methyl esters (Applied Science) with retention time of FAME of samples. Further substantiation of fatty acid identification was provided through mass spectral analysis. This analysis was performed by Dr. Ray Hammond in the laboratory of Dr. Charles Sweeley, Michigan State University. Radioactive fatty acid methyl esters were trapped as they emerged from the column, placed in scintillation fluid and counted. In initial experiments, peak areas were obtained by multiplying peak height times width at half height, i.e. A = H x W (at 1/2 H).

Liquid Scintillation Analysis

Radioactivity was determined in all samples using a Nuclear Chicago Model 6850 liquid scintillation counter. Ten µl aliquots of samples were placed in scintillation vials containing 10 ml of scintillation fluid (PPO, 2,5-Diphenyloxazole, 4 gm/l; POPOP, p-bis 2-(5-Phenyloxazolyl)-Benzene, 0.050 gm/l, and sufficient toluene to obtain a liter solution). All samples were counted in triplicates.

The amount of glucose converted to lipid was computed by dividing the specific activity of various lipid products by the specific activity of labeled precursor, i.e.

$$\frac{\text{specific activity of lipid (cpm/mg)}}{\text{specific activity of glucose (cpm/µg)}} = \frac{\text{µg glucose}}{\text{mg lipid}}$$

In addition, the counts in lipid were expressed on a fat-free tissue and wet weight tissue basis. The amount of glucose converted into lipid on the basis of these two parameters was then calculated.

Glucose Uptake

The uptake of glucose by explants was computed on the basis of the amount of glucose depleted from the medium at termination of culture. Medium glucose was determined by the glucose oxidase method (Glucostat) as described by Worthington Biochemical Co., Freehold, N.J. Aliquots of the upper phase of Folch extracts of glucose-free media (specially prepared by BBL), regular media (contains 1 mg/ml glucose), and cultured media served as blank, standard, and sample, respectively, in spectrophotometric determinations. All readings were made at 415 mμ in a Coleman spectrophotometer.

Histology and Photographs

Representative samples of explants from each treatment and time period were examined histologically to determine viability and secretory development. The explants were fixed in Bouin's solution, serially sectioned at 7 μ, and stained with hematoxyin and eosin. Photomicrographs were made with a Pentax Spotmatic camera mounted on a Bausch and Lomb microscope.

Statistical Analysis

The results of the studies on glucose uptake (cumulative and 4 hour rates), incorporation into total lipid and triglyceride fractions (wet weight and fat-free tissue basis), and the percentage utilization of glucose for lipid synthesis, were tested with a split-plot analysis of variance to determine whether total differences within each parameter varied significantly according to time, treatments and/or

time by treatment interactions. The mean values of all parameters were then subjected to multivariate analysis of variance to determine whether there were significant differences between each hormone combination and time. The 4 hour responses were not included in the statistical analysis because of insufficient samples and thus early differences in time and/or in response to hormones were not statistically verified.

In addition, the data on the distribution of radioactivity in lipid fractions (with the exception of triglycerides), and triglyceride fatty acids, were taken from pooled samples of 4 experiments per time period and, therefore, were not statistically analyzed.

RESULTS

Effects of Hormones on Mammary Lipid Content *In Vitro*

The influence of hormones on total lipid content is summarized in Table I. In the absence of hormones the lipid content of mammary explants increased slightly from 49% to 54% of the total wet weight during 4 days of culture. However, in insulin-containing cultures, total lipid declined as much as 10%. The addition of prolactin and/or corticosterone to insulin-containing cultures had no additional effect on lipid content.

Effects of Hormones on Glucose Uptake by Mammary Explants *In Vitro*

The cumulative effects of hormones over 96 hours on total glucose uptake were compared with their effects during the last 4 hours of each culture interval studied (Tables II-V; Figures 1 and 2). The results are expressed as μg glucose/mg wet weight of tissue.

The total amount of glucose taken up per mg of tissue was significantly different ($P<0.05$) over time, treatments, and time by treatment interactions (Table II). However, it is important to note that since the medium was changed at 48 hours, cumulative uptake thereafter was determined by adding that measured at 72 and 96 hours to 48 hour values (Table III). In addition, the statistical analysis of time influences did not include initial 4 hour results. Therefore,

differences in cumulative glucose uptake, compared to 24 hour values, were not significant until later in time. The amount measured at termination of cultures is presented in Figure 1.

Although the results of the 4 hour experiments were not statistically analyzed, the data clearly indicate that hormones do not stimulate glucose uptake during this period. In fact, the uptake of glucose in hormone-containing cultures was less than that found in no hormone controls. Further analysis of the 24 to 96 hour data showed that without hormones, cumulative glucose uptake increased significantly ($P<0.05$) by 72 hours of culture (Table III). In insulin-containing cultures the responses at 24 hours (71 μg/mg) and at 48 hours (220 μg/mg) were significantly different ($P<0.05$). The addition of prolactin or corticosterone did not change the pattern produced by insulin alone. However, with the combination of 3 hormones, the significance of difference from 24 hour values was not obvious until 72 hours ($P<0.05$).

In comparison with glucose uptake by cultures without hormones, insulin-containing cultures increased total uptake ($P<0.05$) at 72 hours of culture by 45%. The addition of prolactin or prolactin + corticosterone did not significantly alter the response to insulin alone ($P>0.05$). However, corticosterone in combination with insulin delayed the increase above that produced in the absence of hormones until 96 hours (Table III).

Analysis of the 4 hour rate of glucose uptake showed that in the absence of hormones, with the exception of the initial 4 hour rate, the rate at 24 hours (11 μg/mg) remained fairly constant over time ($P>0.05$) (Table V; Figure 2). Table V shows that insulin increased

the 24 hour rate (11 μg/mg) by 2-fold at 72 hours (22 μg/mg) ($P<0.05$), whereas prolactin and/or corticosterone in combination with insulin had no effect ($P>0.05$).

Effects of Hormones on Rates of Lipid Synthesis by Mammary Explants *In Vitro*

Total Lipid Synthesis

The effects of hormones on ^{14}C-glucose incorporation into total mammary lipids are presented in Tables VI, VIII-XI, and Figures 3 and 4. In Table VI the results are expressed as specific radioactivity of wet tissue weight (cpm/mg). However, since interpretations of these data are dependent upon the changing specific activity of media glucose, i.e. cpm/μg (Table VII), the results are expressed as amounts of glucose converted to lipid on a wet weight (Tables VIII-IX; Figure 3) and a fat-free tissue basis (Tables X-XI; Figure 4). The pattern of response did not vary according to the method by which data were expressed; however, significant differences in hormone effects were not apparent until later in time when results were expressed on a fat-free tissue basis.

Tables VIII and X indicate that the total differences in the rate of conversion of ^{14}C-glucose to total lipid, whether expressed on a wet weight or fat-free tissue basis, varied significantly according to time, treatments and time by treatment interactions ($P<0.01$). This fact indicates that the rate of lipid synthesis increased and declined between 24 to 96 hours of culture.

In hormone-free cultures, the initial 4 hour rate of ^{14}C-glucose incorporation into total lipid expressed on a wet weight basis

was 0.28 μg/mg tissue. This rate was essentially maintained over time ($P>0.05$). Table IX indicates that in all insulin-containing cultures, i.e. insulin, insulin + prolactin, insulin + prolactin + corticosterone, and insulin + corticosterone, the rate of conversion of glucose to total lipid, expressed on a wet weight basis, was increased by 24 hours ($P<0.01$) above that produced in the absence of hormones. This effect was maintained over 96 hours in all hormone-containing cultures except those containing insulin alone. In these cultures, after 72 hours, the rate of lipid synthesis declined to minimal values not significantly different from that produced by hormone-free cultures ($P>0.05$). The 3 hormone combination significantly increased the response ($P<0.01$) above all other hormone combinations as early as 24 hours and maintained it over 96 hours of culture ($P<0.05$). The maximal stimulation of lipid synthesis (1.6 μg/mg) was induced by the 3 hormone combination at 48 hours of culture ($P<0.01$). Table IX also shows that prolactin had no effect above that of insulin alone ($P>0.05$). However, insulin + corticosterone increased the response above insulin and insulin + prolactin-containing cultures by 50% at 48 hours ($P<0.01$) and maintained the effect over 72 hours ($P<0.05$).

The effects of time on the rate of total lipid synthesis (Tables IX and XI) in hormone-free and insulin + prolactin-containing cultures were not significantly different ($P>0.05$). However, the greater responses of insulin + prolactin-containing cultures above that of no hormone cultures ($P<0.01$) indicates that the influence of time occurred earlier than 24 hours in insulin + prolactin-containing cultures. In contrast, the effects of time on insulin + prolactin-

containing cultures were significantly different from its effect on all other insulin-containing cultures.

Table XI shows that expressing the incorporation of ^{14}C-glucose into total lipid on a fat-free tissue basis altered the significance of treatment difference with the result that the effect of insulin-containing cultures, with the exception of the 3 hormone combination, was not apparent until 48 hours ($P<0.01$).

Triglyceride Synthesis

The effects of hormones on ^{14}C-glucose incorporation into triglycerides are presented in Tables XII-XIII and Figure 5. The results are expressed as μg glucose/mg fat-free tissue. The percentage of ^{14}C-glucose radioactivity in the triglyceride fraction of total lipid was multiplied by the amount of glucose converted to total lipid to obtain the amount of glucose converted to triglyceride per unit weight of fat-free tissue. The amount of glucose converted to triglyceride was significantly different over time ($P<0.05$), treatments, and time by treatment interactions ($P<0.01$) (Table XII). Table XIII and Figure 5 show that triglyceride synthesis followed the same general pattern of treatment and temporal response as seen with total lipid.

In this regard, the rate of triglyceride synthesis at 4 hours (.3 μg/mg), without hormones, did not change significantly over time. Insulin doubled the effect at 4 hours, maintained the difference at 48 hours ($P<0.05$), and declined thereafter ($P>0.05$). The addition of prolactin to insulin-containing cultures did not alter the response produced by insulin alone ($P>0.05$), whereas corticosterone enhanced the

response by 48 hours ($P<0.05$) with subsequent decline ($P>0.05$). The maximal increase (1.7 μg/mg) was produced by the 3 hormone combination at 48 hours ($P<0.01$) and was maintained over 72 hours. These results indicate that insulin stimulates triglyceride synthesis, while prolactin has no effect. In addition, corticosterone increases triglyceride synthesis, but the maximal response requires the 3 hormone combination.

Effects of Hormones on Glucose Utilization for Lipid Synthesis

The effects of hormones on glucose utilization for lipid synthesis are summarized in Tables XIV and XV, and Figure 6. The results are expressed as the percentage of ^{14}C-glucose taken up by tissues which was incorporated into lipids. Analysis of the data showed that glucose utilization for lipid synthesis varied significantly ($P<0.01$) over time, treatments, and time by treatment interactions (Table XIV). In the absence of hormones, the percent of glucose converted to lipid at 4 hours of culture was more than doubled by 24 hours (from 1 to 2.8%), but was not significantly different ($P>0.05$) from 24 to 96 hours (Table XV; Figure 6). Insulin alone or in combination with prolactin and/or corticosterone enhanced glucose utilization for lipid synthesis as early as 4 hours of culture. The greatest effect was produced in insulin and insulin + prolactin-containing cultures (2.8% and 3.2%, respectively), while the 3 hormone combination and insulin + corticosterone were less effective (approximately 2.1% and 1.6%). Cultures containing insulin and insulin + prolactin utilized 4% to 5% of the glucose for lipid synthesis at 24 hours (Table XV). In insulin-containing cultures the effect was maintained for 48 hours but declined

thereafter ($P<0.05$), whereas prolactin inhibited the decline ($P<0.05$). At 24 hours, cultures containing corticosterone, i.e. insulin + corticosterone, and insulin + prolactin + corticosterone, utilized a slightly greater percentage of glucose for lipid synthesis than all other cultures. Insulin + corticosterone increased slightly the effect at 48 hours over 24 hours, but declined thereafter. The 3 hormone combination, however, increased the response maximally at 48 hours (9.4%) ($P<0.05$), and maintained it over 72 hours ($P<0.05$) before allowing the decline at 96 hours ($P<0.05$). These results are in accord with the absolute amount of glucose incorporated into lipid. These observations further indicate that the 3 hormones in combination are required to maximally stimulate the utilization of glucose for lipid synthesis, and that this action requires 48 hours *in vitro*. Furthermore, prolactin has no effect greater than insulin along during the first 48 hours, but it inhibits the decline produced by insulin in longer cultures. Corticosterone intensifies the response to insulin between 24 and 48 hours, but is ineffective thereafter.

Effects of Hormones of the Distribution of Radioactivity in Mammary Lipids

Total Lipid Fractions

The effects of hormones on the incorporation of ^{14}C-glucose into lipid fractions are presented in Table XVI. The results are expressed as the percentage distribution of radioactivity in various lipid fractions. In the absence of hormones the percent of radioactivity in the triglyceride fraction increased with time until 48 hours and declined thereafter. In hormone-containing cultures the data

suggest that only the 3 hormone combination increased the effect above that observed without hormones. However, since the amount of glucose converted to triglycerides was much greater in cultures containing hormones than in those without, it follows that triglyceride synthesis was increased by all hormone-containing cultures above that observed without hormones. The maximal response (66%) was produced by the 3 hormone combination at 72 hours of culture.

In hormone-free cultures, approximately 90% of the ^{14}C-glucose converted to triglyceride was localized in the glycerol fraction. In all insulin-containing cultures the percent of glucose in triglyceride fatty acids increased until 48 or 72 hours and declined thereafter. The maximal response (43%) was produced with the 3 hormone combination at 48 hours of culture.

The percent of radioactivity in the phospholipid fraction of total lipid varied over time in all cultures. Without hormones, 23% of the ^{14}C-glucose was converted to phospholipid in the first 24 hours of culture. The response increased to 28% at 24 hours and fluctuated thereafter. In all hormone-containing cultures, the effect at 4 hours (approximately 15%) was less than that observed in the absence of hormones. In general, phospholipid synthesis increased at 24 hours, declined at 48 hours, and increased again by 96 hours of culture. The addition of prolactin or corticosterone to insulin-containing cultures had little effect on the magnitude or pattern of response produced by insulin alone. The greatest decline in rate of synthesis was produced by the 3 hormone combination at 72 hours of culture.

The percent of radioactivity in the other lipid fractions, i.e. monoglycerides, diglycerides, cholesterol and free fatty acids, was determined from the difference between the total counts spotted on the TLC plate and the triglyceride + phospholipid fractions. This method was used since recovery of total counts was shown to be 100%, but standard errors were not computed. In the absence of hormones the percent of ^{14}C-glucose in these fractions decreased from 31% to 9% over 96 hours of culture. A similar decrease of a lesser magnitude was produced in insulin-containing cultures. Cultures containing insulin + prolactin and/or corticosterone showed a decline in response at 24 hours, an increase at 48 hours and a decline at 72 hours.

Triglyceride Fatty Acids

The influence of hormones on the incorporation of ^{14}C-glucose into triglyceride fatty acids is summarized in Table XVII. The results are expressed as the percentage distribution of radioactivity in short (8:0 to 14:0), medium (16:0 and 16:1), and long (18:0 and above) chain fatty acids. Without hormones, the radioactivity in short chain fatty acids decreased from 19% to 9% with time. Insulin inhibited the fall in the radioactivity of short chain fatty acids. Prolactin, or corticosterone with insulin, produced minimal increases (6% and 4% respectively), in the incorporation of short chain fatty acids into triglycerides until 48 hours. The maximal increase in the incorporation of newly synthesized short chain fatty acids into triglycerides (66%) was produced by the 3 hormone combination at 48 hours.

In cultures lacking hormones the radioactivity of the medium chain fatty acids decreased until 72 hours, but increased slightly

thereafter. Radioactivity of the long chain fatty acids increased with time. In insulin-containing cultures, incorporation of the medium chain fatty acids into triglycerides generally increased with time, while the long chain fatty acids decreased with time. The addition of prolactin and/or corticosterone to these cultures had little effect on the patterns observed with insulin alone.

Histology

Representative explants from all cultures were examined histologically to determine cellular viability as well as alveolar maintenance and secretory development. The degree of alveolar maintenance and secretory development was characterized in the following manner:

a. alveolar response

- less than 40% of alveoli maintained

\- - virtually no alveoli maintained

\+ same appearance as initial controls

\+ + increase in size and number of alveoli relative to initial controls

b. secretory response

- same appearance as initial controls; no secretion

\+ slight increase over controls; small amount of stainable secretion in lumina

\+ + moderate secretion, i.e. alveoli distended; intermediate amounts of stainable secretions in lumina; very few cells with intracellular vacuoles

\+ + + highly secretory, i.e. alveoli greatly distended; lumina filled with stainable secretion; 90% of alveolar cells with vacuoles

The results are presented in Table XVIII and Plates I-V. The freshly dissected explants of midpregnant mice were characterized by

small alveoli containing small numbers of cells per alveolus and small lumina. Secretory vacuoles were absent and the alveolar cells were cuboidal in shape, indicating an inactive secretory appearance. After 4 hours, the explants from all cultures were histologically identical to the appearance at the beginning of culture (Plate I).

In the absence of hormones, the initial histological appearance was generally maintained for the first 24 hours of culture. However, by 48 hours, the alveoli were broken down into a disorganized arrangement of cells. By 72 hours, the alveolar appearance was completely lost, and the epithelial cells showed almost complete dissolution (Plate II).

In all insulin-containing cultures the alveolar structure was maintained for 96 hours (Plates II-V). By 24 hours, the lumina were greatly distended and the number of cells per alveolus had increased, as shown by the presence of numerous mitotic figures. By 48 hours, cell division was not obvious in most explants, and the alveolar appearance varied according to the hormone(s) present in the media.

In insulin and insulin + corticosterone-containing cultures, the alveoli were distended but lacked intracellular vacuoles and stainable secretion in the lumina (Plates II and IV). This appearance was maintained for 96 hours of culture. Explants cultured with insulin + prolactin showed small amounts of stainable secretions in the lumina by 48 hours (Plate III). This appearance was also maintained for 96 hours with a slight increase in the amount of secretory material.

The explants taken from cultures containing the three hormones in combination showed a more organized alveolar appearance at 24

hours than other insulin-containing cultures (Plate V). In addition, small amounts of stainable secretions were present, but intracellular vacuoles were absent. By 48 hours, the explants from I + P + B cultures exhibited a highly secretory appearance which was maintained for 96 hours. The alveoli were characterized by large numbers of secretory vacuoles, and substantial amounts of stainable secretion in the lumina.

Table I. Influence of hormones on total mammary lipid *in vitro*.

Hormones**	% Lipid of Mammary Explants at Times Indicated (wet weight)*				
	4 hr.	24 hr.	48 hr.	72 hr.	96 hr.
None	49.18±.03	52.99±2.31	54.36±1.33	53.28±2.57	52.54±4.48
I	50.22±.40	49.40±3.19	46.18±0.75	43.04±2.98	41.43±3.75
IP	51.57±1.86	48.19±1.19	44.02±1.25	47.10±8.67	43.44±2.50
IPB	46.35±3.26	42.15±3.32	44.39±0.56	47.03±2.62	41.68±2.80
IB	43.98±4.88	46.14±2.89	42.05±2.03	48.43±3.75	49.85±2.78
Initial lipid 49.82±3.95 (4)	(2)	(4)	(4)	(4)	(4)

* Means ± S.E.

** I = insulin; P = prolactin; B = corticosterone

( ) = number of experiments done at times indicated

Table II. Effects of hormones on cumulative glucose uptake: Split-plot analysis of variance.

Source	Sum of Squares	D.F.	Mean Square	F Values	P
Hours (H)	1,249,315	3	416,438	22.43	< 0.0005
Reps/Hour (R+HR) (Error a)	222,843	12	18,570		
Treatments (T)	64,718	4	16,179	12.56	< 0.0005
HXT	35,722	12	2,976	2.31	0.020
RT+HRT (Error b)	61,819	48	1,287		
Total	1,634,418	79			

Table III. Effects of hormones on cumulative glucose uptake: Multivariate analysis of treatment variance.

Hormones	Mean values of cumulative glucose uptake*			
	24 hr.	48 hr.	72 hr. ✓	96 hr. ✓
None	64a ^{A}	164a ^{AB}	245a ^{BC}	330a ^{C}
I	71a ^{A}	220a ^{B}	352b ^{BC}	494b ^{C}
IP	77a ^{A}	225a ^{B}	350b ^{BC}	430b ^{C}
IPB	90a ^{A}	198a ^{AB}	318b ^{BC}	379b ^{C}
IB	68a ^{A}	202a ^{B}	303a ^{BC}	408b ^{C}

* Any two means in the same column (small letters), or row (large letters) with the same letter(s) are not significantly different at $\alpha = 0.05$.

✓ Cumulative glucose uptake at 72 and 96 hrs. was obtained by adding uptake at these times to 48 hr. values.

Table IV. Effects of hormones on glucose uptake per 4 hours: Split-plot analysis of variance.

	Sum of Squares	D.F.	Mean Square	F Values	P
Hours (H)	451.13	3	150.38	1.35	0.303
Reps/Hour (R+HR) (Error a)	1331.99	12	110.99		
Treatments (T)	304.26	4	76.06	5.94	0.001
HXT	181.15	12	15.09	1.18	0.325
RT+HRT (Error b)	614.56	48	12.80		
Total		79			

Table V. Effects of hormones on glucose uptake per 4 hours: Multivariate analysis of treatment variance.

Hormones	Rates of Glucose Uptake per 4 Hours*			
	24 hr.	48 hr.	72 hr.	96 hr.
None	10.6a ^{A}	13.7a ^{A}	13.6a ^{A}	13.8a ^{A}
I	11.8a ^{A}	18.4a ^{A}	22.0b ^{A}	22.8b ^{A}
IP	12.8a ^{A}	18.8a ^{A}	21.0b ^{A}	17.1ab ^{A}
IPB	15.0a ^{A}	16.5a ^{A}	20.0ab ^{A}	15.1ab ^{A}
IB	11.3a ^{A}	16.9a ^{A}	16.8ab ^{A}	17.2ab ^{A}

* Any two means in the same column (small letters), or row (large letters) with the same letter(s) are not significantly different at $\alpha = 0.05$.

Table VI. Effects of hormones on ^{14}C-glucose incorporation into lipids by mammary explants.

Hormones**	Specific Radioactivity of Mammary Explants (cpm/mg).*				
	4 hr.	24 hr.	48 hr.	72 hr.	96 hr.
None	82±4	97±16	116±9	60±5	87±14
I	150±5	168±18	390±63	147±16	170±28
IP	167±9	183±10	347±49	163±16	177±27
IPB	170±18	273±11	824±134	586±48	334±23
IB	123±15	182±18	522±130	202±12	235±49

* Means ± S.E.

** I = insulin; P = prolactin; B = corticosterone

Table VII. Changes in specific activity of media glucose.

Hormones	Computed Specific Radioactivity at Termination - 4 hrs. (cpm/μg).*			
	20 hr.	44 hr.	68 hr. ✓	92 hr.
None	320±4	371±25	325±7	348±9
I	335±5	467±26	360±10	494±62
IP	338±8	468±18	367±7	415±21
IPB	353±8	523±18	444±16	628±87
IB	333±9	426±28	372±11	445±19

Initial sp. act. (cpm/μg)
290±10

* Specific radioactivity at times indicated was determined by measuring the amount of glucose remaining in the medium at termination, assuming linearity of uptake, and computing the hourly rate.

✓ = Media changed at 48 hours

Table VIII. Effects of hormones on lipid biosynthesis per unit wet tissue: Split-plot analysis of variance.

Source		Sum of Squares	D.F.	Mean Square	F Values	P
Hours	H	2.95	3	.98	9.14	0.002
Reps/Hour (Error a)	R+HR	1.29	12	.11		
Treatments	T	5.42	4	1.36	66.38	< 0.0005
Hours X Trts.	HT	1.91	12	.16	7.80	< 0.0005
Reps. X Trts. (Error b)	RT+HRT	.98	48	.02		
Total		12.55	79			

Table IX. Effects of hormones on lipid biosynthesis per unit wet tissue: Multivariate analysis of treatment variance.

Hormones	Mean values of glucose in lipid/unit tissue (g/mg)*			
	24 hr.	48 hr.	72 hr.	96 hr.
None	.3a A	.3a A	.2a A	.2a A
I	.5b AB	.8b A	.4b B	.3ab B
IP	.5b A	.7b A	.5bd A	.4bd A
IPB	.8c A	1.6c B	1.3c B	.6c A
IB	.6b A	1.2d B	.6d A	.5cd A

* Any two means in the same column (small letters), or row (large letters) with the same letter(s) are not significantly different at $\alpha = 0.05$.

Table X. Effects of hormones on lipid biosynthesis per unit fat-free tissue: Split-plot analysis of variance.

Source	Sum of Squares	D.F.	Mean Square	F Values	P
Hours (H)	8.87	3	2.96	8.44	0.003
Reps/Hour (R+HR) (Error a)	4.20	12	.35		
Treatments (T)	15.85	4	3.96	44.45	< 0.005
HXT	6.79	12	.57	6.35	< 0.005
RT+HRT (Error b)	4.28	48	.09		
Total	39.99	79			

Table XI. Effects of hormones on lipid biosynthesis per unit fat-free tissue: Multivariate analysis of variance.

Hormones	Mean Values of Glucose in Lipid/Unit Fat-Free Tissue (µg/mg).*			
	24 hr.	48 hr.	72 hr.	96 hr.
None	.7a ^{A}	.7a ^{A}	.4a ^{A}	.5a ^{A}
I	1.0ab ^{AB}	1.5bd ^{A}	.7a ^{B}	.6a ^{B}
IP	1.1ab ^{A}	1.3b ^{A}	.9b ^{A}	.8a ^{A}
IPB	1.4b ^{A}	2.8c ^{B}	2.5c ^{B}	1.0a ^{A}
IB	1.1ab ^{A}	2.0d ^{B}	1.1d ^{A}	1.1a ^{A}

* Any two means in the same column (small letters), or row (large letters) with the same letter(s) are not significantly different at $\alpha = 0.05$.

Table XII. Effects of hormones on triglyceride biosynthesis per unit fat-free tissue: Split-plot analysis of variance.

Source	Sum of Squares	D.F.	Mean Square	F Values	P
Hours (H)	2.88	3	.96	5.10	0.017
Reps/Hour (R+HR) (Error a)	2.26	12	.19		
Treatment (T)	6.17	4	1.54	44.06	< 0.005
HXT	2.72	12	.23	6.49	< 0.005
RT+HRT (Error b)	1.68	48	.03		
Total	15.71	79			

Table XIII. Effects of hormones on triglyceride biosynthesis per unit fat-free tissue: Multivariate analysis of variance.

Hormones	Mean Values of Glucose in Triglycerides/ Unit Fat-Free Tissue (µg/mg).*			
	24 hr.	48 hr.	72 hr.	96 hr.
None	.3a ^{A}	.5a ^{A}	.2a ^{A}	.3a ^{A}
I	.6ab ^{A}	.9bd ^{B}	.4a ^{A}	.3a ^{A}
IP	.6ab ^{A}	.7ab ^{A}	.6a ^{A}	.4a ^{A}
IPB	.8b ^{A}	1.7c ^{B}	1.6b ^{B}	.6a ^{A}
IB	.6ab ^{A}	1.1d ^{A}	.6a ^{A}	.7a ^{A}

* Any two means in the same column (small letters) or row (large letters) with the same letter(s) are not significantly different at $\alpha = 0.05$.

Table XIV. Effects of hormones on glucose utilization for lipid synthesis: Split-plot analysis of variance.

Source	Sum of Squares	D.F.	Mean Square	F Values	P
Hours (H)	91.67	3	30.56	7.10	0.005
Reps/Hour (R+HR) (Error a)	51.62	12	4.30		
Treatment (T)	151.55	4	37.89	32.83	< 0.0005
HXT	49.91	12	4.16	3.60	0.001
RT+HRT (Error b)	55.39	48	1.15		
Total	400.15	79			

Table XV. Effects of hormones on glucose utilization for lipid synthesis: Multivariate analysis of variance.

Hormones	Mean Percent Glucose in Lipid*			
	24 hr.	48 hr.	72 hr.	96 hr.
None	2.9a ^{A}	2.5a ^{A}	1.9a ^{A}	1.8a ^{A}
I	4.4ab ^{AB}	4.6a ^{A}	2.4a ^{AB}	1.7a ^{B}
IP	4.8ab ^{A}	3.9a ^{A}	2.7a ^{A}	2.9a ^{A}
IPB	5.6b ^{A}	9.4b ^{B}	6.7b ^{AB}	3.8a ^{A}
IB	5.2b ^{AB}	6.9c ^{A}	3.4a ^{B}	3.0a ^{B}

* Any two means in the same column (small letters) or row (large letters) with the same letter(s) are not significantly different at $\alpha = 0.05$.

Table XVI. Effects of hormones on ^{14}C-distribution in mammary lipids.

Hormones	Fraction*	Percent Radioactivity in Lipid Fractions				
		4 hr.	24 hr.	48 hr.	72 hr.	96 hr.
None	TG	46.1 ± 1.1	48.6 ± 3.3	64.1 ± 6.6	59.6 ± 1.6	56.8 ± 4.1
	FAME	8.4	9.8	11.9	11.2	10.8
	GLY	91.5	90.2	88.0	88.7	89.1
	OF	31.3	22.9	10.0	12.9	9.0
	PL	22.5	28.4 ± 1.8	25.7 ± 2.7	27.4 ± 2.9	34.1 ± 7.2
I	TG	49.8 ± 1.6	54.9 ± 3.2	58.4 ± 3.2	52.8 ± 1.2	55.1 ± 5.1
	FAME	15.6	22.6	28.3	23.2	18.0
	GLY	84.3	77.3	71.6	76.7	81.9
	OF	35.6	20.9	20.2	16.1	11.6
	PL	14.4	24.1 ± 1.8	21.3 ± 2.8	31.0 ± 3.1	33.2 ± 0.6
IP	TG	54.3 ± 1.0	57.0 ± 3.3	54.9 ± 4.1	58.1 ± 4.3	55.1 ± 3.9
	FAME	15.1	26.6	25.0	27.2	20.0
	GLY	84.8	73.3	74.9	72.7	79.9
	OF	30.2	14.6	23.8	14.1	12.2
	PL	15.4	28.2 ± 1.7	21.1 ± 2.8	25.7 ± 3.4	32.6 ± 1.6

	TG	57.7 ± 0.7	55.1 ± 1.6	58.4 ± 4.0	65.6 ± 3.0	63.3 ± 3.8
	FAME	19.5	36.1	42.9	40.6	19.3
IPB	GLY	80.4	63.8	57.0	59.3	80.6
	OF	25.9	22.0	25.6	21.0	15.1
	PL	16.3	22.7 ± 1.9	15.9 ± 1.2	13.3 ± 1.6	21.5 ± 1.7
	TG	56.5 ± 1.6	57.5 ± 5.2	53.7 ± 1.9	56.8 ± 2.7	58.5 ± 6.2
	FAME	15.9	27.2	34.4	20.0	20.5
IB	GLY	84.0	72.7	65.5	79.9	79.4
	OF	28.4	16.7	24.4	20.6	14.6
	PL	15.0	25.7 ± 4.1	21.8 ± 2.9	22.5 ± 1.8	26.7 ± 3.5

* TG: triglycerides

FAME: triglyceride fatty acid methyl esters

GLY: triglyceride glycerol

OF: other fractions: monoglycerides, diglycerides, cholesterol and free fatty acids

PL: Phospholipids

Table XVII. Effects of hormones on the distribution of radioactivity in triblyceride fatty acids.

Hormones	Chain** Length	Percent distribution of radioactivity in fatty acids*				
		4 hr.	24 hr.	48 hr.	72 hr.	96 hr.
None	8:0-14:0	19.5	16.0	17.6	15.4	9.0
	16:0-16:1	59.3	57.3	43.8	44.9	47.3
	18:0 >	21.0	26.6	38.5	39.6	42.6
I	8:0-14:0	18.9	17.1	16.2	17.5	14.0
	16:0-16:1	52.7	55.2	59.3	60.1	57.5
	18:0 >	28.2	27.5	22.4	22.2	28.3
IP	8:0-14:0	22.6	22.4	25.1	22.8	19.4
	16:0-16:1	48.1	51.5	52.0	55.4	56.3
	18:0 >	29.1	26.0	22.7	21.6	24.1
IPB	8:0-14:0	32.2	41.2	66.3	58.3	29.7
	16:0-16:1	42.5	38.6	24.1	32.1	40.8
	18:0 >	25.2	20.1	9.4	9.4	29.4
IB	8:0-14:0	21.3	23.0	21.9	19.4	16.9
	16:0-16:1	55.5	59.4	60.6	59.9	58.2
	18:0 >	23.0	17.5	17.4	20.5	24.8

* All percent values represent pooled samples of 4 replications of each hormone treatment at each time period.

** The number to the right of the colon represents the number of double bonds. 8:0-14:0 includes 8:0, 10:0, 12:0, 14:0 and other minor components. 18:0 > includes 18:1, 18:2, 18:3 and minor amounts of 20:4.

Table XVIII. Histological response of mammary explants to hormones *in vitro*.

Hormones	Response at Time Intervals Indicated*							
	24 hrs.		48 hrs.		72 hrs.		96 hrs.	
	alveolar response	secretory response	alveolar response	secretory response	alveolar response	secretory response	alveolar response	secretory response
None	+	-	-	-	- -	-	- -	-
I	+ +	-	+ +	-	+ +	-	+ +	-
IP	+ +	-	+ +	+	+ +	+ +	+ +	+ +
IPB	+ +	+	+ +	+ + +	+ +	+ + +	+ +	+ + +
IB	+ +	-	+ +	-	+ +	-	+ +	-

* No detectable treatment differences were observed at 4 hour.
See text for description of alveolar response and secretory response.

EXPLANATION OF FIGURES*

Figure 1. The cumulative effects of hormones on total glucose uptake by mouse mammary explants *in vitro*.

* The results for all figures were obtained from mammary gland explants taken from midpregnant mice and cultured in media 199 for the times indicated. NH (no hormone), I (insulin, 5 μg/ml), P (prolactin, 5 μg/ml), and B (corticosterone, 1 μg/ml). Values given are means ± S.E. of 4 experiments, except for 4 hour experiments, which were done twice. Glucose uptake was based on depletion of media glucose (initial conc. = 1 mg/ml).

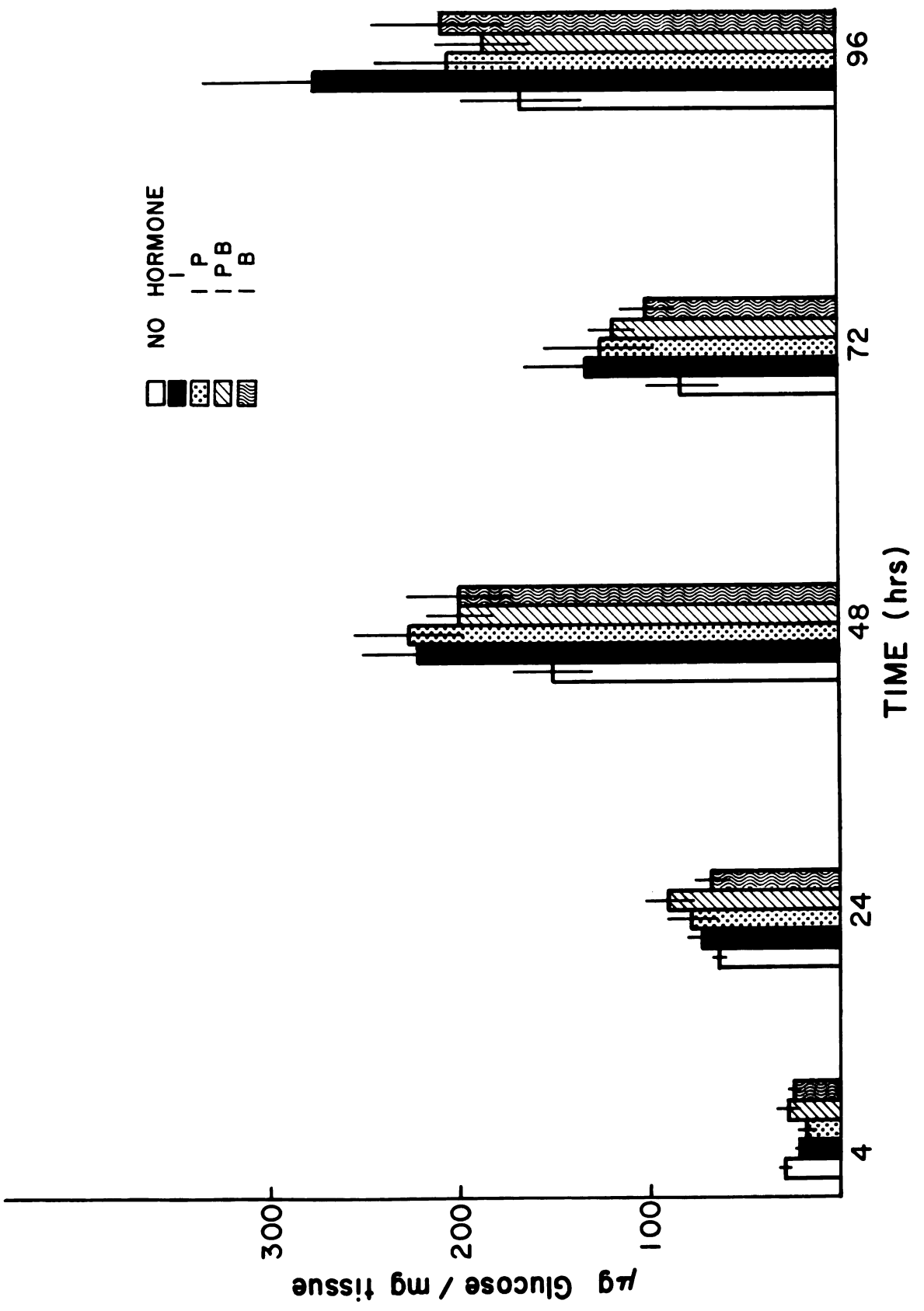

NO HORMONE
P
PB
B
300
200
100
μg Glucose / mg tissue
4
24
48
72
96
TIME (hrs)

Figure 2. The effects of hormones on glucose uptake by mouse mammary gland explants during the last 4 hours of each culture interval studied.

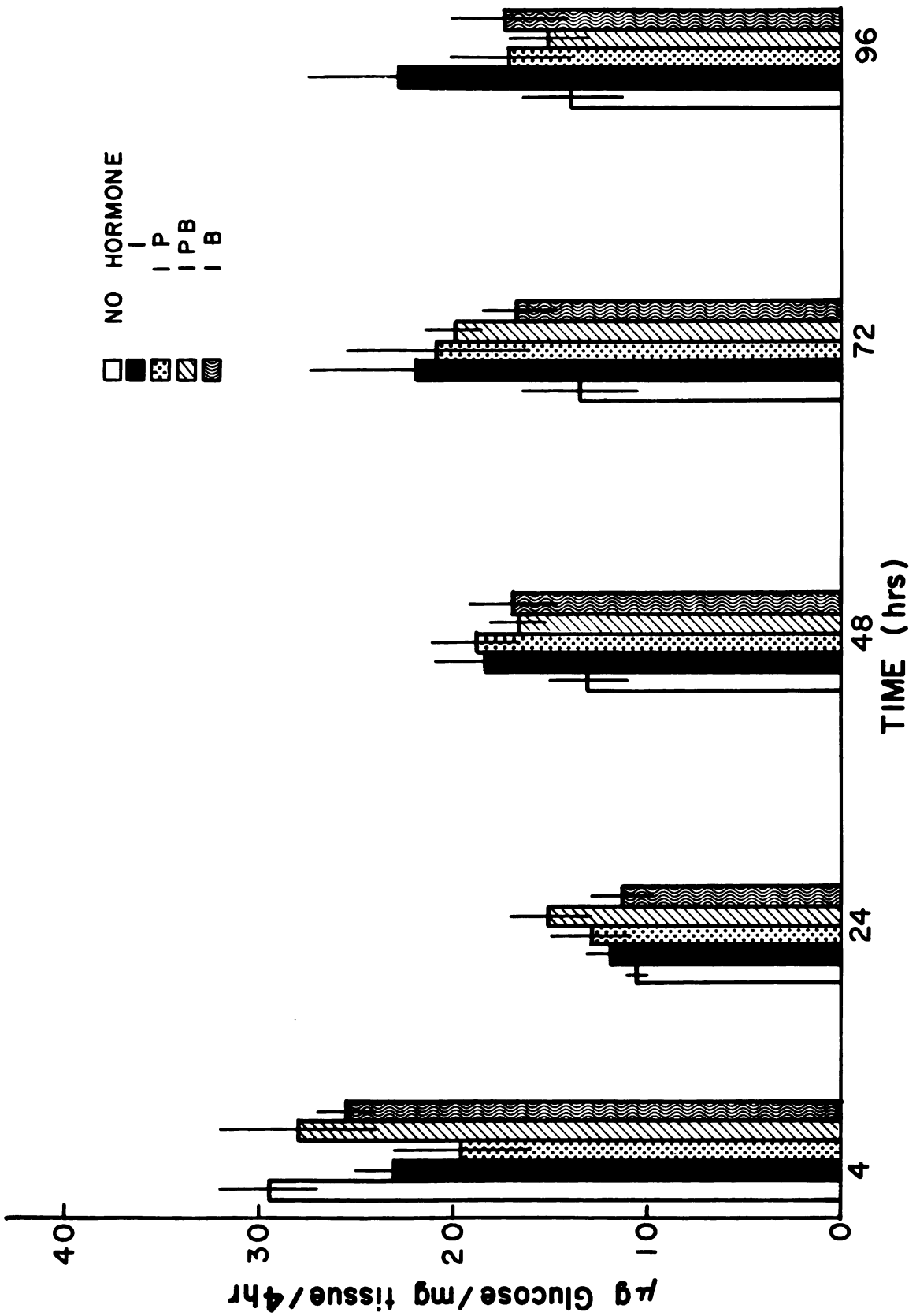

μg Glucose/mg tissue/4hr
0
10
20
30
40
4
24
48
72
96
TIME (hrs)
NO HORMONE
P
PB
B

Figure 3. The effects of hormones on lipid biosynthesis from ^{14}C-glucose by mouse mammary explants in vitro. The results are expressed per mg wet weight of tissue. (See text for method of computing the amount of glucose converted to lipid).

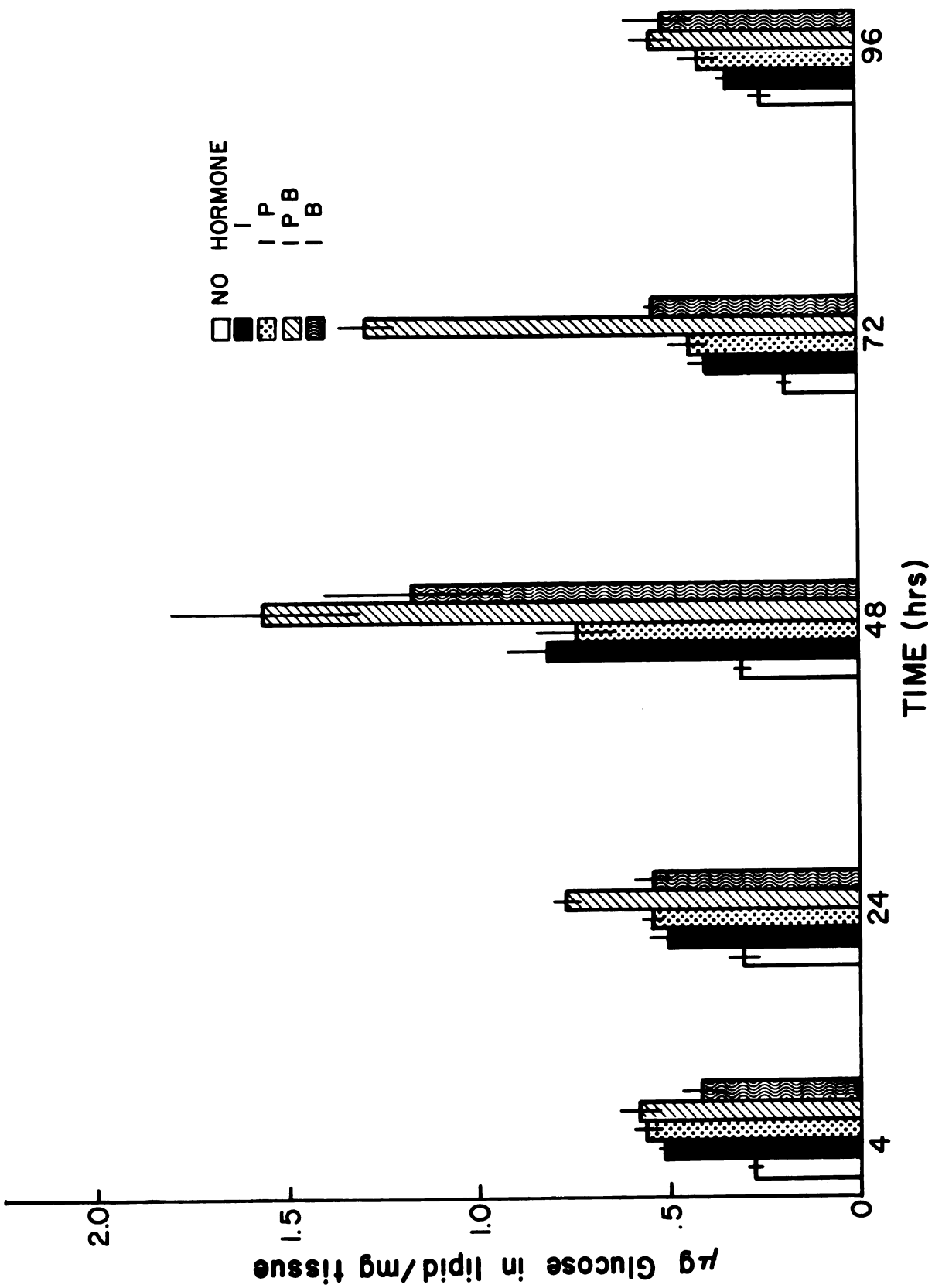

μg Glucose in lipid/mg tissue
2.0
1.5
1.0
.5
0
4
24
48
72
96
TIME (hrs)
NO HORMONE
P
P B
B

Figure 4. The effects of hormones on lipid biosynthesis from ^{14}C-glucose by mouse mammary explants *in vitro*. The results are expressed per mg fat-free tissue. Compare with Figure 3.

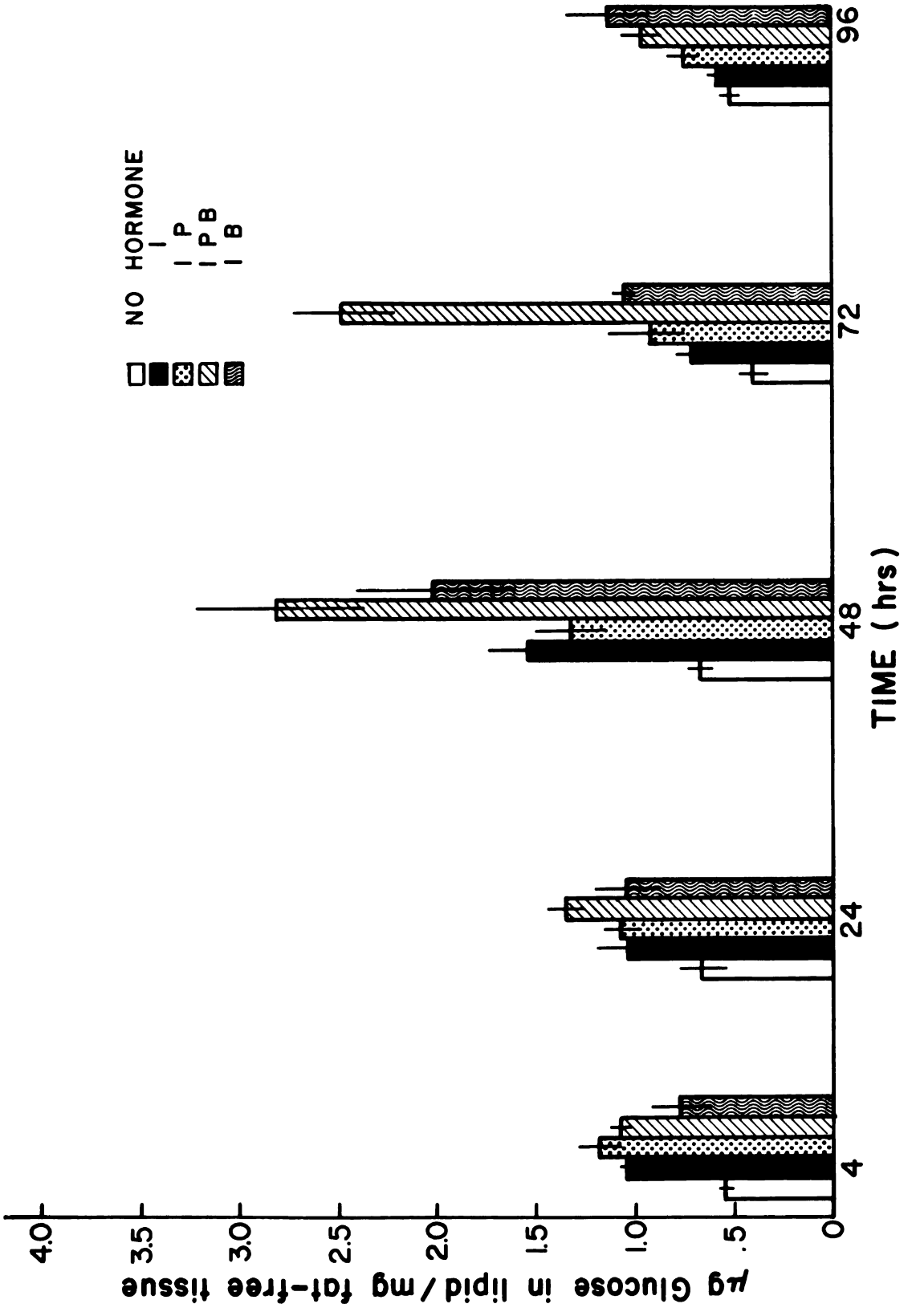

μg Glucose in lipid / mg fat-free tissue
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0
4
24
48
72
96
TIME (hrs)
NO HORMONE
I
I P
I P B
I B

Figure 5. The effects of hormones on triglyceride synthesis by mouse mammary explants *in vitro*.

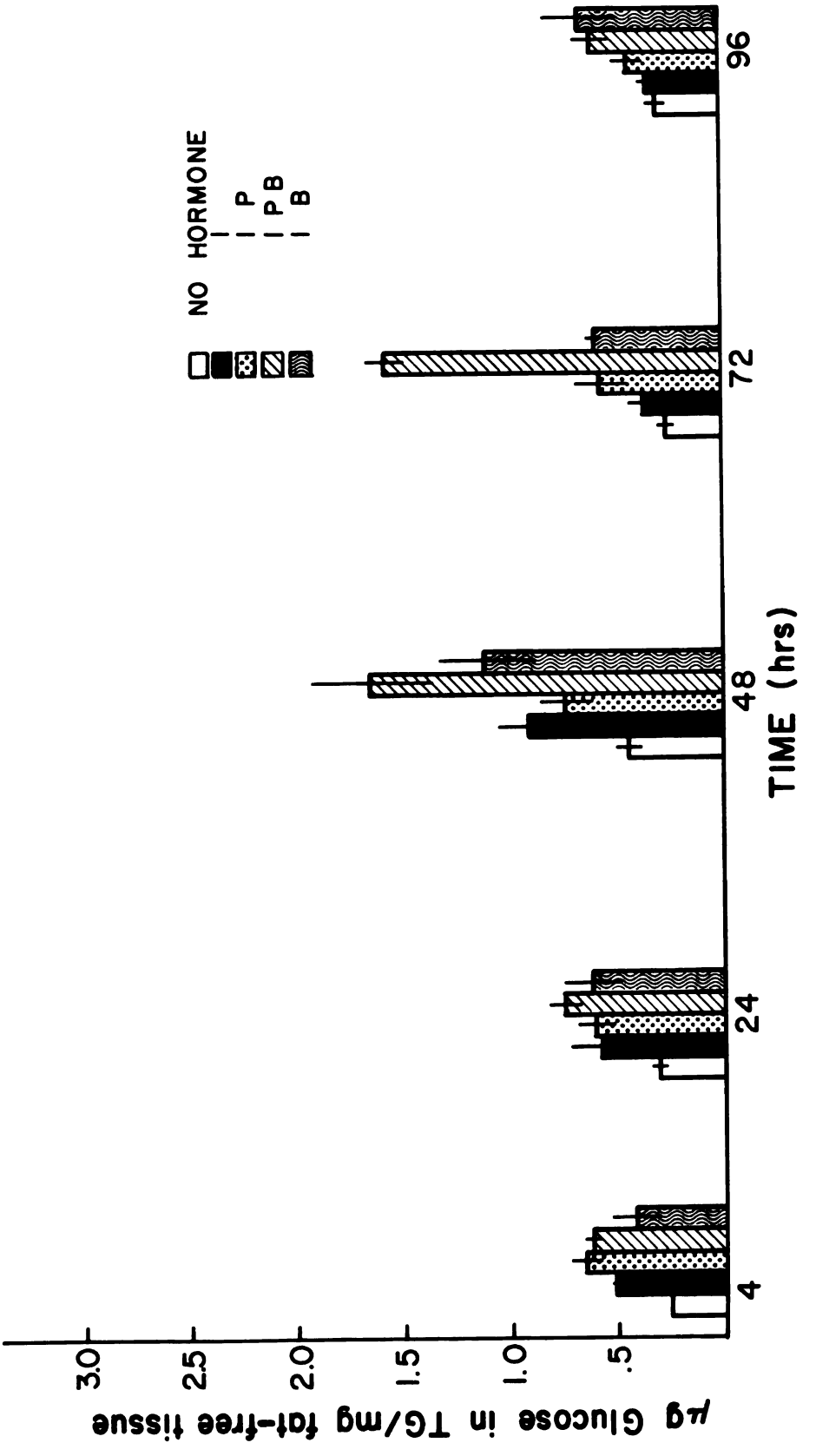
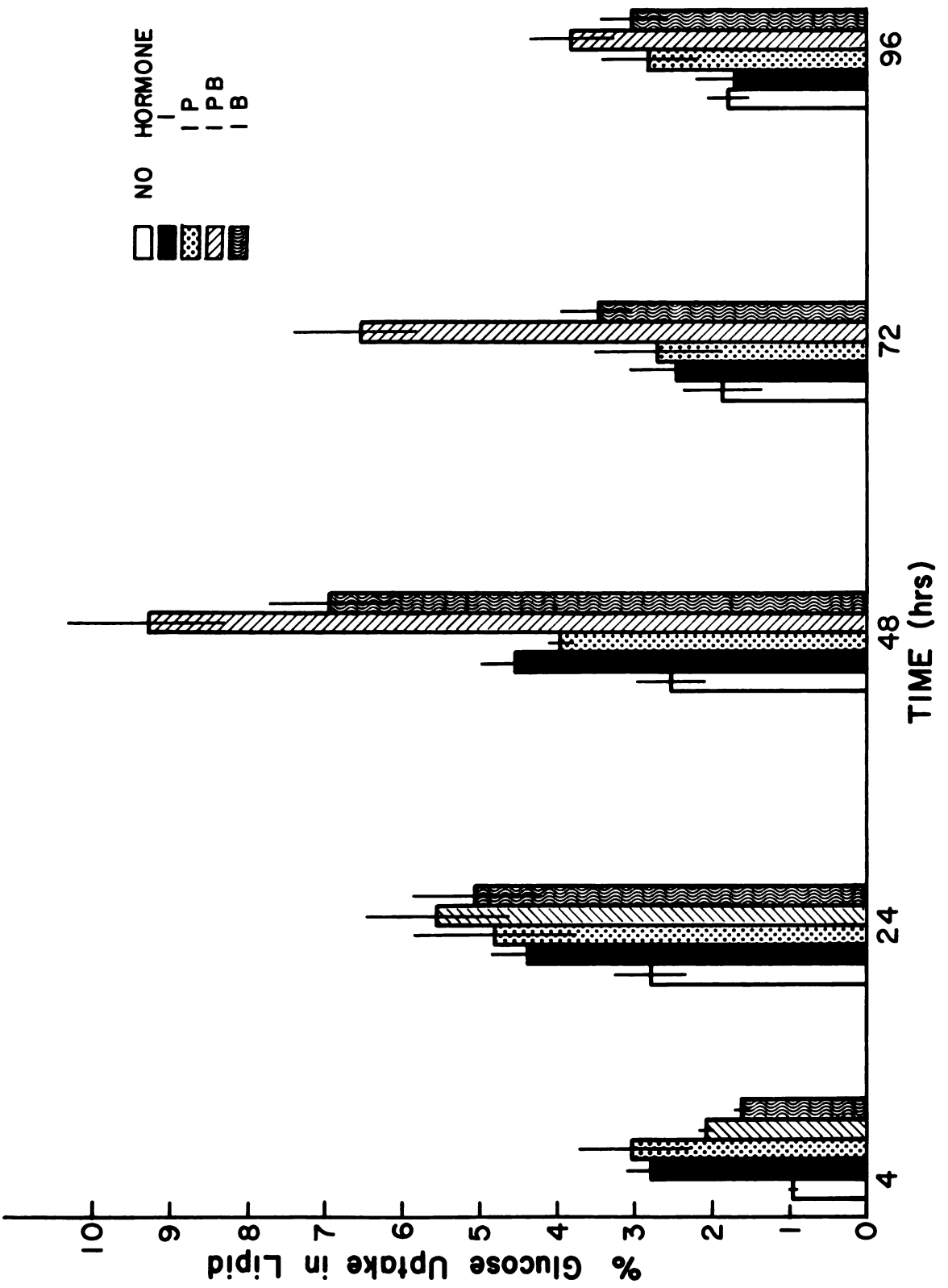

μg Glucose in TG/mg fat-free tissue
3.0
2.5
2.0
1.5
1.0
.5
4
24
48
72
96
TIME (hrs)
NO HORMONE
P
P B
B

Figure 6. The effects of hormones on the utilization of glucose for lipid synthesis by mouse mammary explants *in vitro*.


% Glucose Uptake in Lipid
0
1
2
3
4
5
6
7
8
9
10
4
24
48
72
96
TIME (hrs)
NO HORMONE
I
I P
I PB
I B

DISCUSSION

The overall objective of this study was to determine the effects of insulin, prolactin, and corticosterone on glucose uptake and lipogenesis in mouse mammary explants *in vitro*. The results indicate that these hormones, in various combinations, are capable of inducing qualitative and quantitative changes on the parameters studied.

Influence of Hormones on Total Mammary Lipids

In the absence of hormones, mammary lipids increase slightly from 49% to 54% during 96 hours of culture. With insulin alone, total lipids declined by as much as 10%. The addition of prolaction and/or corticosterone to insulin-containing cultures had no additional effect over that obtained with insulin alone. These data are supported by histological observations of a loss of epithelial and connective tissue components in the absence of hormones. Epithelial proliferation occurs in insulin-supplemented cultures by 24 hours and is maintained for 96 hours. The decrease in total mammary fat in the presence of hormones suggests that any increase in lipid synthesis is minimal relative to the total amount of adipose tissue present.

Total lipid of virgin, pregnant, and lactating rat mammary glands was determined by Wrenn *et al*. (1966), who found that the percentage of fat in the mammary gland declined steadily from 67% in virgin rats to 15% during lactation. At mid-pregnancy, mammary fat represented

approximately 53% of the total wet weight. Similar observations were reported for mouse mammary tissues (Smith et al., 1969), where it was found that mammary glands of virgin and lactating mice contained 48% and 11% fat, respectively.

Effects of Hormones on Glucose Uptake, Lipid Synthesis, and Glucose Utilization for Lipid Synthesis

The uptake and conversion of glucose into lipid by mouse mammary explants appear to be under hormonal regulation. Considerable variation was observed in this study, which may largely reflect the inherent variability in lipid turnover rates and tissue responsiveness to hormones, as well as technical difficulties involved in lipid extraction and analysis. The greatest variability was observed at the 48 hour time period, but primarily in one of the four experiments in which all treatments showed low rates of lipid synthesis. The lower rates of synthesis introduced much greater variation in those hormone treatments with the greatest effects, i.e. I + P + B and I + B rather than those causing smaller responses, i.e. NH, I and I + P.

The early effects of hormones at 4 hours of culture were not statistically analyzed because of the small sample size. However, the data suggest that in the first 4 hours, insulin alone and in combination with prolactin and/or corticosterone does not increase glucose uptake above that produced in the absence of hormones. In contrast, insulin stimulated a two-fold increase in the amount of ^{14}C-glucose incorporated into tissue lipids when compared to controls without hormones. The addition of prolactin or prolactin + corticosterone to insulin-

containing cultures enhanced slightly this response, whereas corticosterone appeared inhibitory to the effect of insulin.

Interpretations of these initial 4 hour results require consideration of the small sample size, the fact that the explants were freshly removed from an in vivo environment, and acclimitization to the culture medium may require some time. Beyond these considerations, however, the data do suggest that the effects of insulin on glucose transport and lipogenesis in mammary explants involve different mechanisms of action that are temporally distinguishable. Further support for this idea is provided by the greater effect of insulin on the percentage utilization of glucose for lipid synthesis than on glucose uptake.

Comparable results on glucose uptake and lipogenesis in response to insulin were reported by Moretti and Abraham (1966). These workers showed that in mid-pregnant mammary explants, insulin stimulated the incorporation of ^{14}C-glucose into lipids in the first 3 hours of culture, whereas its effect on glucose uptake required 48 hours. This early action of insulin on lipid synthesis by mammary explants has also been reported by Mayne and Barry (1970). These authors also found that the addition of prolactin and corticosterone to insulin-containing cultures had no influence on glucose uptake and induced only minor changes (15% greater than insulin alone) in lipid synthesis.

The effects of hormones at 24, 48, 72, and 96 hours of culture were subjected to split-plot and multivariate analysis of variance. The results show that the uptake of glucose in prolactin-supplemented cultures, i.e. I + P and I + P + B, whether expressed on a cumulative

or 4 hour rate basis, did not vary significantly from uptake in the absence of hormones. Furthermore, insulin alone increased the uptake of glucose by 72 hours, whereas corticosterone inhibited the response. In contrast to its delayed action on glucose transport, insulin increased the incorporation of ^{14}C-glucose into lipid as early as 24 hours ($P<0.01$). The addition of prolactin or corticosterone to insulin-containing cultures did not change the response from that produced by insulin alone ($P>0.05$) at 24 hours. However, corticosterone increased lipid synthesis more than did insulin alone at 48 hours ($P<0.01$). The 3 hormone combination increased significantly the incorporation of labeled glucose into lipid compared with the results obtained with all other hormone combinations at 24 hours ($P<0.01$). The maximal response (1.6 μg/mg tissue) occurred with the 3 hormone combination at 48 hours.

The above observations support the notion of a preferential action of insulin on lipogenesis rather than glucose transport, especially notable from results of 4 hour cultures. Moreover, the fact that prolactin and corticosterone failed to alter significantly the effect of insulin on glucose uptake suggests that the action of these hormones on lipid synthesis and other metabolic and developmental processes is not mediated through glucose transport. That prolactin does not alter the lipogenic response of mammary explants to insulin, but significantly enhances it in combination with insulin plus corticosterone, suggests that its action may be dependent upon prior or simultaneous action of insulin plus corticosterone. The observation that corticosterone acted synergistically with insulin to increase the rate of

lipid synthesis suggests an action different from that obtained with prolactin.

Insulin's influence on glucose uptake over 96 hours of culture is in agreement with the results of Moretti and Abraham (1966), in that 48 hours were required to effect an increase in glucose uptake relative to hormone-free controls. According to Mayne and Barry (1970), the addition of prolactin + corticosterone to insulin-containing cultures had no effect on glucose uptake above that produced by insulin alone over 48 hours of culture. The results of this study support this observation and extend it over 96 hours. In addition, prolactin and insulin tended to increase glucose uptake, although the increase was not significantly different from that caused by insulin. Corticosterone and insulin, on the other hand, significantly inhibited cumulative glucose uptake in 72 hour cultures ($P<0.05$).

The influence of insulin on lipid synthesis demonstrated in this study is in general accord with the observations of Moretti and Abraham (1966), who showed that insulin increased the cumulative incorporation of ^{14}C-glucose into total fatty acids until 48 hours, after which there was a decline. On the other hand, the present study shows some discrepancy with the results of Mayne and Barry (1970). These authors, using a 3 hour pulse label of ^{14}C-glucose in insulin and insulin + prolactin + corticosterone-containing cultures, showed a peak rate of lipid synthesis at 3 hours followed by an immediate decline over the remaining 48 hours of the study. An explanation for this discrepancy is not readily available. However, it should be noted that Mayne and Barry (1970) transferred explants to fresh medium containing

the labeled glucose for 3 hours before measuring rates of incorporation (the medium was changed at 48 hours in the present study) and the labeled glucose was added to the medium in which the explants had been cultured for the entire time period. Therefore, it is possible that fluctuations in glucose transport and, thus, the intracellular pool size, due simply to mass transport, might account for our different results. Furthermore, the fact that Mayne and Barry (1970) were able to maintain constant specific activity may have been counteracted by possible shock effects caused by placing explants in fresh medium during the 3 hour labeling period. In contrast, the present study required corrections for changes in specific activity of media glucose, but labeling was carried out in medium to which explants had become acclimatized, thereby preventing shock effects during the period of ^{14}C incorporation. In addition, the present study utilized an experimental design different from that of Mayne and Barry (1970) in that each experiment contained tissues from two mice distributed evenly among all treatments, while Mayne and Barry (1970) used explants from different animals for each treatment. Differences in strain of mice (C3H) used might also have attributed to our divergent results.

Mayne and Barry (1970) also noted that the 3 hormone combination, while increasing the rate of lipid synthesis above that produced by insulin alone, failed to inhibit the decline in rate observed at 48 hours in insulin-containing cultures. Again, the reasons for the discrepancy in our results are not clear. However, since the proportion of fat in milk fat is fairly constant, it would appear that its synthesis should follow the pattern of response shown for other milk

components, i.e. casein, whey proteins, and lactose. In this regard, the evidence is generally consistent that these components reach maximal rates of synthesis at 48 hours of culture in the presence of the 3 hormone combination (Juergens et al., 1965; Lockwood et al., 1967a,b; Turkington et al., 1968). The histological observations in this and other studies (Rivera and Bern, 1961; Rivera, 1964; Stockdale et al., 1966) show that the 3 hormone combination produced large numbers of intracellular secretory vacuoles, indicative of lipid synthesis and secretion by 48 hours.

The synergism of corticosterone and insulin in increasing total lipid synthesis has also been reported for fatty acid synthesis in explants from midpregnant mice (Wang et al., 1972) and rats (Hallowes et al., 1973). Using 4 hour pulse labels of ^{14}C-acetate, these workers demonstrated that corticosterone + insulin enhanced its incorporation into mammary fatty acids to an extent greater than that produced by insulin alone. In contrast to the results reported herein, Wang et al. (1972) noted that in mouse mammary explants, the peak effect of insulin + prolactin, insulin + cortisol, and insulin + prolactin + cortisol, occurred at 24 hours and declined only slightly at 48 hours. The reason for this difference in peak response observed in the present study and theirs is not apparent; however, it should be noted that the effect of insulin alone in their study was maximal at 48 hours and the decline observed by them may not be significant. Hallowes et al., (1973) reported that the peak response to the 3 hormones in midpregnant rat mammary explants occurred at 48 hours and was maintained for 72 hours.

However, they also reported a peak response to I and I + B at 24 hours with subsequent decline.

From this study and others (Moretti and DeOme, 1962; Moretti and Abraham, 1966; Mayne and Barry, 1970), it is clear that insulin increases glucose uptake in mammary explants from midpregnant mice and that the maximal response requires 48 to 72 hours of culture. Prolactin and/or corticosterone do not enhance the insulin-induced response. Moreover, the observation that insulin increases the rate of lipid synthesis by 4 hours of culture as found in the present study is supported by the work of Moretti and Abraham (1966) and Mayne and Barry (1970). Whereas prolactin has little effect on the insulin-induced response, corticosterone significantly enhances it. However, the maximal response in lipid synthesis requires the 3 hormones in combination and occurs between 24 to 48 hours of culture.

The above observations are further supported by the percentage utilization of glucose which showed a pattern of response to hormones similar to that found for the amount of glucose incorporated into lipids. The 3 hormones were again required to produce a maximal effect at 48 hours of culture.

Hormonal Influences on Triglyceride Synthesis and the Distribution of ^{14}C-Glucose in Lipid Fractions

The effects of hormones on the incorporation of ^{14}C-glucose into triglycerides followed the same general pattern observed for total lipid synthesis. The increase in the amount of ^{14}C-glucose incorporated into the triglyceride fractions suggests that the increased rate of total lipid synthesis is due to a large extent to increased triglyceride

synthesis. In general, the rate of total lipid synthesis declined at 72 hours, and the amount of ^{14}C-glucose incorporated into triglycerides was less than at 48 hours in all cultures. However, the percentage of ^{14}C-glucose incorporated into triglycerides increased in insulin + prolactin, insulin + prolactin + corticosterone, and insulin + corticosterone-containing cultures. In these three cultures, therefore, the decline in total lipid synthesis at 72 hours is probably not primarily due to a decreased rate of triglyceride synthesis. Although the literature contains no recent reports of the effects of hormones on the rate of triglyceride synthesis in mouse mammary explants, Strong et al. (1972) have shown that insulin + prolactin + corticosterone increase the proportion of ^{14}C-labeled fatty acids incorporated into triglycerides of mammary explants from pseudopregnant rabbits over 7 days of culture. Moreover, the results suggest that with the 3 hormone combination, the decline in total lipid synthesis may be primarily due to a decrease in phospholipid synthesis. In this regard, Hillyard and Abraham (1972) found that the incorporation rate of ^{14}C-methyl-choline into the phosphatidylcholine fraction of mammary slices from lactating mice is only 11% of that in glands from pregnant animals. In addition, Wellings et al. (1960) report that there is very little proliferation of the endoplasmic reticulum in lactating mammary cells. Strong et al. (1972) also reported that the 3 hormone combination resulted in a decrease in the proportion of phospholipid synthesized by mammary explants from pseudopregnant rabbits.

The above observations indicate that phospholipid synthesis might be expected to decline in actively secreting explants. Histological

observations show that this is the case in 48 and 72 hour cultures containing the 3 hormones. Further support for this suggestion is provided by the fact that phospholipid synthesis remains high in all other cultures.

The increase in phospholipid synthesis during the first 24 hours, both in the presence and absence of hormones, is not readily explainable. However, it is important to note that although the percentage of ^{14}C-glucose incorporated into phospholipids increased in the absence of hormones, the absolute amount of glucose incorporated is much less than when hormones are present. With hormones, cellular proliferation, as determined histologically, can account for some of the increase in phospholipid synthesis.

The distribution of ^{14}C-radioactivity in the glycerol and fatty acid components of triglycerides was taken from pooled samples of 4 experiments per time period and as a result, no statistical analysis was done. However, the data do suggest that in hormone-free cultures, turnover of preformed glycerides may be primarily involved, with very little incorporation of newly-formed fatty acids into triglycerides. In all of the hormone-containing cultures, the percentage of fatty acids into triglycerides increased with time. Prolactin did not increase the effect of insulin alone, whereas corticosterone enhanced the effect of insulin on the incorporation of labeled fatty acids into triglycerides. The maximal response was produced by the 3 hormone combination at 48 hours of culture.

Effects of Hormones on Triglyceride Fatty Acids in Mouse Mammary Explants

The percentage distribution of ^{14}C-radioactivity in triglyceride fatty acids was obtained from GLC analysis of pooled samples from four experiments per time period, but the results were not statistically analyzed. However, the data suggest that in the absence of hormones, the short (8:0-14:0) and medium (16:0-16:1) chain fatty acids decreased from 19% and 59% over 96 hours *in vitro*.

Insulin prevented the decline in short chain fatty acids in hormone-free cultures at 72 hours. Insulin also increased the radioactivity of medium chain fatty acids from 53% at 4 hours to 60% at 72 hours of culture, but decreased long chain fatty acids by 8% over the 72 hour time period. The addition of prolactin and/or corticosterone to insulin-containing cultures enhanced the incorporation of short chain fatty acids into triglycerides until 48 hours, after which the effect decreased. The maximal response (66%) occurred with the 3 hormone combination at 48 hours. Neither prolactin nor corticosterone combined with insulin had significant effect on the distribution of radioactivity in medium and long chain fatty acids at any time.

From the above results it is obvious that the 3 hormone combination exerts a qualitative and quantitative change in the pattern of fatty acids incorporated into mouse mammary triglycerides. In addition, the peak effect occurred at a time (48 hours) when triglyceride and total lipid synthesis were also maximal. The results indicate, therefore, that the induction of a specific lipogenic response in mouse mammary explants requires the same complement of hormones essential for

other biochemical changes, i.e. casein (Juergens et al., 1965; Turkington et al., 1965; Topper, 1968, 1970) and lactose synthesis (Turkington et al., 1968; Palmiter, 1969). In addition, the temporal response in these biochemical changes provides additional support for the initial histological observations (Rivera and Bern, 1961; Rivera, 1964; Stockdale, et al., 1966) that all 3 hormones are required to induce maximal secretion in vitro.

Although the literature indicates no direct evidence for the effects of hormones on the distribution of ^{14}C-radioactivity in triglyceride fatty acids of mouse mammary gland in vitro, the total fatty acid composition of mouse milk triglycerides was studied by Smith et al., (1968). These workers injected oxytocin into 12 day lactating mice to obtain milk and found that up to 40% of the triglyceride fatty acids were 8 to 14 carbons in length, while 16 and 18 carbon fatty acids accounted for approximately 35% and 25%, respectively, of the triglycerides.

In contrast to the lack of information on the pattern of triglyceride fatty acids, the pattern of total fatty acids were examined in mammary glands of virgin and lactating mice (Smith et al., 1969; Abraham et al., 1972). Furthermore, the capacity of hormones to alter patterns of total fatty acid synthesis has also been analyzed in mice (Wang et al., 1972), rats (Hallowes et al., 1973), and rabbits (Forsyth et al., 1972; Strong et al., 1972).

Smith et al. (1969) reported that mammary slices from virgin C3H mice contained no fatty acids less than 14 carbons in length and that only 2% were 14 carbon atoms in length. Twenty-six percent of the

fatty acids were 16 carbons in length and 63% possessed 18 carbon atoms or more. In slices from the lactating gland, a shift was found in the pattern of fatty acids, such that 11% were of the 10 and 12 carbon variety and 7% were 14 carbons in length. The medium chain fatty acids remained about the same, but the long chain fatty acids decreased to 49%.

Smith et al. (1969) observed that while slices of lactating rat mammary glands possessed only 18% of the shorter chain variety, they synthesized 50% containing 8 to 14 carbons from 6-^{14}C-glucose. They found 22% and 28% of the radioactivity in the medium and long chain fatty acids, respectively. Since mouse milk contained 38%, 25%, and 37% of the short, medium, and long chain fatty acids, it appears that mammary explants in vitro synthesize a greater percentage of shorter chain fatty acids than in vivo. Further support for this observation is provided by Abraham et al. (1972), who found 70%, 15%, and 6% of ^{14}C-glucose radioactivity in the short, medium, and long chain fatty acids of lactating mammary slices. A similar pattern was found in dissociated parenchymal cells (Abraham et al., 1972).

The effects of hormones on the distribution of ^{14}C-glucose radioactivity in triglyceride fatty acids observed in the present study are in accord with those of Wang et al. (1972) in mice. The latter authors found that in the first 4 hours of culture without hormones, 25%, 60%, and 14% of ^{14}C-acetate was located in fatty acids containing 8-14, 16, and 18 carbons, respectively. After 48 hours of culture, the pattern changed to 20%, 51%, and 26%. Insulin caused a decline in the percentage of radioactivity in shorter chain fatty acids even further (12%). Cortisol inhibited this decline in shorter chain fatty acids

and increased the percentage of medium chain fatty acids. The maximal response of shorter chain fatty acids was produced with the 3 hormone combination (43%) at 48 hours. In lactating mammary explants the distribution of radioactivity was 51%, 42%, and 6% in the 3 groups of fatty acids (Wang et al., 1972).

Hallowes et al. (1973) reported similar results in explants from midpregnant and lactating rats. In midpregnancy explants cultured without hormones, ^{14}C-acetate radioactivity in the shorter chain fatty acids decreased from 50% to 20% by 48 hours of culture (Hallowes et al., 1973). Insulin + corticosterone failed to prevent this decline. However, in the presence of the 3 hormone combination, they found 63%, 20%, and 15% in the short, medium and long chain fatty acids, respectively (Hallowes et al., 1973).

Again, it appears from this and other studies that 3 hormones in combination are required to induce a specific pattern of fatty acid synthesis in mid-pregnant explants. The response is maximal at 48 hours of culture. The decline seen after 48 hours may be due to functional inhibition resulting from stored secretions in the alveolar lumina (Rivera, in press).

The observations in this study that insulin alone or in combination with prolactin or corticosterone inhibited the decline in percentage of short chain fatty acids are not in complete agreement with those of Wang et al. (1972) or Hallowes et al. (1973). No explanation for this difference is apparent. However, in the present study, the prevention by insulin of the decline in incorporation of short

chain fatty acids into triglycerides was not obvious until 72 hours. Experiments of the above workers were not carried beyond 48 hours of culture

Correlation of Biochemical and Histological Observations

The histological observations in this study are in agreement with those of Rivera and Bern (1961), Rivera (1964), and Stockdale et al. (1966). Without hormones the mammary epithelial components degenerate by 72 hours of culture. Insulin alone induces cellular proliferation in 24 hour cultures as was noted by the large number of mitotic figures observed. Cultures with insulin + prolactin produced small amounts of stainable secretion, while cultures with insulin + corticosterone elicited no morphological response different from that observed with insulin alone. Only the 3 hormone combination induced a highly secretory condition at 48 hours that was maintained for 96 hours.

The maximal rate of lipid synthesis was temporally correlated with the maximal secretory appearance. The explants were characterized by large numbers of intracellular vacuoles indicative of lipid secretion and substantial amounts of stainable secretion in the alveolar lumina.

In contrast, histological observations of insulin + prolactin and insulin + corticosterone-containing cultures did not support the biochemical findings. Biochemically, insulin + prolactin, with the exception of the pattern of triglyceride fatty acids had no effect on lipogenic parameters above that produced by insulin alone, whereas insulin + corticosterone enhanced the effects of insulin on all lipogenic parameters. Yet insulin + prolactin-containing cultures produced

small amounts of stainable secretions over time, whereas insulin + corticosterone-containing cultures contained no secretion. These contradictory biochemical and histological results imply that corticosterone may exert its action more directly on adipose rather than epithelial tissue. If this is the case, it is possible that the epithelial cells are insensitive to corticosterone until prolactin sensitizes them to produce a specific set of responses. Two alternative explanations are equally possible: (1) corticosterone imprints on the cells a specific set of instructions to be activated by prolactin, and (2) the simultaneous action of both hormones in conjunction with insulin is required to induce the specific response.

From recent observations in the rabbit (Forsyth et al., 1972; Strong et al., 1972) it appears that prolactin may be both the specifier and inducer agent which produces a specific pattern of fatty acids. Insulin enhances this response but is not strictly required. The addition of corticosterone to insulin + prolactin-containing cultures increases the rate of synthesis and the percentage of long-chain fatty acids produced. It is interesting that the effectiveness of prolactin is limited to a short time period (16 to 23 days of pregnancy), after which it requires the action of insulin and corticosterone (Forsyth et al., 1972).

In explants from mouse mammary glands, the 3 hormones, insulin, prolactin, and corticosterone in combination are required to induce the synthesis of caseins (Juergens et al., 1965; Topper, 1968, 1970), whey proteins (Lockwood et al., 1966; Turkington et al., 1967a) and lactose synthetase (Turkington et al., 1968; Palmiter, 1969). The results of

this study, in conjunction with those of Wang et al. (1972) and Hallowes et al. (1973), suggest that the same 3 hormone combination is required to induce the maximal rate of total lipid and triglyceride synthesis as well as a specific pattern of fatty acids different from that produced by other hormone combinations and more closely resembling that found in milk fat. The maximal response occurs at a time when the other biochemical parameters are at a maximal rate and the tissue has its most active secretory appearance.

Although inhibitor studies were not done in this study, Wang et al. (1972) reported that inhibition of RNA synthesis with actinomycin D decreased the rate and altered the pattern of fatty acids produced by the 3 hormone combination. In contrast, this inhibitor had no effect on these parameters in medium containing insulin + cortisol. These results suggest that, although the synthesis of milk fatty acids is dependent upon RNA synthesis, RNA synthesis may not be required for fatty acids produced by insulin + cortisol. These observations (Wang et al., 1972) imply that prolactin may act at the level of transcription to induce the synthesis of specific types of RNA that are required for the production of specific enzymes necessary for the synthesis of shorter chain fatty acids. However, the results of the present study suggest that prolactin may be incapable of carrying out this action in the absence of corticosterone. It is also possible that prolactin initiates the synthesis of RNA and that corticosterone is required at a subsequent phase of the response, e.g. for the translation of the RNA messages into proteins (induction of enzymes) or transformation of preformed inactive proteins into active enzymes (activation of enzymes).

The addition of puromycin to the 3 hormone-containing cultures at 16 hours had no effect on fatty acid synthesis (Wang et al., 1972). This information failed to resolve the above question of the 3 hormone requirement for maximal response, but it did suggest to those workers that once the enzymes involved in fatty acid synthesis are induced or activated, their turnover is relatively slow. It would be interesting to determine whether explants cultured for 16 hours with insulin + prolactin + cortisol required cortisol thereafter for fatty acid synthesis, since this might indicate whether their actions involve separate mechanisms.

The work of Mayne and Barry (1970) is interesting in that these authors showed that corticosterone had no effect on RNA synthesis induced by insulin + prolactin, but significantly enhanced the action of these hormones on the incorporation of ^{32}P-orthophosphate into casein in 48 hour cultures. These observations do not agree completely with those of Juergens et al. (1965) and Stockdale et al. (1966) who showed that all 3 hormones were required to induce the above responses. The stimulation of RNA synthesis by insulin + prolactin was also observed by El-Darwish and Rivera (1971); however, these authors found that corticosterone enhanced RNA synthesis in 48 hour cultures and was necessary to maintain the response in longer cultures. Moreover, the finding of Mayne and Barry (1970) that the 3 hormone combination had only 29% greater effect on lipid synthesis than insulin alone is not in accord with the results of the present study. Although the reasons for these differences are not known, it is generally accepted that corticosterone enhances the effect of insulin + prolactin on protein

and carbohydrate synthesis, and in this and other studies (Wang et al., 1972; Hallowes et al., 1973), corticosteroid is necessary to achieve the maximal rate of lipid synthesis in mammary explants from mid-pregnant mice and rats.

The question of the coupling of cell proliferation and functional activity was not approached in the present study. However, the work of Juergens et al. (1965), Stockdale et al. (1966), and Lockwood et al. (1967a,b) for casein synthesis, and Turkington et al. (1967a) for whey protein synthesis and, more recently, those of Wang et al. (1972) for fatty acid synthesis, suggest that DNA synthesis and mitosis require only insulin. The 3 hormone combination, while producing the maximal functional response post-mitotically, appears to have no unique effect on DNA synthesis in 48 hour cultures. However, the triad of hormones is apparently required for maintenance of DNA synthesis in longer cultures (El-Darwish and Rivera, 1970).

SUMMARY

1. Without hormones, mammary gland explants from midpregnant mice utilize glucose and synthesize lipids *in vitro* over a 96 hour period. After 48 hours this activity is primarily due to adipose tissue since the epithelial and connective tissues degenerate.

2. Insulin increases glucose uptake but requires 48 to 72 hours to elicit this effect. It stimulates total lipid as well as triglyceride synthesis within the first 4 hours of culture and maintains the response over 48 hours. Insulin also maintains the percentage of short chain and increases the percentage of medium chain fatty acids incorporated into triglycerides. This hormone stimulates cellular proliferation by 24 hours and maintains the alveolar structure for 96 hours.

3. Prolactin does not significantly enhance the effect of insulin on either glucose uptake or total lipid synthesis. It does not increase triglyceride synthesis, but it does increase the percentage of shorter chain fatty acids in triglycerides. Prolactin also produces small amounts of stainable secretions in alveolar lumuna that are not observable with insulin alone.

4. Corticosterone inhibits glucose uptake induced by insulin at 72 hours. It synergizes with insulin to increase significantly total lipid and triglyceride synthesis within the first 24 hours of culture. The maximal effect occurs at 48 hours. Corticosterone also

increases the incorporation of short and medium chain fatty acids into triglycerides. It has no apparent effect on the morphology of explants.

5. The complete hormone system, i.e. insulin + prolactin + corticosterone, does not enhance the effect of insulin on glucose transport. It significantly increases total lipid and triglyceride synthesis above that produced by all other hormone combinations by 4 hours of culture. Its maximal effect occurs at 48 hours followed by a decline. It stimulates a 3-fold increase in the proportion of shorter chain fatty acids incorporated into triglycerides. It induces a highly secretory condition in explants, characterized by large numbers of intracellular vacuoles and substantial amounts of stainable secretions in the alveolar lumina.

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APPENDIX

APPENDIX

PLATE I. Photomicrographs of Mammary Explants Cultured in the Absence of Hormones.

Figure 1.* Mammary alveoli from 13 day midpregnant mice showing initial histological appearance at beginning of culture. Note the small size of lumina, lack of stainable secretion and intracellular vacuolation.

Figure 2.** Mammary explant cultured for 24 hours in the absence of hormones. Note overall maintenance of alveolar structure.

Figure 3. NH at 48 hours. Note breakdown of alveolar structure.

Figure 4. NH at 72 hours. Note gross dissolution of alveolar structure and epithelial tissue.

* All tissue were stained with hematoxylin-eosin and photographed at 430 X.

** Explants were taken from 13 day midpregnant mice and cultured for various time periods. NH (no hormone), I (insulin, 5 μg/ml), P (prolactin, 5 μg/ml), and B (corticosterone, 1 μg/ml).

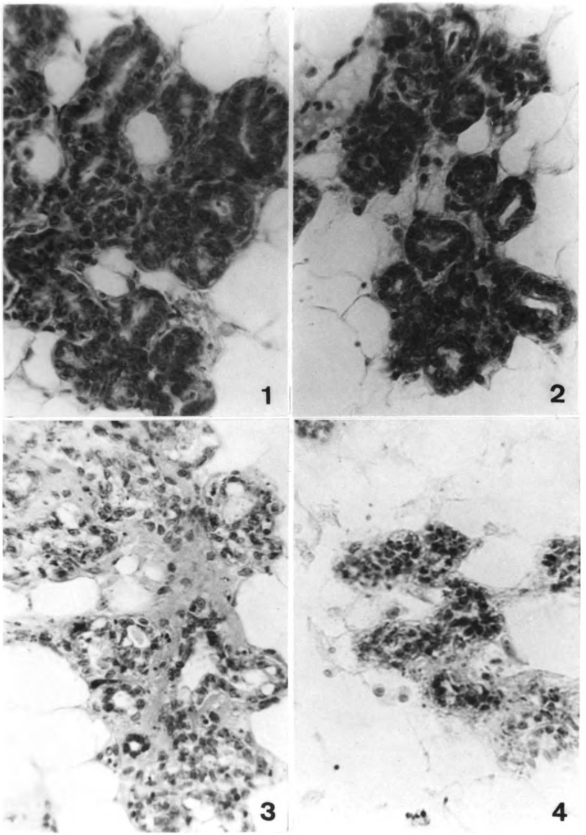
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PLATE II. Photomicrographs of Mammary Explants Cultured in the Presence of Insulin.

Figure 1. Insulin for 24 hours. Note maintenance of alveolar structure, distended lumina, lack of stainable secretion, and mitotic figure.

Figure 2. Insulin for 48 hours. Similar to 24 hours with more alveolar distention. Mitotic figures are not obvious in this section.

Figure 3. Insulin for 72 hours. Alveolar structure maintained, secretion absent.

Figure 4. Insulin for 96 hours. Appearance similar to that seen at 72 hours, alveoli less distended.

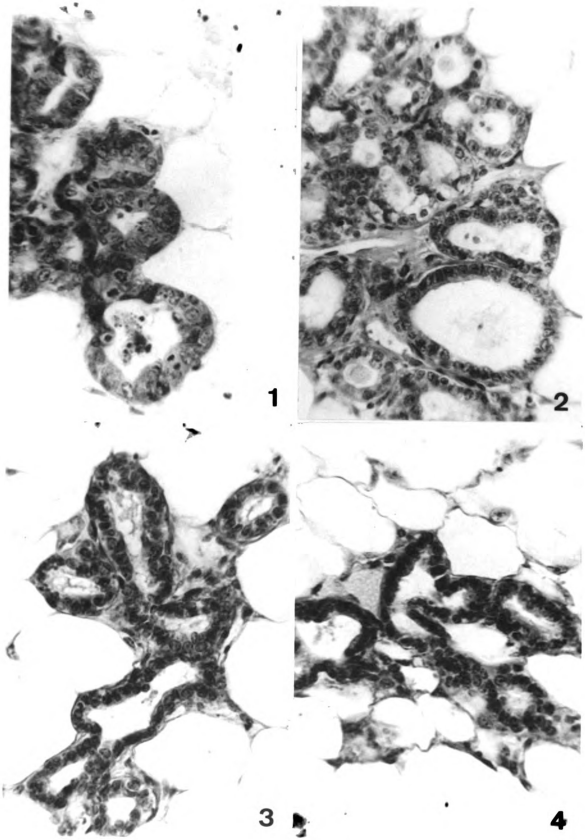
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PLATE III. Photomicrographs of Mammary Explants Cultured in the Presence of Insulin + Corticosterone.

Figure 1. I + B at 24 hours. Maintenance of alveolar structure, distended lumina, and lack of stainable secretion.

Figure 2. I + B at 48 hours. Maintenance of alveolar structure, distended lumina, and lack of stainable secretion.

Figure 3. I + B at 72 hours. Overall maintenance of alveoli, distended lumina, and inactive secretory appearance.

Figure 4. I + B at 96 hours. Appearance similar to that at 72 hours.

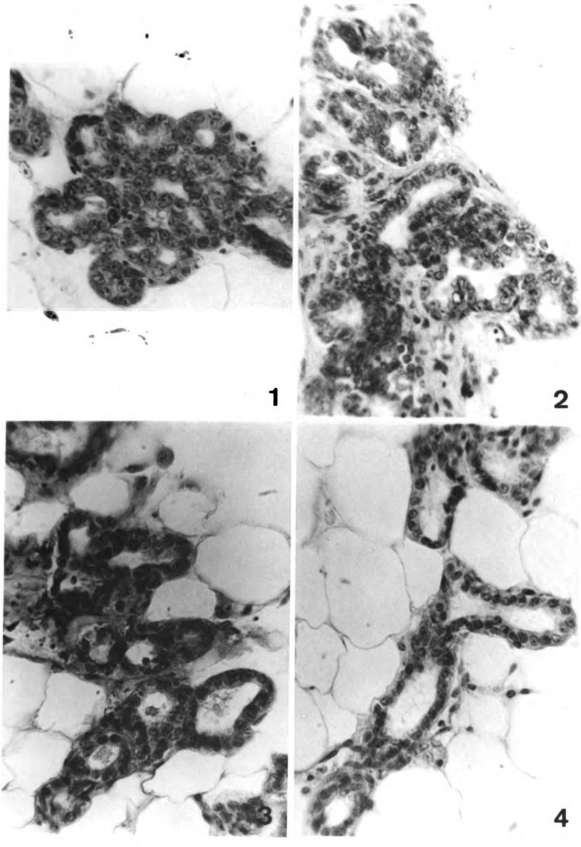
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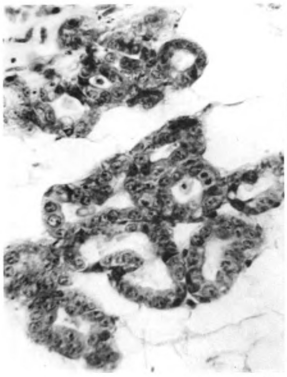
PLATE IV. Photomicrographs of Mammary Explants Cultured in the Presence of Insulin + Prolactin.

Figure 1. I + P at 24 hours. Maintenance of alveolar structure, distended lumina, scanty stainable secretion, and mitotic figure.

Figure 2. I + P at 48 hours. Maintenance of alveolar structure, distended lumina, and small amounts of stainable secretions.

Figure 3. I + P at 72 hours. Increased stainable secretion compared with 48 hours, but absence of intracellular vacuoles.

Figure 4. I + P at 96 hours. Small increase in stainable secretion, intracellular vacuoles conspicuously absent.



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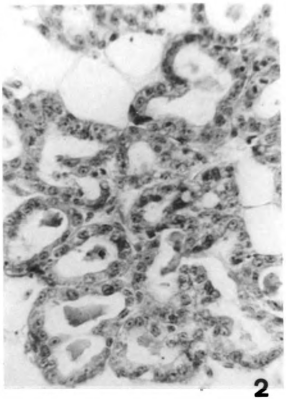

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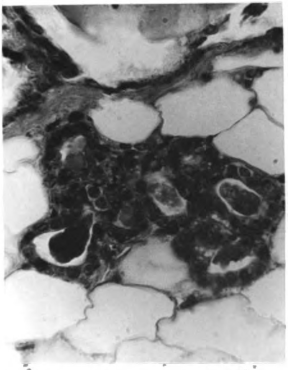

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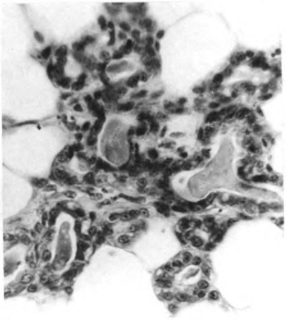

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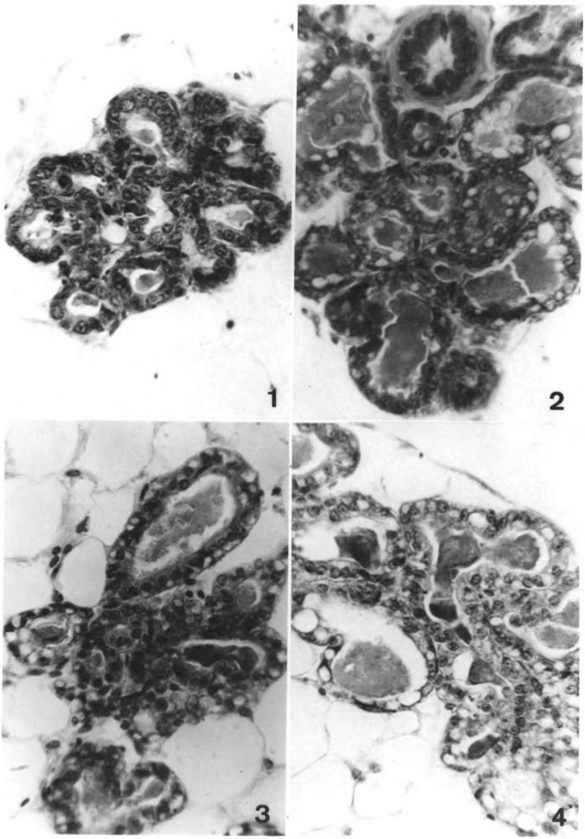
PLATE V. Photomicrographs of Mammary Explants Cultured in the Presence of Insulin + Prolactin + Corticosterone.

Figure 1. I + P + B at 24 hours. Maintenance of alveolar structure, distended lumina, and small amounts of stainable secretions. Intracellular vacuoles absent.

Figure 2. I + P + B at 48 hours. Active secretory appearance, i.e. large number of secretory vacuoles, abundant secretion in lumina (maximal secretion).

Figure 3. I + P + B at 72 hours. Overall development and secretory conditions not different from 48 hours (maximal secretion).

Figure 4. I + P + B at 96 hours. No difference from appearance at 48 hours, i.e. induced secretion maintained.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