ACTIVATION OF UDP-GLUCOSE PYROPHOSPHORYLASE AND EVALUATION OF THE LACTOSE SYNTHETASE ASSAY

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CHAPTER I

INTRODUCTION

Lactose (4-O-β-D-galactosyl-α-D-glucose) is the major carbohydrate component of the milk of most mammals, constituting about 2-7% of the whole milk (1). Free lactose is normally synthesized only in the mammary gland of lactating mammals and is considered to be unique to milk and mammary tissue. Both the development of the mammary gland and the secretion of milk require complex influences of hormones (2,3). Because the mammary gland of mammals develops during pregnancy and rapidly assumes a secretory state after parturition, the mammary gland of small mammals is an attractive model system for the study of differentiation and hormone action. The functional characteristic of the mammary gland is the production of milk which contains several biochemically unique products. Since lactose is one of the unique components of milk, the enzymatic capacity of the mammary gland to synthesize lactose is a useful criterion of the secretory state of the tissue.

Further interest in the mammary gland exists beyond its hormonal responses since the current leading cause of death due to cancer in women in the United States is breast cancer (4). Therefore, many investigators interested in the origin and prevention of cancers and tumors in mammary tissue are investigating mammary metabolism in order

to understand the function of the normal mammary gland so that the malfunction of abnormal tissue is better understood.

One technique widely used for investigating the effects of hormones on mammary tissue is the in vitro organ culture technique. The general procedure is to remove small pieces of tissue (explants) and to incubate them in chemically defined media where the hormonal complement is known. Thus the tissue is removed from the complex interactions of the neuro-endocrine system of the whole animal, and the effects of the hormones added to the culture medium may be evaluated without interference from other hormones or from neural factors.

Elias (5) and Rivera (6) used primarily histological criteria to evaluate the effects of hormones on the organ cultures. More recently, investigators have used biochemical criteria such as the synthesis of milk components to determine whether the tissue maintained in culture with a defined hormonal environment could attain a secretory condition. However, no enzymatic criteria had been used to assess secretory activity when these investigations were initiated. The initial approach was to use the levels of activity of the enzymes required for lactose biosynthesis as a criterion for studying which hormones were required to stimulate secretory activity. However, before evaluation of the enzymatic levels found in the tissue explants could be made it was necessary to have standard values of these enzymatic activities for comparison. Thus the activities of the three enzymes of lactose biosynthesis were assayed in homogenates prepared from the mammary glands of rats at different stages of pregnancy and lactation.

The three enzymes closely associated with lactose biosynthesis catalyze the following reactions:

1) UTP* + glucose-1-P $\rightleftharpoons$ UDP-glucose + PP_i

2) UDP-glucose $\rightleftharpoons$ UDP-galactose

3) UDP-galactose + glucose $\longrightarrow$ lactose + UDP

The enzyme which catalyzes Reaction 1 is UDP-glucose pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9), Reaction 2 is catalyzed by UDP-galactose 4-epimerase (EC 5.1.3.2), and lactose synthetase (UDP-galactose:D-glucose 1-galactosyltransferase, EC 2.4.1.22) catalyzes Reaction 3. Lactose synthetase requires two proteins designated as the A protein and the B protein (α-lactalbumin) for enzymatic activity.

To develop techniques for preparing tissue explants, culturing, and assaying for the enzymes in rat mammary tissue, UDP-glucose pyrophosphorylase was chosen as an indicator enzyme for these initial studies since it was easiest to assay and was thought to be the most stable of the three enzymes. In organ culture experiments, a time-dependent increase in the activity of the enzyme was observed in both the medium and the tissue explants. This increase was not affected by the presence of hormones (insulin, hydrocortisone, and prolactin), did not follow the same time course as reported for the synthesis of the milk protein casein (7), and was not inhibited by puromycin. These results suggested that the increase in enzymatic activity was due to

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* All abbreviations are in accordance with the IUPAC-IUB Combined Commission on Biochemical Nomenclature. J. Biol. Chem., 241, 527 (1966).

an *in vitro* activation of the enzyme rather than *de novo* synthesis. Accordingly, the increase in activity of UDP-glucose pyrophosphorylase in crude homogenates of lactating rat tissue was investigated and an increase in the activity of the enzyme in extracts, was observed. The increase in activity had a similar time course to that observed in the tissue explants. Investigation of the activity of UDP-galactose 4-epimerase also showed a time-dependent increase which was dependent on the presence of NAD^+ in the crude extract. The first part of this thesis is concerned mainly with preliminary investigations: the relative activities of the lactose synthesizing enzymes in pregnant and lactating rats, the organ culture experiments, and the increase in enzymatic activity of UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase in crude mammary gland extracts.

The second major concern of this thesis research was devoted to a detailed investigation of the mechanism of the "activation" of UDP-glucose pyrophosphorylase in crude homogenates of rat mammary tissue. The results suggested a rather slow conformational change or a slight structural modification resulting in an increase of a more active form of the UDP-glucose pyrophosphorylase.

The study of the activity levels of the three enzymes of lactose biosynthesis in pregnant and lactating rats was in progress when Brodbeck and Ebner (8) observed that the lactose synthetase enzyme required two proteins for activity. The assay for lactose synthetase was originally developed by Babad and Hassid (9) before it was known that the enzyme required two nonidentical proteins. In general, the assay for one protein component of a two-protein system is performed

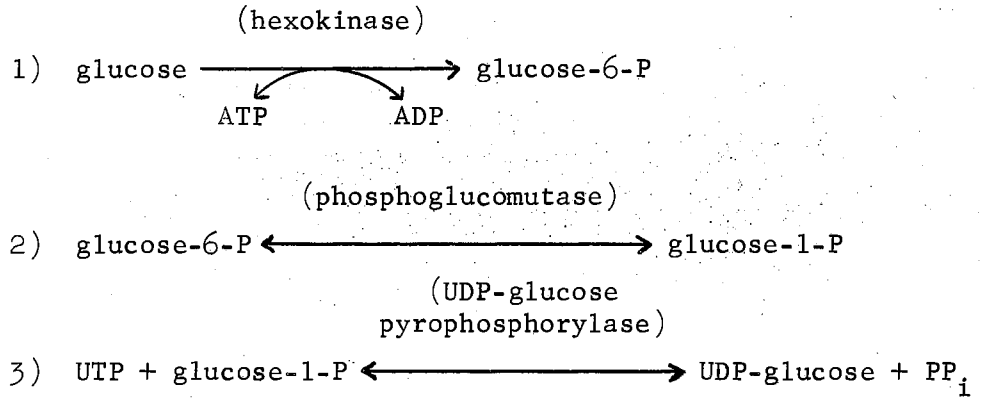
by using saturating amounts of the second protein. It was observed that the specific activity of the B protein of lactose synthetase increased from 150 to 1000 mμmoles/min/mg protein at 20° as a more purified A protein was used in the assay (10). Clearly, the assay required reinvestigation and no thorough investigation of the interactions between the two proteins and substrates of a two-protein enzyme was available. The third part of this thesis deals with the detailed investigation of the assay for lactose synthetase and the investigation of substrate interactions and certain kinetic parameters of the enzyme system. These experiments resulted in an assay with the assay components at an optimum level to provide maximum enzymatic activity and hence maximum sensitivity in the assay for one or the other of the counterpart proteins of lactose synthetase.

CHAPTER II

LITERATURE REVIEW

The Biosynthesis of Lactose

In 1952, Reithel et al. (11) first observed the formation of lactose in homogenates from mammary tissue of lactating rats and guinea pigs. A number of enzymatic steps appeared to be involved in the biosynthesis of lactose. Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) (12), phosphoglucomutase (α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) (13). UDP-glucose pyrophosphorylase (14,15), and UDP-galactose 4-epimerase (15,16) activities have been observed in lactating rat mammary glands. Gander et al. (17,18) reported that a crude preparation from bovine mammary tissue would synthesize lactose-1-phosphate from UDP-glucose and glucose-1-phosphate. They suggested that lactose is synthesized by the following scheme:



$$4)\ \text{UDP-glucose} \xleftrightarrow{\text{(UDP-galactose 4-epimerase)}} \text{UDP-galactose}$$

$$5)\ \text{UDP-galactose} + \text{glucose-1-P} \xrightarrow{\text{(galactosyl transferase)}} \text{lactose-1-P} + P_i$$

$$6)\ \text{lactose-1-P} \xrightarrow{\text{(phosphomonoesterase)}} \text{lactose} + P_i$$

However, this scheme has not been verified. Also, studies of the incorporation of ^{14}C-precursors into lactose are not consistent with this pathway. These studies have been reviewed by Malpress (19), Folley (20), and Watkins and Hassid (21).

In 1962, Watkins and Hassid (21) described a particulate fraction isolated from rat and guinea pig mammary glands which catalyzed the synthesis of lactose from UDP-galactose and glucose. A similar galactosyl transferase activity was found in bovine milk (22) and the soluble lactose synthetase of bovine milk was purified and investigated by Babad and Hassid (9). Their preparations contained no galactosyl transferase activity with glucose-1-phosphate and 25% of the activity with N-acetylglucosamine as the galactosyl acceptor respectively (9). Thus, the scheme for lactose biosynthesis currently accepted is:

$$3)\ \text{UTP} + \text{glu-1-P} \xleftrightarrow{\text{(UDP-glucose pyrophosphorylase)}} \text{UDP-glucose} + PP_i$$

$$4)\ \text{UDP-glucose} \xleftrightarrow{\text{(UDP-galactose 4-epimerase)}} \text{UDP-galactose}$$

$$7)\ \text{UDP-galactose} + \text{glu} \xrightarrow{\text{(galactosyl transferase)}} \text{lactose} + \text{UDP}$$

Further Enzymatic Studies

With the knowledge that the biosynthesis of lactose proceeds by the action of three enzymes: UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase, and a galactosyl transferase (lactose synthetase), further investigation of the role of these enzymes in lactation proceeded.

UDP-Glucose Pyrophosphorylase

UDP-glucose pyrophosphorylase is a ubiquitous enzyme which catalyzes a reaction important in the biosynthesis of glycogen, glycolipid, and glycoprotein as well as lactose. The enzyme has been purified 300-fold from calf liver and crystallized by Hansen and coworkers (23). Steelman and Ebner (24) purified (50-fold) the UDP-glucose pyrophosphorylase from bovine mammary tissue. Emery and Baldwin (25) purified the enzyme about 200-fold from lactating rat mammary glands and also prepared rabbit antibody to the rat enzyme.

UDP-Galactose 4-Epimerase

UDP-galactose 4-epimerase functions in the mammalian cell for the interconversion of glucose and galactose and for the synthesis of the galactosyl moiety of oligosaccharide-containing compounds such as lactose, galactolipids, and blood group substances. Partial purification (30-fold) of the enzyme from bovine mammary gland acetone powder was obtained by Holmberg (26). Further purification of the mammary enzyme is in progress (27).

Lactose Synthetase

As previously discussed, the active lactose synthetase found in bovine mammary tissue and milk has established the route for lactose biosynthesis (21,22,28). Babad and Hassid (9) achieved a 70-fold purification of the soluble milk enzyme.

Brodbeck and Ebner (8) have resolved the soluble lactose synthetase of bovine milk into two proteins, designated as A and B, which individually did not exhibit any catalytic activity for lactose formation in the assay used at that time. They also resolved the lactose synthetase of bovine mammary tissue into the same two proteins and determined the subcellular distribution of the tissue enzyme after homogenizing in a Virtis overhead homogenizer. The A protein was found mainly in the microsomal fraction while the B protein was in both the microsomal and soluble fractions (29). However, Reithel and Coffey (30) have shown that the apparent subcellular distribution of the enzyme is a function of the homogenizing method. When gentler techniques such as homogenizing with a glass homogenizer were used, intact lactose synthetase activity (assayed without added B protein) sedimented in the lysosomal fraction. Further centrifugation of the lysosomal fraction and comparison of the distribution of lactose synthetase with certain marker enzymes such as thiamine pyrophosphatase suggested that the lactose synthetase was associated with the Golgi apparatus (31). Electron micrographs of preparations with high lactose synthetase activity revealed particles that were similar in size and appearance to those of lysosomes and secretory granules (31).

Enzymatic Activities and the Pregnancy-Lactation Cycle

Other investigators have studied the mechanism of lactose synthesis in pregnant and lactating animals. In 1958, McLean (36) provided evidence that the major pathway of glucose metabolism in lactating rat or rabbit mammary tissue occurs *via* the pentose-phosphate pathway rather than glycolysis. Further, she measured the activity levels of hexokinase and phosphoglucomutase during lactation in the rat mammary gland. Hexokinase and phosphoglucomutase activities increased rapidly during the early part of lactation reaching a maximum at the 10th day. Malpress (19) pointed out the need for further enzymatic studies of the lactose biosynthetic enzymes at different times of pregnancy and lactation. Consequently, several research groups have determined the levels of parameters such as DNA, RNA, protein, and specific enzymatic activities in the mammary glands of animals during pregnancy, lactation or after weaning.

Malpress (37) assayed phosphoglucomutase, UDP-glucose pyrophosphorylase, and inorganic pyrophosphatase in rats lactating 6 days and 15 days. (The lactation period in the rat is 21 days.) The enzymatic activities varied directly with the milk-secretory activity which supports a role for these enzymes in lactose synthesis. Bartley *et al.* (38) found that the catalytic activities of glucokinase, hexokinase, glucose-6-phosphate dehydrogenase, malic enzyme, and citrate-cleavage enzyme which respond to dietary and hormonal changes in liver also increase during pregnancy and lactation in the liver and adipose tissue as well as in the mammary gland. These data indicate that lactation results in metabolic changes in other tissues as well as in the mammary

gland. Mumford (39) reviewed the quantitative measurements and the comparison studies of biochemical and structural changes which occur during the lactational cycle. A study of the water, fat, and lactose concentrations during pregnancy in primiparous rats has been presented by Wrenn et al. (40).

The first comprehensive comparative study of the levels of the lactose biosynthetic enzymes in rat mammary glands during the lactational cycle was by Shatton et al. (41). They assayed the levels of UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase, and hexokinase of pregnant and lactating rats. They observed the same pattern of activity for all three enzymes: very low level of activity in unstimulated glands, a moderate increase in activity during pregnancy, an activity maximum during full lactation, and a rapid decrease after weaning (41).

While this thesis research was in progress Baldwin (42) also observed large increases in enzymatic activities of enzymes from several metabolic pathways, including UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase, and phosphoglucomutase. Also, DNA levels during early lactation in the rat and guinea pig were measured. However, equivalently high increases were not seen in the cow. In a similar study in rats Baldwin and Milligan (43) observed that some twenty enzymatic activities increased rapidly after a 3-4 fold increase in the number of nuclei and DNA levels. The increase in the DNA levels followed soon after parturition. The relative increases in the activity levels of the enzymes (with respect to one enzyme, glucose-6-phosphate dehydrogenase, given a relative activity of 1) during

early lactation (1-3 days) were very similar to the relative increases observed between 15-17 days of lactation. Thus, Baldwin suggests that the new secretory cells formed early in lactation have an enzymatic complement similar to that of fully developed secretory cells. Also, he suggested that the enzymatic complement of virgin and pregnant rats probably reflects the adipose and connective tissue of the mammary gland, whereas the enzymes extracted from later lactating tissue probably represent primarily secretory tissue (43). Furthermore, the similarity between the early and late lactating enzymatic compositions coupled with the similar patterns of increase for practically all the enzymes investigated does not support the proposal of specific hormonal "induction" of certain preferred enzymes during lactation (37). Rather, Baldwin proposes that the hormones exert a passive influence by providing an environment in which secretory cells survive and develop an inherent enzymatic complement (42).

Emery and Baldwin (25) have purified UDP-glucose pyrophosphorylase and other enzymes from lactating rat mammary tissue and have prepared rabbit antisera to these enzymes. They investigated the turnover of the enzymes and established that the increases in enzymatic activity were due to increased rates of synthesis.

Kuhn and Lowenstein (44) expanded the work of Baldwin and Milligan (43) with a comparative enzymatic study primarily concerned with the time periods immediately preceding and following parturition. They observed a striking increase in the UDP-glucose pyrophosphorylase activity but a more gradual increase with the UDP-galactose 4-epimerase. They concluded that the changes in these enzymes are not abrupt

enough to account for the very rapid appearance of lactose (12-13 μmoles/g fresh weight) at parturition which is not present 24 hours prior to parturition. Also, while this thesis research was in progress, Kuhn (45) reported that the activity of lactose synthetase in rat mammary tissue homogenates was very nearly zero until just prior to parturition and has concluded that this enzyme is rate limiting for lactose biosynthesis. However, Kuhn assayed only inherent lactose synthetase activity. Turkington et al. (46) have assayed the separate A and B subunits of lactose synthetase in pregnant and lactating mice. They reported a substantial increase in the A subunit activity during pregnancy in contrast to the B subunit activity which increased significantly only at parturition. Thus, lactose biosynthesis is limited by the amount of lactose synthetase present and more specifically, by the amount of the B subunit of lactose synthetase.

In Vitro Action of Hormones on the Enzymes of Lactose Biosynthesis

For example, Jones (47) has studied the effect of hypophysectomy and weaning on the activities of UDP-glucose pyrophosphorylase and other enzymes in rats. He observed that hypophysectomy caused UDP-glucose pyrophosphorylase levels to decline to about 50% of control values during the first 12 hours after hypophysectomy. Also, the pattern of change in the mammary gland with respect to the enzymatic activities measured seems to be similar when the mother is hypophysectomized or the young are weaned. In hypophysectomized rats the administration of oxytocin, cortisol, and prolactin only partially replaced the pituitary influences as measured by the weight gain of the

litter of the hypophysectomized mothers (48). Baldwin and Martin (48) further observed that the administration of cortisol and prolactin to hypophysectomized rats would maintain the activity levels of several of the enzymes with respect to non-hypophysectomized controls but would not maintain other enzymes. However, casein synthesis was maintained at normal rates with prolactin or prolactin plus hydrocortisone treatment.

Heitzman (49,50) has measured UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase activities in pregnant and lactating rabbits. He reported that the patterns of change for the pyrophosphorylase and epimerase in rabbits are similar to those described for rats. Further, he was able to show significant increases of these enzymes upon treatment with human chorionic gonadotropin, prolactin, and hydrocortisone (49,50). Lactose synthetase activity was also observed in lactating and hormonally treated rats.

Enzymatic Activities and Mammary Tumors

Several investigators have begun enzymatic studies on mammary tumors. For example, Shatton et al. (41) assayed for UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase activities in normal rats as a prerequisite to studying the retention of functional enzymatic activities in neoplastic tissue. They reported that both rat mammary UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase activities were low in benign tumors in comparison to the values obtained with lactating rats but that the activities were elevated in malignant tumors. Archer and Orlando (51) investigated the enzymatic

patterns of alkaline phosphatase and β-glucuronidase as well as classified the growth patterns and histological appearance of rat mammary tumors induced by 7,12-dimethylbenzanthracene (DMBA). They found that the activity of alkaline phosphatase was significantly reduced in static tumors compared with actively growing tumors and that a small group of tumors with histological secretory appearance contained elevated β-glucuronidase activity.

Hilf and coworkers (52) have obtained a transplantable adenocarcinoma (mammary tumor) which undergoes a lactational response in non-lactating rats when treated with estrogen. They have shown that estrogen treatment of this tumor resulted in increased glucose-6-phosphate dehydrogenase, malate dehydrogenase (decarboxylating), and phosphoglucomutase activity and decreased activity of glucose-phosphate isomerase, isocitrate dehydrogenase, and glycerolphosphate dehydrogenase. However, the rate of growth of the tumor was inhibited by estrogen treatment (52). All the above enzymes increased when normally lactating tissue was treated with estrogen. After treatment with estrogen this same adenocarcinoma, R3230AC, produces a whitish fluid which has certain similarities to rat milk. It contains casein, similar whey proteins, and similar fatty acids but its lactose content is only about 0.06% compared with 3% for rat milk (53). Thus, the enzymes of lactose synthesis may provide a useful means of studying the hormonal control of this tumor which produces a milk-like fluid when it is stimulated by estrogen even though the tumor-bearing rat is not lactating.

Enzymes Requiring Two Proteins

Lactose synthetase is of interest not only because of its position as the terminal step in the reactions of lactose biosynthesis but also because of its rather unique protein structure. The enzyme apparently consists of two separate non-identical protein subunits, designated A and B, both of which are required for significant enzymatic activity (8). Thus, lactose synthetase is a member of a group of enzymes which require two separate proteins for activity and at present it is the only mammalian enzyme of this type.

At this time it is appropriate to define clearly the properties of the enzymes which are classified as requiring two proteins for activity, since this terminology does not unequivocally describe the enzymes. First, the enzyme may be resolved into two non-identical proteins which have different properties such as molecular weight, heat stability, or chromatographic behavior. In general, this dissociation occurs with mild treatment such as molecular sieve filtration differential gel adsorption, or ammonium sulfate fractionation and the proteins are recovered in an apparently unaltered state. That is, the protein fractions may be recombined to regenerate full or nearly full activity. Other enzymes may be dissociated into non-identical proteins with rather harsh treatments such as 8 M urea. However, these enzymes are excluded from the definition of two-protein enzymes since this dissociation does not appear to be a naturally occurring process. Next, the proteins appear to act in combination as a functional unit to catalyze a specific reaction. Neither protein exhibits significant catalytic activity in the reaction under consideration in the absence

of the counterpart protein. Perhaps these protein interactions may be interpreted as an extension of the models of Monod et al. (54,55) in which one protein acts as an allosteric effector of the other protein to give rise to the catalytic activity.

These criteria of non-identical protein "subunits" for catalytic activity characterize the enzymes which require two proteins. Currently there are nine enzymes which meet these criteria: tryptophan synthetase, lipoic acid-activating system, glutamate mutase, glycine decarboxylase, citramalate hydroylase, acetyl-CoA carboxylase, nucleoside diphosphate reductase, Qβ-replicase, and lactose synthetase.

There are certain technical problems associated with the two-protein subunit enzymes not found in single-protein enzymes which are pertinent to this thesis. Certainly the involvement of two proteins in the catalytic activity complicates the enzymatic assay. That is, in developing an assay for enzymatic activity, one must consider not only the effect of substrates and metals on the assay but also the interaction of the proteins with one another and with the substrates. Much of the data concerning the involvement of the two subunits suggests that the catalysis of the reaction occurs through the interaction of the proteins. The implication is that the two proteins react to form a complex which then can carry out the catalysis. The requirements for the formation of the complex vary with the enzyme system and generally complex formation requires substrates or cofactors. Such a complex has been found and characterized with the tryptophan synthetase of Escherichia coli (56,57,58). Another point of interest with the two-protein enzyme systems is an attempt to understand the bio-

logical role of the proteins. Do the two separate proteins provide a control mechanism or do they increase the versatility of enzymes in the organism? Some of the properties and possible functions of the two-protein enzymes are discussed in the following sections.

Tryptophan Synthetase

E. coli tryptophan synthetase (L-serine hydrolyase (adding indole), EC 4.2.1.20) was shown to consist of two non-identical and readily separable protein subunits by Crawford and Yanofsky (59). This enzyme catalyzes the terminal step in tryptophan biosynthesis according to the following reactions:

9) indoleglycerol-phosphate $\rightleftharpoons$ indole + glyceraldehyde-3-phosphate

10) indole + L-serine $\xrightarrow{\text{pyridoxal phosphate}}$ L-tryptophan

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11) indoleglycerol-phosphate $\xrightarrow{\text{pyridoxal phosphate}}$ L-tryptophan + glyceraldehyde-3-phosphate

Reaction 9 is catalyzed by the lower molecular weight subunit, α (A protein), and Reaction 10 is catalyzed by the higher molecular weight subunit, β (B protein), and requires pyridoxal phosphate (PLP). However, the isolated subunits have only trace catalytic activities, and maximum activity in Reaction 9 or 10 or any activity in the net reaction (Reaction 11) requires physical contact of the two proteins (59).

The β subunit consists of a dimer (108,000 molecular weight) of two polypeptide chains and the α subunit has a molecular weight of 29,500 (58). The amino acid sequence of the A protein (267 amino acids) is known (60). Sephadex G-200 chromatography and sucrose density gradient studies have indicated that two α-subunits and one β dimer form a complex designated as $\alpha_2\beta_2$ with a molecular weight of 159,000-163,000 (58,56,57). The association of the subunits was dependent on the presence of PLP and serine and the apparent association constant (K_a) was determined to be 4×10^6 - 2.6×10^9 $\underline{M}^{-1}$ (56).

Several other reactions have also been ascribed to the β subunit (61,62,63):

12) L-serine $\xrightleftharpoons{\text{PLP, } \beta}$ pyruvate + ammonia

13) Mercaptoethanol + L-serine $\xrightleftharpoons{\text{PLP, } \beta}$ S-hydroxyethyl-L-cysteine + H_2O

14) R-SH + L-serine + PLP $\xrightleftharpoons{\beta}$ R-S-mercaptopyruvate + PMP + H_2O

Recently Crawford and coworkers (63) have shown that the α subunit alters the relative activity of the β protein towards the possible reactions by altering the fate of the enzyme-bound phosphopyridoxyl-aminoacrylic acid intermediate. The protein completely inhibits transamination (Reaction 14) and deamination (Reaction 12), and greatly stimulates the indole β-addition (Reaction 11 of the tryptophan synthetase scheme). Thus a regulatory function of the α-protein with the possible reactions of the β subunit has been described.

The tryptophan synthetase of the mold Neurospora crassa behaves as a single component upon purification and is believed to be a single component (64,65). However, a recent description of the tryptophan synthetase of the tobacco plant Nicotiana tabacum var. Wisconsin 38 indicates that the enzyme of this species exists as two separate protein components separated by ammonium sulfate fractionation (66). Both the N. tabacum proteins can substitute for the analogous E. coli proteins in Reaction 11 but sucrose density gradient studies with substrates and cofactor were unable to reveal complex formation.

Lipoic Acid-Activating System

Extracts prepared from lipoic-acid deficient cells of Streptococcus faecalis contain an apopyruvate dehydrogenation system which is "activated" when lipoic acid is added to the extracts (67). After "activation" the extracts carry out the dehydrogenation of pyruvate (Reaction 15).

15) Pyruvate + NAD^+ + CoA $\rightleftharpoons$ acetyl-CoA + CO_2 + NADH + H^+

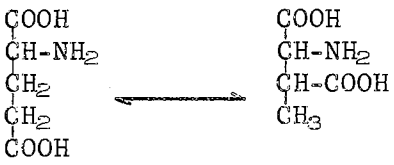
The apopyruvate dehydrogenation system was separated from the lipoic-acid activating system by protomine sulfate fractionation. Furthermore, the lipoic-acid activating system was separated into two essential components (PS-2A and PS-2B) when fractionated with ammonium sulfate at an alkaline pH. Both fractions PS-2A and PS-2B had to be incubated simultaneously with the apopyruvate dehydrogenation system, lipoic acid, and ATP to obtain an active pyruvate dehydrogenase system. The lipoic-acid activating system from E. coli would replace that from S. faecalis but the E. coli system was not resolved into components.

Fraction PS-2A was heat-labile and formed lipohydroxamic acid when incubated with lipoic acid, ATP, and hydroxylamine. Fraction PS-2B was stable to boiling water for 10 min, relatively stable to treatment with TCA, and partially inactivated by trypsin. Reed et al. (67) suggested that fraction PS-2A functions by activating the lipoic acid through an ATP-dependent reaction involving the formation of lipoyl adenylate and that fraction PS-2B may function as a carrier of the lipoyl moiety between a lipoyl adenylate-fraction PS-2A complex and the apopyruvate dehydrogenation system.

Glutamate Mutase

The bacterium Clostridium tetanomorphum initiates the degradation of glutamate by the action of glutamate mutase (EC 5.4.99.1) which catalyzes the conversion of glutamic acid to β-methylaspartic acid (68):

16)



In the purification of glutamate mutase, treatment with calcium phosphate gel yielded two protein fractions which had to be recombined for enzymatic activity (69). The gel supernatant fraction was designated as Component S and the gel eluate fraction as Component E. Component E has been further purified and has a molecular weight of 128,000 as determined from sucrose density gradient studies. Component E exhibited no activity without adding Component S to the reaction mixture but superoptimal levels of S resulted in inhibition of the reaction; the

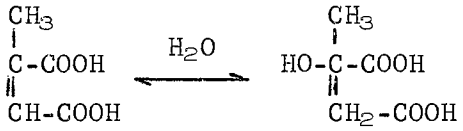
magnitude of the inhibition appeared to increase with the S:E ratio. Also, at higher levels of S, sigmoidal curves of activity vs. concentration of E were observed at low levels of E (69). Further purification of Component S, however, eliminated these effects and no inhibition by Component S was observed at any concentration examined (70); the authors suggested that the previous inhibitions had been due to impurities. The molecular weight of Component S was estimated by Sephadex G-100 gel filtration to be 18,000 for the monomer and 34,400 for the dimer (70).

While investigating the interactions of the E and S proteins Switzer and Barker (70) observed that Component E bound the coenzyme, dimethylbenzimidazolylcobamide, and that the amount of coenzyme bound was significantly increased in the presence of excess S protein. Thus, a function of Component S in the catalysis can be ascribed to its ability to decrease the apparent K_m of Component E for the cobamide coenzyme, although it was observed that increases in the S:E ratio which no longer resulted in further increases in mutase activity did continue to decrease the K_m for the coenzyme.

Experiments to obtain evidence for complex formation from an effluent of Sephadex G-100 were unsuccessful, but Switzer and Barker (70) suggested that the effects of Component S on the coenzyme binding by Component E do establish that the components interact. They proposed that the two proteins bind to each other rather weakly in a rapid, reversible reaction.

(+)-Citramalate Hydrolyase

Another enzyme in the glutamate degradation pathway of C. tetanomorphum which requires two proteins for activity is (+)-citramalate hydrolyase, also known as mesaconase (EC 4.2.1), which catalyzes Reaction 17:

17) 

Blair and Barker (71) reported the partial purification of this enzyme and its resolution into two protein fractions designated Component I and Component II by elution from DEAE-cellulose. The assay for 1-2 units of Component I was performed with 2.5 units of Component II and conversely 2.5 units of I were used in the assay of 0.2-0.9 units of II; both assays were linear at the one level of the counterpart component although both protein fractions contained small amounts of the other fraction. From Sephadex G-100 chromatography studies the molecular weight of I was 20,000-40,000 and the molecular weight of II was above 100,000; both proteins were heat labile.

Component II was inactivated by exposure to oxygen and required activation with a sulfhydryl reagent and Fe^{++}. No direct evidence for complex formation or for the role of either of the two protein fractions was presented, but a higher level of activity (two to five-fold) for Component II was observed when it was activated in the presence of Component I (71).

Glycine Decarboxylase

Another bacterial enzyme which requires two non-identical proteins is the glycine decarboxylase of Peptococcus glycinophilus (72). This enzyme catalyzes the labilization of the carboxyl group of glycine and is assayed according to the following reaction:

18) $HCO_3^- + \underset{\underset{NH_2}{|}}{CH_2}\text{-}{}^{14}COOH \longleftrightarrows H^{14}CO_3^- + \underset{\underset{NH_2}{|}}{CH_2}\text{-}COOH$

Klein and Sagers (72) separated the enzyme into two protein fractions, P_1 and P_2, with Sephadex G-100 chromatography. P_1 is heat labile and contains tightly bound pyridoxal phosphate; P_2 is a heat-stable, low molecular weight component which is inactivated by proteolytic enzymes. Further purification of both proteins was obtained, and a saturation curve of one level of P_1 and P_2 required relatively large quantities of P_2 (5-8 mg/ml) (73). No definitive function for either protein was described.

Acetyl-CoA Carboxylase

Alberts and Vagelos (74) recently described a two-protein, biotin-containing enzyme, acetyl-CoA carboxylase (EC 6.4.1.2) of E. coli. This requirement for two proteins has not been described for enzymes from other sources (74,75). Two protein fractions (E_a and E_b) were separated by alumina C_γ gel fractionation, and both protein fractions were required for enzymatic activity. E_a was sensitive to avidin and contained biotin and the carboxylate binding site. Studies with the two proteins established the following reaction sequence (74):

19) $E_a + HCO_3^- + ATP \overset{Mn^{++}}{\rightleftharpoons} E_a\text{-}CO_2^- + ADP + P_i$

20) $E_a\text{-}CO_2 + \text{acetyl-CoA} \overset{E_b}{\rightleftharpoons} E_a + \text{malonyl-CoA}$

---

21) $HCO_3^- + ATP + \text{acetyl-CoA} \rightleftharpoons ADP + P_i + \text{malonyl-CoA}$

Thus, a rather unique function is ascribed to protein E_a since it catalyzes the binding of CO_2 to biotin and also acts as a partial substrate for the transfer of the carboxylate group to acetyl-CoA by the protein E_b.

Assays of the proteins were shown to be linear with excess counterpart protein. Evidence was presented that the proteins do interact since E_b enhances the sensitivity of E_a to avidin inactivation and sigmoidal curves were obtained with activity *vs.* E_b plots when $E_a\text{-}CO_2^-$ was used as the substrate (74).

Ribonucleoside Diphosphate Reductase

Reichard (76) first reported the separation of *E. coli* ribonucleoside diphosphate reductase (fractions B1 and B2) with C_γ-alumina in 1962. The following scheme was proposed for the synthesis of deoxyCDP from CDP (77):

22) $\text{CDP} \xrightarrow[\text{B1, B2}]{\text{ATP, } Mg^{++}} \text{deoxy CDP}$

23) $\text{Thioredoxin-}(SH)_2 \rightleftharpoons \text{Thioredoxin-}S_2$

24) $TPN^+ \xrightarrow{\text{thioredoxin reductase}} TPNH + H^+$

Further study showed that the same four purified proteins (thioredoxin, thioredoxin reductase, B1, and B2), catalyzed the reduction of ADP and GDP as well as CDP and UDP, although the specificity was influenced by different nucleotide effectors (78,79). That is, ATP stimulated the

reduction of the pyrimidine ribonucleotides, dGTP the purine ribonucleotides, dTTP both, and dATP inhibited enzymatic activity with all four substrates (79).

Recently Reichard's group have demonstrated the existence of a complex of B1 and B2 by sucrose density gradient studies which required the presence of Mg^{++} for complex formation (80). They also showed that protein B1 binds radioactive nucleotides during Sephadex G-25 chromatographic studies (80). Thus it appears that the non-identical protein subunit B1 of ribonucleoside diphosphate reductase serves a regulatory function through its ability to bind the allosteric effectors.

Qβ-Replicase

Purified Qβ-replicase (a RNA-dependent RNA-polymerase induced by Qβ-bacteriophage) will catalyze the synthesis of biologically competent RNA (81,82). The active enzyme appears to exist as a "complex" which is dissociated into two protein fractions by successive and prolonged centrifugation in sucrose density gradients (83). The heavier component (molecular weight approximately 130,000) also has poly-C dependent poly-G synthetic activity; the lighter component has a molecular weight of approximately 80,000. Neither of the isolated proteins can initiate or complete the reaction catalyzed by Qβ-replicase in the absence of the other protein. This suggests that the proteins act as a functional unit.

The paucity of genetic information in the Qβ-RNA suggested that one of the proteins of the replicase might be derived from the E. coli host, and indeed Eikhom, Stockley, and Spiegelman (84) have isolated

a protein from uninfected cells which has all the properties of the "light" component. Furthermore, the "light" component was isolated from E. coli B, an F^- strain which cannot be infected by the Qβ phage. Thus, they have shown that one of the protein components of Qβ-replicase is host-specified. Current investigations are underway to determine the function of the "light" protein in uninfected cells (84).

Lactose Synthetase

Lactose synthetase (EC 2.4.1.22) from bovine skim milk was first observed to require two proteins by Brodbeck and Ebner (8). The proteins, designated A and B, were separated on Biogel P-30 into a larger molecular weight fraction (A) and a smaller molecular weight fraction (B). Similar elution patterns of the enzyme from Biogel P-30 indicated that the lactose synthetase from bovine mammary tissue was identical to the enzyme obtained from milk, and the subcellular distribution study of the tissue enzyme indicated the A protein was located mainly in the microsomal fraction while the B protein was located in both the microsomal and soluble fractions (29). The B protein has been purified to homogeneity and crystallized. It was subsequently identified as the well characterized milk protein α-lactalbumin (32), which then ascribed to α-lactalbumin a biological role as a subunit of lactose synthetase.

Further insight into the role of the two proteins has been presented by Brew et al. (34). These workers have found that the A protein alone will catalyze the formation of N-acetyllactosamine when N-acetylglucosamine is substituted for glucose:

25) UDP-galactose + N-acetyl-D-glucosamine $\xrightarrow{A,\ Mn^{++}}$ UDP + N-acetyllactosamine

Furthermore, this activity is inhibited by α-lactalbumin (B protein). They suggested that the B protein modifies the substrate (acceptor) specificity of the galactosyl transferase from N-acetylglucosamine to glucose, and designated α-lactalbumin a "specifier" protein (34).

Mammary Gland Response to Hormones: *In Vitro* Studies

The development of the mammary gland and maintenance of lactation require complicated hormonal interactions (2,3). Estrogen is believed to stimulate growth of the duct system in early pregnancy while progesterone and estrogen are later required for full alveolar growth. Prolactin is also thought to be influential at the end of pregnancy and at parturition.

Milk secretion is believed to be initiated by the withdrawal of estrogen and progesterone and the continuation of prolactin and adrenal corticoid influence (3). Turner has reviewed the *in vivo* experimentation which implicate these hormonal influences in the development of the mammary system and lactation (3). It was believed that the hormones may increase the rate of synthesis of certain selected enzymes at parturition (2), but the results of Baldwin and coworkers (43) show that the activity levels of many enzymes rise during lactation. They suggested that the hormones may exert more of a passive, permissive influence.

Because of the complex interactions of the neuro-endocrine system which may occur with whole-animal *in vivo* experiments, several researchers have turned to *in vitro* experiments with organ cultures in a chemically defined medium to study hormonal effects and differentiation. With this type of experiment the chemical environment, especially the hormonal environment, of the tissue may be controlled and definitive effects of the hormones may be sought. Generally, a small amount of tissue is incubated with the defined medium in a modified Petri dish with controlled atmosphere and sterile conditions.

Using such a technique, Elias (5) has successfully maintained adult mouse mammary tissue in a synthetic medium for five days. Histological studies indicated that cortisol and prolactin in Medium 199 with explants of 14-day pregnant mice maintained and stimulated the secretory activity of prelactating mammary lobules. Rivera (6) has since determined the minimal levels of hormones required to maintain and initiate secretion in C3H prelactating (10- to 13-day pregnant) lobules to be aldosterone (1 μg/ml), insulin (5 μg/ml) and prolactin (1 μg/ml).

Juergens *et al.* (7) measured the incorporation of ^{32}P into rennin-insoluble material (casein) as a biochemical criterion of differentiation. The mammary cultures from C3H/HeN mice (11-12 days pregnant) responded to insulin, hydrocortisone, and prolactin at a concentration of 5 μg/ml each; the peak in the casein synthesis was at 48 hours. The biochemical synthesis of casein as a criterion of differentiation has been correlated with the previously established histological changes by Stockdale *et al.* (85).

Further biochemical identification of the effects of the insulin-hydrocortisone-prolactin (IFP) medium has been pursued. Lockwood et al. (86) showed a 300-400% increase of α-lactalbumin and 125-225% increase of β-lactoglobulin in explants in the IFP medium; insulin alone caused only 100% stimulation of both proteins. Furthermore, the IFP medium caused a stimulation of casein, α-lactalbumin, and β-lactoglobulin synthesis of similar magnitudes and with similar time courses (86,87). Thus, the possibility of using the organ culture techniques to investigate the hormonal influences of lactose biosynthesis was established, since α-lactalbumin is a protein subunit of lactose synthetase. Also Brew and Campbell (88) established that the milk protein α-lactalbumin was synthesized during the incubation of whole cells. Guinea pig slices were incubated in a defined salt medium containing leucine-^{14}C, and 3.3 mg of α-lactalbumin-^{14}C were isolated from the medium.

Topper's group have further investigated the role of hormones in the differentiation of mammary tissue in explants. Stockdale and Topper (89) showed that DNA synthesis and/or cell division in the mammary epithelium is necessary for casein synthesis, and that the daughter cells have different functional properties when formed in different hormonal environments.

Lockwood et al. (90) have attempted to determine the site of action of the three hormones in the IFP system by transferring the cultures from media with certain combinations of hormones to media containing different hormones at different times in the culture period. They indicated that hydrocortisone acts during proliferative

periods (preceding or during mitosis), but that prolactin can act after mitosis has occurred. Insulin is required for the action of both hydrocortisone and prolactin, and the proposed model suggests that insulin is involved in the initiation of DNA synthesis. Insulin is also required during the phase after mitosis (90).

Turkington et al. (91) have provided evidence that prolactin acts to induce casein synthesis in the daughter cells formed in vitro in the presence of insulin and hydrocortisone rather than to increase the rate of synthesis of casein by pre-existing differential cells. Turkington (92) has also shown that the casein-producing cells previously formed in vivo lose their capacity to synthesize detectable amounts of casein after 96 hours in insulin-hydrocortisone (IF) medium, though they still can synthesize α-lactalbumin and β-lactoglobulin. The cells which had been incubated with the IF medium then synthesized casein when exposed to either human placental lactogen (HPL) or prolactin, but this required the continuing presence of insulin in the medium. Associated with the induction of milk protein synthesis is a rise in the rate of RNA synthesis which is inhibited by actinomycin D. This effect suggests that HPL or prolactin stimulates DNA-directed RNA synthesis (92).

The effects of in vitro incubations of mammary tissue on various enzymatic activities have been examined. Ebner et al. (93) assayed enzymatic activities in bovine mammary cell cultures to compare with enzymatic activities in the tissue from which the cultures were derived. Their results indicated a marked decrease in the specific activities of most of the enzymes, including UDP-galactose 4-epimerase,

in the cell cultures compared with the activities in the original tissue. They pointed out that the cells appear to lose their specialized functional characteristics in culture even though they remained visually in a "secretory" state.

In a preliminary communication Leader and Barry (94) have reported an increase in the activity of glucose-6-phosphate dehydrogenase after 12-24 hours in explants cultured in Medium 199 supplemented with insulin. This two-fold increase was inhibited by actinomycin D and cycloheximide but not by hydroxyurea; therefore, the increase is believed to require protein and RNA synthesis but not DNA synthesis.

The lactose synthetase system has been investigated by Turkington et al. (46) in both organ culture and in pregnant and lactating mice. Both A and B proteins in explants responded to the IFP medium, and they both showed the specific induction by prolactin after prior incubation with insulin and hydrocortisone. When the proteins were assayed in mice at different stages of pregnancy and lactation, the A activity reached a maximum just prior to parturition; the B activity, however, was low during pregnancy and increased rapidly after parturition. They suggested that the differential expression of the A and B proteins may be due to a unique control mechanism, but the nature of this mechanism is yet unknown.

Increases in Enzymatic Activity In Vitro

During this investigation time-dependent increases in enzymatic activity of UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase were observed in rat mammary gland homogenates maintained at room

temperatures. That is, an increase in the total enzymatic units as measured by a standard assay was observed as a function of time while the homogenates were allowed to stand at room temperature. The activity of UDP-glucose pyrophosphorylase was previously shown to increase in rat mammary gland homogenates with freezing and thawing (25). Similar time-dependent increases in the enzymatic activity of glycogen synthetase, 5'-nucleotidase, acetyl-CoA carboxylase, pyruvate kinase, and xanthine oxidase have been observed.

Gold and Segal (95) described an increase in the activities of both glucose-6-P "dependent" and "independent" glycogen synthetase (UDP-glucose:glycogen α-4-glucosyltransferase, EC 2.4.1.11) of rat liver. This increase (3- to 4-fold and 10-fold for the glucose-6-P dependent and independent activities, respectively) was observed in an 8,000 xg supernatant solution after transfer from 0° to 24°. The activation has been related to a conversion of the glycogen synthetase from one form (b) to an activated form (a) which has increased affinities for glucose-6-P, UDP-glucose, and inorganic phosphate (96). Gold (97) has observed that the two forms differ in the effects of EDTA and Mg^{++} on the stimulation and suggested that the activation produces a form of the enzyme with an increased affinity for Mg^{++} (an effective activator of glycogen synthetase). No further evidence concerning the mechanism of interconversion of the two forms was presented.

The activity of the 5'-nucleotidase of *E. coli* cell extracts may be increased by diluting the extracts with water and incubating them at 37° (98). Dvorak *et al.* (99) have shown that this activation is due to a gradual destruction of an proteinaceous inhibitor of the 5'-

nucleotidase and they have partially separated the inhibitor-enzyme complex from the inhibitor by Sephadex G-100 chromatography.

Rat liver acetyl-CoA carboxylase (acetyl-CoA:CO_2 ligase (ADP), EC 6.4.1.2) is activated by incubating either the partially purified enzyme or a 100,000 xg rat liver supernatant solution at 37° for 3-4 hours (100). This activation is accelerated when citrate is present and is associated with the aggregation of protomeric subunits as determined by sucrose density gradient studies. However, trypsin treatment also effects an activation of the acetyl-CoA carboxylase which is not accompanied by an increase in the molecular weight. The mechanism of the trypsin activation is not yet known.

Bailey et al. (101) have observed that preincubation of rat liver pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) at 25° for 5 min results in marked increases in the cooperativity of the enzyme with respect to the substrate phosphoenolpyruvate and the allosteric activator fructose-1,6-diphosphate. Hence an increase in activity occurs when these compounds are present in the assay. Bailey et al. (101) suggested that liver pyruvate kinase exists in two forms - L_A and L_B - and that the L_A form is converted to the L_B (activated) form during the activation. The mechanism for converting L_A to L_B was not discussed.

Corte and Stirpe (102) have reported that xanthine oxidase (xanthine-oxygen oxidoreductase, EC 1.2.3.2) activity with O_2 as the electron acceptor increases about 4- to 6-fold after storage at -20° for 24 hours. A study of activity vs. storage time at -20° resulted in a sigmoidal curve, but the activation mechanism was not clarified.

Thus, increases in activity upon incubation ("activation") have been described for five enzymes other than those described in this study: rat liver glycogen synthetase, E. coli 5'-nucleotidase, rat liver acetyl-CoA carboxylase, rat liver pyruvate kinase, and rat liver xanthine oxidase. Of the increases investigated, satisfactory mechanisms to explain the increase in enzymatic activity have been described for the 5'-nucleotidase (destruction of an inhibitor) and for the acetyl-CoA carboxylase (aggregation of subunits), although the action of trypsin on the acetyl-CoA carboxylase increase has not been explained. For the other enzymes, however, the physico-chemical basis of the enzymatic increases remains obscure.

CHAPTER III

PRELIMINARY EXPERIMENTS

Experimental Procedure

Preparation of Extracts

Animals were killed in a CO_2 chamber and the tissue was removed, minced finely with sharp-pointed scissors and homogenized in a buffer at 4° containing 0.15 M KCl, 0.005 M $MgCl_2$, and 0.005 M EDTA, pH 7.5 with a Sorvall Omnimixer (Model OM-1150), setting 8, at 0° for 30-60 sec. The organ culture explants of rat inguinal mammary glands (50 mg) were homogenized in 2 ml of the homogenizing buffer using the microattachment to the Sorvall Omnimixer. The homogenates were centrifuged at 10,000 xg for 10 min in a Sorvall RC-2 centrifuge at 4°. The same procedure was also used to prepare extracts from L-cells (0.2 g/ml of homogenizing buffer).

For the study of the activities of the mammary enzymes during the pregnancy-lactation cycle 1 g of rat abdominal mammary tissue was homogenized with 2 ml of the homogenizing buffer using the microattachment, and the homogenates were centrifuged at 30,000 xg for 15 min. The supernatant solution (S-30 supernatant) between a heavy layer of fat and the cellular debris was removed with a syringe fitted with a 4" needle. Microsomes were prepared by taking a portion of the

S-30 supernatant solution and centrifuging at 105,000 xg for 60 min or 80,000 xg for 120 min in a Spinco Model L refrigerated centrifuge. The precipitated microsomes were suspended in 0.26-2.0 ml of 0.25 M sucrose depending on the quantity of microsomes.

To prepare extracts for the activation studies usually 1 g of tissue was homogenized with 10 or 20 ml of the homogenizing buffer. The homogenates were centrifuged at 10,000 xg for 20 min and were filtered through glass wool. Bean, pea, and mung bean seeds were ground to a powder in a mortar and pestle and the powders were homogenized (see Table I for concentration) as were the animal tissues. After filtration through cheese cloth the plant extracts were centrifuged at 30,000 xg for 20 min and the supernatant solutions were passed through glass wool. E. coli K-12 wild type cells grown on minimal salts-glucose medium (103) to late log phase were harvested by centrifugation. One gram of the cells was suspended in 5 ml of the homogenizing buffer and disrupted for 15 min in a Raytheon Sonic Oscillator at maximum amperage. This mixture was then centrifuged at 30,000 xg for 15 min and the supernatant solution was used for the enzymatic extract.

Preparation of Organ Explants

Explants for organ culture were prepared similarly to the method of Juergens et al. (7). Three or four small pieces of tissue (50 mg total) were placed on a stainless steel grid in a 20-ml beaker so that they were just covered by 3 ml of Medium 199, Hank's salt base (104). The cultures were maintained at 37° in 95% O_2, 5% CO_2 at approxi-

TABLE I

INCREASE IN UDP-GLUCOSE PYROPHOSPHORYLASE ACTIVITY IN A VARIETY OF TISSUE HOMOGENATES AT 20° C

Source	Homogenate Concentration (mg/ml)	Initial Enzyme Activity (units/g tissue)	Time For Maximum Increase (Hr.)	Fold Increase At Maximum Value
1. Mammary Glands (lactating)				
Rat	50	2.63	12.3	8.9
Rabbit	100	0.29	12.7	6.2
Opossum	50	7.44	5.4	3.4
Guinea Pig	50	0.53	27.3	50.4
Bat	50	0.37	6.3	15.6
Hamster	100	0.18	11.7	2.7
Mouse	100	0.37	13.0	37.9
Bovine	50	1.33	4.7	2.1
2. Livers				
Rat	100	3.40	22.2	2.8
Rabbit	100	2.04	8.4	4.1
Opossum	50	7.59	12.5	2.8
Guinea Pig	50	0.56	31.0	17.1
Bovine	100	0.50	4.5	2.1
Hamster	50	2.98	2.7	1.7
Chicken	200	0.11	9.7	5.1
3. Others				
Rat Brain	50	0.003	3.5	55.6
Bean Seeds	200	0.11	6.0	10.2
Pea Seeds	200	0.65	6.0	2.3
Mung Bean Seeds	200	0.14	4.2	4.9
E. coli Cells	200	0.25	2.6	1.3
L-Cells	100	0.07	6.0	2.5

mately pH 7.5 up to 24 hr. Hormones (insulin, hydrocortisone, or prolactin) were added to the medium where indicated at a final concentration of 5 μg/ml and K-penicillin G (50 μg/ml) was added to all media. The media were sterilized by filtration through Millipore filters (0.45 μ).

Enzymatic Assays

UDP-glucose pyrophosphorylase was assayed by coupling the formation of UDP-glucose with UDP-glucose dehydrogenase as previously described (24). One unit of enzyme is the amount which catalyzes the formation of 1 μmole of UDP-glucose per minute.

UDP-galactose 4-epimerase was also measured by coupling the formation of UDP-glucose from UDP-galactose with UDP-glucose dehydrogenase (26). One unit of enzyme is the amount which catalyzes the conversion of 1 μmole of UDP-galactose to UDP-glucose per min. Care was taken with both assays to ensure that the amount of UDP-glucose dehydrogenase present in the reaction cuvette was not limiting. The change in absorbance for all the spectrophotometric assays used in these studies was followed at 340 mμ with a Cary-14 or Beckman DB recording spectrophotometer.

Lactose synthetase was assayed in the crude extracts by measuring the incorporation of UDP-galactose-^{14}C into lactose-^{14}C (8). The UDP-galactose-^{14}C was removed from the incubation mixture by passage through BioRad AG 11A8 and Dowex-1-formate. The lactose-^{14}C was collected in scintillation vials, Bray's scintillation fluid was added (105), and the samples were counted with a Packard Tri-Carb

liquid scintillation counter. One unit of lactose synthetase is the amount which catalyzes the synthesis of 1 mμmole of lactose per minute incubation.

Materials

UTP, glucose-1-P, K-penicillin G, puromycin, and NAD^+ were purchased from Sigma Chemical Co., St. Louis, Missouri. Medium 199 was from Baltimore Biological Lab.; insulin and hydrocortisone from Mann Research Laboratories; prolactin from the Endocrinology study section, National Institutes of Health; and Dowex-1 x 8 (50-100 mesh) and BioRad AG 11A8 (50-100 mesh) were from BioRad Laboratories. UDP-galactose-^{14}C (galactose-^{14}C) (120 mcuries/mmole) was purchased from New England Nuclear Corp., Boston, Mass. Non-radioactive UDP-galactose was synthesized by the method of Moffatt and Khorana (106,107). Any other chemicals were of reagent grade.

UDP-glucose dehydrogenase was prepared from bovine liver by the method of Strominger et al. (108) through Step 5. Rats were from Holtzman Co., Wisconsin or from the rat colony of the Biochemistry Department which were originally from Holtzman; guinea pigs and hamsters were from Don B Lab Animals, California. The lactating mouse was a gift from Dr. Ernest B. Hodnett, Chemistry Department. Beans (Kentucky Wonder Pole) and peas (Miragreen) were from Ferry Morse Seed Co., California; mung beans (Berken M-339) were obtained from the Agronomy Department. The L-cells were donated by Dr. M. L. Higgins of the Biochemistry Department, and the E. coli cells were supplied by Dr. Jerry Chandler of the Biochemistry Department.

Results

Activities of the Lactose Synthesizing Enzymes in Rat Mammary Gland Homogenates

Before an evaluation of the enzymatic activities in organ culture explants could be made, the base levels of these activities had to be determined for the mammary glands of control rats. Thus, a study of the activities of UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase, and lactose synthetase in mammary glands of normal, pregnant, lactating, and post-weaned rats was undertaken and the results are presented in Figure 1. UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase were assayed in the S-30 supernatant solution and lactose synthetase was assayed in the microsomal preparation as described in the Experimental Procedure. The activities of UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase increased somewhat during pregnancy and increased rapidly after parturition and in early lactation. The maximum activity is seen in mid- to late-lactating glands. Also, some activity of these enzymes could be detected in nonpregnant rats and in those of early pregnancy, even though this is not readily seen in Figure 1. Lactose synthetase, on the other hand, was not detected at all until prior to parturition. The activity of this enzyme appeared to increase rapidly after parturition and was at a maximum in late lactation. These results are consistent with previously published data (41,46,50).

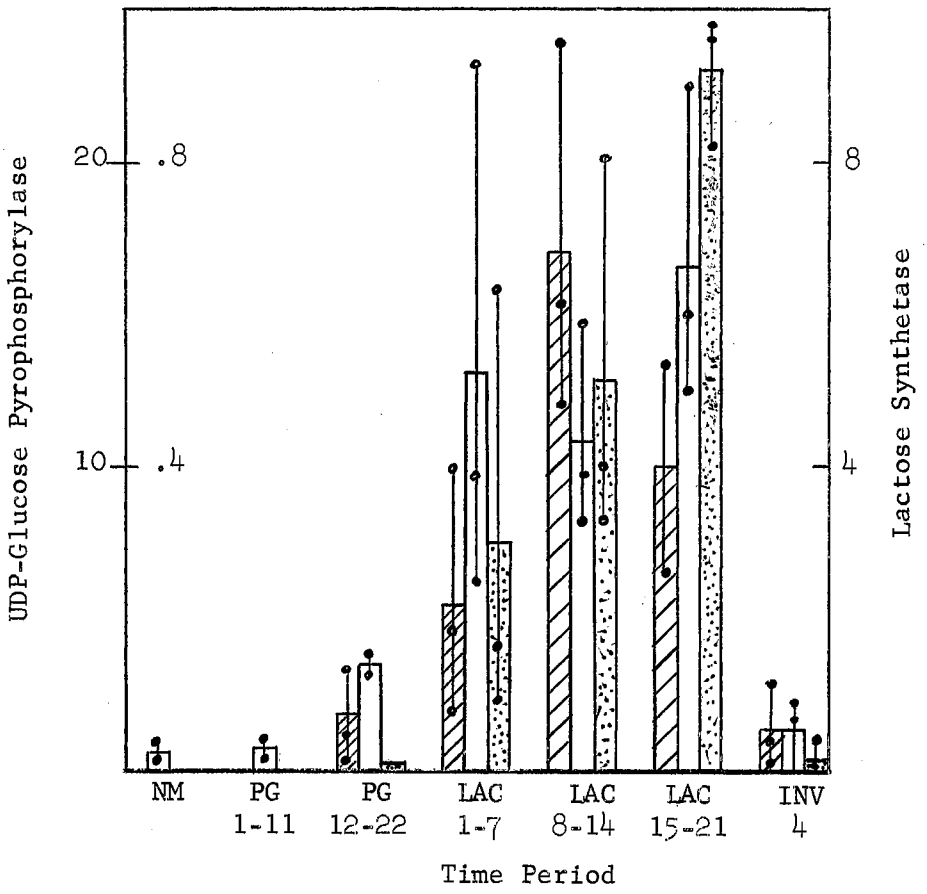


Figure 1. The Activities (Units/g Tissue) of the Enzymes of Lactose Biosynthesis in Homogenates of Rat Mammary Glands.

Time periods: normal; pregnant 1-11 and 12-22 days; lactating 1-7, 8-14, and 15-21 days; and involuted 4 days. The bar plots represent the mean of the values and the closed circles represent the values from individual rats: UDP-glucose pyrophosphorylase (cross-hatched), UDP-galactose 4-epimerase (unfilled) and lactose synthetase (dotted).

Organ Cultures of Rat Mammary Tissue

To develop the techniques for preparing the tissue explants, culturing them, and assaying for the enzymes, UDP-glucose pyrophosphorylase was chosen as an indicator enzyme. The total UDP-glucose pyrophosphorylase activity in the explants and the culture medium of the explants of a 16-day lactating rat with or without added hormones as a function of time is shown in Figure 2. The enzymatic value at time zero is the activity found in freshly prepared explant homogenates at the initiation of incubation. Both the explants and the medium contained similar levels of enzymatic activity which indicates that a considerable amount of the enzyme was leaking into the medium. The total amount of activity increased in a nearly linear manner for 10 hours and then decreased at 24 hours. The data in Figure 2 suggest that hormones (cortisol, prolactin, and insulin) increased enzymatic activity but this single experiment is misleading; other experiments have shown that the increase in enzymatic activity is independent of hormones. That is, the differences seen in Figure 2 are not significant since there was considerable variation in the extent of enzymatic increase in the explants (6- to 10-fold for late lactating tissue) and other experiments analogous to that represented in Figure 2 showed less increase in enzymatic activity for explants and medium incubated with hormones. Furthermore, the time of maximum increase (10 hr) observed for the rat mammary explants does not coincide with the time of maximum synthesis of casein (24-48 hr) observed in mice mammary explants (7). Also, the synthesis of casein in the mouse explants was dependent on the presence of hormones in the medium and was inhi-

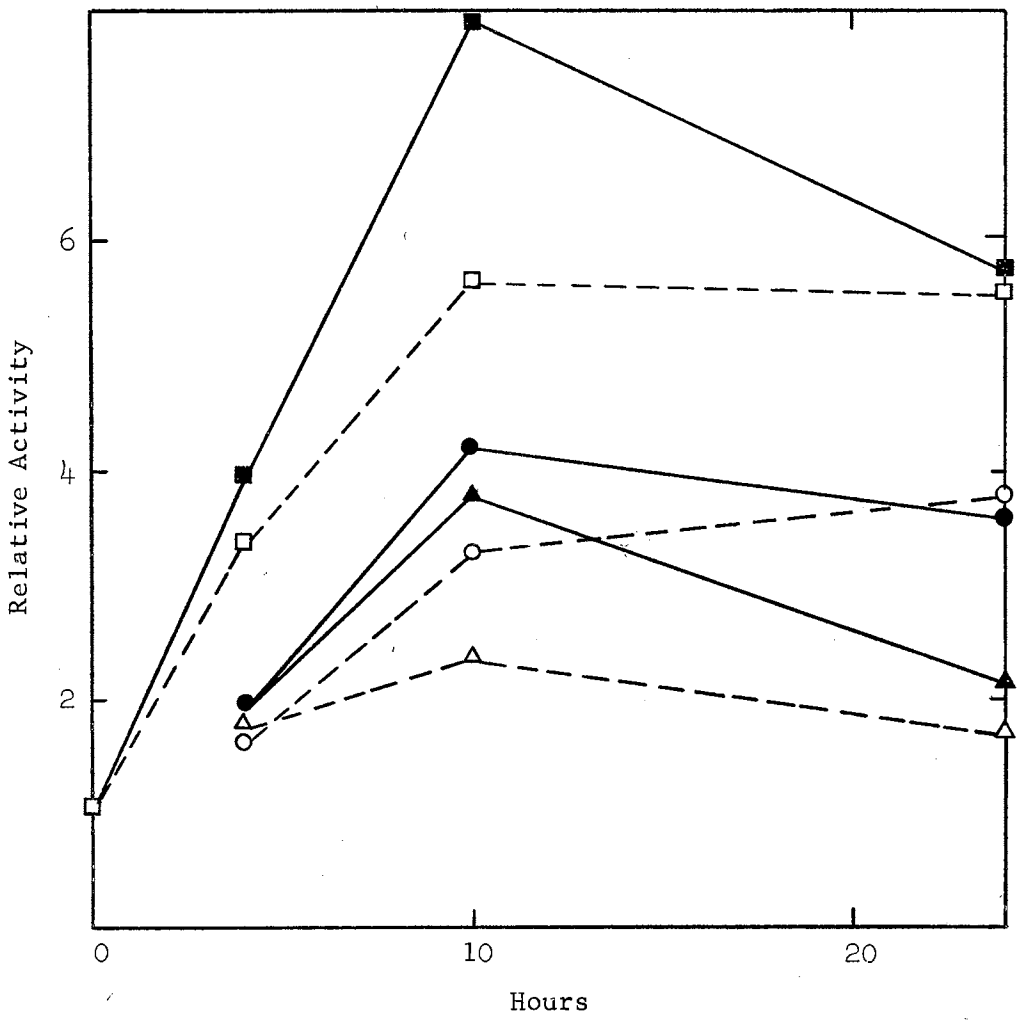


Figure 2. Activity of UDP-Glucose Pyrophosphorylase in Explants and Medium from 16-Day Lactating Rat Mammary Gland.

Open figures are in the absence of hormones and closed figures are in the presence of hormones (prolactin, hydrocortisone, and insulin; 5 μg/ml). ● - ●, Medium; ▲-▲ , tissue; ■-■ , total of medium and tissue with hormones. O - O, Medium; △−△, tissue; □−□ , total of medium and tissue without hormones. Relative activity 1.0 = 0.15 units/g tissue. Cultures were maintained at 37°.

bited by puromycin (7). However, the UDP-glucose pyrophosphorylase increase in rat mammary explants (Figure 2) does not require hormones, and the addition of 0.2 mM puromycin to the medium did not inhibit the time-dependent increase in enzymatic activity. Thus, the increase in enzymatic activity appears to be from a process other than enzyme-protein synthesis.

A similar investigation of the activity of UDP-galactose 4-epimerase in 20-day lactating rat mammary explants cultured in the presence or absence of hormones is presented in Figure 3. No activity of this enzyme was detected in the medium. Figure 3 demonstrates a time-dependent decay of UDP-galactose 4-epimerase in the mammary tissue explants with no significant effect of hormones. Thus, it appears that organ cultures of rat mammary gland with insulin, hydrocortisone, and prolactin with the techniques described do not exhibit increased synthesis of UDP-galactose 4-epimerase or UDP-glucose pyrophosphorylase.

Enzymatic Activity Increase in Crude Homogenates

The time-dependent increase in UDP-glucose pyrophosphorylase activity observed in the medium and tissue explants was not affected by the presence of insulin, hydrocortisone, and prolactin (IFP); did not follow the time course reported for the synthesis of casein (7); and was not inhibited by puromycin. These results suggested that an in vitro "activation" of a pre-existing enzyme form was occurring. Therefore, tissue extracts of rat mammary glands were examined for a time-dependent increase in the activity of UDP-glucose pyrophosphorylase.

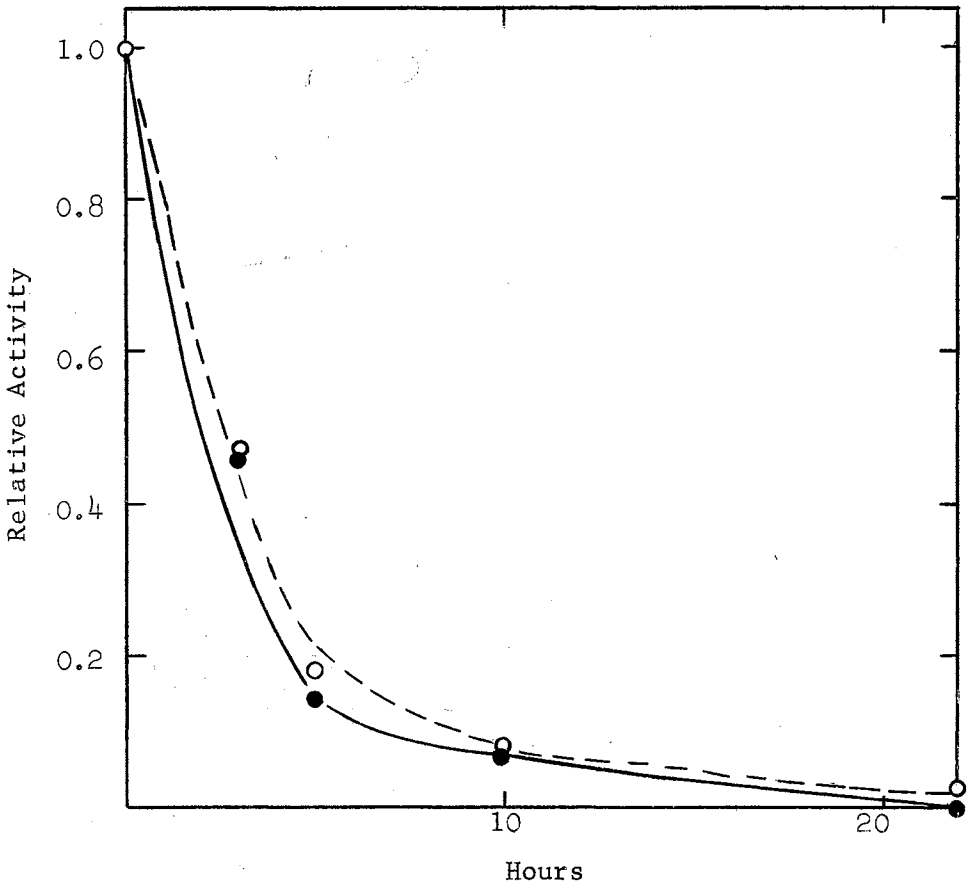


Figure 3. Activity of UDP-Galactose 4-Epimerase in 20-Day Lactating Rat Mammary Explants.

● - ●, activity in explants cultured in Medium 199 containing insulin, hydrocortisone, and prolactin (5 μg/ml each). O - O, activity in explants cultured without hormones. Relative activity 1.00 = 0.599 units/g tissue. The cultures were maintained at 37°.

Figure 4 demonstrates the time-dependent "activation" of UDP-glucose pyrophosphorylase in mammary gland homogenates from a 19-day lactating rat and an 18-day pregnant rat. The time course of the activity increase is consistent with that observed in the organ cultures: a maximum at 10 hours and a decline at 24 hours.

The effect of the temperature of incubation on the increase of the pyrophosphorylase activity in the homogenates is presented in Figure 5. There is a 2-4 hour lag at 0° which decreases at 15° and nearly disappears at 24° or at 37°. Also, the extent of increase is directly related to the temperature of incubation; the highest fold increase is seen at 37°. Thus the increase in activity exhibits some dependence on the temperature of incubation.

The rat mammary homogenates were also examined for an increase in the activity of UDP-galactose 4-epimerase. The time-dependent increase in the epimerase activity in 13-day lactating rat mammary gland homogenates at 0° with or without added NAD^+ is presented in Figure 6. In the homogenate without NAD^+, the UDP-galactose 4-epimerase activity increased 9-fold in about 3.5 hours and then fell rapidly. When 5 mM NAD^+ was present the activation occurred more slowly, reached a maximum at approximately 25 hours and then declined. (The NAD^+ must be added in a buffered solution. Addition of crystalline NAD^+ lowered the pH, and loss of activity of the enzyme probably due to denaturation occurred.) Addition of NAD^+ to the extracts was suggested by the observation of C.M. Tsai (27) that NAD^+ stabilizes the epimerase during purification.

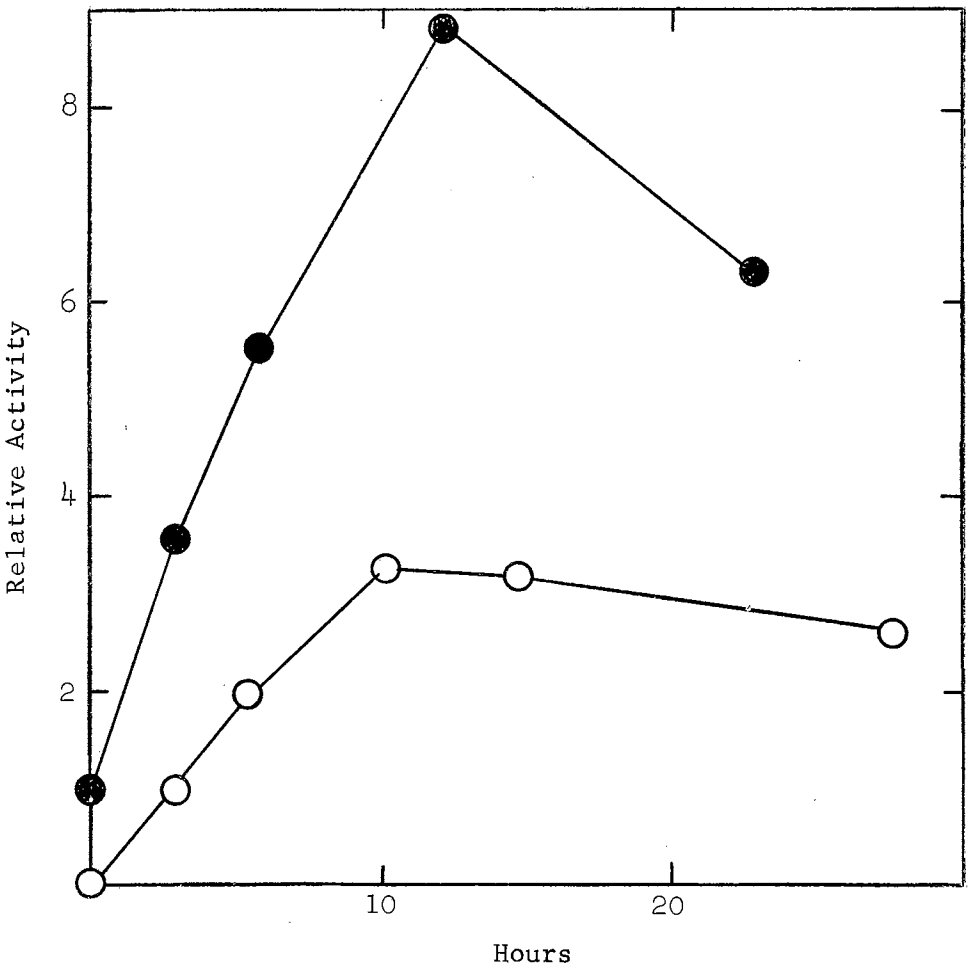


Figure 4. Time-Dependent Increase in UDP-Glucose Pyrophosphorylase Activity in Rat Mammary Gland Homogenates.

● - ●, 19-day lactating rat, 1 g/20 ml homogenate, relative activity 1.0 = 2.6 units/g. O - O, 18-day pregnant rat, 1 g/5 ml homogenate, relative activity 1.0 = 0.29 units/g. Incubations were at 24°.

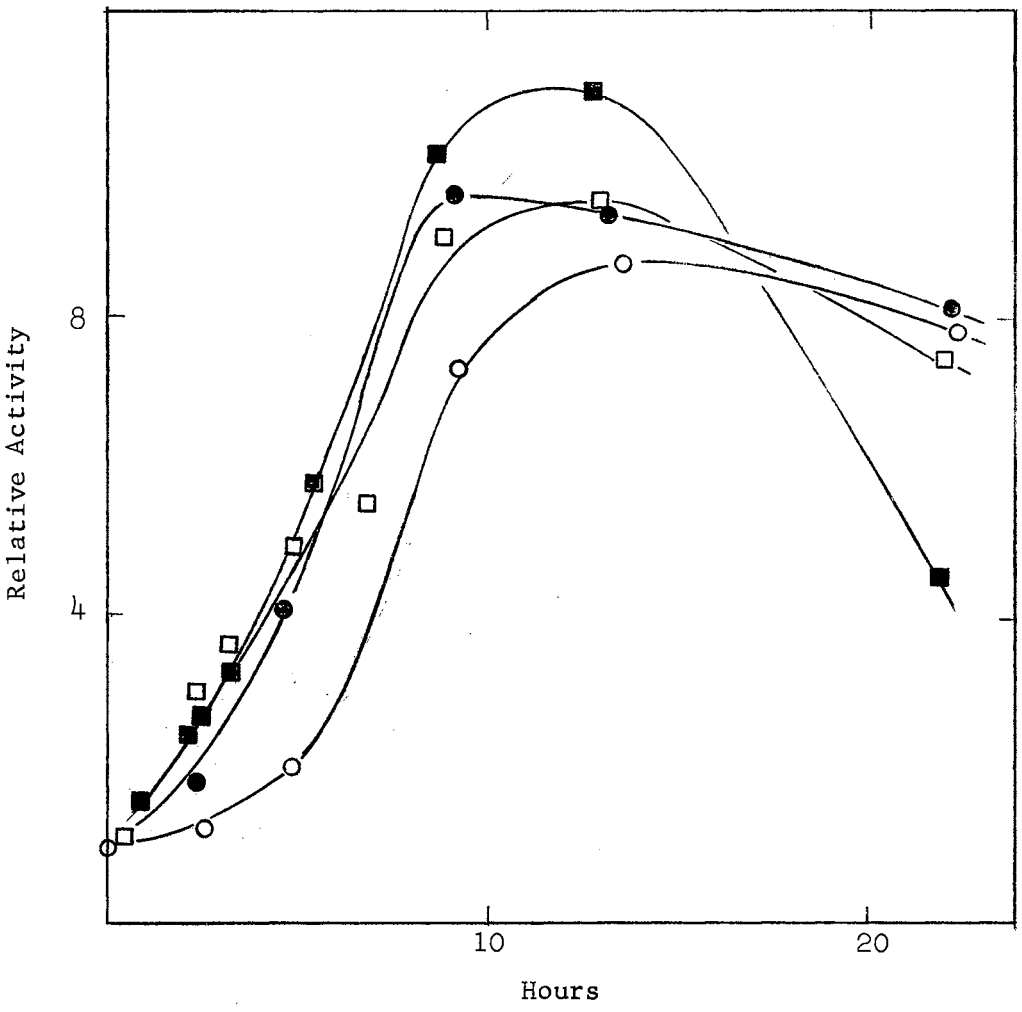


Figure 5. Increase in Activity of UDP-Glucose Pyrophosphorylase in 10-day Lactating Rat Mammary Gland Extracts as a Function of Temperature.

○ - ○, 0°; ● - ●, 15°; □-□ , 25°; and ■-■ , 37°. Relative activity 1.00 = 1.42 units/g tissue.

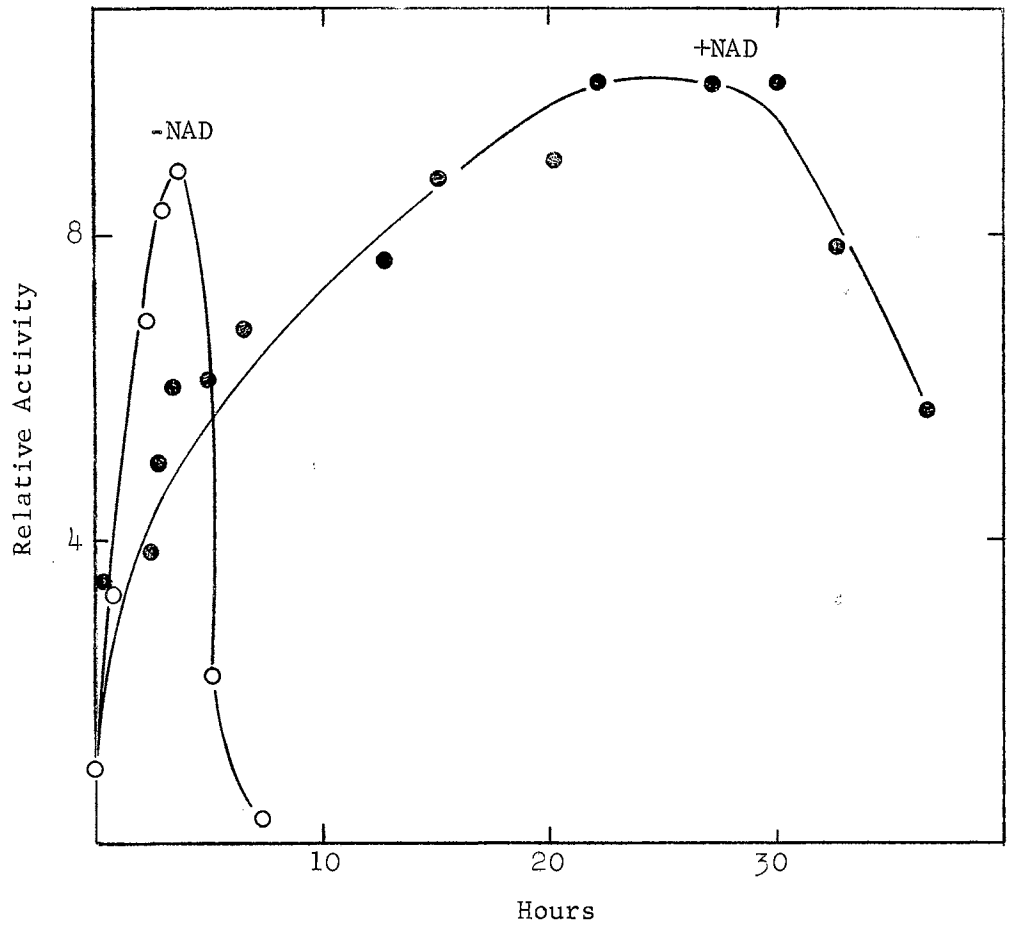


Figure 6. Time-Dependent Increase in UDP-Galactose 4-Epimerase Activity in 13-Day Lactating Rat Mammary Gland Homogenates.

O - O, Activity in homogenates without added NAD^+; ● - ●, activity in homogenate when 5 mM NAD^+ (0.05 ml of 100 mM stock solution in 0.1 M phosphate buffer, pH 7.6 added to 0.95 ml of extract) was added. Relative activity 1.00 = 0.121 units/g tissue and incubation was at 0°.

The response of UDP-galactose 4-epimerase "activation" to temperature is illustrated in Figure 7. The rate of increase in activity and subsequent decay is temperature-dependent both in the presence and absence of NAD^+. The activity increased and decreased very rapidly at 24°, more slowly at 15°, and most slowly at 0°. Moreover, the extent of activation is greater at lower temperatures. Also, the presence of NAD^+ in the homogenate retards the rate of increase in enzymatic activity and the rate of decay. Thus, the increase in activity of UDP-galactose 4-epimerase is a function of both temperature and the presence of NAD^+ in the homogenate, although the effect of the incubation temperature seems to be somewhat different from that of the UDP-glucose pyrophosphorylase.

To determine whether the increase in activity of UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase upon incubation was found in other tissues and species or if the increase was restricted to rat mammary glands, a variety of tissue extracts were examined for "activation". Table I shows the results of incubating the homogenates from various tissue sources on the activity of UDP-glucose pyrophosphorylase. Almost all of the homogenates showed some increase in activity although the extent of activation varied somewhat as did the time for maximum activity. Also, the initial level of the pyrophosphorylase activity showed considerable variation from different sources.

Similar increases in activity in other species are also seen with the UDP-galactose 4-epimerase (Table II). Again, the increase in activity is dependent on NAD^+. Thus, the increase in the activi-

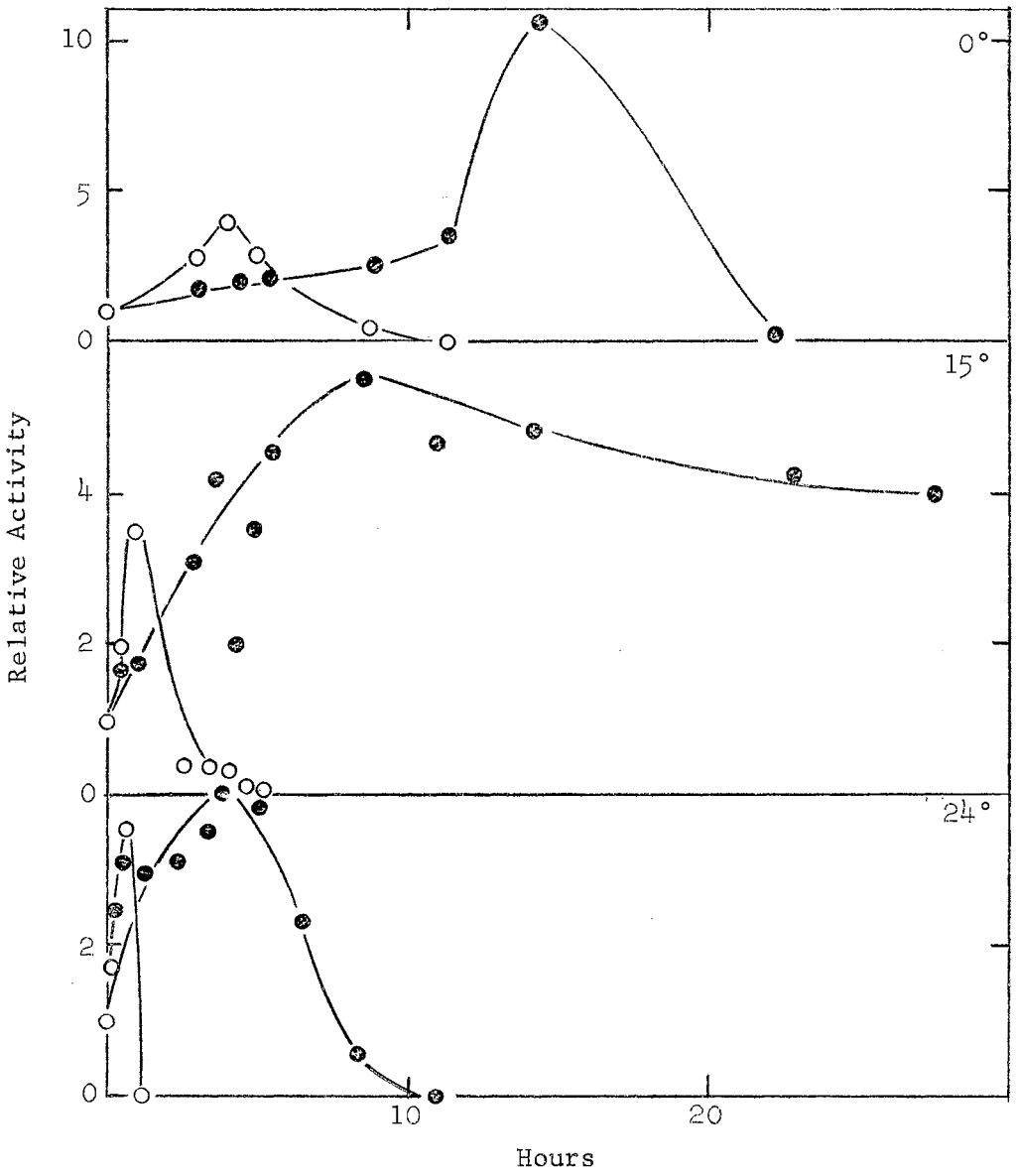


Figure 7. Increase in UDP-Galactose 4-Epimerase Activity in 16-Day Lactating Rat Mammary Gland Extracts at 0°, 15°, and 24°.

O - O, Activity in homogenate without added NAD^+.
● - ●, Activity in homogenate with 5 mM NAD^+ in the tissue extract. Relative activity 1.00 = 0.605 units/g tissue.

TABLE II

INCREASE IN UDP-GALACTOSE 4-EPIMERASE ACTIVITY IN A VARIETY OF TISSUE HOMOGENATES AT 20° C

Source	Homogenate Concentration (mg/ml)	Initial Enzyme Activity (units/g tissue)	Time For Maximum Increase (Hr.)		Fold Increase At Maximum Value	
			$-NAD^+$	$+NAD^+$	$-NAD^+$	$+NAD^+$
1. Mammary Glands (lactating)						
Rat	100	0.61	2.2	3.9	3.7	4.0
Mouse	100	2.0	2.7*	4.5	---	2.2
Hamster	100	0.1	2.4*	5.9	---	1.7
Guinea Pig	50	46.3	3.0*	12.7	---	2.9
Rabbit	100	0.4	2.6*	12.7	---	3.8
Opossum	50	2.8	4.1*	6.1	---	2.0
Bat	50	6.4	2.1*	5.0	---	1.2
2. Other						
L-Cells	200	0.05	5.1	3.5	2.6	2.4

* In some cases, a decrease in activity was observed the first time the homogenate was assayed after zero time. It is possible that the first assay was performed after the activation had occurred and only the decline in activity was observed.

ties of UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase with incubation is not restricted to rat mammary tissue but occurs in many tissue extracts.

CHAPTER IV

INVESTIGATION OF THE INCREASE IN UDP-GLUCOSE PYROPHOSPHORYLASE ACTIVITY IN RAT MAMMARY GLAND HOMOGENATES

Experimental Procedure

Homogenates of lactating rat mammary glands were prepared and UDP-glucose pyrophosphorylase was assayed as described in Chapter III. In a few experiments another homogenizing medium such as 4% sucrose or 0.1 M potassium phosphate, pH 7.6 was substituted for the homogenizing buffer (0.15 M KCl, 0.005 M $MgCl_2$, 0.005 M EDTA, pH 7.5) but the homogenization procedure was the same as in Chapter III. Acetone powders were prepared from a lactating rat mammary gland by twice extracting 1.4 g of tissue with 15 volumes of acetone at -15°. The yield of the acetone powder was 16.5%.

Sucrose density gradient studies were carried out by the method of Martin and Ames (109) in 5-20% linear sucrose gradients containing 0.15 M KCl, 0.005 M $MgCl_2$, and 0.005 M EDTA, pH 7.5. The tissue extract (0.1 ml of the supernatant solution from 37,000 xg centrifugation for 20 min) was layered onto the gradients which were centrifuged at 65,000 r.p.m. for 1.5 hours in a Spinco L2-65 ultracentrifuge using the SW-65 rotor. The bottoms of the tubes were punctured and 10-drop fractions were collected. The K_m for UTP was determined as described by Steelman and Ebner (24). UDP-glucose pyrophosphorylase was also

measured in a few cases by determining glucose-1-phosphate formation as previously described (24).

Phenylmethylsulfonyl fluoride and UDP-glucose were purchased from Sigma Chemical Co.; Sephadex G-25, medium, from Pharmacia; and sodium cholate was from Mann Research Laboratories. All other materials were previously described in Chapter III.

Results

The results presented in Chapter III demonstrate that a time-dependent increase in the activity of UDP-glucose pyrophosphorylase was observed in the medium and tissue explants of rat mammary organ cultures. This increase was not affected by the presence of hormones (IFP), did not follow the same time course as reported for the synthesis of casein (7), and was not inhibited by puromycin. These results suggested that the time-dependent increase in the activity of UDP-glucose pyrophosphorylase was not due to hormone-mediated increase in the synthesis of enzyme protein but rather to an in vitro "activation" of a pre-existing enzyme form. This suggestion was supported by the finding that a similar time-dependent increase in the activity occurred in crude homogenates of mammary glands and other tissues. Thus, an investigation of the nature of the "activation" phenomenon observed in crude extracts was carried out. UDP-glucose pyrophosphorylase was chosen for these studies since the investigation of the increase in activity of UDP-galactose 4-epimerase was complicated by the role of NAD^+.

General Nature of the Activity Increase

Several experiments were performed to obtain information about the general nature of the "activation" process. The effects of treating the rat mammary tissue extract with phenylmethylsulfonyl fluoride (an inhibitor of certain types of proteolysis), the detergent sodium cholate, 4 M urea, and 0.2 M potassium phosphate, pH 6.2 are illustrated in Figure 8. Neither phenylmethylsulfonyl fluoride nor sodium cholate affected the increase in activity; however, the activity remained essentially constant in 0.2 M potassium phosphate, pH 6.2. Treatment with 4 M urea, however, resulted in an initial rapid increase followed by a decrease in activity. Thus, the increase in activity is not affected by the presence of phenylmethylsulfonyl fluoride or sodium cholate but is inhibited at pH 6.2 or in 4 M urea.

The extent of activation was dependent on the ratio of tissue to homogenizing buffer and the effect of diluting the original extract 5-, 10-, and 25-fold with the homogenizing buffer is seen in Figure 9. The samples which were diluted increased in activity to only half the extent of the undiluted control. For more actively lactating rats (15-21 days) a greater extent of increase in activity was found with 1 g tissue/20 ml homogenizing buffer, and for the less actively lactating rats (1-14 days) the ratio of 1 g tissue/10 ml of homogenizing buffer yielded a higher extent of increase.

Examination of Possible Ionic Effects on Interactions with Low Molecular Weight Compounds

The homogenizing medium might play a role in the activation pro-

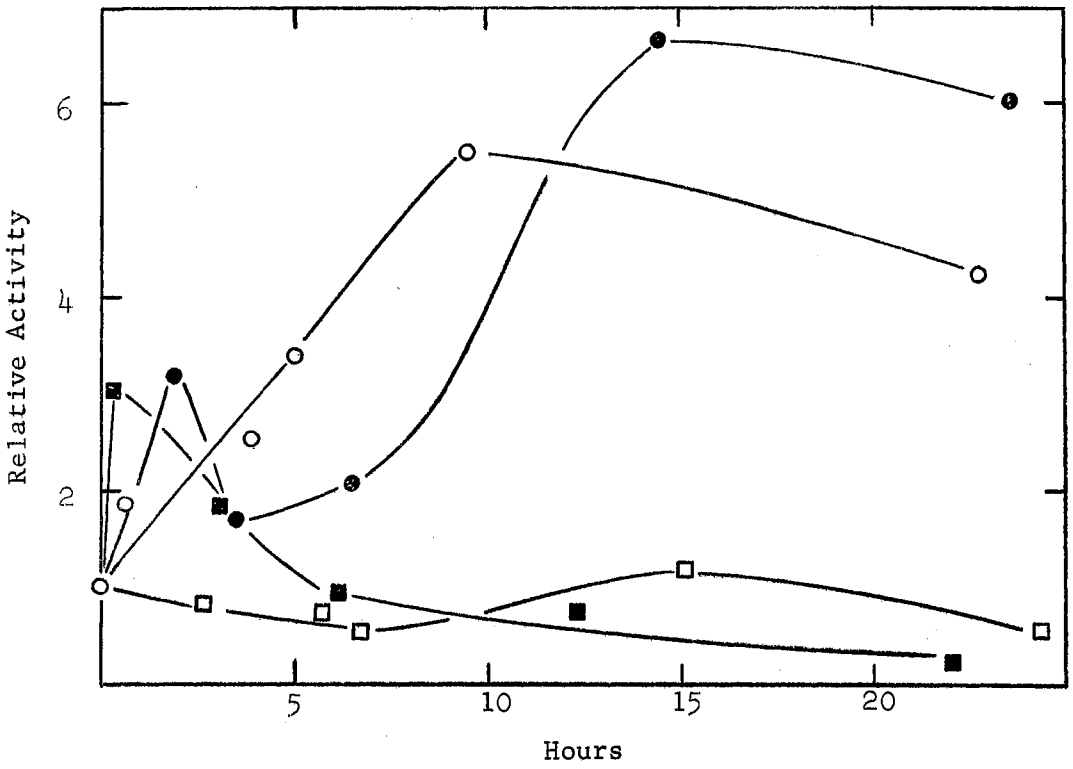


Figure 8. Effect of Treatment on the Activity of UDP-Glucose Pyrophosphorylase.

(○ - ○), 0.5 mM Phenylmethylsulfonylfluoride added to a 20-day lactating rat mammary homogenate (50 mg tissue/ml). Relative activity 1.0 = 2.91 units/g tissue. (● - ●), 1% sodium cholate; (■-■), 4 M urea; and (□-□), pH 6.2 of 0.2 M potassium phosphate added to an equal volume of the homogenate. The homogenate (1 g tissue/20 ml) was from a 19-day lactating rat. Relative activity 1.0 = 2.63 units/g tissue. All incubations were at 24°.

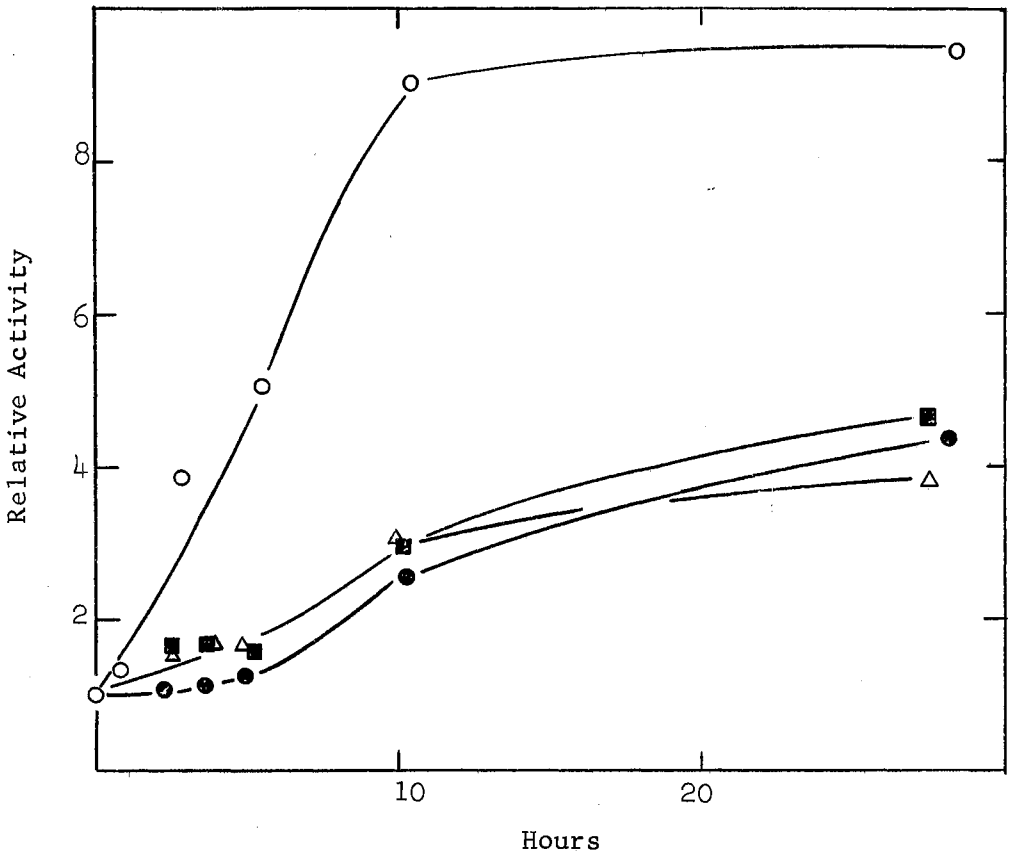


Figure 9. Activity of UDP-Glucose Pyrophosphorylase in Diluted Extracts from 16-Day Lactating Rat.

(O - O), Normal control, undiluted (100 mg tissue/ 2 ml homogenizing buffer); (● - ●), diluted 5-fold with the homogenizing buffer; (■ - ■), diluted 10-fold; (Δ-Δ), diluted 25-fold. Relative activity 1.00 = 2.91 units/g tissue. Incubations were at 24°.

cess. Extracts prepared in 0.15 M KCl alone, however, also showed an increase in enzymatic activity with time. Homogenizing the tissue with different media did not significantly alter the increase in activity. For example, extracts prepared in 4% sucrose or in 1 M potassium phosphate, pH 7.6 exhibited the same characteristic increase in UDP-glucose pyrophosphorylase activity.

Further experiments were carried out to study possible ionic effects on the activity increase. Figure 10 demonstrates that the time-dependent increase also occurred in an extract prepared from an acetone powder of lactating rat mammary gland and in ammonium sulfate fractions of an homogenate.

It was possible that low molecular weight materials had some effect on the increase in activity. Therefore, a portion of the crude homogenate was passed through Sephadex G-25. As seen in Figure 11, there was no significant difference in the time course of the activity increase between the control (normal homogenate) and the effluent from the Sephadex G-25 column. These experiments, therefore, suggest that ionic effects or interactions with low molecular weight materials were not responsible for the increase in activity.

pH and Urea Studies

It was hoped that some condition could be found in which the activity of UDP-glucose pyrophosphorylase would remain constant (inhibition of the activation without denaturation of the enzyme) while retaining the ability to show activation by alteration of the condition. Therefore, the dependence of the increase in activity on the

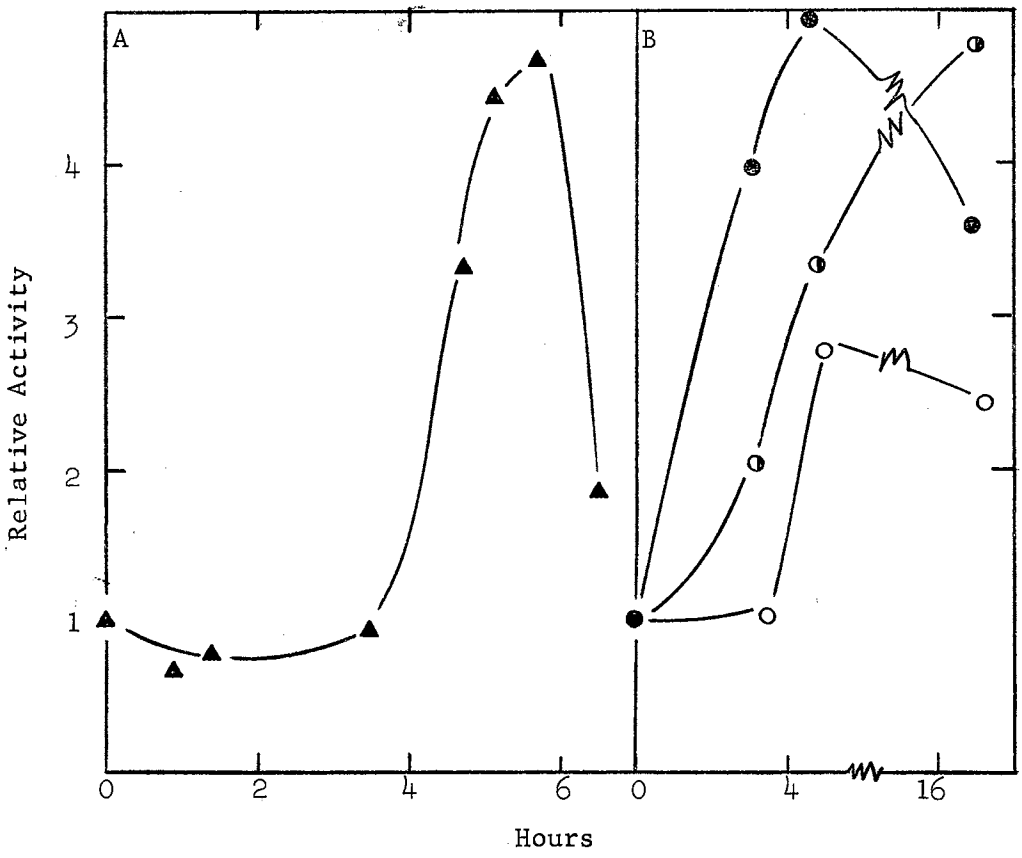


Figure 10. UDP-Glucose Pyrophosphorylase Activity in an Extract from an Acetone Powder and Ammonium Sulfate Fractions.

A. ▲—▲ , Extract from 0.025 g acetone powder/2 ml homogenizing buffer from mammary gland of 12-day lactating rat. Incubation was at 0° and relative activity 1.0 = 0.29 units/g tissue.

B. Ammonium sulfate fractions from mammary extract of 1 g tissue/20 ml homogenizing buffer from 20-day lactating rat. ● - ●, Control, relative activity 1.0 = 5.16 units/g tissue; ◑ - ◑, 0-30% ammonium sulfate supernatant, relative activity 1.0 = 2.32 units/g tissue; ○ - ○, 30-50% ammonium sulfate precipitate, relative activity 1.0 = 5.01 units/g tissue. Incubations were at 24°.

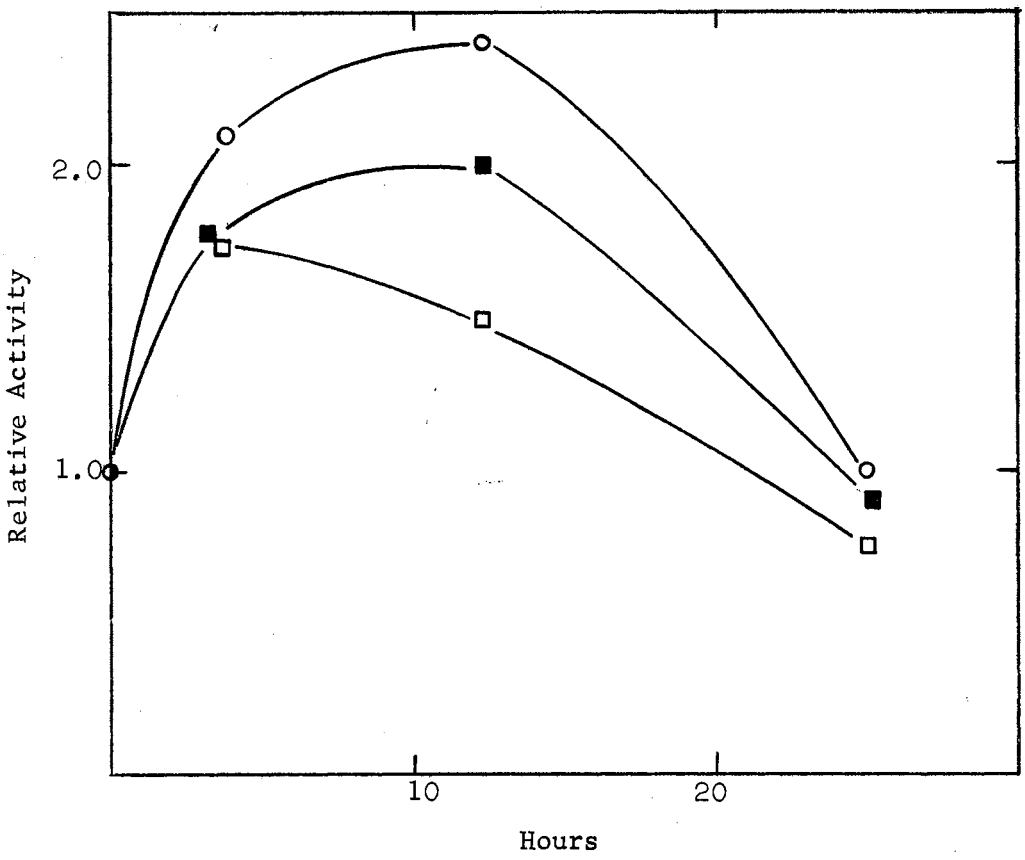


Figure 11. UDP-Glucose Pyrophosphorylase Activity in Effluents from a Sephadex G-25 Column.

A 5-ml aliquot of the 37,000 x g supernatant solution of the mammary homogenate (1 g/10 ml) from a 16-day lactating rat was applied to a 2 x 20 cm Sephadex G-25 column and was eluted with the homogenizing buffer. The fractions from the protein peak were pooled and the absorbance of the eluate at 280 mμ was measured. A diluted control was prepared in which a portion of the control homogenate was diluted to an absorbance at 280 mμ equivalent to that of the eluate. O - O, Normal homogenate; ■ - ■, eluate from G-25 column; □ - □, diluted control.

pH of the extract was studied. Figure 12 presents the increase in activity of UDP-glucose pyrophosphorylase as a function of pH. At pH's 7.5 to 9.0 no significant difference occurred; the activation progressed according to the normally observed time course. At pH 6.2 no significant increase in activity was observed and the activity remained relatively constant. Other experiments have shown that the activity may increase slightly or remain relatively constant between pH 5.5 and 6.5 although this may vary somewhat with different experiments. At pH 5.0 there was a slight initial increase which rapidly declined.

Several other experiments were performed in an attempt to reverse the inhibition of activation by pH's below 6.0. Figure 13 presents the results of incubating the extract at pH 5.5 for increasing periods of time and then readjusting the pH of the extract to 7.1 (the pH of the control). No reversal of the inactivation was observed, and the final level of activity was proportional to the length of time the extract was at pH 5.5. Several attempts in other experiments were made to reverse the inhibition of the activation at low pH's but none were successful.

As seen in Figure 8, 4 M urea prevents the increase in pyrophosphorylase activity. A concentration of urea was sought at which the activation could be retarded so that the initial activity remained constant. The effect of varying concentrations of urea on the increase in activity is seen in Figure 14. This experiment indicates that 0.5 M to 4 M urea progressively inhibits both the extent of activation and the time for maximum increase in activity. Efforts to

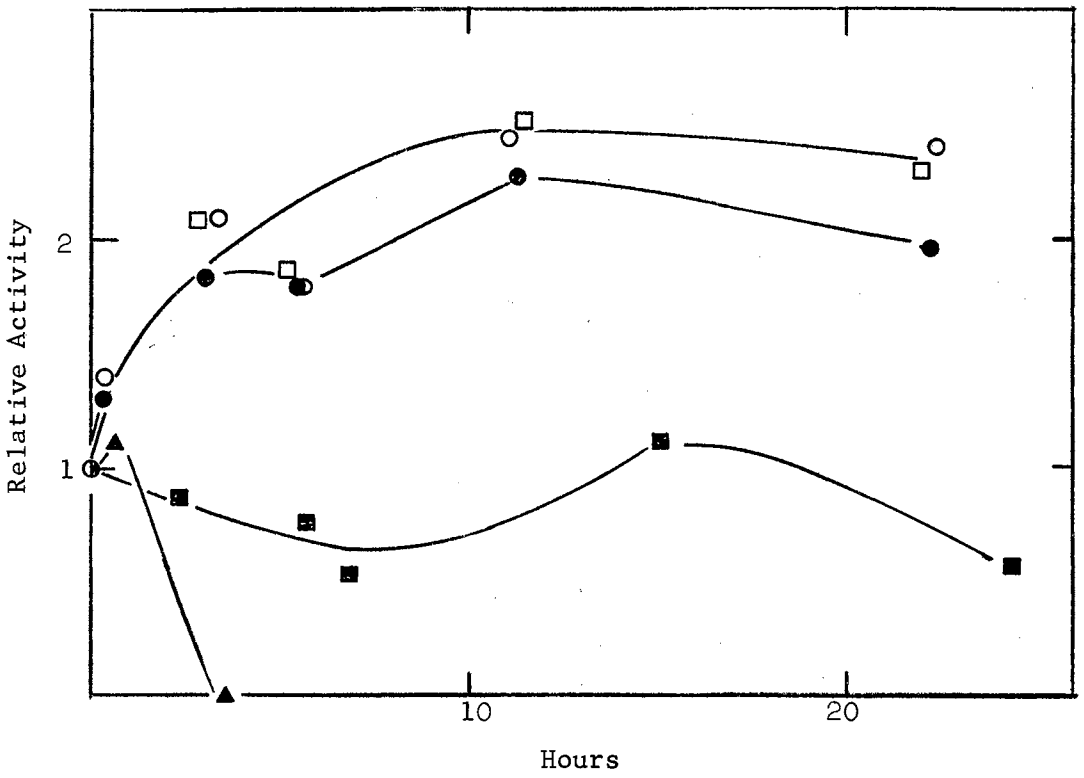


Figure 12. Activity of UDP-Glucose Pyrophosphorylase as a Function of pH.

An aliquot of the extract was diluted with an equal volume of the indicated buffer and maintained at 24°: 0.2 M glycine, pH 9.0 (O - O); 0.2 M Tris-HCl, pH 8.0 (● - ●); 0.15 M KCl, 0.005 M $MgCl_2$, 0.005 M EDTA, pH 7.5 (homogenizing buffer), (□–□); 0.2 M potassium phosphate, pH 6.2 (■–■); and 0.2 M sodium acetate, pH 5.0, (▲–▲). The extract at pH 6.2 was 1 g tissue/20 ml homogenizing buffer from a 19-day lactating rat (relative activity 1.0 = 2.63 units/g tissue); the extract at the other pH's was 1 g tissue/10 ml homogenizing buffer from a rat lactating 7 days (relative activity 1.0 = 0.32 units/g tissue.

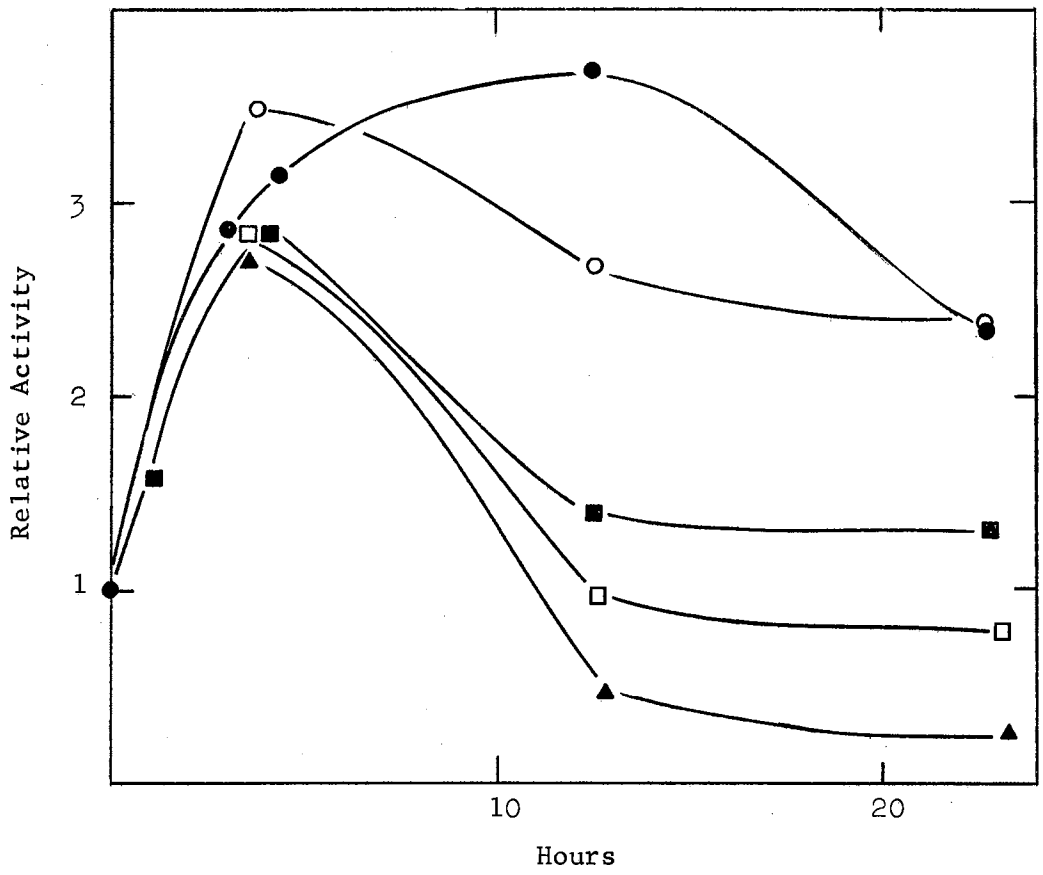


Figure 13. UDP-Glucose Pyrophosphorylase Activity at pH 5.5 for Different Extents of Time.

A portion of the extract (1 g tissue from 20-day lactating rat/10 ml of homogenizing buffer) was adjusted to pH 5.5 for the indicated period of time, and then the pH was readjusted to 7.1. (● - ●), Control, pH 7.1; (○ - ○), pH 5.5 for 15 min; (■-■), pH 5.5 for 1 hr; (□-□), pH 5.5 for 2 hr; (▲-▲), pH was maintained at 5.5. Relative activity 1.0 = 0.28 units/g tissue.

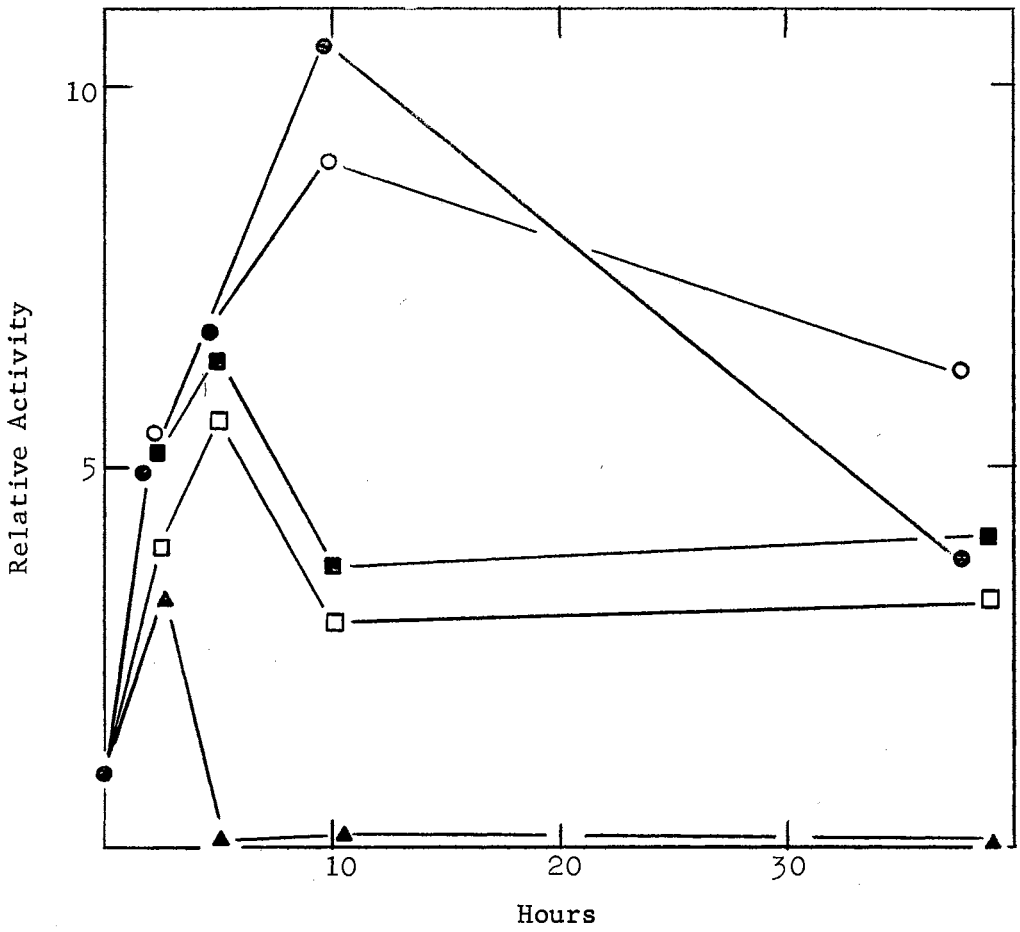


Figure 14. Effect of Urea Concentration on the Activity of UDP-Glucose Pyrophosphorylase.

The extract (1 g tissue/20 ml of homogenizing buffer) was prepared from the mammary gland of an 11-day lactating rat. (● - ●), Control, no urea added; (○ - ○), 0.5 M urea; (■-■), 1 M urea; (□-□), 2.5 M urea; (▲-▲), 4 M urea. The extracts were maintained at 24°, and relative activity 1.0 = 5.91 units/g tissue.

reverse the effects of urea also were not successful. That is, the enzymatic activity of an extract made 4 M in urea and immediately sieved through Sephadex G-25 to remove the urea did not increase as did the control to which no urea was added. Chromatography of the extract on Sephadex G-25 was previously shown not to inhibit the activation (Figure 11). Apparently the effects of both low pH and high urea concentrations to inhibit the increase in UDP-glucose pyrophosphorylase activity can not be easily reversed.

K_m and Molecular Weight Studies

The increase in activity might be due to an alteration in the structure of the enzyme. Consequently, K_m and sucrose density gradient studies were carried out on initial extracts and on extracts after incubation. Figure 15 presents the double reciprocal plot (110) for the determination of the K_m of UTP for UDP-glucose pyrophosphorylase in the initial extract (zero time) and after incubation at 24° for 10 hours. There was no significant difference in K_m between the values obtained at zero time and after activation. The value of the K_m for UTP is approximately 2.1×10^{-4} M. During the course of the investigation for the K_m, UDP-glucose pyrophosphorylase was found subject to potent substrate inhibition by levels of UTP higher than 0.2 mM. (The concentration of UTP in the standard assay (24) determined for bovine UDP-glucose pyrophosphorylase is 1.0 mM). Perhaps the increase in the activity was due to some change in the enzyme toward the substrate inhibition by UTP. However, investigation of the activity in extracts where different levels of UTP were utilized showed

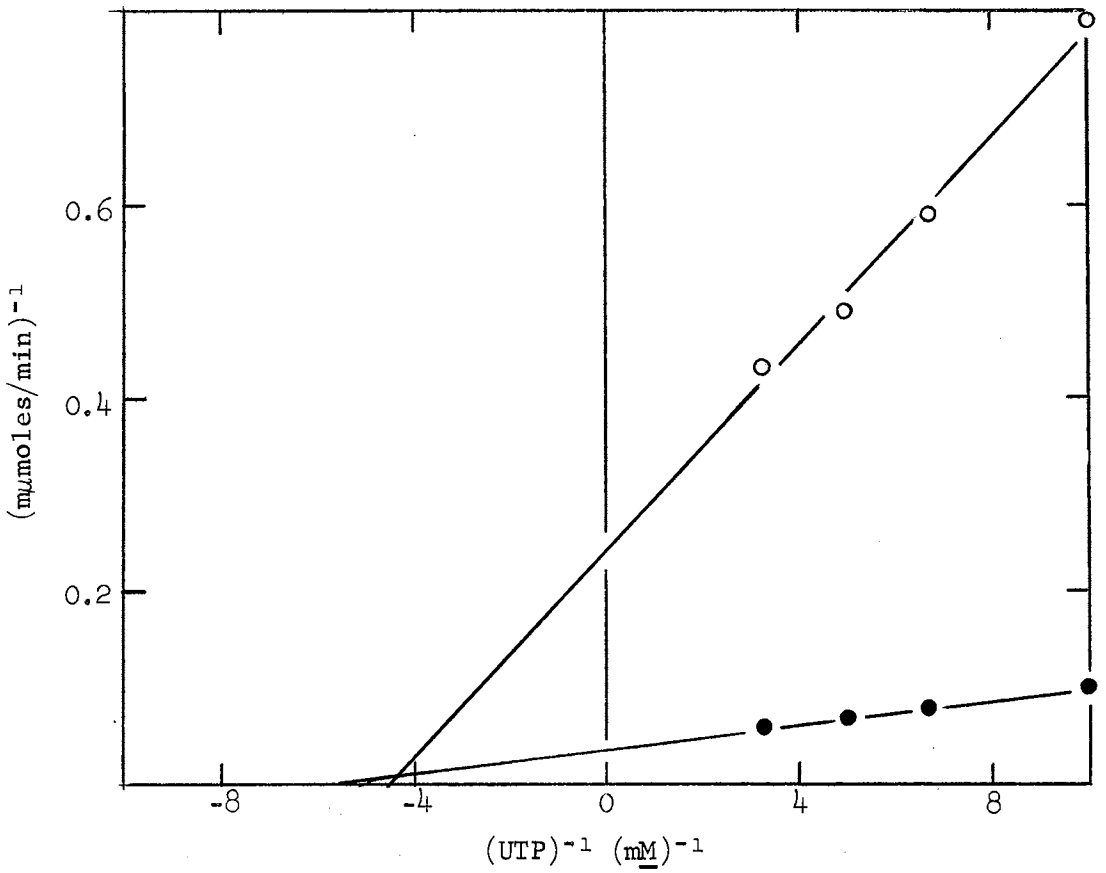


Figure 15. Lineweaver-Burk Plot for UDP-Glucose Pyrophosphorylase Activity at Zero Time and after Incubation at 24°.

Homogenate (0.5 g tissue/10 ml homogenizing buffer) was prepared from a 14-day lactating rat. (O - O), Zero-time values; (● - ●), values obtained after 10-hr incubation. The K_m at zero time was 2.3×10^{-4} M and at 10 hr was 1.9×10^{-4} M.

that activation also occurred with all levels of UTP used, even when the UTP concentration was not inhibitory. Thus, the increase in activity does not appear to be due to a loss of substrate inhibition by UTP.

Another possible mechanism for the increase in activity could be a molecular weight change as would occur with the aggregation or disaggregation of subunits. Therefore, sucrose density gradient studies were performed to determine if there was a change in molecular weight of the enzyme. Figure 16 illustrates the pattern of enzymatic activity from centrifugation of a portion of the extract in a 5-20% linear sucrose gradient at zero time and after 13 hours incubation at 24°. The results of this experiment indicate that the enzymatic activity of UDP-glucose pyrophosphorylase before and after incubation was found in the same area of the sucrose gradient when centrifuged under identical conditions. Therefore, the activation process of the enzyme does not occur by a marked molecular weight change of the active enzyme to another species which has activity.

In summary, the time-dependent increase in activity of UDP-glucose pyrophosphorylase upon incubation most probably occurs by a process involving protein interactions or by a slow modification of the protein structure of the enzyme which does not affect the molecular weight of the active enzyme or the K_m for UTP.

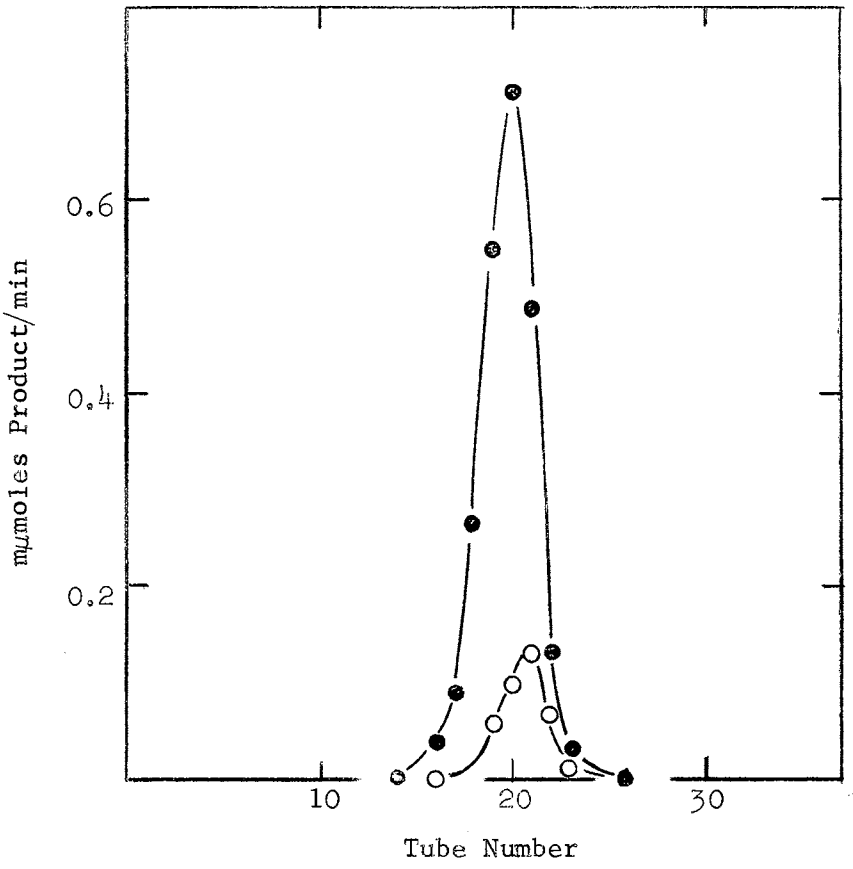


Figure 16. UDP-Glucose Pyrophosphorylase Activity in Sucrose Density Gradient.

The gradient (5-20% sucrose) were centrifuged at 65,000 r.p.m. for 1.5 hr at 4° in SW-65 Rotor. 0.1 ml of homogenate (1g/10 ml) of 12-day lactating rat mammary gland was layered onto the sucrose gradients which also contained 0.15 M KCl, 0.005 M $MgCl_2$, 0.005 M EDTA, pH 7.5. (O - O), Activity from extract at zero time, 1.03 units/g tissue; (● - ●), activity from extract after incubation at 24° for 13 hr, 2.52 units/g tissue.

CHAPTER V

CRITICAL EVALUATION OF THE ASSAY FOR LACTOSE SYNTHETASE

Experimental Procedure

Spectrophotometric Assay for Lactose Synthetase

Lactose synthetase activity may be measured by the incorporation assay as described in Chapter III, or by coupling the UDP formed to the oxidation of NADH via the reactions of nucleoside diphosphokinase (EC 2.7.4.6), pyruvate kinase (EC 2.7.1.40), and lactic dehydrogenase (EC 1.1.1.27) (8). The reagents used to measure the formation of UDP were 0.15 mM NADH, 1.0 mM phosphoenolpyruvate (PEP), and 0.05 ml of a 1 to 10 dilution of pyruvate kinase (Sigma Chemical Co., Type I, 25 mg protein/ml and 2.4 international units of pyruvate kinase/mg protein). This commercial preparation of pyruvate kinase also contains the nucleoside diphosphate kinase and lactic dehydrogenase for coupling of UDP formation to NADH oxidation. For a stock solution the NADH and PEP were dissolved in 2 mM NaOH.

Preparation of Homogenates

In general, the homogenation procedure for preparing the tissue extracts was the same as described in Chapter III. There were some differences in centrifugation speeds, but the majority of the experi-

ments dealing with the development of the assay for lactose synthetase in rat mammary gland homogenates were performed with the supernatant solution obtained from centrifugation at 1,000 xg for 10 min. After centrifugation, these extracts were filtered through glass wool to remove fat.

Particles from rabbit mucosa were prepared by the method of Ziderman et al. (111). The opossum, guinea pig, and hamster extracts were prepared as described above. The assays for UDP-galactose galactosyl transferase activity of the bat mammary tissue represent the sum of activities assayed in the 10,000 xg supernatant solution and precipitate.

Materials

N-Acetylglucosamine, NADH, PEP, glycylglycine, and pyruvate kinase (Type I) were purchased from Sigma Chemical Co. Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer was from Calbiochem Laboratories and Tween 80 was from Mann Research Laboratories. The purified bovine A protein was isolated as previously described (112) and a flow diagram of the purification is shown in Figure 17. B protein (α-lactalbumin) was prepared through Step 5 of the method of Brodbeck et al. (8).

All other experimental procedures and chemicals were described in Chapter III.

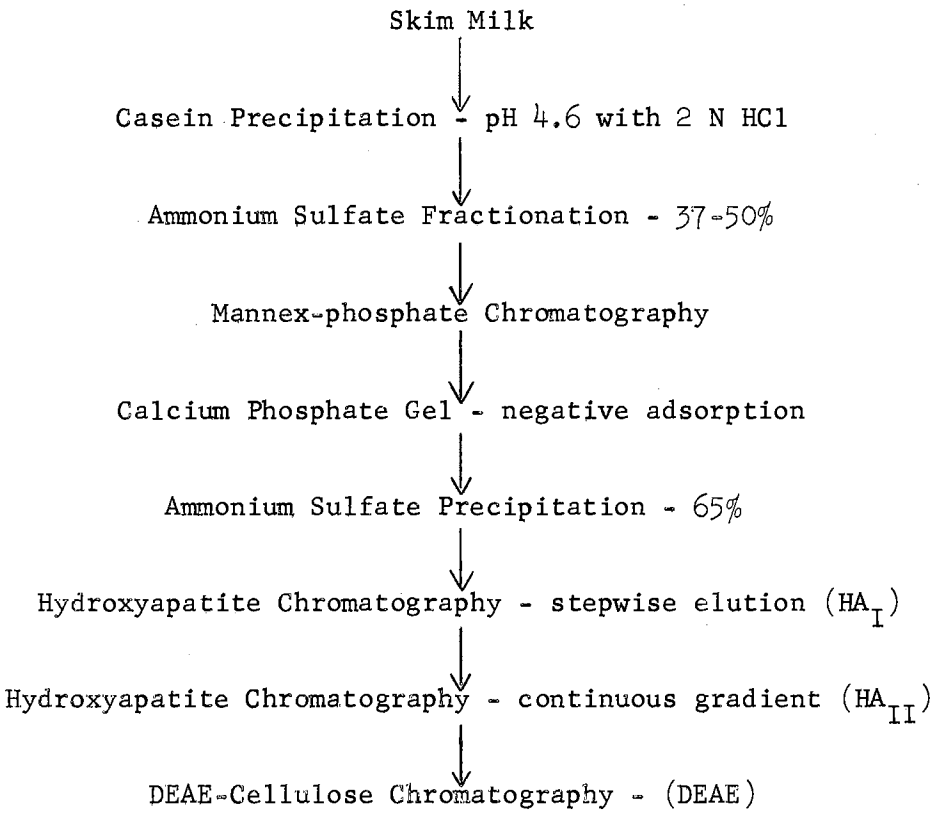


Figure 17. Flow Diagram of the Purification Procedure of the A Protein

Results

The Lactose Synthetase Assay and Associated Problems

As previously discussed, lactose synthetase catalyzes the terminal step of lactose biosynthesis:

$$1)\quad \text{UDP-galactose} + \text{glucose} \xrightarrow[Mn^{++}]{A,\ B} \text{lactose} + \text{UDP}$$

This reaction requires two proteins (A and B) and Mn^{++} for significant activity (8). The A protein in the absence of added B protein catalyzes the formation of N-acetyllactosamine when N-acetylglucosamine is substituted for glucose in the reaction (34):

$$2)\quad \text{UDP-galactose} + \text{N-acetylglucosamine} \longrightarrow \text{N-acetyllactosamine} + \text{UDP}$$

The synthesis of N-acetyllactosamine with N-acetylglucosamine as the substrate and no added B protein is designated the A-protein activity (Reaction 2). Assay for lactose synthesis in the presence of glucose with or without added B is designated as LS_A activity (Reaction 1). Activity for the B protein with excess A protein and glucose would then be LS_B activity (Reaction 1). The units of A protein were determined with the LS_A assay and one unit is defined as that amount of A protein which catalyzes the formation of 1 mμmole of lactose per min under the defined assay conditions. Any designation of units of LS_A activity is accompanied with the assay conditions in which the units were determined.

Generally, the assay for one protein component of a two-protein enzymatic system is performed by using saturating amounts of the

second or counter-part protein. This technique has been used to assay the separate proteins of the enzymes requiring two proteins (56-84) including the lactose synthetase A and B proteins (8,34,46). However, there are no reported detailed investigations of assays of this type.

Two assay procedures may be used for lactose synthetase. The incorporation assay as previously described measures the conversion of UDP-galactose-^{14}C-(galactose-U-^{14}C) into lactose-^{14}C. The spectrophotometric assay measures UDP formation in the lactose synthetase reaction by determining the oxidation of NADH using nucleoside diphosphokinase, pyruvate kinase, and lactate dehydrogenase. The spectrophotometric assay is more convenient since it is more rapid than the incorporation assay and does not require radioisotopic techniques. This assay, however, is limited to partially purified systems because interfering enzymes in crude preparations catalyze endogenous oxidation of the NADH. For crude systems the incorporation assay must be used.

The original assay for lactose synthetase was developed by Hassid and coworkers (9,21) before it was known that the enzyme required two nonidentical proteins. Basically this assay was adopted by Brodbeck and Ebner (8) in their investigations with the modification that "saturating" levels of A protein were used to assay low levels of B protein and vice versa. These original assay conditions which are designated Assay System I included 20 mM $MnCl_2$, 0.25 mM UDP-galactose, and 20 mM glucose. The A protein was dissolved in 20 mM Tris-HCl, pH 7.4 so that the final Tris concentration in the assay was about 2 mM. The B concentration was usually 100 μg/ml.

Questions arose concerning the values for saturating concentrations of the B protein in the LS_A assay and results of experiments to determine these values are presented in Figure 18. This figure shows the investigation of the activity of the A protein (LS_A assay) at several levels of B protein in the spectrophotometric assay under initial assay conditions (Assay System I). Two important observations are present in this figure: 1) linearity may be observed with respect to A concentration even though the level of B is not saturating and hence activity is not maximum; 2) higher levels of B are inhibitory though the assay is still linear (a criterion of a functional enzymatic assay). Maximum LS_A activity was found with 200 μg B/ml.

Further difficulties with the original assay system were found when attempts were made to determine saturation values of B for different amounts of the A protein in the incorporation assay. The results were expected to be similar to those presented in Figure 18 with the spectrophotometric assay. That is, there should be a linear response between the amount of A protein and rate at varying but constant levels of B protein. However, the data presented in Figure 19 demonstrate that this was not observed. At one level of B (0.05 mg), an S-shaped curve was found instead of linearity. At a level of B 10 times greater (0.50 mg), the plot is linear but also shows linear inhibitions at the higher amounts of A as previously found in the spectrophotometric assay (Figure 18). With 25 times the level of B (1.25 mg), the activity is further inhibited in a linear manner. The last three points of the 0.05 mg B curve will extrapolate through the origin.

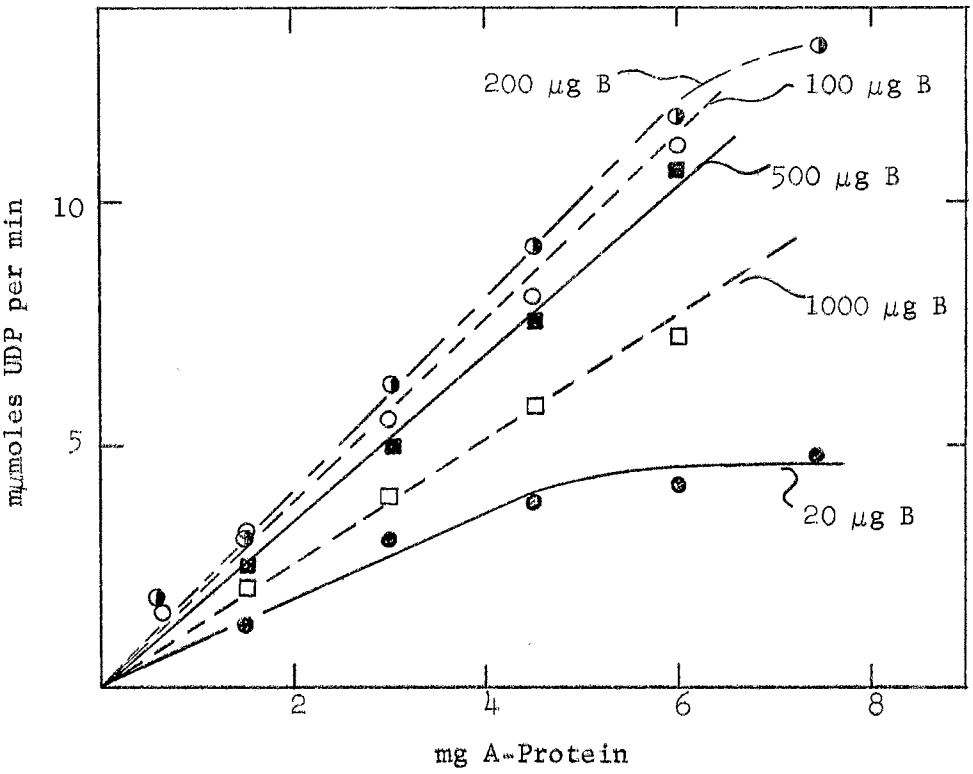


Figure 18. Spectrophotometric Assay for LS_A Activity (Bovine) at Several Levels of B-Protein (Bovine).

Assay conditions: A-protein off Mannex-P, dissolved in 20 mM Tris-HCl, pH 7.4; 20 mM $MnCl_2$; 20 mM glucose; and 0.25 mM UDP-galactose. ● - ●, 20 μg B/ml; O - O, 100 μg B/ml; ◑ - ◑, 200 μg B/ml; ■ - ■, 500 μg B/ml; and □ - □, 1,000 μg B/ml.

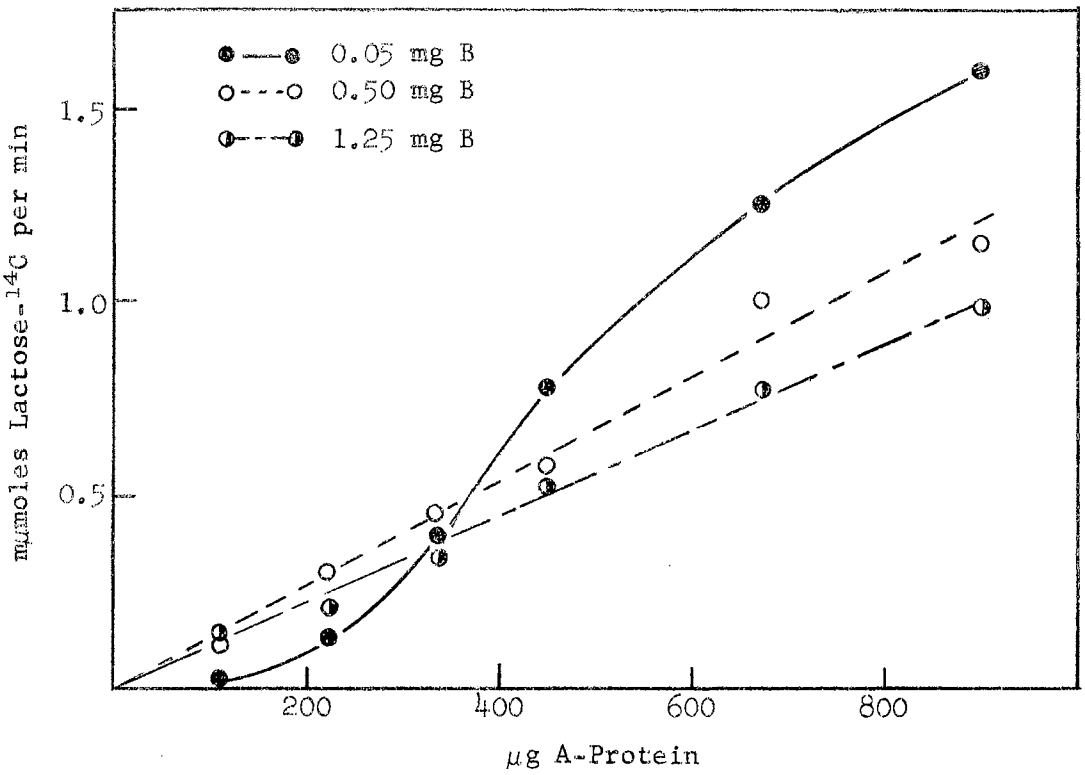


Figure 19. Incorporation Assay for LS_A at Three Levels of B Protein.

Assay conditions: 50 mM Tris-HCl, pH 7.2; 5 mM ATP; 20 mM $MnCl_2$; 20 mM glucose; and 0.62 mM UDP-galactose-^{14}C. The final volume was 0.1 ml. Incubation time was 20 min at 37° with a preincubation of 60 min before UDP-galactose-^{14}C was added. The A protein was off Mannex-P. ● - ●, 0.05 mg B protein per assay; O - O, 0.50 mg B; and ◑ - ◑, 1.25 mg B.

Furthermore, the specific activity of the B protein of lactose synthetase had increased from 150 to 1,000 mμmoles/min/mg protein at 20° as a more purified A protein was used in the assay for the B protein (10). The higher specific activity was obtained with a higher A/B ratio. Thus, the assay for lactose synthetase required a thorough investigation.

Investigation of the Assay for LS_A and A-Protein Activities with Purified Bovine Proteins

The initial attempts to redefine the assay conditions were undertaken with the spectrophotometric assay. The approach taken was to establish optimal conditions with purified bovine A and B proteins in the spectrophotometric assay and then return to the incorporation assay to examine assay conditions in the crude systems.

There were five parameters which required investigation for maximum lactose synthetase activity: buffer and pH; metal ion concentration; concentrations of the substrates, UDP-galactose and glucose; and the ratio of B protein to A protein.

The first parameter investigated was the buffer-pH optimum but it was soon discovered that buffer, pH, and metal concentration were intimately related in the assay. That is, 50 mM Tris-HCl precipitated and hastened the oxidation of Mn^{++} at pH's greater than 8. Even at pH 7.5, 50 mM Tris inhibited lactose synthetase activity about 30% compared with Hepes buffer. With a combination buffer of Hepes-glycylglycine, the pH optimum was determined to be at pH 8.0 and 50 mM glycylglycine was chosen as the buffer for the assay.

The rapid precipitation and apparent oxidation of Mn^{++} which occurred in the presence of Tris was not observed in the presence of glycylglycine. However, apparent oxidation of the Mn^{++} accompanied by a brownish color did occur after standing 10-15 min. In order to minimize the oxidation of Mn^{++} which is known to occur at alkaline pH's (113), the $MnCl_2$ was added to the cuvette just prior to starting the reaction with the substrates. This procedure resulted in an optimum concentration of 6 mM $MnCl_2$. Previous work by Babad and Hassid (9) indicated 13.3 mM.

With certain preparations of pyruvate kinase endogenous NADH oxidase activity prevented an accurate determination of the rate of UDP formation. This oxidase activity was somewhat inhibited by $MnCl_2$ and was minimized by making a new blank for each assay or by omitting the pyruvate kinase from the blank cuvette since the oxidase activity did not appear for at least 10 min. In either case the pyruvate kinase was added just prior to adding substrate.

After conditions of 50 mM glycylglycine, pH 8.0 and 6 mM Mn^{++} were chosen, the K_m values for UDP-galactose and glucose were investigated. Conditions were sought so that the requirements for UDP-galactose would be minimized while retaining maximum enzymatic activity. However, the Lineweaver-Burk plots obtained did not yield reproducible values for the K_m of UDP-galactose. Thus, to obtain an optimum UDP-galactose concentration, the enzymatic activity as a function of UDP-galactose concentration was determined. 0.4 mM UDP-galactose was found to be saturating at about 20 and 40 mM glucose (Figure 20) and this UDP-galactose concentration was used in the standard assay.

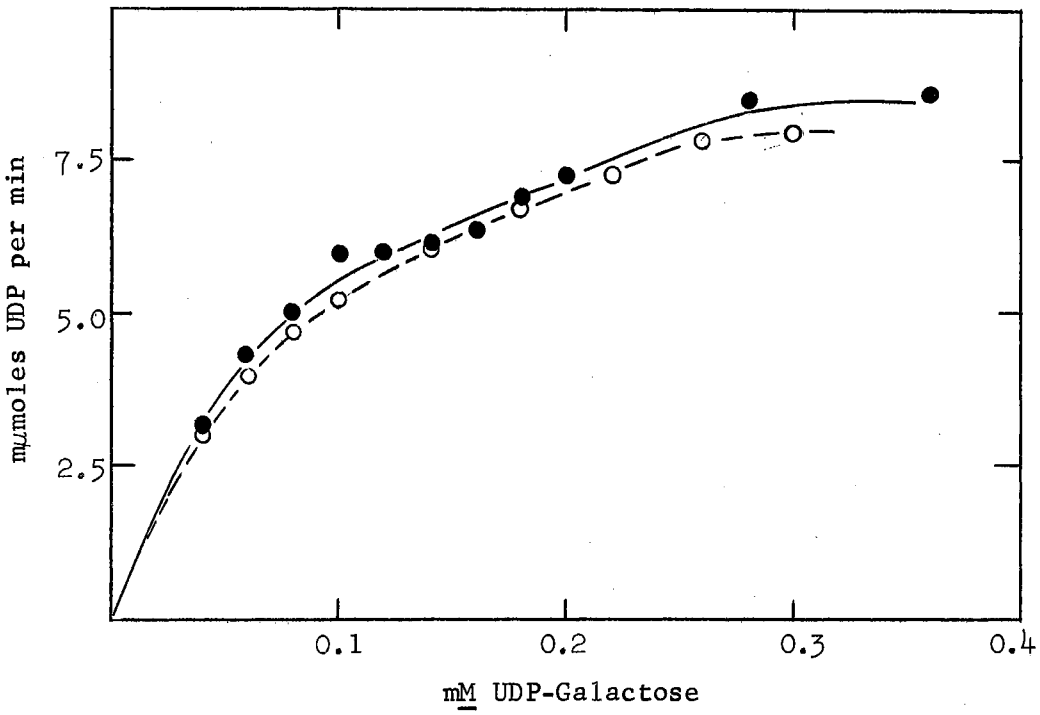


Figure 20. Saturation Curve of UDP-Galactose.

Assay conditions: 50 mM glycylglycine, pH 8.0; 6 mM $MnCl_2$; 200 μg B protein/ml; ● - ●, 40 mM glucose; and O - O, 20 mM glucose. A protein (A-3) was from HA_{II}.

Similarly the enzymatic rate was determined as a function of glucose concentration. On the basis of the data with 0.4 mM UDP-galactose in Figure 21 the glucose concentration for the assay was set at 50 mM. At this point the optimum pH and manganous ion concentration were determined again and further adjusted to pH 8.5 and 5 mM $MnCl_2$.

The assay conditions at that time (50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 50 mM glucose; and 0.4 mM UDP-galactose) were then used to examine the saturation levels of B protein. These results, presented in Figure 22, indicated that the optimum B concentration was again 200 μg B/ml. Furthermore, the previously observed inhibition of activity with high levels of B protein was diminished. Thus, the optimal assay conditions after this evaluation were 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 50 mM UDP-galactose; and 200 μg B/ml. These conditions are designated Assay System II.

Nonlinearity and sigmoidal curves were previously observed for increasing A concentrations in the incorporation assay (Figure 19). At that time the reaction mixtures were incubated usually for 60 min or for 20 min plus a prior incubation before UDP-galactose-^{14}C was added. However, when the concentration of enzyme was increased and the incubation period shortened and the prior incubation omitted, the S-shaped curves were eliminated and a linear response of activity to concentration of A protein was obtained. Figure 23 is a plot of lactose synthesized *vs.* time for two levels of A protein determined with Assay System II. This plot indicates that the incorporation of ^{14}C into lactose is linear with time from 2 to 5 min for both levels of A protein.

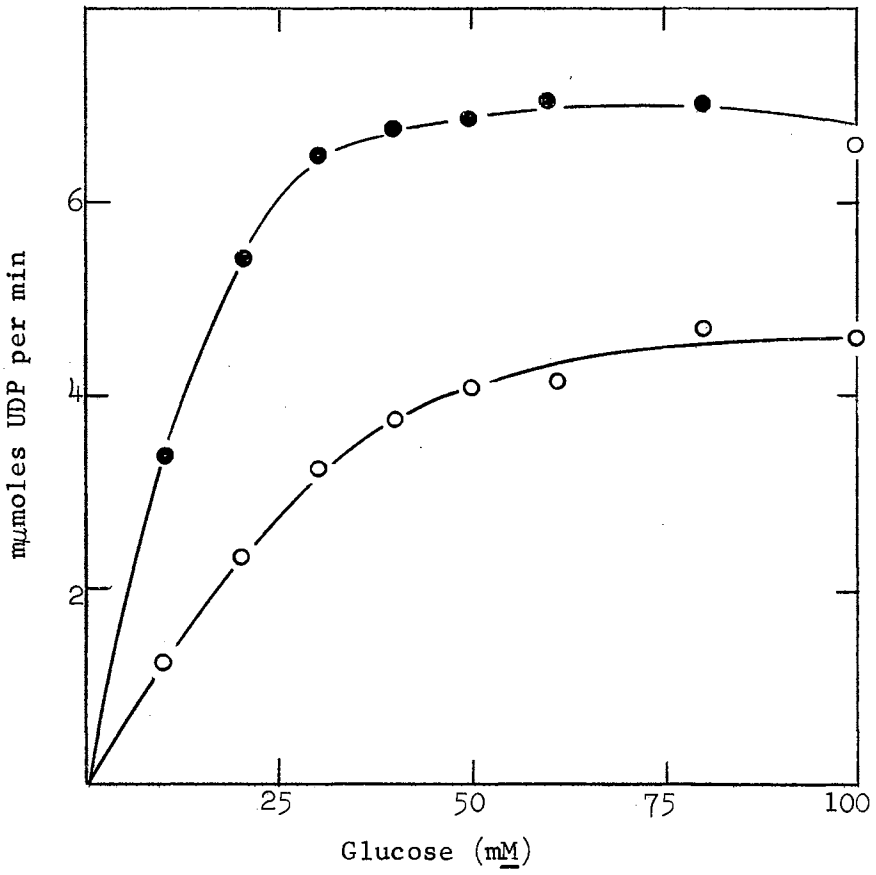


Figure 21. Dependence of LS_A Assay on Glucose Concentration.

Assay conditions: 50 mM glycylglycine, pH 8.0; 6 mM $MnCl_2$; 0.4 mM UDP-galactose; ● - ●, 200 μg B/ml; and O - O, 50 μg B/ml. A protein (A-5) was from DEAE chromatography step.

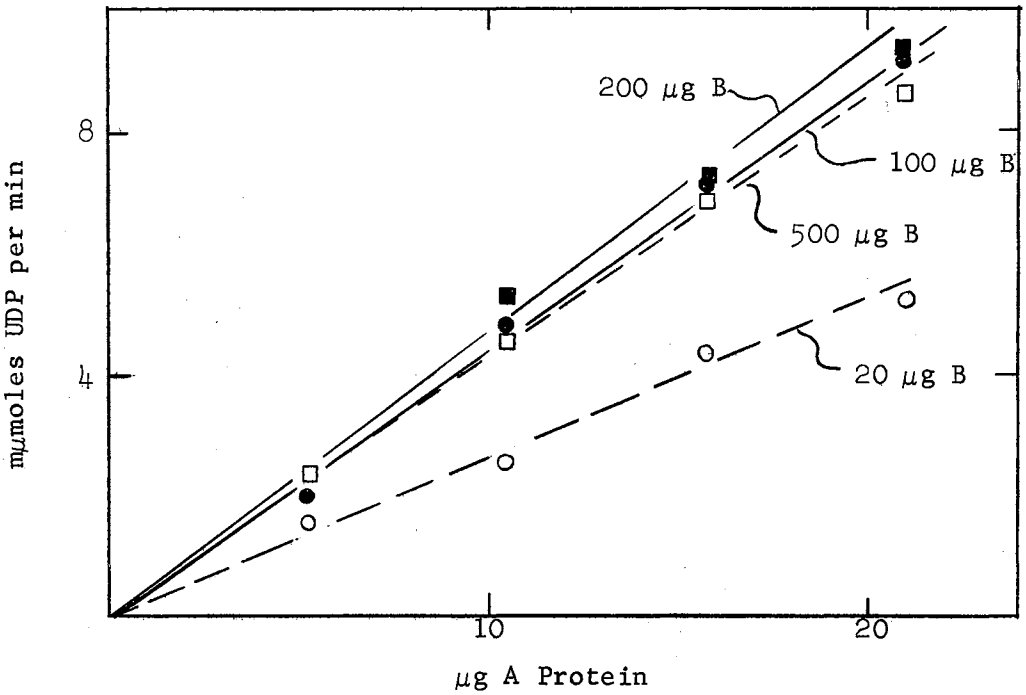


Figure 22. Spectrophotometric Assay for A Protein with Several Levels of B Protein after Re-evaluation.

Assay conditions: 50 mM glycylglycine, pH 8.5; 60 mM glucose, 5 mM UDP-galactose; 5 mM $MnCl_2$, and 5-21 μg A-5 (off DEAE-cellulose). O - O, 20 μg B/ml; ● - ●, 100 μg B/ml; ⊡-⊡ , 200 μg B/ml; □-□ , 500 μg B/ml.

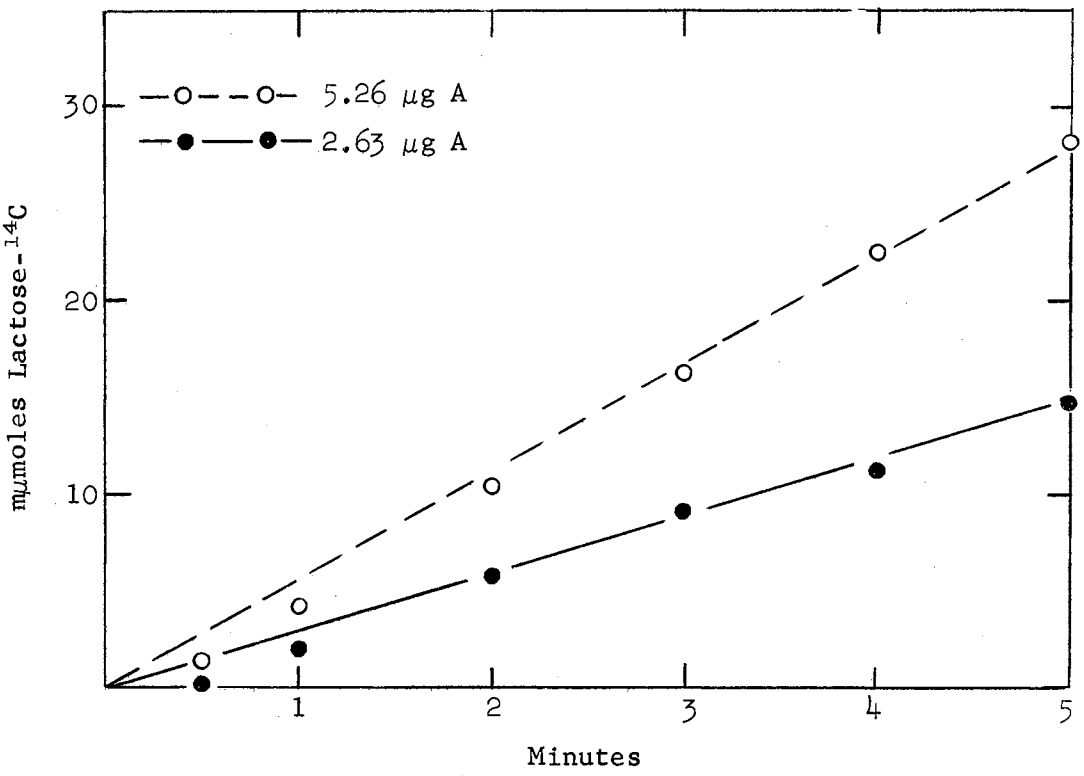


Figure 23. Linearity of LS_A Assay (Incorporation) with Time.

Assay conditions: 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 0.68 mM UDP-galactose; 50 mM glucose; 200 μg B/ml. ● - ●, 2.63 μg A protein (A-5, off DEAE-cellulose) and O - O, 5.26 μg A protein.

A plot of enzyme units (mμmoles lactose/min incubation) vs. μg A protein in Figure 24 indicates that linearity to at least 4 units of activity was obtained with a 4-min incubation time. Similar plots were obtained with several different levels of B protein. Therefore, the sigmoidal curves previously observed were eliminated.

With Assay System II a series of kinetic experiments were performed. Figure 25 shows the optimum UDP-galactose concentration in the LS_A assay at several glucose concentrations. These plots indicate that UDP-galactose was saturating at 0.4 mM. Furthermore, the glucose concentration did not affect the saturation level for UDP-galactose although the glucose concentration did affect the maximum rate. Lineweaver-Burk plots were made of these data and other similar data, but no distinction between ordered or ping-pong reaction mechanisms could be made (114).

Similarly, an investigation of the effect of B protein concentration on the saturation value for UDP-galactose was carried out at 20 mM glucose (Figure 26). Thus, the concentration of B protein in the assay did not significantly affect the optimum UDP-galactose concentration although 200 μg B/ml resulted in increased activity over 50 μg B/ml.

Conversely, similar studies of the effects of UDP-galactose and B protein concentrations on the optimum glucose concentration were performed. The data in Figure 27 indicate that 0.1 mM to 0.4 mM UDP-galactose did not alter the optimum glucose concentration (20 mM) although the maximum activity was obtained with 0.4 mM UDP-galactose.

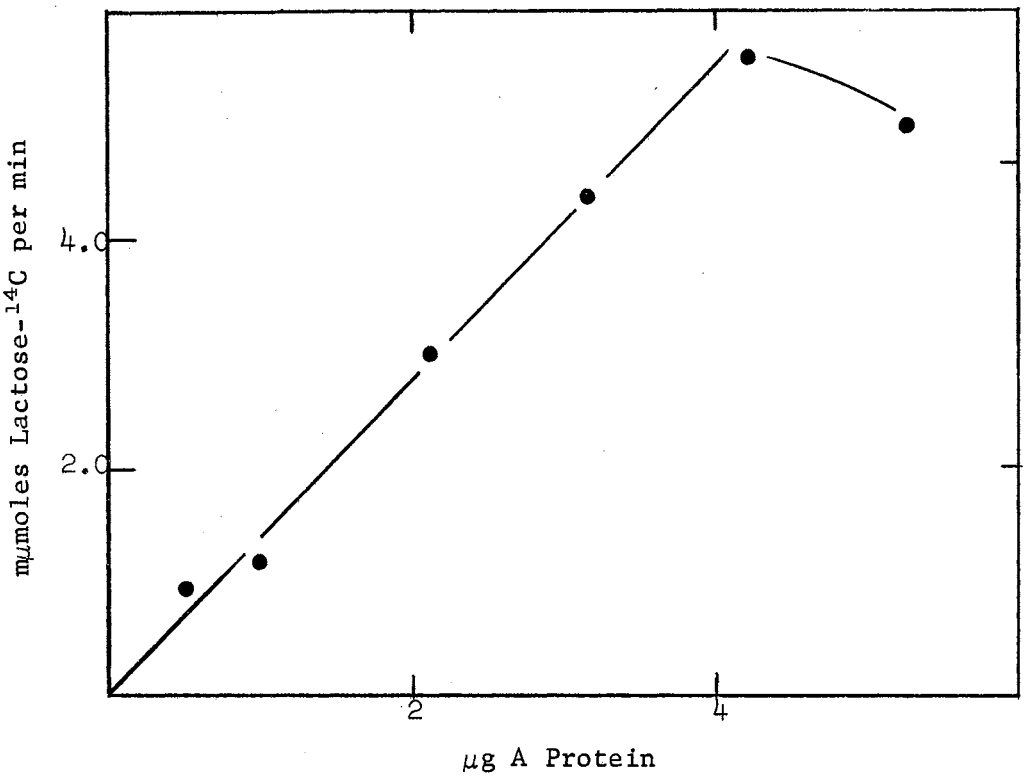


Figure 24. Linearity of the Incorporation Assay for LS_A.

Assay conditions: 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 0.68 mM UDP-galactose; 50 mM glucose; 200 μg B/ml; and incubation time, 4 minutes. A protein (A-5) is off DEAE. Rate is in mμmoles lactose-^{14}C per min.

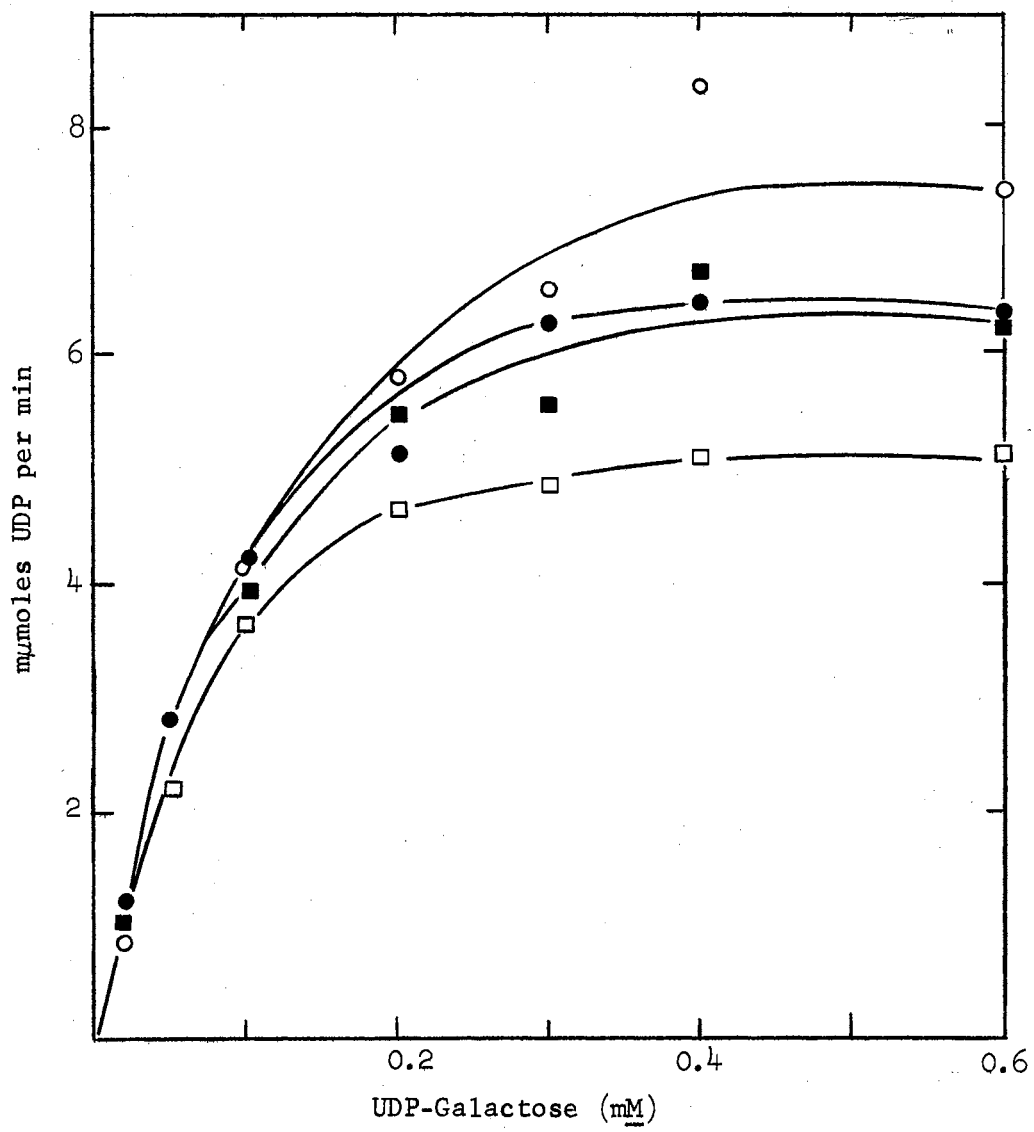


Figure 25. Optimum UDP-Galactose Concentration in LS_A Assay (Spectrophotometric) at a Variety of Glucose Concentrations.

Assay conditions: 5 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; and 200 μg B/ml. A protein was from HA_I chromatography (A-7). Glucose concentrations: ● - ●, 10 mM; O - O, 20 mM; ■-■, 50 mM; and □-□, 100 mM.

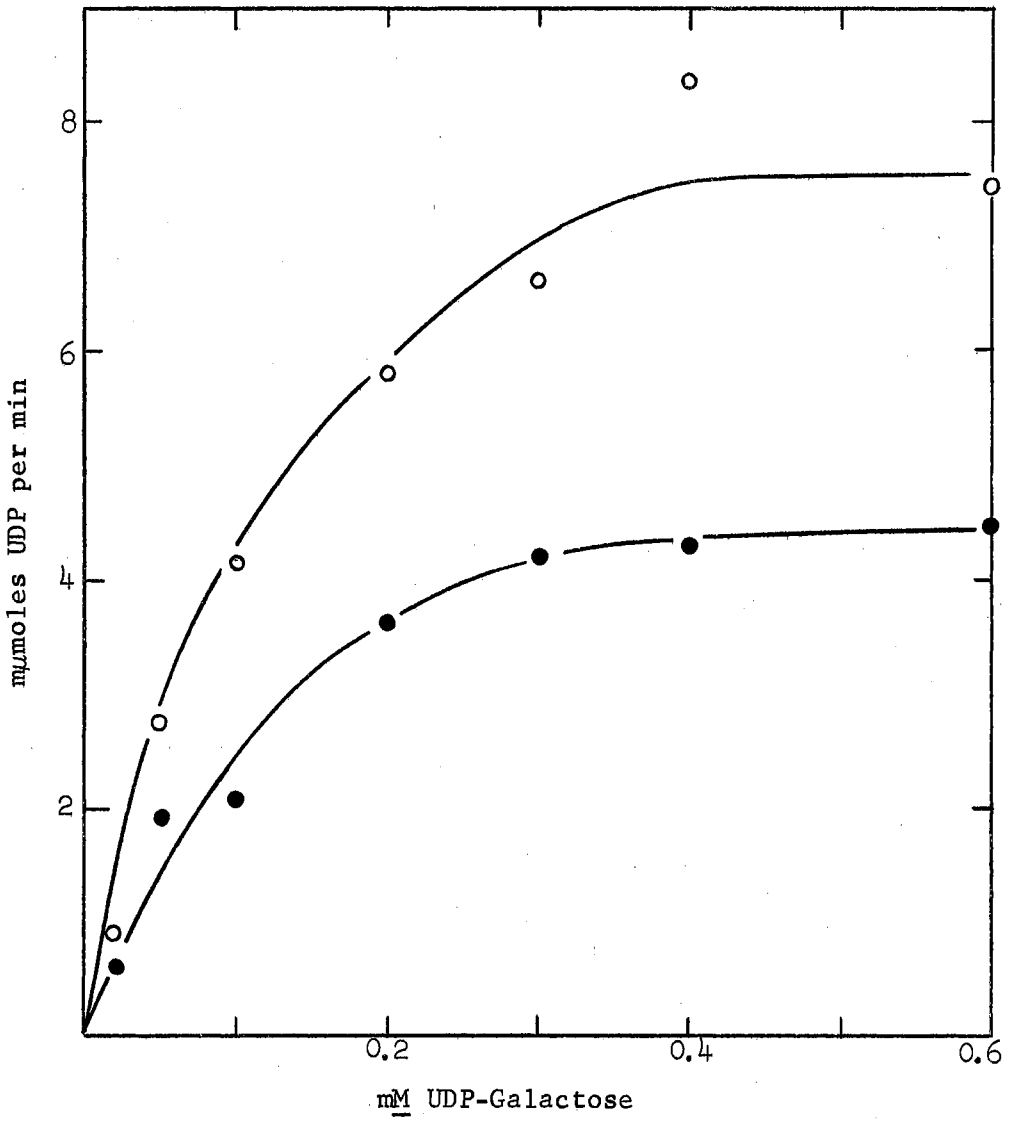


Figure 26. UDP-Galactose Optimum in LS_A Assay (Spectrophotometric) at Two Levels of B Protein.

Assay conditions: 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; and 20 mM glucose. A protein was from HA_I (A-7). ● - ●, 50 μg B protein/ml and O - O, 200 μg B/ml.

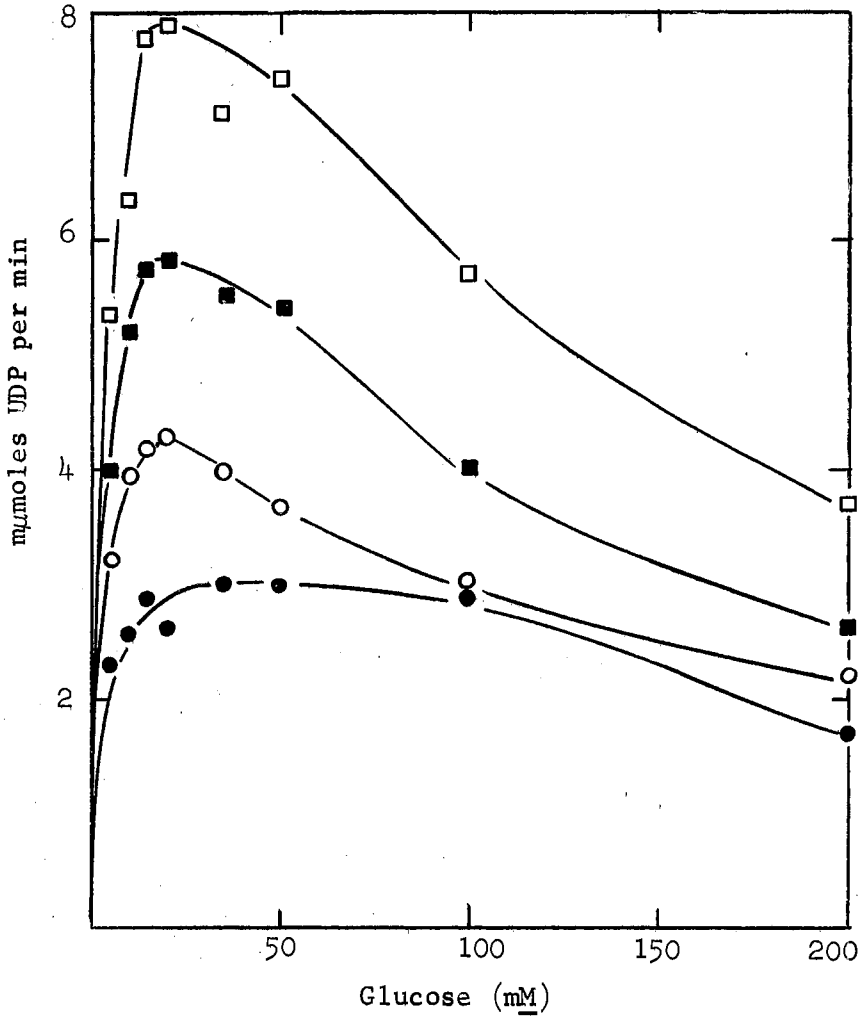


Figure 27. Optimum Glucose Concentration for LS_A Assay at Several Levels of UDP-Galactose.

Assay conditions: 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; and 200 μg B protein/ml. A protein (A-7) was from HA_I. UDP-galactose concentration: ● - ●, 0.05 mM; ○ - ○, 0.1 mM; ■-■, 0.2 mM; □-□, 0.4 mM.

However, when the B protein concentration was altered from 50 μg/ml to 200 μg/ml the optimum glucose concentration was shifted from 50 mM to 20 mM, respectively (Figure 28). Figure 28 also illustrates that the enzymatic activity at 200 μg B/ml and 20 mM glucose is greater than at 50 μg B/ml and 50 mM glucose. The B protein, therefore, affects the glucose concentration at which maximum LS_A activity occurs and also functions in a saturating manner (Figure 22) to alter enzymatic activity.

The Lineweaver-Burk plots for glucose at the two levels of B and 0.4 mM UDP-galactose are presented in Figure 29. These plots yield values of the K_m for glucose at 50 μg B/ml and 200 μg B/ml of 15.4 mM and 5 mM, respectively. Similar effect of the B concentration on the K_m for glucose was found with other levels of UDP-galactose. These results are: 0.05 mM UDP-galactose - 33 mM (50 μg B/ml) and 4 mM (200 μg B/ml); 0.1 mM UDP-galactose - 11 mM (50 μg B/ml) and 3 mM (200 μg B/ml); and 0.2 mM UDP-galactose - 14 mM (50 μg B/ml) and 4 mM (200 μg B/ml). These plots also clearly demonstrate the marked substrate inhibition of enzymatic activity by glucose.

Previously, differences were observed in the glucose optima even though the experiments were performed with the same B protein concentration. These differences were due to the preparation of the A protein used in the experiments (Figure 30). This figure presents the effect of glucose concentration on the enzymatic activity of three A-protein preparations from different steps of the purification procedure (See Figure 17). As the A protein was purified - from the first hydroxyapatite chromatography (HA_I) to the second hydroxyapatite

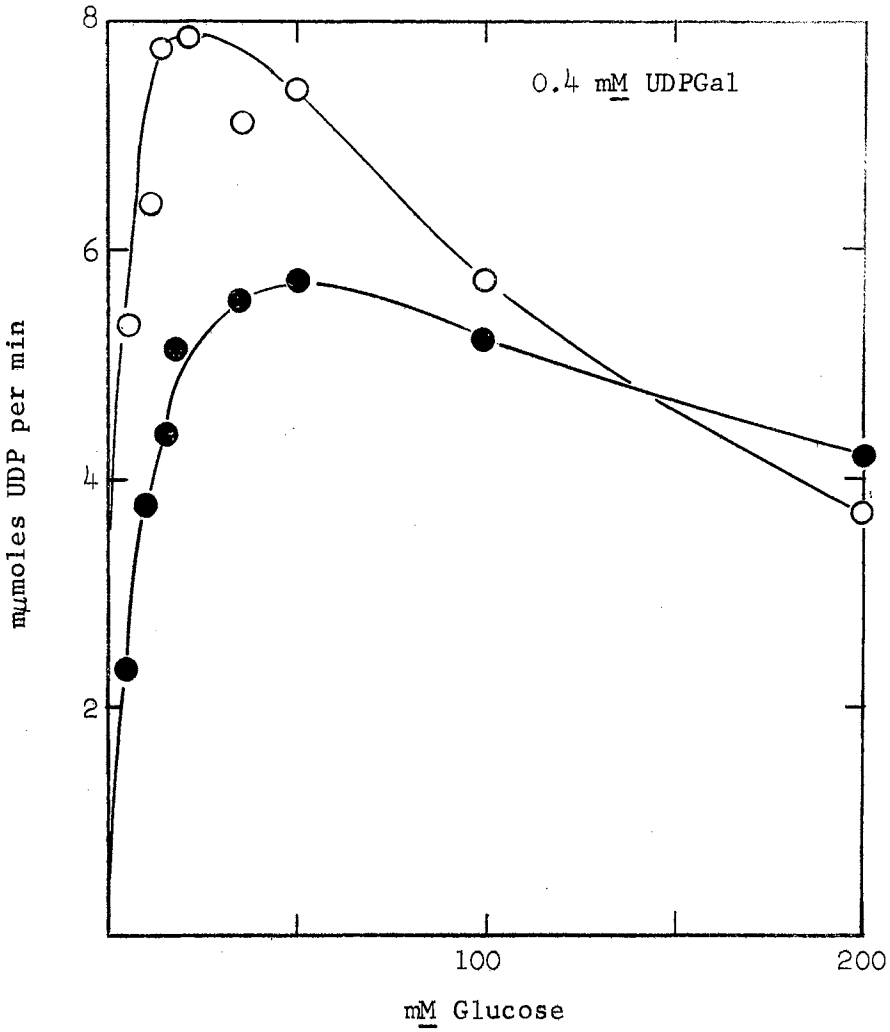


Figure 28. Glucose Optimum in LS_A Assay with Two B-Protein Concentrations.

Assay Conditions: 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; and 0.4 mM UDP-galactose. A-protein was from HA_I (A-7). ● - ●, 50 μg B/ml and O - O, 200 μg B/ml.

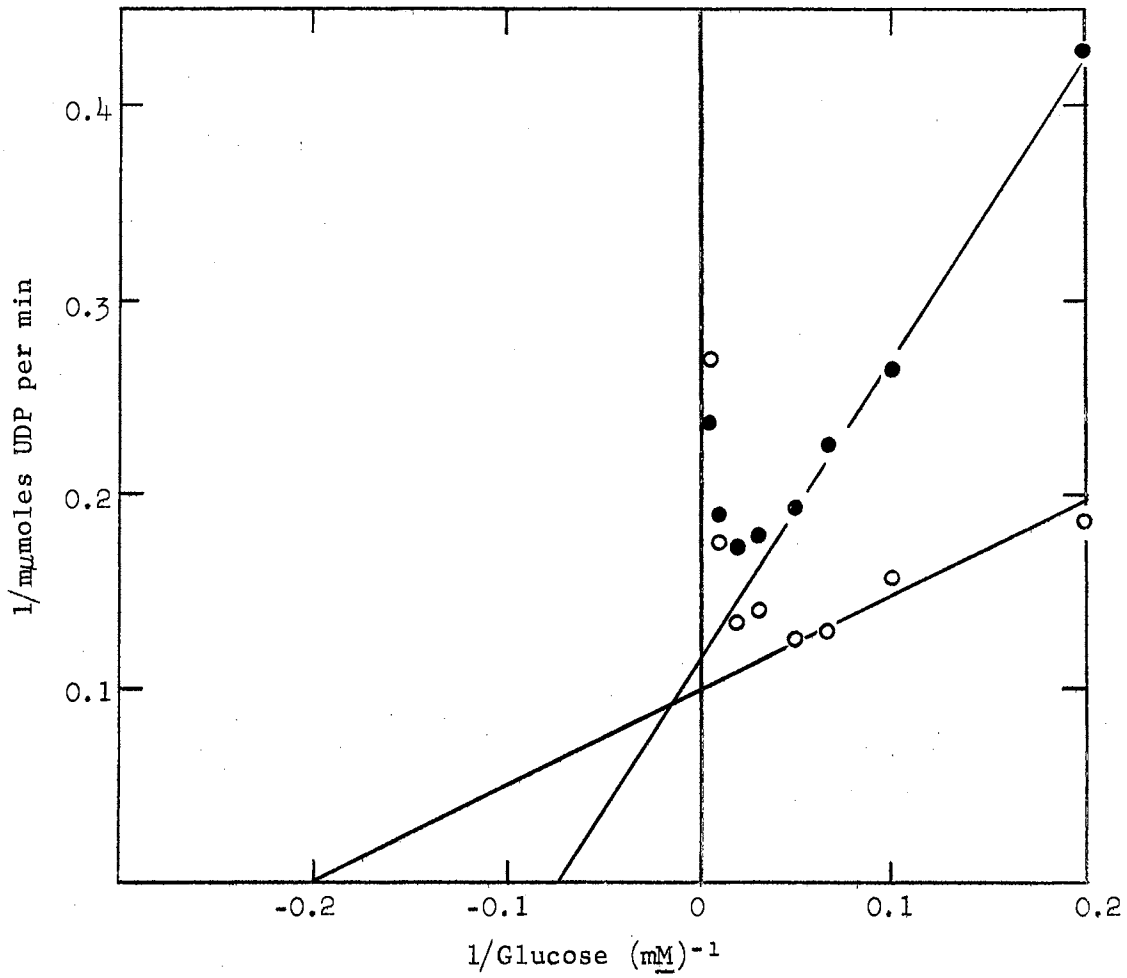


Figure 29. Lineweaver-Burk Plot for Glucose at 50 μg and 200 μg B Protein/ml.

Data are derived from Figure 28. ● - ●, 50 μg B/ml; 0 - 0, 200 μg B/ml. The K_m for 50 μg B was 15.4 mM and for 200 μg B was 5 mM. UDP-galactose was 0.4 mM. Similar differences in K_m were observed with other levels of UDP-galactose (see text).

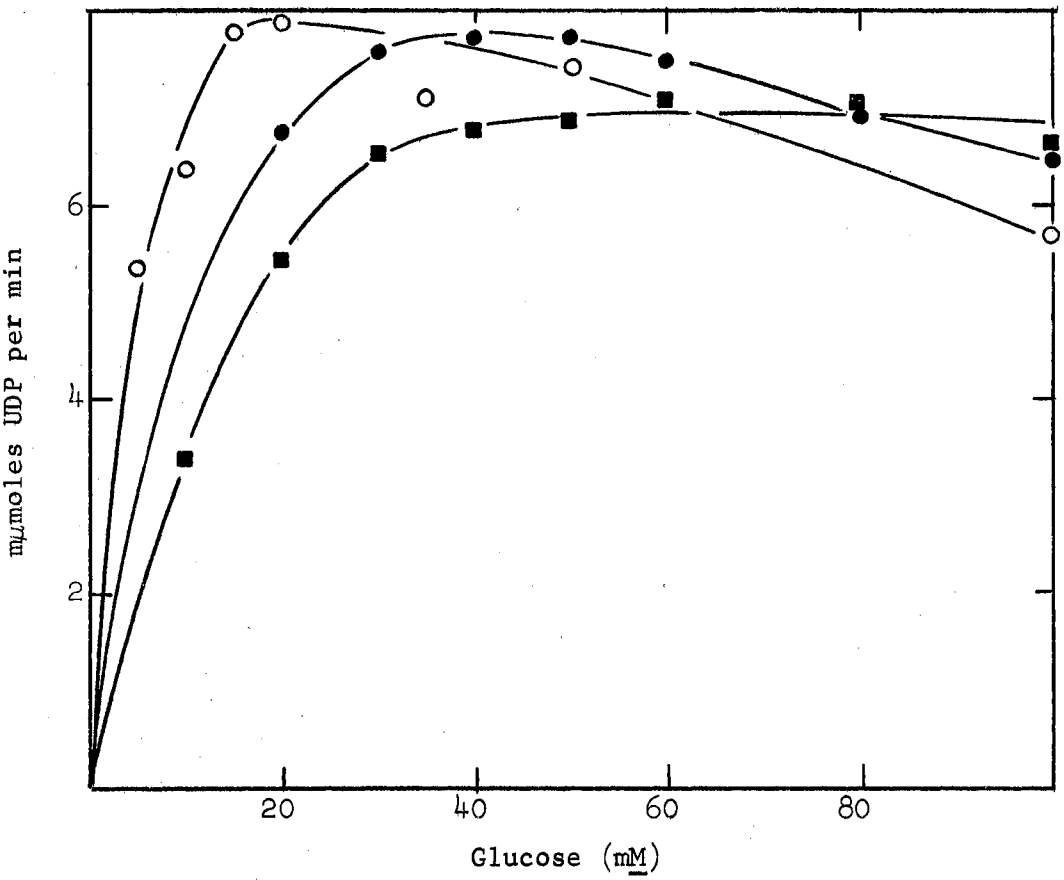


Figure 30. Glucose Optimum for Different Purification Steps of A protein.

O - O, A-7, from HA_I - assayed with 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 0.4 mM UDP-galactose; and 200 μg B/ml. ● - ●, A-3, from HA_{II} - assayed with 50 mM glycylglycine, pH 8.0; 6 mM $MnCl_2$; 0.25 mM UDP-galactose; and 200 μg B/ml. ■-■, A-5, from DEAE - assayed with 50 mM glycylglycine, pH 8.0; 6 mM $MnCl_2$; 0.4 mM UDP-galactose; and 200 μg B/ml.

chromatography (HA_{II}) to DEAE-cellulose chromatography (DEAE) - the concentration of glucose for maximum activity increased and the substrate inhibition by glucose diminished. The difference is probably not due to assay conditions since the UDP-galactose concentration did not affect the optimum glucose concentration (Figure 27) and other differences in the assay were minor. Furthermore, several A-protein preparations from different stages of purification which were assayed for maximum activity as a function of glucose concentration exhibited the same pattern of glucose optimum corresponding to the step of purification as illustrated in Figure 30. This was observed even while the assay was being developed and slight modifications to the assay were being made.

The A protein was stored at -10° and the glucose optimum was determined after two weeks for one preparation and after two months for another preparation. However, no alteration of the glucose optimum was observed within any one preparation of A protein. Thus, these experiments suggest that the optimum concentration of glucose in the LS_A assay depends on the concentration of the B protein in the assay and on the purity of the A protein.

Since the optimal conditions were not all determined with the same A protein, the assay parameters were reexamined with A protein from the same step of purification (from HA_I). Also, the optima were examined for the assay of A-protein activity since no thorough investigation of this assay had yet been performed. Figure 31 illustrates the pH optima for LS_A activity (25 m$\underline{M}$ glucose and 200 μg B/ml) and for A-protein activity (25 m$\underline{M}$ N-acetylglucosamine). Both acti-

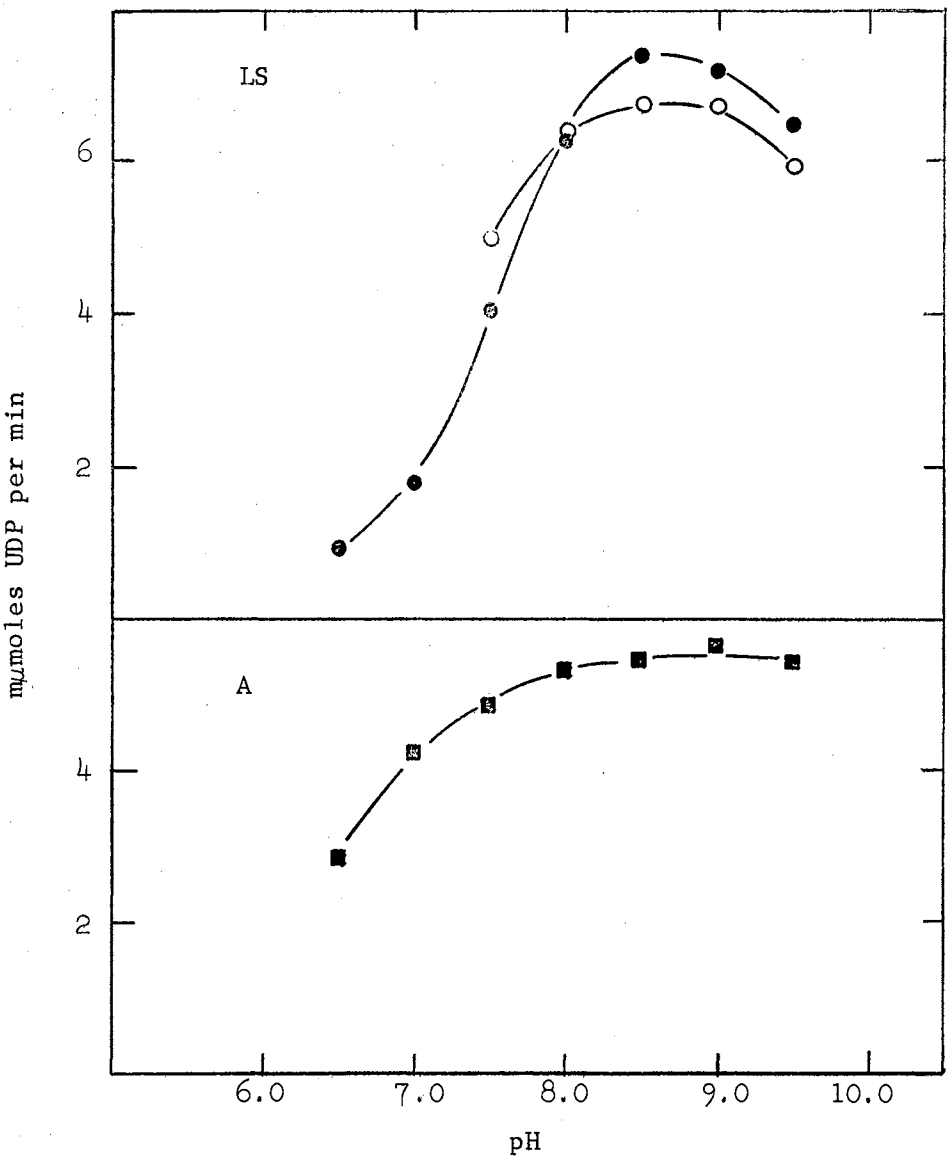


Figure 31. pH Optimum for LS_A and A-Protein Assays.

● - ●, LS_A assay: 50 mM glycylglycine, 5 mM $MnCl_2$, 0.4 mM UDP-galactose, 50 mM glucose, 200 μg B/ml and A-3 (off HA_{II}). O - O, LS_A assay: 50 mM glycylglycine, 5 mM $MnCl_2$, 0.4 mM UDP-galactose, 25 mM glucose, 200 μg B/ml, and A-10 (off HA_I). ■ - ■, A-protein assay: 50 mM glycylglycine, 5 mM $MnCl_2$, 0.4 mM UDP-galactose, 25 mM N-acetylglucosamine, and A from HA_I.

ties have a broad pH optimum between pH's 8.0 and 9.5 though the A-protein has slightly more activity at the acid side. The A-protein activity shows slightly more activity at pH 9.0 but pH 8.5 was chosen for future assays to minimize oxidation of Mn^{++} which occurs at higher pH's. The optimum $MnCl_2$ concentration was determined to be 5 mM for both assays and the data are presented in Figure 32.

Figure 33 indicates that UDP-galactose becomes saturating in the assay for both LS_A and A-protein activity at 0.4 mM. In Figure 34 the glucose/N-acetylglucosamine optima for the LS_A activity and the A-protein activity are 20 to 30 mM. Substrate concentration of 25 mM was chosen for both activities in the standard assay.

The effect of B protein on the assays is presented in Figure 35. The B protein is saturating in the LS_A assay (13 units) at 200 μg/ml. In the A-protein assay, however, the B protein inhibits A-protein activity (12 units) 100% with 100 μg/ml and inhibits 50% with 5 μg/ml.

After the development of Assay System II this system was used to assay UDP-galactose galactosyl transferase activity in certain tissues. It was recognized that the conditions had not been completely optimized for each enzymatic source. Nevertheless, the qualitative results were considered significant. Table III indicates that lactose synthetase and A-protein activities are present in homogenates of opossum, hamster, and bat mammary glands. Rabbit gastric mucosal particles synthesized lactose only after addition of B protein although some N-acetyllactosamine synthesis was observed. The guinea pig mammary gland was most unusual in that the absence of glucose or N-acetylglucosamine in a reaction incubation (normally used as an incubation blank to monitor hydrolysis of UDP-galactose) did not

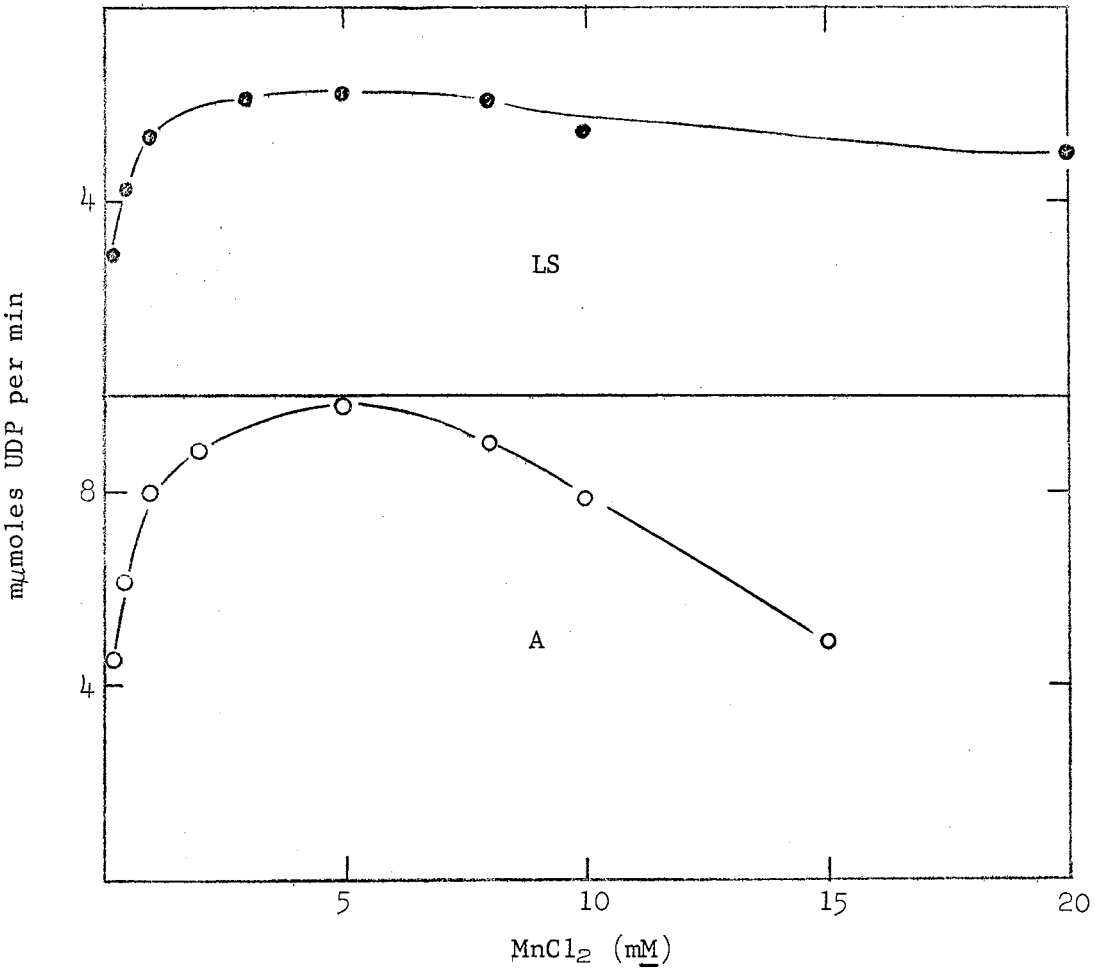


Figure 32. Optimum Mn^{++} Concentration for LS_A and A-Protein Assays.

● - ●, LS_A assay; 50 mM glycylglycine, pH 8.5; 0.4 mM UDP-galactose; 25 mM glucose; 200 μg B/ml; and A-10 (from HA_I). ○ - ○, A-protein assay: 50 mM glycylglycine, pH 8.5; 0.4 mM UDP-galactose; 25 mM N-acetylglucosamine; and A-10 (from HA_I).

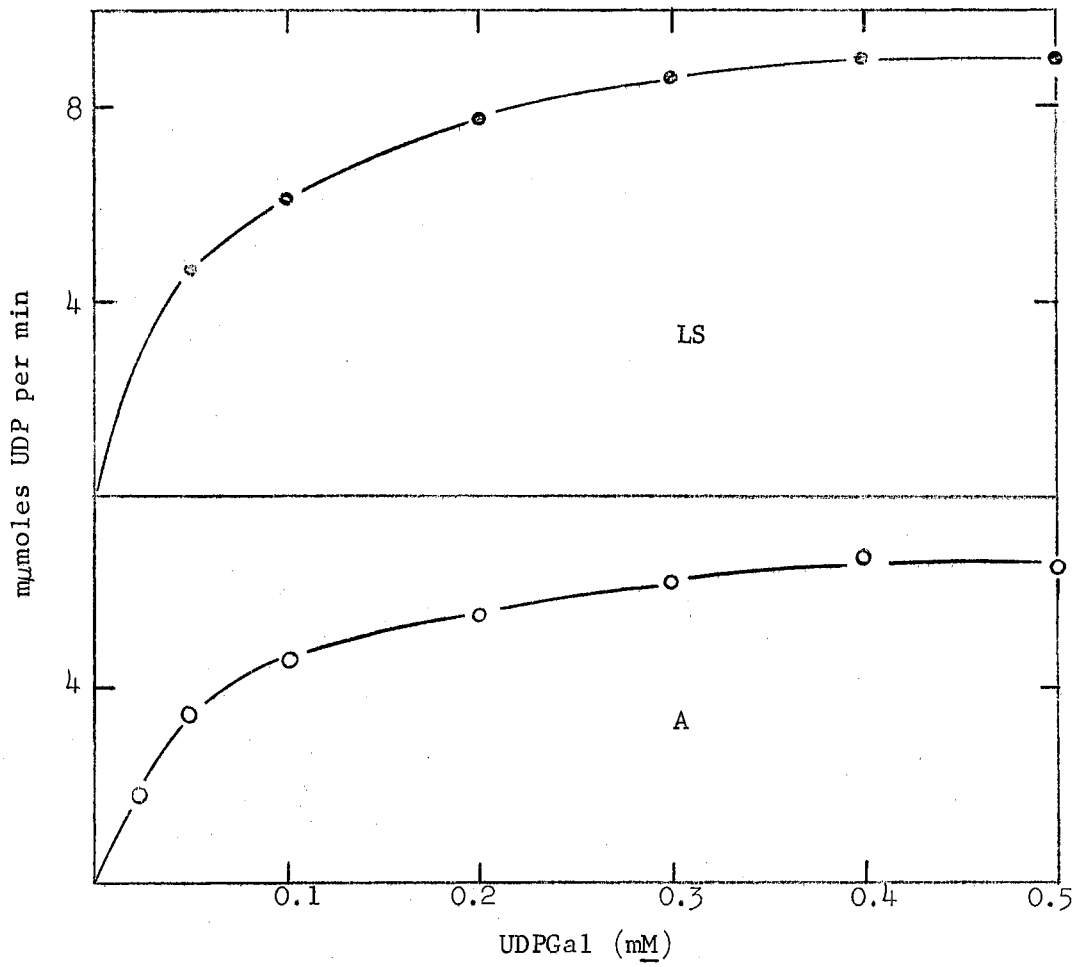


Figure 33. Optimum UDP-Galactose Concentration for LS_A and A-Protein Assays.

● - ●, LS_A assay: 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 25 mM glucose; 200 μg B; and A-10 (from HA_I). O - O, A-protein assay: 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 25 mM N-acetylglucosamine; and A-10. The curve represents the average of two replicate assays.

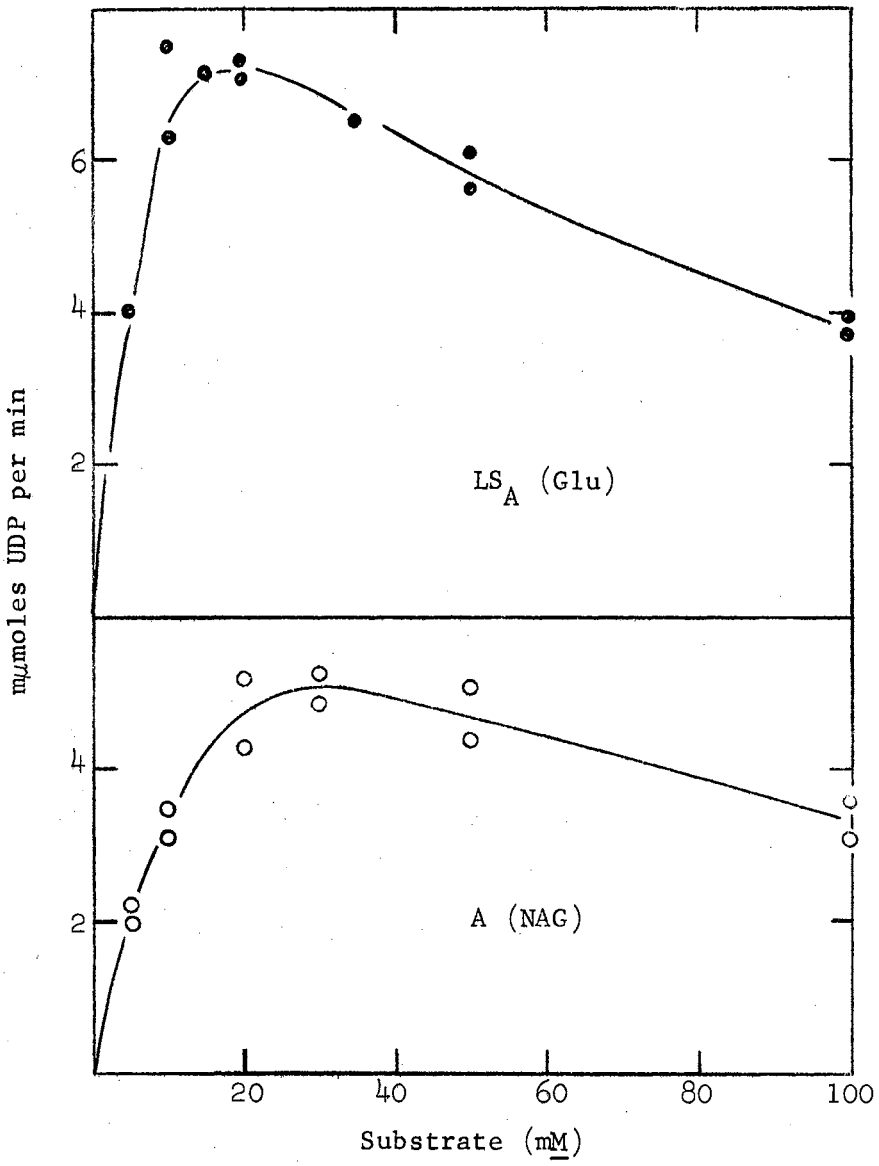


Figure 34. Substrate (Glucose/N-Acetylglucosamine) Optima for LS_A and A-Protein Assays.

● - ●, LS_A assay: 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 0.4 mM UDP-galactose; 200 μg B/ml; and A-10 (from HA_I). O - O, A-protein assay: 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 0.4 mM UDP-galactose; and A-10 (from HA_I).

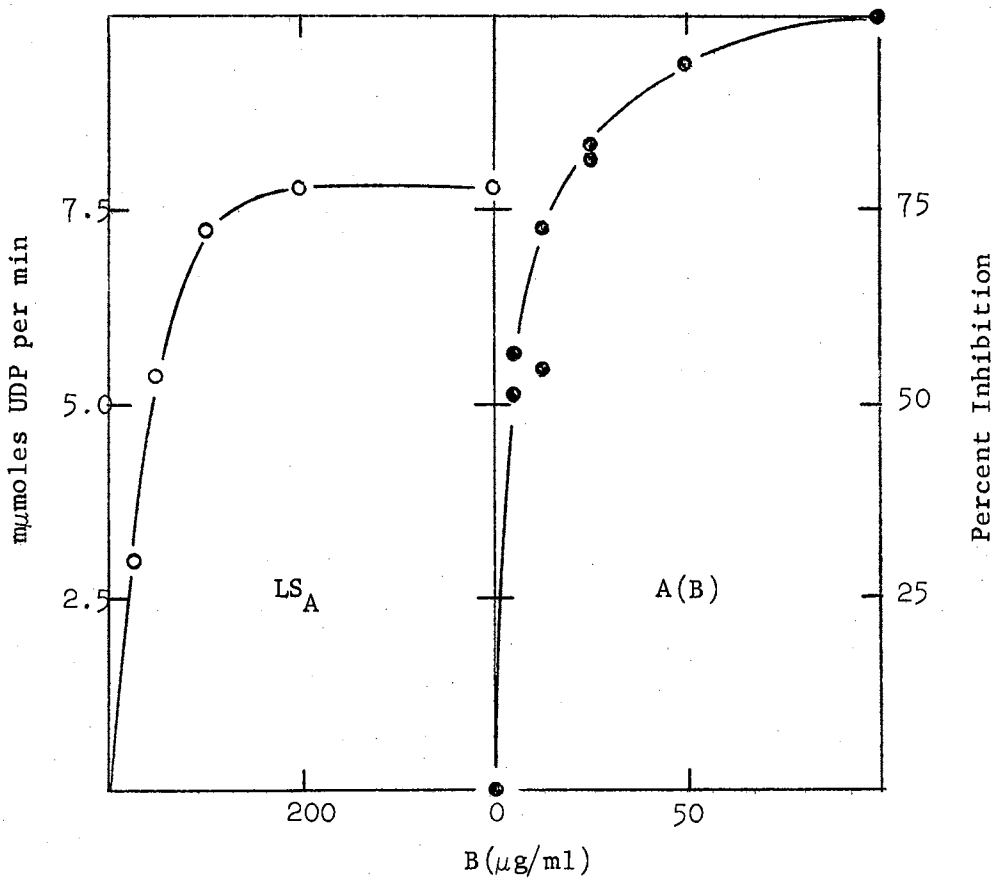


Figure 35. The Role of the B Protein in LS_A and A-Protein Assays.

O - O, LS_A activity (rate vs B concentration): 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 0.4 mM UDP-galactose; 25 mM glucose; and 13 units of A from HA_I. ● - ●, A-protein activity (percent inhibition vs. B concentration): 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 0.4 mM UDP-galactose; 25 mM N-acetylglucosamine; and 12 units of A from HA_I.

TABLE III

GALACTOSYL TRANSFERASE ACTIVITIES IN A VARIETY OF TISSUES

Source	Substrate	-B (units/g tissue)	+B (200 μg/ml) (units/g tissue)
Rabbit Gastric Mucosal Particles	Glucose	0	8.51
	N-acetylglucosamine	16.2	4.7
Opossum Mammary Gland	Glucose	76.5	186.3
	N-acetylglucosamine	267.3	176.0
Guinea Pig Mammary Gland	Glucose	105	558
	N-acetylglucosamine	380	180
Bat Mammary Gland	Glucose	50	266.4
	N-acetylglucosamine	288	72.5
Bat Milk	Glucose	14.5 *	27.5 *
	N-acetylglucosamine	12.5 *	2.0 *
Hamster Mammary Gland	Glucose	106	758
	N-acetylglucosamine	579	386

* Activity is in units per ml.

Preparation of homogenates (1 g/5 ml of 0.15 M KCl, 0.005 M $MgCl_2$, 0.005 M EDTA) was described in Experimental Procedure, Chapter V. The incorporation assay was used.

affect the synthesis of either lactose or N-acetyllactosamine. However, the effect of B protein on the activities of LS_A and A-protein produced the effect normally observed - stimulation of lactose synthesis and inhibition of N-acetyllactosamine synthesis. These results with the guinea pig mammary tissue require further verification and investigation.

Thus, reactions involving the two catalytic activities of the A protein require very similar conditions for optimum enzymatic activity with purified bovine proteins. The optimum conditions for LS_A activity are 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 0.4 mM UDP-galactose; 25 mM glucose; and 200 μg B/ml. The optimum conditions for A-protein activity are identical except for substituting N-acetylglucosamine for glucose at the same concentration and omitting the B protein. These conditions are designated Assay System III for LS_A activity or A-protein activity. However, this system applies only to A-protein preparations purified through the first hydroxyapatite chromatography step or less. More purified A proteins require higher glucose concentration for maximum activity. The optimum N-acetylglucosamine concentration with different A-protein preparations was not investigated due to a lack of available A protein purified beyond the first hydroxyapatite chromatography step.

Assay for LS_A Activity in Rat Mammary Gland Homogenates

In preparing to assay for lactose synthetase in rat mammary gland tissue the conditions for obtaining maximum LS_A activity in homogenates of rat mammary gland were investigated using the incor-

poration assay. Not only were the previously discussed parameters of pH, metal concentration, and substrate concentration important but also such treatments as sonic oscillation, detergents, and centrifugation were considered.

Studies by Brodbeck et al. (29) indicated the A protein of lactose synthetase to be primarily in the microsomal fraction. The assays for lactose synthetase presented in Chapter III were indeed with the "microsomal" fraction (sedimenting between 37,000 xg and 100,000 xg). However, preliminary observations suggested that more vigorous homogenation of the tissue such as with the Sorvall Omnimixer used in these experiments compared with the VirTis homogenizer used by Brodbeck et al. (29) resulted in disruption of the particles containing the A protein. An experiment was performed to investigate the LS_A activity in fractions from centrifugation at 10,000 xg for 10 min and 85,000 xg for 120 min. These results are in Table IV and indicate that significant LS_A activity is found in both the 10,000 xg precipitate and in the 85,000 xg supernatant solution. Therefore, assays using microsomal preparations do not measure the total LS_A activity but the whole homogenate is required.

Brodbeck (115) found that anionic detergents such as sodium cholate and lauryl sulfate inhibited lactose synthetase but that the non-ionic detergent Tween 80 did not inhibit. Consequently, an investigation of the effect of Tween 80 on the LS_A activity in crude homogenates was carried out. Table V presents the effects of 0.2% Tween 80 on the LS_A activity of the 1,000 xg supernatant solution and precipitate. The Tween 80 not only increased the total activity of

TABLE IV

LS_A ACTIVITY IN VARIOUS CELLULAR FRACTIONS BASED ON CENTRIFUGATION

Fraction	Units of Activity
Crude Homogenate	2304
10,000 xg for 10 min.	
Supernatant	1661
Precipitate	274
85,000 xg for 120 min.	
Supernatant	556
Precipitate	1340

Homogenates (1 g fresh mammary tissue - 14-day lactating rat/10 ml of 0.2 M Tris, pH 8.0) was prepared as in Chapter III. Assays were with Assay System III using the incorporation assay.

TABLE V

EFFECT OF CENTRIFUGATION AND TWEEN 80 ON LS_A ACTIVITY IN RAT MAMMARY GLAND HOMOGENATES

Fraction	Units of Activity
Minus TWEEN 80	
Crude Homogenate	7490
1,000 xg Precipitate	233
1,000 xg Supernatant	7095
Plus TWEEN 80	
Crude Homogenate	9400
1,000 xg Precipitate	32
1,000 xg Supernatant	9745

Assays were with Assay System III using the incorporation assay. Homogenate (1 g frozen mammary gland - 18-day lactating rat/10 ml of homogenizing buffer) was prepared as in Chapter III. Centrifugation was at 1,000 xg for 10 min. 0.2% Tween 80 was added to the crude homogenate as indicated.

the crude homogenate but also minimized the activity in the 1,000 xg precipitate.

Further experiments with Tween 80 at 0.02%, 0.2%, and 2.0% indicated maximal activity with 0.2%. Table VI shows that when the 0.2% Tween 80 was added to the homogenizing buffer before homogenation 69% of the activity was destroyed. When the Tween 80 was added after the 1,000 xg centrifugation the activity was increased by 77%.

Brodbeck et al. (29) indicated that sonic treatment of the particulate preparation of bovine mammary tissue released the A protein from the particulate fraction. Therefore, sonic oscillation of the 1,000 xg supernatant solution was performed and the results are presented in Table VII. This table shows that sonic oscillation of the 1,000 xg supernatant solution either with or without Tween 80 did not increase the total activity of A protein as measured by the LS_A assay. Again addition of 0.2% Tween 80 increased the activity in the 1,000 xg supernatant solution by 49%.

The mechanism by which Tween 80 increases the activity is not known. In some experiments the Tween 80 did not significantly "solubilize" the A protein by causing release from the particulate fraction to the soluble fraction. Experiments with purified bovine A protein indicated that the purified bovine A protein is not activated by Tween 80 in the LS_A assay even though Tween 80 does effect some increase in activity in a homogenate of fresh bovine lactating mammary tissue.

Thus, the maximum activity of LS_A activity occurred with a 1,000 xg supernatant solution with 0.2% Tween 80. Sonic oscillation did not increase the total LS_A activity when assayed with Assay System III.

TABLE VI

EFFECT OF 0.2% TWEEN ON LS_A ACTIVITY IN RAT MAMMARY GLAND HOMOGENATES

Treatment	Units of Activity
Normal Extract	1.3
Normal Extract + 0.2% Tween 80 added after centrifugation	2.3
Extract with 0.2% Tween 80 added to homogenizing buffer	0.4

Homogenates (1 g frozen mammary tissue/10 ml of 0.15 M KCl, 0.005 M $MgCl_2$, and 0.005 M EDTA) was prepared as described in Chapter III and centrifuged at 1,000 xg for 10 min. Assays were with Assay System III by the incorporation assay.

TABLE VII

EFFECT OF TWEEN 80 AND SONIC OSCILLATION ON LS_A ACTIVITY IN RAT MAMMARY GLAND HOMOGENATE

Treatment	Units of Activity
Normal Extract	1.75
+ 15 min. oscillation	1.75
+ 30 min. oscillation	1.6
Extract with 0.2% TWEEN 80 added after centrifugation	2.6
+ 15 min. oscillation	2.0
+ 30 min. oscillation	2.25

Homogenate (1 g/10 ml homogenizing buffer) was prepared as in Chapter III and was centrifuged at 1,000 xg for 10 min. Oscillation was in a Raytheon Sonic Oscillator at 0°. Assays were with Assay System III by the incorporation assay.

A study of the LS_A activity was carried out to determine if a time-dependent increase in LS_A activity occured with incubation as was seen with the UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase. Figure 36 indicates that no significant change in activity was observed when the crude homogenage (no centrifugation) of lactating rat mammary gland was incubated at 0°.

Further investigation of the pH, substrate concentrations, and B protein requirements were carried out with the supernatant solution from 1,000 xg centrifugation containing 0.2% Tween 80 *via* the incorporation assay. The pH optimum for LS_A activity was found to be between 8.5 and 9.5 (Figure 37). For a standard assay pH 9.0 was chosen. The data of Figure 38 indicate the optimum $MnCl_2$ concentration was 5 mM.

The optimum B concentration is presented in Figure 39. With the rat mammary homogenate the bovine B requirement increased to 1 mg/ml compared with 0.2 mg/ml in the assay with purified bovine proteins. With 1 mg B/ml the glucose optimum was determined to be 25 mM (Figure 40). A previous experiment with 0.2 mg B protein/ml resulted in 50 mM glucose for maximum activity. Thus, the dependence of the optimum glucose concentration on the B protein concentration was observed with the rat mammary homogenates as well as with the purified bovine proteins. Figure 41 shows the UDP-galactose concentration to be saturating at 0.6 mM.

UTP inhibition of the UDP-galactose hydrolase of bovine mammary tissue has been reported (21,30). Experiments were therefore carried out to assess the effect of UTP and ATP on the standard assay for LS_A

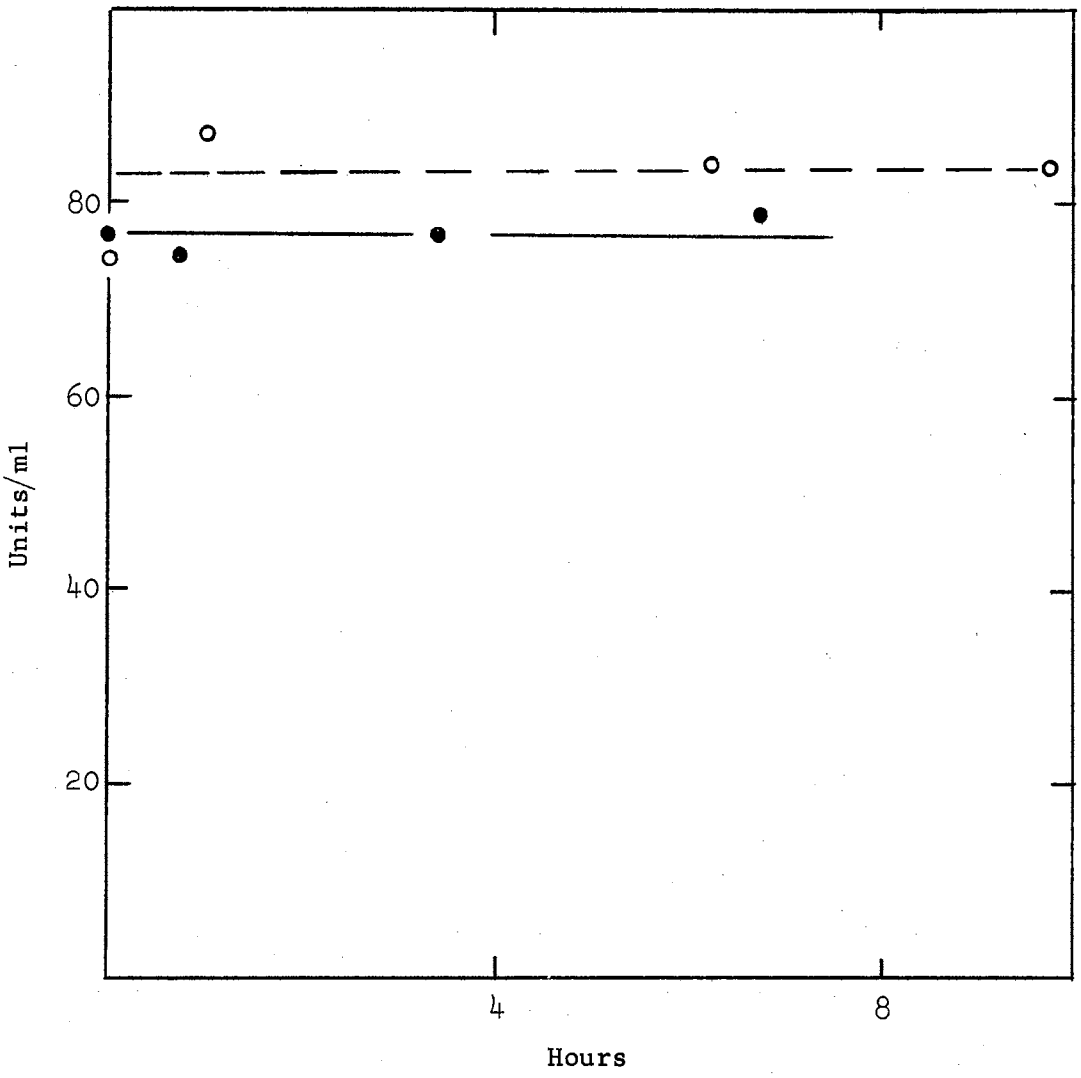


Figure 36. LS_A Activity of a Homogenate Incubated at 0° for Various Time Periods.

(● - ●), 1 g tissue (mammary gland from 14-day lactating rat/10 ml of 0.2 M Tris, pH 8.0. (○ - ○), 1 g tissue (frozen tissue from above)/10 ml of homogenizing buffer. Assays in both cases were of the crude homogenate (no centrifugation) with Assay System III using the incorporation assay.

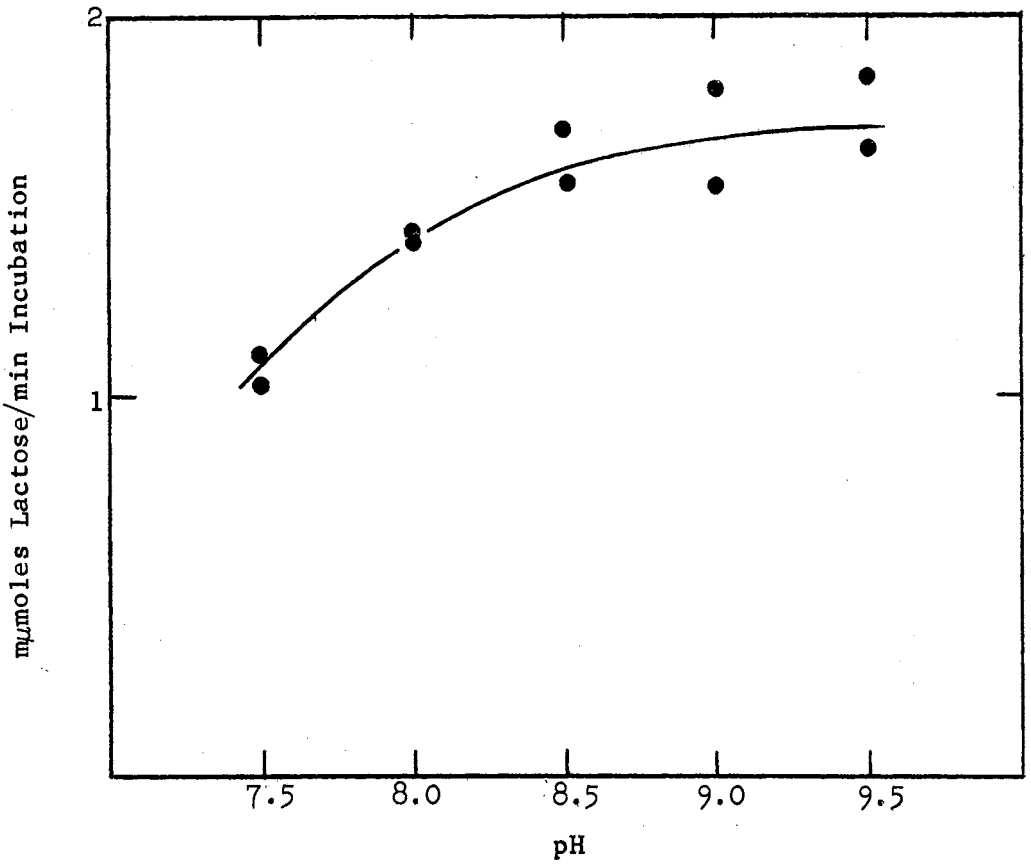


Figure 37. pH Optimum for LS_A Assay in Lactating Rat Mammary Gland Homogenates.

Assay conditions: 50 mM glycylglycine; 5 mM $MnCl_2$, 25 mM glucose, 1 mg B/ml, 0.4 mM UDP-galactose-^{14}C, and 20 μl of the homogenate (200 mg/ml of homogenizing buffer) after 1,000 xg centrifugation. The incorporation assay was used.

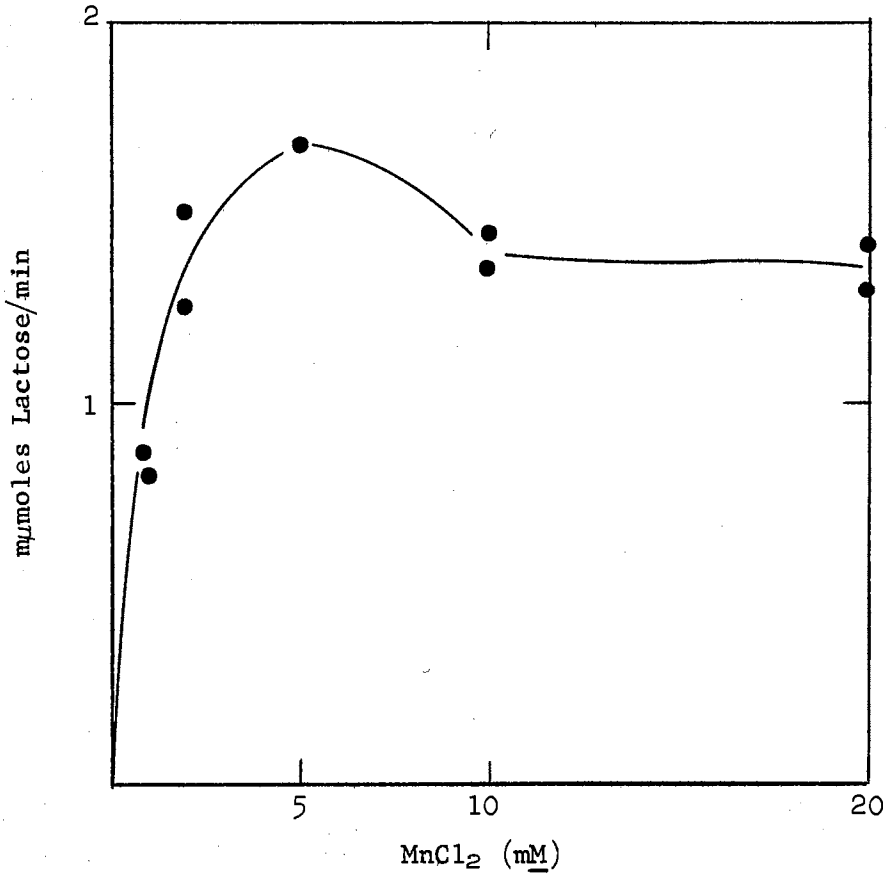


Figure 38. Optimum $MnCl_2$ Concentration for LS_A Assay in Lactating Rat Mammary Gland Homogenate.

Assay conditions: 50 mM glycylglycine, pH 9.0; 0.4 mM UDP-galactose-^{14}C; 25 mM glucose; 1 mg B protein/ml; and 20 μl of the homogenate (200 mg tissue/ml of homogenizing buffer) after 1,000 xg centrifugation. The incorporation assay was used.

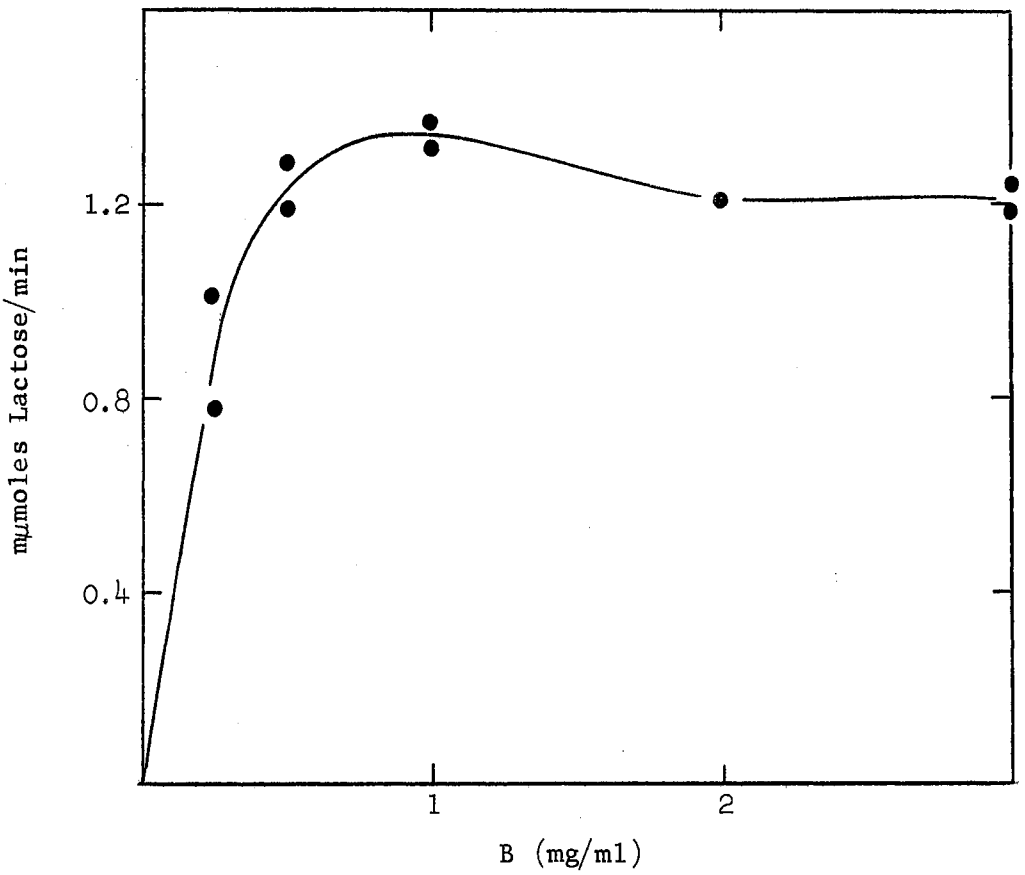


Figure 39. Optimum B Protein Concentration for LS_A Assay in Lactating Rat Mammary Gland Homogenates.

Assay conditions: 50 mM glycylglycine, pH 9.0; 5 mM $MnCl_2$; 25 mM glucose; 0.4 mM UDP-galactose-^{14}C; 1 mg B/ml and 20 μl of the homogenate (200 μg/ml homogenizing buffer) after 1,000 xg centrifugation. The incorporation assay was used.

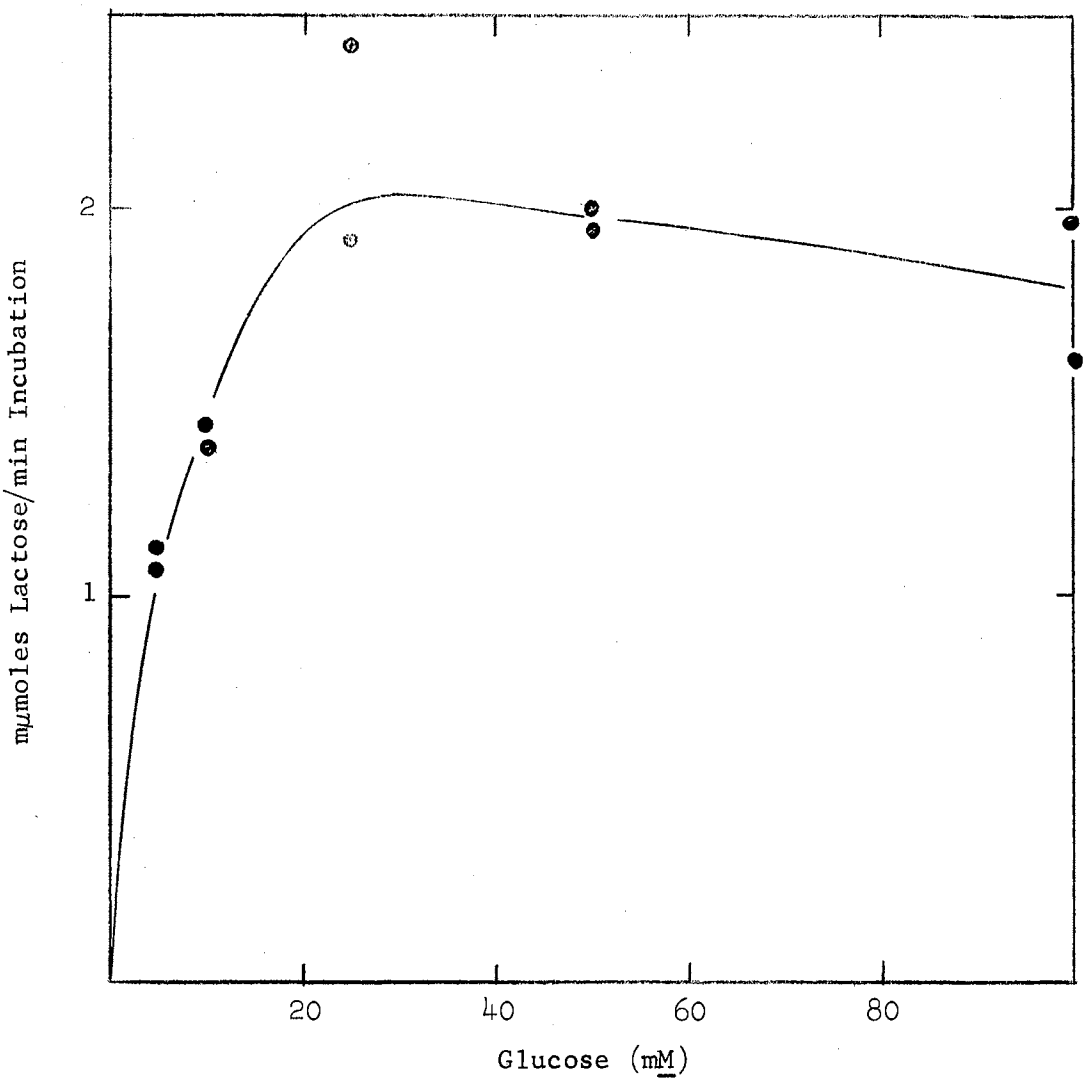


Figure 40. Glucose Optimum for LS_A Assay in Lactating Rat Mammary Gland Homogenate.

Assay conditions: 50 mM glycylglycine, pH 9.0; 5 mM $MnCl_2$; 0.4 mM UDP-galactose-^{14}C; 1 mg B/ml; and 20 μl of the homogenate (200 mg/ml of homogenizing buffer) after 1,000 xg centrifugation. The incorporation assay was used.

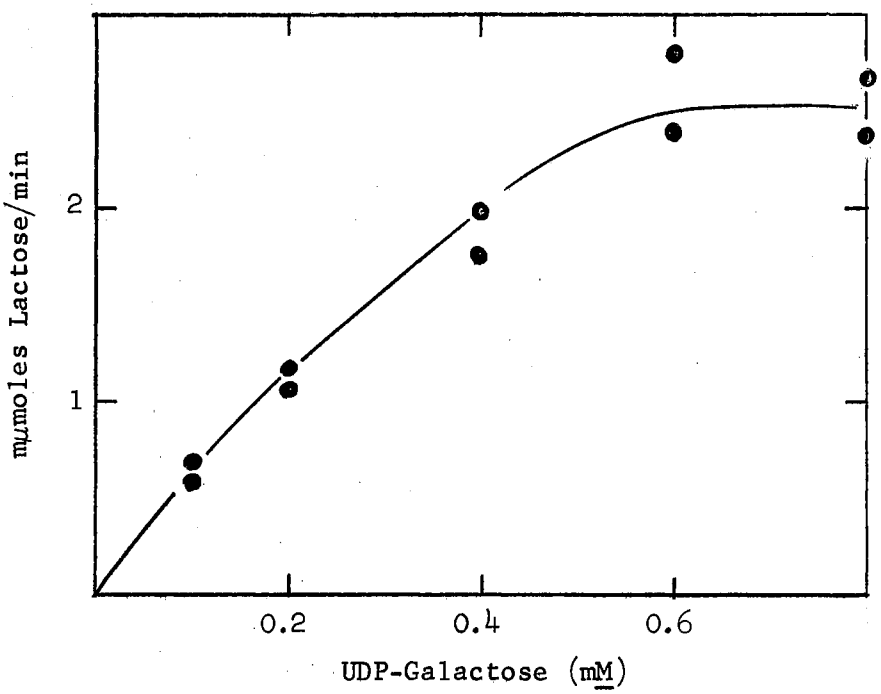


Figure 41. Optimum UDP-Galactose Concentration in LS_A Assay of Rat Mammary Homogenate.

Assay conditions: 50 mM glycylglycine, pH 9.0; 5 mM $MnCl_2$; 1 mg B/ml; 25 mM glucose; and 20 μl of rat mammary gland homogenate (400 mg/ml of homogenizing buffer) after 1,000 xg centrifugation. The incorporation assay was used.

activity in rat mammary tissue. However, these compounds even at 1 mM concentration not only did not increase the LS_A activity but tended to inhibit. Consequently, ATP and UTP were not included in the standard assay.

The limits of linearity of the assay for LS_A activity is presented in Figure 42. This figure indicates the rate (mμmoles lactose/min incubation) is proportional to the amount of the extract in the assay up to 3 mμmoles lactose/min incubation.

Thus, the assays for LS_A activity and A-protein activity have been reevaluated with purified bovine A and B proteins in the spectrophotometric assay. The conditions in the assay of both activities were nearly identical except for the substitution of substrates; these conditions were designated Assay System III. The conditions for obtaining maximum LS_A activity in homogenates of rat mammary tissue have been developed using the incorporation assay. The assay conditions for the rat mammary homogenate (Assay System IV) are: 50 mM glycylglycine, pH 9.0; 5 mM $MnCl_2$; 25 mM glucose; 0.6 mM UDP-galactose; 1 mg/ml of B protein; and aliquots of a 1,000 xg supernatant solution to which 0.2% Tween 80 has been added. A summary of the assay conditions are presented in Table VIII.

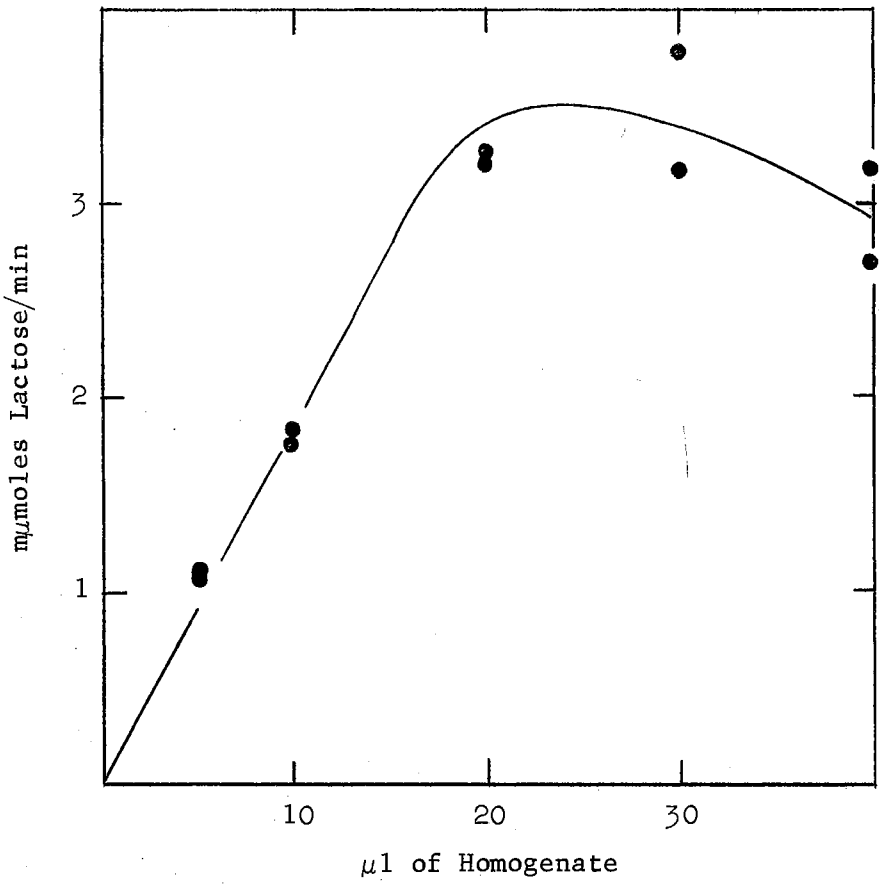


Figure 42. Linearity of LS_A Assay with Homogenate Concentration.

Assay conditions: 50 mM glycylglycine, pH 9.0; 5 mM $MnCl_2$; 25 mM glucose; 0.6 mM UDP-galactose; and 1 mg B/ml. Homogenate (2 g tissue from 6-day lactating rat/5 ml of homogenizing buffer) was from 1,000 xg centrifugation. The incorporation assay was used.

TABLE VIII

SUMMARY OF ASSAY CONDITIONS FOR LS_A ASSAY

Assay System	Assay Conditions	Application of Assay
I	2 mM Tris-HCl, pH 7.4 20 mM $MnCl_2$ 0.25 mM UDP-galactose 20 mM glucose 100 µg B protein/ml	Initial assay; derived from Babad and Hassid (9)
II	50 mM glycylglycine, pH 8.5 5 mM $MnCl_2$ 0.4 mM UDP-galactose 50 mM glucose 200 µg B protein/ml	Used for bovine A-protein preparations more purified than the HA_{II} Step (Figure 17)
III	50 mM glycylglycine, pH 8.5 5 mM $MnCl_2$ 0.4 mM UDP-galactose 25 mM glucose 200 µg B protein/ml	Useful for bovine A-protein preparations purified through HA_I or less (Figure 17)
IV	50 mM glycylglycine, pH 9.0 5 mM $MnCl_2$ 0.6 mM UDP-galactose 25 mM glucose 1 mg B protein/ml	Assay for LS_A activity in rat mammary gland homogenate

CHAPTER VI

DISCUSSION

The Enzymes of Lactose Biosynthesis in Rats and in Rat Organ Cultures

The data in Figure 1 show the activities in rat mammary gland extracts of three enzymes associated with and responsible for lactose biosynthesis - UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase, and lactose synthetase. UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase activities increased somewhat during pregnancy and rapidly increased after parturition to a maximum at mid to late lactation. These results are similar to those of Shatton et al. (41), Baldwin and Milligan (43), and Kuhn and Lowenstein (44) in rats and Heitzman (49) in rabbits. No statistical evaluation of the data was made since there were only three animals in each group. The variation observed within the groups could be due to variation among the animals within the rather extensive time periods (e.g., lactating 1 to 7 days) or to some in vitro activation in the extracts before assays were performed. Nevertheless, the trends are consistent with the published studies.

No lactose synthetase activity was detected in normal or pregnant animals until a day or two before parturition. Similar results for lactose synthetase in rat mammary homogenates were described by

Kuhn (45). Thus, the levels of enzymatic activity observed for UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase, and lactose synthetase in pregnant and lactating rat mammary homogenates support the studies of other investigators.

Glycogen synthetase (UDP-glucose:α-1,4-glucan α-4-glucosyl-transferase EC 2.4.1.11) activity has been observed in bovine mammary tissue and the enzyme was partially purified (116). Therefore, an attempt was made to assay rat mammary gland homogenates for glycogen synthetase activity by measuring the incorporation of glucose-^{14}C into glycogen from UDP-glucose-^{14}C (116). However, no significant incorporation was observed with the homogenates except those from normal and early pregnant rats. Moreover, glycogen incubated with the homogenates from lactating glands decreased or disappeared in the 10-minute incubation period. Thus, lactating rat mammary gland appears to have a very active glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase EC 2.4.1.1) which may account for low values of glycogen synthetase activity and the disappearance of glycogen. Such results support the view that during lactation the mammary gland is directed toward the formation of lactose. For this process to be efficient the activity of glycogen synthetase should be greatly reduced so that UDP-glucose formed from the UDP-glucose pyrophosphorylase reaction would be directed toward lactose formation.

The organ culture experiments suggest that certain precautions and limitations need to be recognized when using enzymes as indicators of metabolic function. As indicated in Figure 2, greater than half of the total enzymatic units after 10 and 24 hours in organ culture

were found in the medium. Thus, a considerable portion of the UDP-glucose pyrophosphorylase was leaking into the medium and permeability characteristics of the cells in culture may have been altered. Some leakage into the medium is expected due to damaged cells from the excision process, but it seems unlikely that loss of enzyme from the damaged cells on the exterior of the explants would account for 50% of the total activity in the medium. It is also tenuous to speculate that the high amount in the medium represents a "secretory process".

No correction was made for the possible decay of UDP-glucose pyrophosphorylase activity in the medium at 37°. Very limited decay studies of UDP-glucose pyrophosphorylase activity in medium maintained at 37° suggested that the enzymatic activity decreased. The reliability of the data, however, is uncertain and requires further investigation. These studies were not pursued after the increase in activity in homogenates was observed. Bovine mammary UDP-glucose pyrophosphorylase is stable for one hour at 50° (117) and it is possible that a loss in activity could reflect proteolysis. Nevertheless, for organ culture studies using enzymatic activities as indicators it seems desirable to determine the amount of indicator proteins in the medium under the culture conditions.

The increase in activity of the UDP-glucose pyrophosphorylase in the explants and crude homogenates occurred with a similar time course. This suggests that a similar process(es) was occurring with both systems and that the tissue explants may not reflect the metabolic activity of the mammary gland *in vivo*. Thus, organ culture studies utilizing enzymatic activities require interpretation with caution and may be subject to limitations not previously recognized.

The decay of UDP-galactose 4-epimerase activity in the organ explants (Figure 3) may be due to the increased lability of this enzyme compared with the lability of UDP-glucose pyrophosphorylase. The increase in activity of UDP-galactose 4-epimerase is very sensitive to the temperature of incubation (Figure 7) and an increase in activity in the explants might have occurred before assays were performed. Thus, it appears that neither UDP-glucose pyrophosphorylase nor UDP-galactose 4-epimerase is a suitable enzyme to use as a biochemical criterion of mammary function in rat organ cultures.

The different temperature dependencies (Figures 6 and 7) for the "activation" of UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase might suggest that the method of activation of the two enzymes may be different and also may reflect differences in stability. The role of NAD^+ (Figures 5 and 7) in allowing the observed increase of activity of UDP-galactose 4-epimerase is unknown. The increase in epimerase activity occurred not only in rat mammary glands but in a variety of mammary tissues (Table II) and the increase in lactating tissues was dependent on the presence of NAD^+ in the extracts. NAD^+ is required to determine significant UDP-galactose 4-epimerase activity in adult human red cell hemolysates but is not required in red cell hemolysates of newborns (118). The mechanism of the increase in activity of UDP-galactose 4-epimerase and the role of NADH in the process require further investigation and present very interesting problems. It should be pointed out that Tsai (27) has shown that NAD^+, UDP-glucose, UDP-galactose and other nucleotide di- and triphosphates protect the enzyme from inactivation.

Increase in Activity of UDP-Glucose Pyrophosphorylase

The results from Chapter IV indicate that the increase in UDP-glucose pyrophosphorylase activity upon incubation is due to a proteinaceous process or to a slight modification of enzymatic structure.

The removal of low molecular weight material by chromatography with Sephadex G-25 did not prevent the increase in activity, and the increase in activity occurred in an acetone powder extract, in ammonium sulfate fractions of a normal extract, and in a homogenate prepared in 4.0% M sucrose. These results would argue against ionic effects or interactions with low molecular weight compounds or lipids being responsible for the increase in activity and suggest that the process is proteinaceous in nature.

There are several models involving protein-protein interactions which could account for the observed increase in activity. One possible model is limited proteolysis. The failure of phenylmethylsulfonyl fluoride to prevent the increase in activity reduces the probability that the increase occurred by limited proteolysis of the enzyme to a more active form although this possibility cannot be excluded. The phenylmethylsulfonyl fluoride is not a universal inhibitor of proteolytic enzymes and generally inhibits proteases having a reactive seryl group in the active site (119).

Another mechanism by which the increase in enzymatic activity could occur is a slow time-dependent destruction of an inhibitor. However, the results of Chapter IV suggest that if this were the case the inhibitor would be most likely a protein. The increase by incu-

bation of 5'-nucleotidase activity of E. coli was accompanied by destruction of a protein inhibitor with a molecular weight very similar to that of the 5'-nucleotidase (99). Thus, destruction of a protein inhibitor of UDP-glucose pyrophosphorylase with time is a possible method of increasing the total units of enzymatic activity.

Certain enzymes may be converted to more active forms by the action of different enzymes. For example, glycogen synthetase D may be converted to the more active glycogen synthetase I by the removal of a phosphate group from a seryl reside with a specific phosphatase (120,121) and adenylated glutamine synthetase (L-glutamate: ammonia ligase (ADP) (EC 6.3.1.2) is converted to a more active form when the AMP moiety is hydrolyzed by the action of a deadenylating enzyme (122). It seems likely that such a process with UDP-glucose pyrophosphorylase would not involve any low molecular weight compounds. However, the data presented here are consistent with some type of limited hydrolytic enzymatic action (since there is no gross change in molecular weight), perhaps reflecting the function of a control mechanism analogous to the control of glycogen synthetase.

The increase in activity could be accounted for by aggregation or disaggregation of subunits. Such a mechanism has been described for the increase in activity of rat liver acetyl-CoA carboxylase (100). If a similar process occurs with the UDP-glucose pyrophosphorylase, however, one of the subunits apparently does not have enzymatic activity since no marked changes in molecular weight were observed after the activation (Figure 16).

UDP-glucose pyrophosphorylase might undergo a slow time-dependent alteration of enzymatic structure allowing for the formation of a more active form of the enzyme. Such a process must occur, however, without significant alteration of the K_m for UTP (Figure 15). This view is supported in part by the observations that the extent of increase of enzymatic activity depended on the concentration of the initial extract and the initial level of enzyme, and that the increase in activity was progressively inhibited by urea, exhibited a temperature dependence, and was markedly dependent upon pH. However, these observations do not exclude an increase in activity occurring by the mechanisms described above. Thus, the data in Chapter IV are consistent with the view that the increase in UDP-glucose pyrophosphorylase activity is due to a structural change of the enzyme by some process that results in a more active form of the enzyme.

Lactose Synthetase: Assay and Role in Control of Lactose Biosynthesis

Chapter V indicates that assays for LS_A and A-protein activities have been developed whereby the assay conditions were optimized for maximum enzymatic activity. The results of this critical investigation of LS_A activity are summarized in Table VIII.

The LS_A and A-protein activities have nearly identical optimal assay conditions with respect to pH, manganese concentration, UDP-galactose concentration, and substrate (glucose or N-acetylglucosamine) concentration. This supports the suggestion by Hill and coworkers (34) that the A protein is involved in the synthesis of both

lactose and N-acetyllactosamine and that both catalytic activities are carried out by a single A protein. Also, neither activity could be observed when Mg^{++} at a concentration of 5 m$\underline{M}$ was substituted for Mn^{++} in the reaction mixture. With the enzymatic preparation of Babad and Hassid (9), however, 25% of the activity with $MnCl_2$ was observed with $MgCl_2$. This difference was not accounted for.

The inhibition of A-protein activity by α-lactalbumin indicated that the A-protein activity was inhibited 50% by 5 μg B protein/ml and with 100 μg B/ml the A-protein activity was inhibited 100%. Thus, on the basis of α-lactalbumin concentration in bovine milk (1 mg/ml) (123) and in bovine tissue (34 μg/ml) (10), the significant catalytic activity of A protein would likely be toward the synthesis of lactose.

One finding from the kinetic studies is that the B-protein and glucose concentrations appear to be reciprocally related with respect to maximum activity. That is, lower levels of B protein require a higher glucose concentration for maximum activity (Figure 28). This finding is supported by the recent studies of Andrews (124) which indicate that the apparent K_m for B protein is less at higher glucose concentrations. K_m studies (Figure 29) indicated that the K_m for glucose is affected by the level of B protein in the assay. With conditions which lead to maximum activity in the assay (Figure 28) the K_m for glucose is about 5 m$\underline{M}$. Previously, Babad and Hassid (9) reported a K_m for glucose of 25 m$\underline{M}$. The K_m for N-acetylglucosamine was about 8 m$\underline{M}$.

The assay for LS_A in rat mammary gland homogenates has also been developed to detect maximum LS_A activity (Table VIII). This assay

now seems quite adequate to measure LS_A activity in rat tissue homogenates. A modification of the assay was used to measure LS_A and A-protein activity in the opossum and bat mammary glands and the results indicated that opossum and bat mammary glands and bat milk contained LS_A activity. No data concerning lactose synthetase in bats have been reported. It has been reported that the milk of the opossum contains no detectable lactose after paper chromatography of unhydrolyzed protein-free filtrates of the milk (125). However, the opossum apparently has the capacity to synthesize lactose from UDP-galactose and glucose (Table III).

The assay for LS_A activity in homogenates of rat mammary gland maintained at 0° for 9 hours indicated that no time-dependent increase in the LS_A activity occurred (Figure 36). Thus it may be possible that LS_A activity can be used as a biochemical criterion of mammary gland function in rat mammary gland organ cultures. Such culture experiments would still require the precautionary considerations previously described.

It was somewhat surprising to discover that the precipitate from a very low speed centrifugation (1,000 xg for 10 min) still contained some detectable amounts of LS_A activity. Previous studies indicated that the A protein was in the microsomal fraction. This requirement for the whole homogenate to measure total LS_A activity, however, is consistent with the suggestion of Coffey and Reithel (31) that the lactose synthetase is found in lactose synthetase particles which have characteristics similar to membranes from the Golgi apparatus. Thus, it appears that the organization of lactose synthetase in

secretory cells is more complex than previously believed.

The question arises as to the validity of the lactose synthetase assays performed with the original assay presented in Figure 1. These assays are probably qualitatively valid relative to one another since the assays and tissue preparations were all done in the same manner. However, the assays do not necessarily reflect quantitative lactose synthetase activity as defined by LS_A or LS_B activity. The data do support the conclusion of Kuhn (45) that the lactose synthetase reaction is the rate-limiting step in lactose biosynthesis. That is, no activity of lactose synthetase was found in the mammary homogenates from pregnant rats until very near to parturition. Also, the maximum rates observed for the three enzymes - 20 μmoles product/min/g tissue (UDP-glucose pyrophosphorylase), 1 μmole product/min/g tissue (UDP-galactose 4-epimerase), and 0.01 μmole product/min/g tissue (lactose synthetase) - support the suggestion that lactose synthetase is rate-limiting.

Furthermore, the studies of Turkington et al. (48) indicate that the synthesis of lactose is really limited by the amount of B protein (α-lactalbumin). No significant B protein activity was observed until after parturition even though the A protein gradually increased during pregnancy (46). Thus, it may well be that the lactose synthetase activity presented in Figure 1 may reflect the presence of B protein in the homogenates. This view is supported by the findings that the A protein is present in other tissues (rabbit gastric mucosa, Table III and rat liver, Brew et al. (34)) and that homogenates from these tissues synthesize lactose in the presence of exogenous B protein.

Thus, the presence or absence of B protein (α-lactalbumin) appears to be critical to the control of lactose biosynthesis.

SUMMARY

Measurements of the activities of the enzymes associated with lactose biosynthesis in rat mammary gland homogenates showed that UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase activities increased gradually during pregnancy and rapidly increased after parturition. No lactose synthetase activity was detected until just prior to parturition; the activity increased rapidly after parturition and reached a maximum during late lactation (15-20 days after parturition). A time-dependent increase in UDP-glucose pyrophosphorylase activity was observed in the medium and explants upon incubation of rat mammary gland explants in organ culture. However, the increase was independent of hormones (insulin, hydrocortisone, and prolactin) added to the medium and was not inhibited by puromycin which suggested that the increase was not due to hormonal-mediated enzyme protein synthesis. The increase in activity of UDP-glucose pyrophosphorylase occurred in a variety of tissue homogenates including rat, guinea pig, mouse, bat, hamster, opossum, and rabbit mammary gland and liver homogenates. The increase also occurred in extracts of bean and pea seeds but little if any occurred in E. coli extracts. Further investigation of the 10-fold increase in UDP-glucose pyrophosphorylase activity in rat mammary gland homogenates indicated that the increase was due to a proteinaceous process resulting in a more active form of

the enzyme. The increase occurred without a marked molecular weight change or a change in the K_m for UTP. The increase was inhibited at pH 6.0 or lower or in 2 M urea or greater.

The assay for lactose synthetase was critically evaluated and was developed so that the assay conditions were optimized for maximum enzymatic activity. The optimum conditions for partially purified bovine A protein of lactose synthetase (LS_A) were 50 mM glycylglycine, pH 8.5; 5 mM $MnCl_2$; 0.4 mM UDP-galactose; 25 mM glucose; and 200 μg B protein/ml. Nearly identical optimal conditions were found for the A-protein assay except N-acetylglucosamine was substituted for glucose and the B protein was omitted. Similar conditions were optimal in the assay of LS_A activity in rat mammary gland homogenates except that the B protein (bovine) requirement was 1 mg/ml.

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