STUDIES ON THE GALACTOSYL TRANSFERASE ACCEPTOR SPECIFICITY OF THE LACTOSE SYNTHETASE A-PROTEIN AND ITS INTERACTION WITH ALPHA-LACTALBUMIN

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CHAPTER I

INTRODUCTION

The secretion of milk by the mammary gland for the nourishment of the young is one of the more interesting organ systems available for the study of biological differentiation and metabolic control. The mammary gland is easily observed and in most cases can be readily obtained in a defined physiological and functional state. It also undergoes differentiation under the appropriate *in vivo* hormonal influence and involution in the absence of this influence. Hence it is an excellent model system for the study of both differentiation and hormone function at the cellular and organ levels. Indeed, the mammary gland is a paradoxical organ worthy of study, for while it gives sustenance to the young, it is on the other hand prone to malignancy which is the single greatest cause of death from cancer in adult women.

Milk, the postpartum secretory product of the mammary gland, also has a number of distinguishing features worthy of investigation. One of these is its carbohydrate content. The disaccharide lactose (4-O-β-D-galactosyl-D-glucose) is the only major carbohydrate constituent of milk and the capacity for lactose biosynthesis is restricted to the mammary gland and a few plants (1, 2). The biosynthesis of lactose by the mammary gland appears to be controlled in a manner similar to the functional control mechanism of the mammary gland itself (3, 4, 5). Therefore, an understanding of the control of lactose

biosynthesis is essential in order to gain insight into the functional control of the mammary gland. Similarly, it seems unlikely that one could understand the control of lactose biosynthesis without a detailed knowledge of the enzymes involved. Lactose biosynthesis occurs via the following series of enzyme catalyzed reactions[1]:

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

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

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

Reaction 1 is catalyzed by the enzyme UDP-glucose pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridyltransferase, E.C.2.7.9). Reaction 2 is catalyzed by the enzyme UDP-galactose-4-epimerase (E.C.5.1.3.2), and reaction 3 by the enzyme lactose synthetase (UDP-galactose: D-glucose 1-galactosyltransferase, E.C.2.4.1.22).

Lactose synthetase is a most interesting enzyme in that it requires two dissimilar proteins for significant lactose synthesis. Brodbeck, et al, (6) demonstrated a complete loss of lactose synthetase activity when the proteins were separated by gel filtration. However, upon recombination of the protein components, full activity was restored. In this respect, lactose synthetase is a member of the newly recognized class of enzymes which require two naturally occurring proteins for activity. The detailed studies on tryptophan synthetase by Yanofsky, et al, (7,8) provide the best example of an enzyme from this group.

The heavier protein of bovine lactose synthetase has been designated as the A-protein (molecular weight approximately 77,000).

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[1] All abbreviations are in accordance with the IUPACIUB Combined Commission on Biochemical Nomenclature, J. Biol. Chem., 241, 527 (1966). except where designated.

The other was designated as the B-protein and was later identified as the abundant milk protein α-lactalbumin (α-LA[2]) (molecular weight 14,437) (9,10). Kinetic studies of the reaction catalyzed by bovine lactose synthetase showed that the presence of both proteins was required for activity, clearly implying an interaction between the subunits. This interaction was further substantiated by the finding that the assay for either of the individual proteins could be performed by using an excess or saturating amounts of the other protein (9).

The mutual saturation of either protein by the other implied that the active enzyme may consist of an association complex between the two subunits. The investigation of this apparent association was undertaken in an attempt to demonstrate any enzymically active subunit complex involved in the enzyme catalyzed reaction. Understanding the control of lactose synthesis is dependent upon understanding the interaction between the protein components of this enzyme, for it is with subunit enzymes that many biological control mechanisms are imposed at the enzyme level (11). A demonstrable complex has been shown for the subunits of tryptophan synthetase (7) and nucleoside diphosphate reductase (12).

The ease of demonstration of complex formation between two interacting proteins is dependent on the association constant of the system and the rate at which the system establishes equilibrium. It seemed most likely that if the protein components of lactose synthetase formed a demonstrable complex during the course of the enzymatic reaction, similar conditions used in transport experiments, e.g.

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[2] α-lactalbumin, α-LA

density gradient centrifugation and gel filtration, should provide a means of detecting interacting species. In particular, the presence of substrates at concentrations used in the assay might enhance association. The results of attempts to demonstrate an A: α-lactalbumin complex are presented in this dissertation.

Two types of transport experiments were used. The first type was sucrose density gradient centrifugation under conditions similar to that used in the enzymic assay, including the presence or absence of substrates. The second type of transport experiment was a series of gel filtration experiments. The method of Hummel and Dreyer (13), which has been used for demonstration of the binding of small molecules to proteins by gel filtration, was applied to the lactose synthetase system. In order to have greater sensitivity for the detection of an association between the two proteins, α-LA was iodinated with ^{125}Iodine to yield enzymically active ^{125}I-α-lactalbumin. Binding of ^{125}I-α-lactalbumin could then be monitored by assaying for radioactivity rather than by the relatively insensitive enzymatic assay for α-LA. Equilibrium dialysis as well as fluorescence techniques were also used in the investigation of any subunit association.

Hill, et al, (3) found that the A-protein alone could catalyze the synthesis of N-acetyllactosamine according to the following reaction:

4) UDP-galactose + N-acetyl-D-glucosamine $\longrightarrow$ N-acetyllactosamine + UDP.

α-Lactalbumin inhibited this reaction under normal assay conditions. Numerous other carbohydrates were tested as acceptors for the galactosyl transferase activity of the A-protein, none of which showed appreciable activity. However, orosomucoid, a glycoprotein, was an

acceptor for the A-protein galactosyl transferase activity. Galactosyl transferase activity identical to that of the A-protein was observed in tissues other than mammary gland and each of these tissues could synthesize lactose only if α-LA were added to the assays (3,4). Thus, it was proposed that the A-protein is a general galactosyl transferase found in many tissues. In the mammary gland, it may be responsible for the transfer of galactose to terminal N-acetylglucosamine residues of glycoproteins. These glycoproteins are present in large quantities in colostrum immediately prior to parturition and in casein (14). At parturition, the synthesis of α-LA is markedly increased and its presence might alter the acceptor specificity of the A-protein from terminal N-acetylglucosamine to glucose, thereby inhibiting glycoprotein synthesis and allowing lactose synthesis to proceed (4). Hence α-lactalbumin has been termed a "specifier protein", a new mode of enzyme control.

The specificity of the A-protein for various carbohydrate acceptors was investigated in order to better understand the true biological function of this complex enzyme. The results of these investigations are also reported in this dissertation.

CHAPTER II

LITERATURE REVIEW

Protein-Protein Associations

The control of metabolic reactions is of utmost importance in the understanding of biochemistry. Several proposals have been made in attempts to explain the control of enzyme-catalyzed reactions. Among the most familiar of these is the allosteric enzyme model of Monod, Wyman, and Changeux (15). Koshland, et al, (16) modified this model. The basis for either of these proposals is the avowed conformational change which occurs upon binding of an effector molecule by the allosteric enzyme. The effector molecule thereby alters the ability of the enzyme to bind and react with substrate. The change in enzyme affinity for its substrate is thereby a means of controlling that particular metabolic reaction via another small molecule, presumably a metabolite though not necessarily structurally related to the substrate. Most enzymes exhibiting this type of control consist of multiple subunits.

Only the influence of one subunit, undergoing a conformational change, on the adjacent subunits of a subunit complex is considered in the allosteric enzyme models. However, another proposal by Nichol (17) attempts to explain kinetic observations on subunit enzymes for which the degree of cooperativity cannot be accounted for on the basis of conformational changes alone. The polymerization of

the subunits was included to account for the full extent of cooperativity. An example of this type cooperativity is found with hemoglobin which exists as a tetramer of two α and two β subunits. The tetramer dissociates into two dimers of αβ. Oxygen binds tighter to the αβ dimer than to the $\alpha_2\beta_2$ tetramer. Oxyhemoglobin thereby exists predominately as the αβ dimer while deoxyhemoglobin exists as $\alpha_2\beta_2$ tetramer. Thus, oxygen influences the association of hemoglobin subunits and the extent of association governs the affinity of the molecule for oxygen. Only if this polymerization is taken into account can the full cooperative effect of oxygen be explained (11). Another example of an associating enzyme is glutamic dehydrogenase. The molecular weight of glutamic dehydrogenase ranges from 400,000 to 2,000,000 due to polymerization. GTP is an inhibitor of glutamic dehydrogenase and the binding of GTP to glutamic dehydrogenase shows a sigmoidal binding curve. This was explained with the finding that GTP binds tighter to the 400,000 molecular weight species than to the two million molecular weight polymer, and thereby causes a shift in the equilibrium of the association-dissociation reaction toward the 400,000 molecular weight species (11).

Polymerizing protein systems and macromolecular interactions have been reviewed. Nichol (18) and Cann and Goad (19) discuss the theoretical aspects of the behavior of interacting macromolecules in transport systems. A more practical review is presented by Reithel (20) in which a number of interacting proteins are discussed as well as a general theoretical treatment of transport experiments on interacting systems. McKenzie reviewed the aspects of interacting protein systems present in milk (14). However, the most lucid discussion of

protein-protein interactions in transport experiments is found in reviews by Nichol (21) and Frieden (11). These reviews treat three general types of association reactions which deserve further discussion.

If a mixture of polymeric species in equilibrium is subjected to an applied field in a transport process involving a concentration plateau region, the species tend to separate. The nature of the resulting separation pattern and hence the success of the method used for the study of this interaction is determined by the rate of re-equilibration following disturbance of the initial equilibrium by mass transport compared to the difference in rate of transport of the individual species. Three general classes of interacting systems are considered: 1) those in which re-equilibration is slow compared to the time required for transport and resolution of the species; 2) those in which equilibrium is established much faster than the rate of transport and hence the system maintains equilibrium throughout the transport system; 3) intermediate systems in which the rate of re-equilibration is comparable to the rate of attempted separation of species. The first class of interacting system is easily interpreted since the transport process will resolve each molecular species as a distinct peak. The results obtained with the second and third types of interacting systems are more difficult to interpret. Observed peaks do not necessarily represent distinct molecular species and interpretation becomes extremely difficult. However, by proper choice of transport conditions, i.e., increasing the magnitude of the applied field, one might obtain definitive information on the behavior of the system (21). A serious disadvantage with studies of this type is that

relatively large amounts of protein must be used since protein concentration is monitored. This precludes their use in systems in which only minute amounts of protein are available and hence only enzyme activity can be monitored.

The behavior of polymerizing systems in zone transport processes such as density gradient centrifugation or gel filtration experiments must also be considered since smaller amounts of protein are required. The first class of interacting systems will again yield a distinct peak for each species present. The second and third classes of interacting systems would most likely yield at least a bimodal elution pattern with a diffuse area in between, indicative not of distinct species but instead of reaction zones which can never be completely resolved. Results from these experiments must be interpreted with caution, since anomalous and artifactual maxima can easily result (21).

The biological implications of enzyme polymerizations are based on the assumption that various polymeric forms of an enzyme exhibit different activities toward particular substrates. This is true for α-chymotrypsin, trypsin, and urease (21). The implication drawn from this is that a rapidly equilibrating association-dissociation system may confer an unusually high sensitivity of the enzyme system to ligand concentration resulting in a sensitive point of metabolic regulation. On the other hand, a slow association-dissociation may act as a buffer to metabolic stress without altering the sensivity to ligand concentration normally characteristic of the enzyme (11). Studies on the interaction of the subunits of lactose synthetase may yield insight into the relationship between the protein interaction and the control of lactose synthesis.

Methods of Studying Associating Systems

Several methods are available for study of interacting protein systems. However, from the above discussion, only a select few of these are suitable for a given interacting system depending on the amount of protein available, method of assay, association constant, rate constant for attainment of equilibrium, etc. The classical methods of analyzing interacting protein systems have been analytical ultra centrifugation, moving boundary electrophoresis, and gel filtration with a sample of sufficient size and concentration to produce a plateau region instead of a finite peak. Although these methods have been used in a number of systems (20), they require a large amount of protein which is not available in the lactose synthetase system. Therefore, only the methods applicable to the lactose synthetase system, i.e., require small amounts of protein, will be discussed further.

Sucrose density gradient centrifugation techniques as described by Martin and Ames (22) demonstrated subunit complex formation with both tryptophan synthetase (7,8) and ribonucleoside diphosphate reductase subunits (12). This appears to be a reliable and useful method for studies of this type. Its application to the lactose synthetase system is reported herein.

Equilibrium dialysis would be another method of detecting protein-protein interactions provided a membrane can be obtained which is permeable to one of the two protein subunits (based on molecular weight differences) and impermeable to the other. Such a system has been used for determining the association constant for lysozyme-conalbumin interaction (23). This might be a practical

approach to association studies if one could assay for each of the protein components and find a suitably selective membrane for the system in question.

Fluorescence techniques appeared promising for use in the lactose synthetase system, since the studies by Kronman (24) show α-LA to undergo two distinct conformational changes as measured by changes in ultraviolet fluorescence intensity. The more subtle of the changes occurs at pH 6 as the temperature is lowered from 25° to 1°. This is thought to be due to the contraction of the "crevices" surrounding the two exposed tryptophan residues. A more drastic change in intensity occurs below pH 4 which is a result of denaturation processes. Thus α-LA appears to have two exposed and three buried tryptophans with a total of five tryptophans. In spite of the fact that α-LA was later shown to contain only four tryptophan residues (25), the concept of the two exposed residues being more sensitive to environmental changes probably remains valid. Binding of lactose synthetase substrates by α-LA or the binding of the A-protein itself might be reflected as a change in ultraviolet fluorescence intensity of α-LA. It is interesting to note that sugars which inhibit lysozyme activity also cause a marked quenching of lysozyme's fluorescence spectra (26). Since lysozyme and α-LA are similar in amino acid composition, the components of lactose synthetase reaction might cause a similar effect on α-lactalbumin's fluorescence spectrum.

The attachment of an environmentally sensitive reporter molecule such as the dansyl group (1-dimethylaminonaphthalene-5-sulfonic acid) to α-LA might provide a more sensitive means of detecting perturbations in α-lactalbumin conformation. The dansylation of proteins and

amino acids has been extensively studied by Gros and Labouesse (27) using lysozyme, ribonuclease, α-chymotrypsin, and several other proteins and amino acids. Horton and Koshland (28) reviewed the methods utilizing environmentally sensitive reporter groups attached to proteins for use in studying protein reactions to environmental change.

Another method used for studying protein interactions is gel filtration. The use of gel filtration to resolve protein mixtures was first described by Flodin (29,30) and has since become one of the most widely used transport processes for separating or studying proteins. Andrews (31,32) developed a reliable gel filtration procedure using Flodin's cross-linked dextran gels (Sephadex) for the determination of protein molecular weights to within ± 10% accuracy for most proteins. Polyacrylamide gels have also been used for molecular weight determination of proteins (33,34). This method should also be applicable to protein-protein interactions in which the resultant complex has a higher molecular weight than either of the interacting proteins alone. Indeed, theoretical studies of interacting protein systems during gel filtration have shown that gel filtration can be treated as a transport process in general such that frontal analysis will detect these interactions (35). This treatment was expanded by Ackers (36,37) to include subunit association reactions, allosteric isomeric states, and isomers representing geometric arrangements of subunits. The success of demonstration of any such complex again depends on the interacting system having a suitably high association constant and a slow rate of re-equilibration.

A method of demonstrating binding of small molecules to large molecules using gel filtration was developed by Hummel and Dreyer (13).

This procedure involves equilibration of the gel filtration column with ligand and then applying a zone of the protein also equilibrated with the same concentration of ligand to the column. The protein zone is then eluted with buffer containing ligand at the same concentration as that used in the initial equilibration. The elution profile is monitored for both ligand and protein. If sufficient binding of ligand occurs, a peak of ligand concentration coincident with the protein will first be observed. There will follow a depression or "hole" in the elution profile corresponding to the elution volume of the ligand. By comparing areas under each of the peaks, the stoichiometry of the association can be determined. This method was used to demonstrate binding of ATP and ADP to glutamine synthetase (38). Although this method has not been used for study of interacting protein systems to date, the originators state that it should be applicable to macromolecular association studies.

Iodination of α-Lactalbumin

Radioactively labeled α-LA was prepared in order to increase the sensitivity of assay. This would allow detection of much smaller amounts of bound α-LA in studies to detect an α-LA: A protein complex by most of the methods discussed above. Iodination of α-LA with ^{125}I was chosen as the most convenient and least destructive means of attaching a label to the protein. Although the iodination of α-LA has not been reported, the results of a number of studies on the oxidation and iodination of lysozyme, which is highly analogous to α-LA, are available.

There exists three general methods for the iodination of proteins.

The first is the iodine manochloride method as used by Reif for the iodination of γG gobulin (39). This procedure requires no special technique and its high efficiency yields 70-90% of the protein labeled to give moderately high specific activities. However, it has the distinct disadvantage of being suitable only for amounts of protein in excess of one milligram, thus limiting its use to relatively large scale applications. Also, IC1 is inactivated by free sulfhydryl groups of the proteins being reacted. Hence, some proteins give poor results. Another serious disadvantage is the finding that enzymes such as papaya lysozyme (40), glyceraldehyde-3-phosphate dehydrogenase (41), and egg white lysozyme (42) are all inactivated by IC1, the inactivation of the latter two enzymes being due to oxidation of a critical tryptophan residue. From this one could expect other enzymes to be sensitive to iodine monochloride as well.

The method of Covelli and Wolff (43) utilized KI_3 to iodinate lysozyme. The procedure requires milligram quantities of protein and iodinates nearly all exposed tyrosyl residues. However, low specific activities are obtained due to the dilution of ^{125}I with unlabeled carrier iodine. It was also observed in lysozyme that iodohistidine (43,44) was formed and tryptophan (42) was oxidized upon iodination with KI_3 at molar ratios of I_2 to protein above two, making retention of enzyme activity questionable after iodination.

The iodination of human growth hormone with sodium iodide and chloramine-T was described by Greenwood, Hunter, and Glover (45). This method appeared to be the method of choice for iodinating α-lactalbumin. Carrier free ^{125}I is used with chloramine-T as the oxidizing agent to produce extremely high specific activity in

microgram quantities of protein. There is no apparent destruction of growth hormone at specific activities up to 300 μc/μg. At specific activities above this, a progressive denaturation is observed. The radio-iodination of submicrogram quantities of tyrosine with very high specific activities was accomplished using this procedure (46). The stability of the reaction products was quite acceptable with either ^{131}I of ^{125}I when stored in the dark in the absence of copper ions.

Studies on lysozyme have shown that iodinations produced a 30% loss in enzyme activity regardless of the extent of iodination (47), possibly because of the oxidation of a tryptophan residue required for activity. Under these conditions, tryptophan 108 is oxidized before any iodination of tyrosine occurs with KI_3 (48,49,50). A loss of enzyme activity accompanies the iodination of muscle fructose diphosphate aldolase after binding only a few atoms of iodine per molecule of aldolase (51). The extent of loss in enzyme activity upon iodination varies from enzyme to enzyme as indicated above, making any conclusion difficult as to the mildness of iodination as a protein modification. Iodination of non-critical tyrosine residues would have little effect on enzyme activity providing no oxidations occurred elsewhere in the protein molecule. Indeed, nitration of α-LA with tetranitromethane resulted in only 20% loss of activity with only one tyrosine being iodinated (52), suggesting that the most available tyrosine residue is probably not required for its participation in lactose synthetase activity. Thus moderate iodination of α-LA may result in a similar retention of activity, allowing radio-iodine labeled α-lactalbumin to be used in detection of lactose synthetase complex.

Enzymes Requiring Two Proteins

Lactose synthetase is of interest for two primary reasons: the first because of its position in the synthetic scheme for lactose, and the second because of its interesting protein structure. It is a member of an expanding class of enzymes which require two naturally occurring dissimilar proteins for activity. The definition of this class of enzymes is rather nebulous but will here be restricted to include only those enzymes with the following two properties. First, the enzyme is easily resolved into two dissimilar proteins differing in properties such as chromatographic behavior, molecular weight, stability, etc. The dissociation of the enzyme occurs under the mild separatory processes employed in their purification such as gel filtration, ammonium sulfate fractionation, gel adsorption, or ion exchange. The protein components are obtained in an apparently native state following any such dissociation procedure and can be recombined to yield essentially complete composite enzymatic activity.

This excludes most subunit enzymes which must be subjected to extremes in pH or ionic strength, reducing agents, or strong denaturing agents such as urea or guanadine hydrochloride before subunit dissociation occurs. Enzymatic activity is difficult to recover upon recombination of subunits separated by such means. It is concluded that these enzymes could rarely exist in a dissociated state under physiological conditions and therefore are not considered here as enzymes requiring two separate proteins.

A second property of these enzymes is that the apparent combination of proteins produces a resultant composite enzymatic activity which is either a regulated or modified catalytic activity of one of

the individual proteins or a summation of the individual activities of the individual proteins although it should be mentioned that most proteins of this class exhibit little catalytic activity individually.

At this time there are at least fourteen enzymes reported to require two proteins. They will be discussed individually with emphasis on those in which the formation of an enzymically active protein-protein complex has been studied.

Lipoic Acid Activating System

An apopyruvate dehydrogenase system found in cell-free extracts of lipoic acid deficient Streptococcus faecalis was activated upon addition of lipoic acid to the extracts (53). The reaction for the pyruvate dehydrogenase system is carried out according to Reaction 5.

5) $\text{Pyruvate} + \text{NAD}^+ + \text{CoA} \rightleftharpoons \text{Acetyl CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$

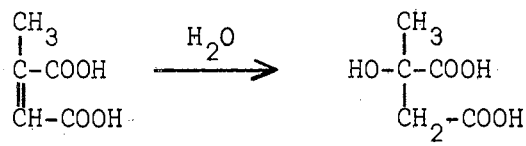
Protamine sulfate fractionation separated the lipoic acid activating system from the apopyruvate dehydrogenase system. The lipoic acid activating system was then fractionated into two components, PS-2A and PS-2B, by ammonium sulfate at pH 8. Both fractions PS-2A and PS-2B had to be incubated simultaneously with the apopyruvate dehydrogenation system, lipoic acid, and ATP to obtain an active pyruvate dehydrogenase system.

E. coli lipoic acid activating system replaced that from S. faecalis but the E. coli system was not separated into components. Fraction PS-2A was heat labile and catalyzed the formation of lipohydroxamic acid from lipoic acid, ATP, and hydroxylamine. Fraction PS-2B was stable after boiling for 10 minutes, relatively stable

to trichloroacetic acid treatment, and partially inactivated by trypsin. Fraction PS-2A was proposed to be a lipoic acid activating enzyme which catalyzes an ATP dependent reaction to produce lipoyl adenylate. Fraction PS-2B was proposed to function as a lipoyl carrier protein, carrying lipoic acid between the lipoyl adenylate-fraction PS-2A complex and the apopyruvate dehydrogenation system.

L-Citramalate Hydrolyase

L-Citramalate hydrolyase, also known as mesaconase (E.C. 4.2.1), catalyzes Reaction 6 below as a step in the catabolism of glutamate.

6) 

This enzyme was separated into two protein components, designated Component I and Component II, by DEAE-cellulose chromatography following partial purification (54). Both components were required for enzymatic activity. Chromatography on Sephadex G-100 showed that the molecular weight of Component II was about 100,000 while the molecular weight of Component I was between 20-40,000. The spectrophotometric assay for activity of either component could be accomplished by preincubation of the desired component in an excess of the other in the presence of Fe^{++} and cysteine. Oxygen rapidly inactivates Component II while Fe^{++} and cysteine reactivate it. Component I is not subject to oxygen inactivation nor does it require activation in any manner, however, it does increase the activation of Component II with Fe^{++} and cysteine, suggesting an interaction between the two proteins must occur to give full enzymatic activity.

Glycine Decarboxylase

Glycine decarboxylase of Peptococcus glycinophilus catalyzed the labilization of the glycine carboxyl group according to the following reaction which is used for the enzyme assay:

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

Two protein fractions, P_1 and P_2, were obtained after chromatography of the enzyme on Sephadex G-100 (55). P_1, purified 60-fold, contained pyridoxal phosphate and was destroyed by boiling. P_2, purified 230-fold, was stabile to boiling, had a low molecular weight, and was inactivated by proteolytic enzymes. The K_m for P_2 in the saturation of P_1 with P_2 was 1.3 mg per ml (56). No function for either individual protein was described.

Acetyl-CoA Carboxylase

Although acetyl-CoA carboxylase (EC 6.4.1.2) preparations from E. coli were initially described as requiring two protein fractions for activity after alumina-C_γ fractionation, later studies showed it to consist of three functionally distinct protein subunits (57) which catalyze the following series of reactions:

8) $ATP + HCO_3^- + \text{Biotin-P} \xrightleftharpoons[\text{Carboxylase, } Mn^{++}]{\text{Biotin}} CO_2\text{-Biotin-P} + ADP + Pi$

9) $CO_2\text{-Biotin-P} + \text{Acetyl-CoA} \xrightleftharpoons{Eb} \text{Malonyl-CoA} + \text{Biotin-P}$

10) $HCO_3^- + ATP + \text{Acetyl-CoA} \rightleftharpoons ADP + Pi + \text{Malonyl-CoA}$

The enzyme was prepared from other sources as a homogeneous protein (58,59). Biotin carboxylase, one of the three required subunits, catalyzes the ATP-dependent carboxylation of the biotin-protein

(Biotin-P, the second required subunit) to form carboxybiotin-protein (Reaction 8). The biotin carboxylase subunit also catalyzes the carboxylation of free biotin. Biotin-protein contains covalently bound biotin and has no known catalytic activity but closely resembles acyl carrier protein. It can thereby be considered a carboxyl carrier protein that functions as a substrate for the third subunit, Eb, which catalyzes the transfer of the carboxyl group of carboxybiotin-protein to acetyl CoA (Reaction9). Thus, one non-catalytic subunit of acetyl CoA carboxylase has the interesting function of being a substrate for the other two catalytic subunits. Although evidence for subunit interaction was presented, no attempt was made to demonstrate a physical complex (58). It is also of interest to note that a biotin containing protein has been found as a subunit of oxalacetic acid transcarboxylase (60). This biotin containing protein is also proposed to function as a biotin-carboxyl carrier protein which may be a common mechanism in all biotin enzymes.

Q ß-Replicase

Q ß-replicase is an RNA-dependent RNA-polymerase purified from Q ß-bacteriophage infected E. coli Q-13 which can be separated into two components by repeated centrifugation in sucrose gradients (61). One component is a light chain of about 80,000 molecular weight, the other has a molecular weight of about 130,000. Neither component alone can initiate extensive polynucleotide synthesis when challenged with intact Q ß-RNA but a combination of the two is active. The heavy component is unique to infected cells and appears to be programmed in the viral RNA. The light component appears to exist in

uninfected cells (62). No function is described for the light component.

Although it appeared that the enzyme was isolated from infected cells as a complex, it was subsequently easily dissociated on sucrose density gradient centrifugation. No attempt was made to study the complex from an association-dissociation point of view, although it should be mentioned that the complex appears to be a slowly equilibrating association-dissociation system with a high association constant since centrifugation for thirty-six hours in 5-20% sucrose density gradients which was also 10% saturated with ammonum sulfate, pH 7.4, at 2° was required for separation.

Two factors may aid the dissociation on the sucrose gradient: 1) low temperature which favors disruption of hydrophobic interactions and 2) high ionic strength (10% ammonium sulfate). Assays for the recombination of subunits (which yields full activity) were done at much lower ionic strength and at 35° for only 20 minutes, indicating a much more rapid association under these conditions. The forces involved in the interaction clearly remain to be delineated.

PEP-dependent Formation of Fructose-1-Phosphate

Fructose metabolism in A.aerogenes proceeds primarily through the pathway: fructose → fructose-1-P → fructose-1, 6-diP. The initial phosphorylation of fructose to produce fructose-1-P uses phosphoenolphyruvate according to the scheme outlined below (63).

11) PEP + HPr $\xrightarrow{\text{Enzyme I}}$ Pyruvate + Phospho-HPr

12) Fructose (Sugar) + Phospho-HPr $\xrightarrow{\text{Enzyme II}}$ Fructose-1-P (Sugar-1-P) + HPr

This system was resolved into four components on Sephadex G-200; one for enzyme I, another for HPr protein, and two for enzyme II. Enzyme II separated into one "heavy" protein and one lighter protein, called the fructose specifier protein. Enzyme II heavy protein phosphorylates both mannitol and fructose in the absence of the fructose specifier protein. However, the K_m of enzyme II heavy protein for fructose is lowered from 65 mM to 1.0 mM or less upon combination with fructose specifier protein, thereby shifting the substrate specificity of the enzyme to fructose. The specifier protein is inducible in cells grown on fructose but the heavy protein appears to be the same regardless of carbohydrate source of growing cells, rendering it a constituitive protein. No further study of the interaction of heavy protein with specifier protein to form a low fructose K_m active enzyme II complex has been reported although such a complex is speculated to exist (63).

Sucrose Synthetase

Sucrose synthetase from Phaseolus aureus catalyzes Reaction 13 (64).

13) XDP Glucose + Fructose $\longrightarrow$ Sucrose + XDP
(where X = Uridine, Cytidine, Adenosine, Guanidine, or Thymidine.)

Purification of the enzyme on DEAE cellulose resulted in stimulation of activity with UDPG but less activity with ADPG, CDPG and GDPG. Addition of another fraction from the same DEAE cellulose column to the fraction containing synthetase activity resulted in marked inhibition of synthetase activity with UDPG but increased activity for ADPG, CDPG, and GDPG; TDPG activity remained unchanged. It was implied that

the second fraction contained a specifier protein which alters specificity of the synthetase for the nucleoside base and thereby governs the interconversion of XDP-sugars. It was not definitely shown whether the modifier fraction contained a protein or was instead other material such as carbohydrate or RNA.

Trehalose Phosphate Synthetase

Preparations from mycobacteria can catalyze the synthesis of trehalose phosphate from either UDP-glucose or GDP-glucose and glucose-6-phosphate according to the following reactions.

14) UDP-glucose + glucose-6-P ⟶ trehalose-P + UDP

15) GDP-glucose + glucose-6-P ⟶ trehalose-P + GDP

A cell free extract of M. smegmatis was separated into two fractions on DEAE cellulose. Fraction 1 catalyzed trehalose-P synthesis from GDP-glucose (Reaction 15) but was relatively inactive with UDP-glucose (Reaction 14). However, upon the addition of Fraction 2 to Fraction1, UDP-glucose served as an effective glucosyl donor. Under these conditions, GDP-glucose was still active but less so than with Fraction 1 alone. Fraction 2 had no detectable enzymatic activity, was heat stable, and appeared to be a low molecular weight RNA (65). Fraction 2 could be replaced by the RNA fraction from M. smegmatis, an RNA isolated from crude α-lactalbumin preparation, and by polyuridylic acid, but not by high molecular weight yeast RNA, yeast t-RNA, polyadenylic acid, UTP, UMP or a number of proteins (66).

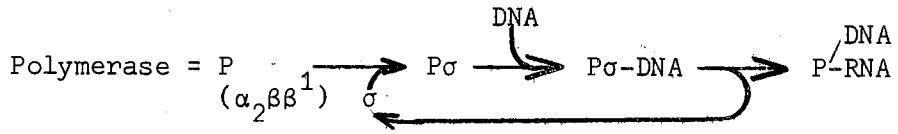
This appears to be a unique enzyme in that the "modifier subunit" is an RNA which functions by altering the specificity of the synthetase for the nucleoside base. The change in nucleoside

specificity of the synthetase in the presence of "modifier subunit" is similar to that observed in sucrose synthetase above and is an intriguing mode of control of carbohydrate reaction and interconversion.

DNA-dependent RNA Polymerase

In E. coli the synthesis of all types of cellular RNA is thought to be mediated by a single enzyme, DNA-dependent RNA polymerase. This enzyme dissociates during purification on phosphocellulose into three protein peaks, A, B, and C. Peaks A and C had very little polymerase activity but Peak B was active with T4 phage DNA. Activity could be increased in either peak A or B by the addition of peak C. The active enzyme exists as a complex of five subunits, $\alpha_2\beta\beta^1\sigma$ (molecular weight 495,000) which could be dissociated into $\alpha_2\beta\beta^1$ (molecular weight 400,000) and σ (molecular weight 95,000) subunits. More rigorous conditions dissociated the polymerase ($\alpha_2\beta\beta^1$) into three subunits, α, β, and β^1, with molecular weights of 40,000, 155,000, and 165,000, respectively. The σ subunit corresponds to peak C and is required for initiation of RNA synthesis on the DNA template. An active one to one complex between polymerase ($\alpha_2\beta\beta^1$) and initiation factor (σ factor) was demonstrated on phosphocellulose columnchromatography, electrophoresis, and glycerol gradient centrifugation (67). No requirement other than the simultaneous presence of both polymerase and σ factor was necessary for complex formations. σ factor somehow stimulates the initiation of RNA synthesis by polymerase on the DNA template. σ factor is then thought to dissociate from the initial polymerase and can then associate with another molecule of polymerase to allow

another initiation (68). No catalytic function is known for σ factor. The proposed reaction mechanism is described below:



Tryptophan Synthetase

The tryptophan synthetase (L-serine hydrolyase [adding indole], EC 4.2.1.20) of E. coli is composed of two nonidentical and readily separable protein subunits, designated as the A and B subunits (8). The A subunit has been renamed the α subunit and is a single polypeptide chain, molecular weight 30,000 (69), whose complete amino acid sequence and genetic mapping locus on the E. coli chromosome is known (70). The B subunit (molecular weight 99,000) is composed of two identical polypeptide chains and has been renamed the β_2 subunit (71). Fully associated tryptophan synthetase consists of one β_2 and two α subunits, $\alpha_2\beta_2$ (molecular weight 159,000) (71,72). The tryptophan synthetase complex catalyzes the following reactions (73).

16) Indole + L-serine $\xrightarrow{\text{Pyridoxal-P}}$ L-tryptophan

17) Indoleglycerol-P $\rightleftharpoons$ INDOLE + glyceraldehyde-3-P

18) Indoleglycerol-P + L-serine $\xrightarrow[\text{L-tryptophan}]{\text{Pyridoxal-P}}$ + glyceraldehyde-3-P

The physiologically significant reactionis probably Reaction 18, which is believed to be a distinct reaction, not merely the sum of the first two (8,74). The α subunit alone can catalyze Reaction 17 at about 1% the rate produced by the $\alpha_2\beta_2$ complex. Reaction 16 can be catalyzed by the β_2 subunit, giving about 3% of the activity found with the $\alpha_2\beta_2$ complex. Full activity in any of the three reactions

above requires physical contact of both subunits to form $\alpha_2\beta_2$ complex. Complexes formed with either mutant α or mutant β_2 subunits and the normal second subunits will catalyze Reactions 16 and 17, respectively, with essentially full activity but will not catalyze Reaction 18 (75).

The association of the α and β_2 subunits of E. coli tryptophan synthetase has been studied by sucrose gradient centrifugation, gel filtration, and enzymatic activity measurements. The fully associated enzyme ($\alpha_2\beta_2$) has a sedimentation coefficient of 6.45 and is in relatively rapid equilibrium with the free subunits. Mass law and kinetic considerations are consistent with the reversible binding of individual α subunits to two identical and independent sites on the β_2 subunit with each combining site contributing the same enzymatic activity according to the scheme below (7):

19) $\alpha + \beta_2 \overset{Ka_1}{\rightleftharpoons} \alpha\beta_2$; $\alpha\beta_2 + \alpha \overset{Ka_2}{\rightleftharpoons} \alpha_2\beta_2$

When mixtures of the two subunits containing an excess of the α subunit were sedimented in sucrose gradients containing pyridoxal-P and L-serine, a rapidly sedimenting peak corresponding to $\alpha_2\beta_2$ complex was observed. Omission of pyridoxal-P and L-serine in a similar experiment resulted in each subunit sedimenting separately with no indication of complex formation. Hence, incubation of the subunits with pyridoxal-P and L-serince was required for complete complex formation. However, limited association of the subunits was observed upon sedimentation in the presence of pyridoxal-P alone but no association was observed with L-serine alone. NaCl (0.04 to 0.1M), in the absence of serine and pyridoxal-P, caused full association of the normal subunits, the extent of which decreased with the age of the extract, but not those from mutant E. coli. The complex $\alpha_2\beta_2$ was

formed when either equal amounts of both subunits or an excess of the α subunit was used, however, an excess of the β_2 subunit produced an intermediate complex, $\alpha\beta_2$. No evidence for dimerization of a subunit alone could be found even in the presence of pyridoxal-P and L-serine, substantiating Reaction 19 (7).

The apparent intrinsic association constant, K_a, of the α and β_2 subunit combining sites for each other was determined by assaying the enzymatic activity of a mixture of approximately equal units of α and β_2 subunits at several concentrations, the difference between observed and expected activity being due to the degree of association at that particular concentration. The values of Ka_1 and Ka_2 of Reaction 19 are 2 x K_a and ½ x K_a, respectively. The K_a for each of the three reactions was found to be 3.2×10^7 (Reaction 16), 4×10^6 (Reaction 17), and 5.4×10^8 (Reaction 18). NaCl (0.1M) slightly increased the K_a for Reactions 16 and 18 to 8×10^8, 2.6×10^9, respectively, but did not affect Reaction 17 (74). The rate constants for the association reaction $\alpha + \beta_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \alpha\beta_2$ were determined as $k_1 = 2 \times 10^4$ $sec^{-1}M^{-1}$ in Reaction 17 and $k_1 = 6 \times 10^5$ $sec^{-1}M^{-1}$ in Reaction 18, indicating a rapid association. The dissociation rate constant, k_2, for Reactions 17 and 18, were 4.8×10^{-3} sec^{-1} and 1.8×10^{-4} sec^{-1}, respectively. These values correspond to half-lives of 2.4 and 64 minutes, respectively. Although the tryptophan synthetase complex is readily dissociable, pyridoxal-P, serine, and NaCl together increased the association rate constant by a factor of 30 and increased the half-life of the complex by a factor of about 27 (7).

Several other reactions have been ascribed to the β subunit (76,77,78).

20) L-serine $\xrightarrow{\text{pyridoxal-P}}$ pyruvate + NH_3

21) mercaptoethanol + L-serine $\xrightarrow{\text{pyriodoxal-p}}$ s-hydroxyethyl-L-cysteine + H_2O

22) RSH + L-serine + PLP $\rightleftharpoons$ R-S-mercaptopyruvate + PMP + H_2O

The catalytic roles of the α and β_2 subunits in tryptophan synthetase complex has been clarified since phosphopyridoxylaminoacrylic acid appears to be a common intermediate in all the reactions of tryptophan synthetase (78). The α subunit is not required for formation of this intermediate but does regulate its fate once formed and thereby directs the further reaction of the β_2 intermediate toward tryptophan synthesis instead of other alternate breakdown pathways. The α subunit completely inhibits transamination (Reaction 22) and deamination (Reaction 20) while greatly stimulating the indole β-addition (Reaction 18), thereby explaining the regulatory or "reaction specifier" role of the α subunit (78). The reaction itself proceeds through two intermediate complexes, termed "aqua" and "amber" in order of appearance, according to recent stopped flow studies (79). The formation and breakdown of the "amber complex" ($\alpha_2\beta_2$-L-serine-indole complex) is the rate limiting step of the reaction.

Interesting observations have been made on tryptophan synthetase from other organisms. That from the mold N. crassa behaves as a single component upon purification although it can be dissociated in guanidine hydrochloride to four subunits (80). Tryptophan synthetase from Nicotiana tabacum (tobacco plant) can be separated into two components, A and B, by differential ammonium sulfate precipitation and resembles the two subunit enzyme of E. coli (81). However, tobacco subunit B alone catalyzes the conversion of indole to

tryptophan and this activity is not stimulated by the addition of component A. The combination of A and B is necessary for the conversion of indoleglycerol-P to tryptophan. Both the A and B tobacco components can substitute for the analogous E. coli proteins in Reaction 18. Attempts to demonstrate complex formation between tobacco A and B proteins were unsuccessful under varying conditions of serine, pyridoxal-P, salt concentration, and buffer concentration on sucrose gradients. Apparently no stable complex is formed under these conditions or that if formed it is rapidly and easily dissociable during sedimentation (81).

Ribonucleoside Diphosphate Reductase

Ribonucleoside diphosphate reductase from E. coli B catalyzes the following reaction.

23) CDP + Thioredoxin-$(SH)_2$ $\xrightarrow[B_1B_2]{Mg^{++},ATP}$ deoxyCDP + Thioredoxin-S_2

Thioredoxin is cyclic in this reaction, being catalytically reduced by thioredoxin reductase and TPNH. All four commonly occurring ribonucleoside diphosphates (ADP, GDP, CDP, and UDP) can serve as substrates in Reaction 23 (82). Reichard first reported the fractionation of ribonucleoside diphosphate reductase into two subunits (B_1 and B_2) on Cø-alumina gel (83). The simultaneous presence of both subunits and Mg^{++} is required for activity; each protein alone is inactive. The specificity of the enzyme toward the different ribonucleoside diphosphates is determined by specific nucleoside triphosphates which act as allosteric effectors. Specifically, pyrimidine ribonucleotides are substrates if ATP is the effector, purine

ribonucleotides with dGTP, and both purine and pyrimidine ribonucleotides with dTTP. dATP is a negative effector, inhibiting activity with all four substrates (84).

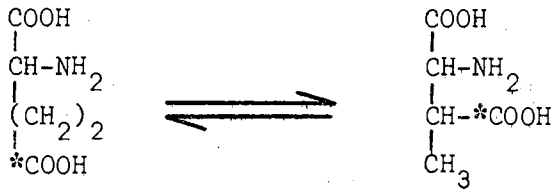
Complex formation between B1 and B2 has been demonstrated on sucrose gradients (12). When B1 and B2 are centrifuged separately in the presence of 10mM Mg^{++}, the resulting $S_{20,w}$ values for the subunits were 7.85 and 5.55, respectively, as monitored by activity, indicating no dimerization. When both B1 and B2 are preincubated and centrifuged together under the same conditions, both activities appeared in a new peak having an $S_{20,w}$ of 8.85. The formation of a complex between proteins B1 and B2 was dependent on the presence of Mg^{++} in the sucrose gradient. The metal requirement could not be replaced by the allosteric effector dTTP (12). No further studies on the nature of the complex are reported.

Binding studies performed on Sephadex G-25 demonstrated binding of the effector, dTTP, to B1 or B1:B2 complex but not to B2 (12). Protein B1, but not protein B2, has a marked capacity to bind effector molecules. Thus protein B1 of ribonucleoside diphosphate reductase appears analogous to the regulatory subunit of aspartate transcarbamylase. However, protein B2, unlike the catalytic subunit of aspartate transcarbamylase, shows no catalytic activity in the absence of B1.

Glutamate Mutase

Glutamate mutase (EC 5.4.99.1) of Clostridium tetanomorphum catalyzes the first step in glutamate degradation via Reaction 24 which requires participation of a cobamide coenzyme.

24)



Two protein fractions were obtained upon fractionation of the enzyme with calcium phosphate gel resulting in concomittant loss in enzymatic activity. Recombination of the fractions was required for recovery of enzymatic activity (85). The gel supernatant fraction was designated Component S (molecular weight 17,000) and the gel eluate fraction as Component E (molecular weight 128,000). Purified Component E contains no known cofactors. Neither component alone has activity, however assay for either component could be performed by using saturating amounts of the other.

The amount of Component S required to saturate Component E in kinetic studies could be markedly decreased by the addition of benzimidazolylcobamide coenzyme, indicating increased mutual affinities of the protein components for each other. The function of Component S was clarified by the observation that Component E alone binds approximately one mole of cobamide coenzyme, whereas, in the presence of a 7-fold molar excess of Component S it bound two moles of cobamide coenzyme. Component S markedly decreased the K_m of Component E for cobamide coenzyme (86).

Attempts to demonstrate complex formation between the two protein components on Sephadex G-100 were unsuccessful even though experiments were tried in the presence of glutamate and cobamide cofactor, which enhances association of the components in the assay. The conclusion regarding complex formation was that the association-dissociation reaction of the two components must be very rapid and

that binding is sufficiently weak that its physical separation by gel filtration is not feasible (86).

Lactose Synthetase

Babad and Hassid were the first to report on the purification of lactose synthetase from bovine milk (87). Brodbeck and Ebner resolved lactose synthetase on BioGel P-30 into two protein components, A and B, which had to be recombined for recovery of activity. Neither component individually had any catalytic activity (6). The larger A component was heat labile while the smaller B protein was heat stable. The A-protein was associated with the microsomal fraction of rat mammary tissue while the B protein was distributed between the microsomal and soluble fractions, being more easily dissociated from microsomes than the A (88). Ebner, et. al., (9,10) identified the B protein of lactose synthetase as α-LA by their interchangeability in the lactose synthetase assay. α-Lactalbumins from other ruminants as well as non-ruminants were enzymatically active with bovine A-protein (89). The biological role of α-LA has been reviewed (90).

It had been reported early that N-acetyl glucosamine (NAG) had 25% and cellobiose had 7.5% the activity of glucose with lactose synthetase (87). Brew, Vanaman and Hill (3) described the A-protein of lactose synthetase as a general galactosyl transferase which used NAG as a galactosyl acceptor in the absence of α-LA. Under normal assay conditions α-LA inhibits this reaction and allows the A-protein to use glucose as an acceptor. The A-protein alone synthesizes little lactose in the absence of α-LA. Hence, α-lactalbumin has been described as a "specifier" protein for the A-protein. The A-protein

also transfers galactose to orosomucoid, however, this reaction was inhibited only 17% in the presence of α-lactalbumin (4).

The A-protein of lactose synthetase resembles the UDP-galactose: N-acetylglucosamine galactosyltransferase described by McGuire, et. al. (91). Indeed, lactose synthetase activity was found in several tissues other than mammary tissue if α-lactalbumin is added to the assay of the tissue preparation (3,4).

Ebner, et. al., (92), recently reported the reciprocal relationship between α-lactalbumin and glucose in lactose synthetase. High concentrations of glucose greatly stimulate lactose synthetase activity of the A-protein (K_m=1.5m) in the absence of α-lactalbumin. Lactose synthetase A-protein as purified from human milk by Andrews (93) showed properties similar to that from bovine milk.

No studies have been reported on the nature of the α-lactalbumin: A-protein interaction in lactose synthetase although kinetic evidence implies that physical interaction is required for activity (9,10). In this respect it is interesting that Coffey and Reithel (94,95) report the isolation and characterization of particles from lactating bovine mammary gland with intact lactose synthetase activity. These particles appeared related to the Golgi apparatus and suggested an *in vivo* particulate complex of A-protein and α-LA. In addition to this, Palmiter (96) has proposed a third component Z, required for lactose synthetase activity in mouse mammary gland, which has the role of preventing rapid dissociation of the A: α-LA complex, although evidence for this component is not convincing.

GLYCOSYL TRANSFERASES

The A-protein of lactose synthetase seems to be a general galactosyl transferase with its acceptor specificity partially determined by α-lactalbumin (3,4). Hence, the A-protein has been implicated in the biosynthesis of glycoproteins. It therefore seems desirable to review glycosyl transferase reactions in general with respect to their acceptor specificity and role in glycoprotein biosynthesis. The topic of complex heteropolysaccharides of animals has been reviewed (97).

Mammalian glycoproteins contain seven carbohydrates: N-acetylglucosamine, N-acetylgalactosamine, galactose, mannose, L-fucose, sialic acid, and glucose. Sialic acid and L-fucose are found as the terminal residue of the nonreducing end of the chain. The initial linkage of carbohydrate to protein occurs through either N-acetylglucosamine linked to the amide of asparagine, N-acetylgalactosamine linked to a serine or threonine hydroxyl, or a galactose linked to the hydroxyl group of hydroxylysine as in collagen (97).

The glycopeptides released from proteolytically degraded glycoproteins are heterogeneous, exhibiting differences in both polysaccharide sequence and mode of linkage to peptide. It appears that most, if not all the sugar residues of glycoproteins are added sequentially after the synthesis and release of the polypeptide from the ribosomes into the membranous portion of the microsomal fraction (97).

Enzymatic studies support the hypothesis of sequential addition of sugars to the preformed polypeptide. Enzymically or chemically degraded glycoproteins freed of one or more of their sugars can serve as acceptors for transfer of sugar from the appropriate sugar

nucleotide by specific glycosyl transferases. The presence or absence of specific glycosyl transferases seems responsible for the genetic control of cellular antigens (glycoproteins) whose structural determinants are carbohydrates. This mechanism is consistent with the biosynthesis of blood group substances, where both genetic and structural studies can be made. It can be inferred that the addition of a given sugar in a given linkage is catalyzed by the same enzyme regardless of whether the product is glycoprotein, glycolipid, or an oligosaccharide, implying that specificity of the transferase for the acceptor lies only in the correct preceeding carbohydrate linkage of the acceptor (97). A brief discussion of the specificity and function of a number of glycosyl transferase follows.

Bosmann and Eylar (98) isolated a UDP-glucose: galactosylcollagen glucosyl transferase from embroynic guinea pig skin. The transferase was almost totally inactive with all glycoprotein receptors other than prepared collagen, nor was it active with the monosaccharides galactose, galactosamine, and N-acetylgalactosamine. Specificity apparently resides in the sequence around the hydroxylysine to which the galactose is attached.

The biosynthesis of the two galactose residues in the carbohydrate sequence gal-β-1,3-gal-β-1,4-xyl-β-1-serine of chondroitin sulfate was studied by Helting and Rodin (99). Transfer of the galactose residues from UDP-gal was shown for several fragments from the carbohydrate linkage region, (i.e., xyl-ser, gal-xyl, gal-xyl-serine, and free xylose). Competition studies showed two transferase activities with UDP-gal; one specific for xyl-ser linkages, the other for gal-xyl. This specificity represented transfer of the first and second galactose

residues, respectively. Endogenous chondroitin sulfate stripped of carbohydrate is also an acceptor.

Transfer of glucuronic acid from UDP-glucoronic acid occurred to the galactose moiety present at the nonreducing end of the disaccharide, 3-O-β-D-galactosyl-D-galactose, or larger, similar fragments from the linkage region of chondroitin sulfate. Free D-galactose was practically inactive as an acceptor (100).

Basu, Kaufman, and Roseman (101) described a UDP-galactose: glycolipid galactosyltransferase from embryonic brain which used Tay-Sachs ganglioside as the lipid acceptor. Various modified glycoproteins were equally good but N-acetylglucosamine was by far the best acceptor. The goat colostrum UDP-galactose galactosyltransferase of McGuire, et.al. (91) was active with the same glycoproteins as acceptors but not with glycolipids.

Hagopian and Eylar (102) showed the polypeptidyl: N-acetylgalactosamine transferase from bovine submaxillary glands to be highly specific for bovine submaxillary mucin stripped of carbohydrate. Terminal N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose, and sialic acid residues would not substitute as acceptors for the specific hydroxyamino acids of the submaxillary polypeptide chain as tested by appropriately degraded glycoproteins. Twenty-five proteins were tested for acceptor activity with negative results. The size requirement of the transferase for acceptors was shown by the inactivity of pronase digestion fragments of bovine submaxillary mucin. Purification of this transferase is described (103).

Glycosyl transferases in human tissues for both N-acetylgalactosamine (104), galactose (105), and α-L-fucose (106) are specific for

oligosaccharide sequences corresponding to the known sequence of the determinant for the donor's ABO blood type.

Watkins (107) described a galactosyl transferase from rabbit gastric mucosa which utilized N-acetylglucosamine, β-methyl-N-acetylglucosamine, and the disaccharide β-N-acetylglucosamine-(1-4)-N-acetylglucosamine equally as acceptors. α-Methyl-N-acetylglucosamine had about half the activity while N-acetylmannosamine and glucose had little activity. The enzyme had a pH optimum of 6 to 8 and required Mn^{++} for full activity (Mg^{++} gave 10% activity). Specificity and activity from human donors was similar, irrespective of donor ABO blood type.

Carlson, et.al. (108) and McGuire and Roseman (109) collectively have delineated the sequence and enzymes for biosynthesis of the disaccharide side chains of sheep submaxillary mucin. A sialyl transferase from sheep submaxillary glands transfers sialic acid to sialidase treated sheep submaxillary mucin yielding the completed carbohydrate sequence, sialic acid-N-acetylgalactosamine-protein (108). The addition of the first sugar residue, N-acetylgalactosamine, is accomplished by an N-acetylgalactosamine transferase (109) which uses carbohydrate-free ovine submaxillary mucin (OSM) as its only acceptor. Peptides from OSM were inactive as were thirty-one other glycoproteins, glycolipids, and mucins, several amino acids (particularly serine, threonine, and glycylserine) and twenty-four low molecular weight sugars and sugar derivatives.

Johnston, et.al. (110), purified a UDP-N-acetylglucosamine: blycoprotein transferase from goat colostrum. This enzyme showed a broad specificity toward a variety of treated glycoproteins with terminal mannose residues as acceptors. No acceptor activity was

found with twenty low molecular weight sugars and derivatives, including glucose, galactose, mannose, and each of their α and β-methyl glycosides nor with several amino acids. Hence, this enzyme is specific for terminal mannose of high molecular weight glycoproteins but the peptide sequence of the glycoprotein makes little difference.

The galactosyltransferase fractionated from goat colostrum reported by McGuire, et al. (91), could transfer galactose from UDP-galactose to both N-actylglucosamine and glycoproteins containing terminal β-N-acetylglucosamine residues, suggesting the presence of more than one galactosyltransferase in the preparation. Specifically, the ratio of activities to both substrates varied widely during purification. However, using either acceptor, UDP-galactose gave 100% activity as the glycosyl donor while UDP-glucose and UDP-N-acetylgalactosamine were 1.6% and 1.3% as active, respectively. The K_m for N-acetylglucosamine was 1.3 mM; K_m for UDP-galactose was 0.25 mM. Other low molecular weight acceptors included di and tri-β-N-acetylglucosamine which gave full activity. The ability of this transferase to use N-acetylglucosamine as an acceptor is similar to the galactosyl transferase activity of the lactose synthetase A-protein (3,4).

Ovarian cyst glycoprotein which had been sialidase treated served as an acceptor for a goat colostrum galactosyl transferase (111), presumably the same transferase as that of McGuire, et al., (91) described above. Intact glycoprotein as well as A, B, and H blood group substances were inactive. It was presumed that the galactose incorporated was attached to a terminal β-N-acetylglucosamine residue.

The function of the various glycosyl transferases in

heteropolysaccharide synthesis clearly seems to be one of obtaining correct sequence of the carbohydrate chains. The specificity of those transferases which attach the first sugar residue to the polypeptide chain of the glycoprotein is very rigid for that particular glycoprotein. However, the specificity of transferases for a specific glycoprotein seems to decrease as more sugar residues are attached to the carbohydrate. Instead specificity seems to lie in the terminal moiety and its anomeric linkage.

The relationship of the A-protein of lactose synthetase to these glycosyl, and particularly galactosyl, transferases is intriguing. It appears similar to the goat colostrum galactosyl transferase reported by McGuire et. al., (91) and Iyer and Carlson (111), and even the gastric mucosal galactosyl transferase reported by Watkins (107), for they are all active with N-acetylglucosamine as an acceptor. Investigations of the acceptor specificity of lactose synthetase are reported in this dissertation.

CHAPTER III

STUDIES ON THE INTERACTION OF LACTOSE SYNTHETASE A-PROTEIN WITH α-LACTALBUMIN

Experimental Procedure

Materials and Reagents

Phosphoenolpyruvate, NADH, Tris (tris-hydroxymethyl-amino methane), glycylglycine, Pyruvate kinase (Type I from rabbit muscle, crystalline ammonium sulfate suspension contains lactic dehydrogenase), Peroxidase (horseradish, Type VI), o-dianisidine, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis. Pronase, Grade B, was purchased from Calbiochem. Potassium iodide, manganese chloride, and D-glucose, were obtained from Baker Chemical Co. Bio-Gel P was obtained from Bio-Rad Laboratories, Sephadex from Pharmacia. Dansyl chloride (1-Dimethylaminonaphthalene-S-sulfonyl chloride, abbv. DNS) was obtained from Calbiochem.

^{125}I was obtained fresh as a carrier free solution of $Na^{125}I$, also free from reducing agents, from Nuclear Chicago Corp., and prepared by the Radiochemical Centre, Amersham, England. UDP-gal was synthesized by the method of Moffat and Khorana (112,113). Bovine α-lactalbumin was prepared from milk according to Brodbeck, et. al. (6) as a lyophilized, salt free, electrophoretically homogeneous protein. Lactose

synthetase A-protein was prepared according to Ebner, et. al., (114) and obtained as an ammonium sulfate solution of partially purified A-protein from either the HA_I or HA_{II} step of the purification. All other materials used were of reagent grade.

Spectrophotometric Assay for Lactose Synthetase Activity

The lactose synthetase proteins can be assayed separately in the presence of saturating amounts of the second protein as described earlier (6). Enzymatic activity is assayed spectrophotometrically at 340 mμ by coupling UDP formation of Reaction 3 to NADH oxidation by means of pyruvate kinase and lactic dehydrogenase. Assays were done in 1.0 ml final volume and contained 0.15 mM NADH, 1.0 mM PEP, 0.05 ml of a 1 to 10 dilution of pyruvate kinase (Sigma, Type I, 25 mg protein/ml with 2.4 IU pyruvate kinase/mg protein), 20 mM Tris-HCl, pH 7.4, 5mM $MnCl_2$, 0.25 mM UDP-gal, and 20 mM glucose. For assay of the A-protein lactose synthetase activity the assay cuvette also contained 100 μg α-lactalbumin. Conversely, for the enzymatic assay of α-lactalbumin (α -LA), each assay cuvette contained approximately 100 units of A-protein. One unit of enzymatic activity is defined as that amount of enzyme which catalyzes the formation of one mμmole of UDP per minute per ml and equals 0.0062 ΔA_{340}/min/ml. α-Lactalbumin was estimated by UV absorption at 280 mμ, assuming an extinction coefficient of 1 A_{280} per mg/ml. All assays were performed on a Beckman Model DB recording spectrophotometer with a water jacketed cell chamber thermoregulated at 25°.

Sucrose Density Gradient Centrifugation

Sucrose density gradient studies were performed by the method of

Martin and Ames (22). Linear sucrose gradients of 5-10%, 5-15%, or 5-20% (w/v) were prepared in the appropriate buffer. If substrates were desired in the gradient, they were added at the appropriate concentration as the gradients were prepared so as to be homogeneous throughout the gradient. Gradients were allowed to equilibrate at least three hours at the desired temperature prior to use. Total gradient volume was 4.6 ml per tube. Samples of 0.1 ml to 0.2 ml containing the desired proteins, substrates, and buffer were layered carefully on top of the gradients immediately before centrifuging. An aliquot of a sample was routinely layered onto 5% sucrose prior to layering on the gradients to visually ensure that the sample did not sink through the gradient. Gradients were centrifuged in a Beckman L2-65 preparative ultracentrifuge at the desired speed and temperature using either the SW-39 (with cellulose nitrate tubes) or SW-65 rotor (with polyallomer tubes). Centrifugation time is reported as total elapsed time from start of the run until the rotor stopped with no correction made for acceleration and deceleration time. Fractions were collected from each of the three centrifuge tubes immediately after stopping by means of piercing through the bottom with a 20 gauge hypodermic needle and forcing the gradient out slowly through the needle with controlled air pressure. Six drop fractions were collected by hand at room temperature for a total fraction volume of approximately 80-90 µl. The fractions were immediately cooled in ice and subsequently assayed for the presence of either or both proteins. Results are reported as % sedimentation down the centrifuge tube with the bottom of the tube being 100%.

Gel Filtration Methods

Gel filtration glass columns of desired length and diameter were silanized according to instructions in Bio-Rad, Price List T (115), using a 1% solution of dichlorodimethyl silane in benzene, prior to packing. Gels were swollen, deareated, and packed in the columns according to the technical manuals (115,116). Samples were applied to the top of the gel in a uniform layer. Absence of zoning and general uniformity of packing was determined visually with a blue dextran sample (approximately 1 mg/ml) as was the void volume. The optimum flow rate determined for each column was the maximum flow rate which would allow migration of a blue dextran band without local tailing or skewing.

Fluorescence Studies Methods

Fluorescence studies were done on an Aminco-Bowman Spectrophotofluorimeter equipped with a water-jacketed sample chamber with thermoregulation at 25°. Spectra were recorded directly with an Aminco-Bowman X-Y-T Recorder. The light source was a continuous spectrum Xenon lamp. An IP-128 photomultiplier tube was the detector. The sample volume was 1.0 ml in a 3.0 ml quartz cuvette with appropriate buffer at the desired concentration and pH. Slit program number 3 as described in the Aminco-Bowman instruction manual was normally used. However, for purposes of attenuation, the photomultiplier tube slit was sometimes varied. Spectra are reported as recorded and are not corrected for photomultiplier and spectrofluorimeter efficiencies. All buffers, reagents, and water were filtered

through a course glass frit prior to use and were found to be free from significant background fluorescence under the conditions used in the study. The instrument was standardized with a 1 μg/ml solution of tryptophan prior to use each day.

Results

The two proteins of lactose synthetase are easily separated on Bio-Gel P-30 with resultant loss in lactose synthetase activity. Recombination of the proteins restored enzymatic activity in the standard assay. The enzymatic assay for each of the proteins is done in the presence of saturating amounts of the other. Thus physical and kinetic data clearly suggest that a physical interaction between the two proteins is necessary for lactose synthetase activity. This would presumably involve an enzymatically active complex of the A-protein and α-lactalbumin. Since the two proteins are easily separated by gel filtration in the absence of substrates (6), the presence of substrates or substrate at or near the concentrations used for assay might enhance complex formation, similar to the conditions required for tryptophan synthetase (71,72) and ribonucleoside diphosphate reductase (12) complex formation. First attempts to demonstrate complex formation were made using sucrose density gradient centrifugation as described in Methods. The A-protein used throughout the studies on complex formation was an ammonium sulfate solution concentrated from the HA_I step of purification from bovine milk as described earlier (114).

Sucrose Density Gradient Centrifugation Studies

Initial experiments were performed under assay concentration of substrates (0.25 mM UDP-gal, 20 mM glucose) when present and all gradients and samples contained 20 mM Tris, pH 7.4, 5mM $MnCl_2$ unless otherwise specified. Centrifuge runs were at 2° unless otherwise noted. As mentioned under Methods, the samples and gradients were identical in substrate content and concentration. Only the sample layers contained either or both A-protein and α-LA in the amounts specified.

Results of the first centrifugation are shown in Figure 1. These were 5-20% sucrose density gradients centrifuged 15 hours at 35,000 RPM in the SW-39 rotor. Sample layers contained 200 units of A-protein, 1000 µg α-LA in 0.1 ml, and substrates as indicated. Tube 1 contained no substrates, Tube 2 contained 0.25 mM UDP-gal, and Tube 3 contained 0.25 mM UDP-gal and 20 mM glucose. The refractive indeces of fractions from Tube 1 established the linearity of the 5-20% sucrose gradient.

The high protein levels contained in each of the samples were chosen for three reasons: 1) to have a maximum amount of both proteins present to aid formation and detection of an A: α-LA complex, since the only means of detection was by enzymatic assay for each of the proteins; 2) stability of the A-protein was questionable at this time and survival of enzymatic activity for the duration of the centrifuge run was in doubt; 3) most importantly, to favor the establishment of complex if it involves an equilibrium reaction.

Location of the A-protein and α-LA peaks by assaying fractions for enzymatic activity showed an appreciable difference in the

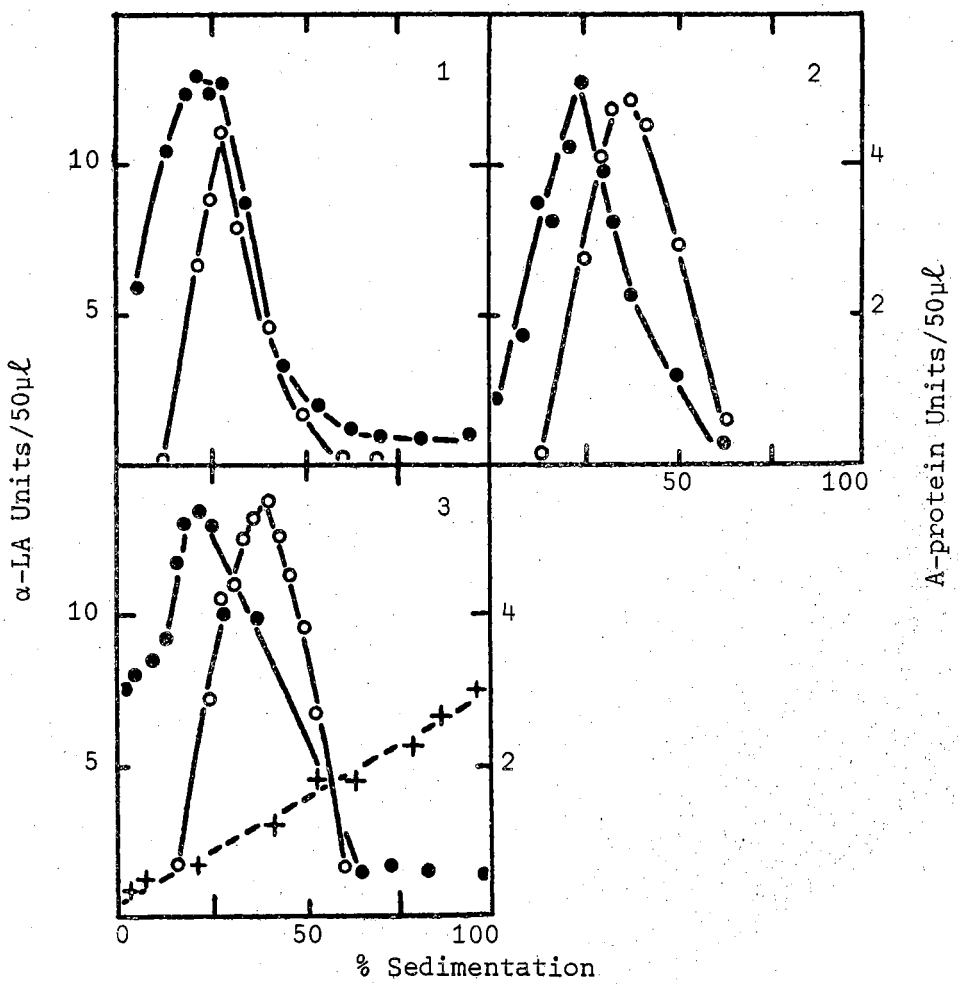


Figure 1. Sedimentation Patterns of the A-Protein (200 Units) and α-LA (1000 μg) in the Presence and Absence of Substrates Using 5-20% Sucrose Gradients.

5-20% sucrose gradients containing 20 mM Tris, pH 7.4 and 5 mM $MnCl_2$ were centrifuged at 35,000 RPM, 2°, for 15 hours. Total fractions collected: Tube 1 = 32, Tube 2 = 24, Tube 3 = 33. O, A-protein activity; ●, α-LA activity; +, Refractive Index

Tube 1: 200 units A, 1000 μg α-LA, no substrates % Sed.; α-LA = 23%, A = 28%.

Tube 2: 200 units A, 1000 μg α-LA, UDP-galactose % Sed.; α-LA = 29%, A = 37%.

Tube 3: 200 units A, 1000 μg α-LA, UDP-galactose, glucose % Sed. α-LA = 23%, A = 39%.

sedimentation of the A-protein in Tube 1 and Tubes 2 and 3. Sedimentation of A-protein was 28% in Tube 1 (containing no substrates), 37% in Tube 2 (containing UDP-gal), and 39% in Tube 3 (with UDP-gal and glucose), suggesting that under these conditions and in the presence of UDP-gal or UDP-gal and glucose, complex formation may have occurred as indicated by the faster sedimenting A peak. The high excess of α-LA over A-protein should favor binding of α-LA to A-protein and hence most if not all of the A-protein might be expected to appear in a faster sedimenting peak indicative of an A: α-LA complex. Assays for α-LA showed no appreciable change in its sedimentation due to substrates.

Since the A-protein has a molecular weight of about 70,000 (114) its S_{20} was estimated to be around 5, based on other proteins of this molecular weight (22). An A: α-LA complex in one to one ratio (as the simplest case) would have as a sedimentation coefficient approximately 6.75 which is the sum of the A-protein S_{20} of 5 and the S_{20} for α-LA of 1.75 (117). The expected % sedimentation of the A: α-LA complex would then be 38% as calculated from S values using 28% (from Tube 1) as the sedimentation of the A-protein alone. The 38% predicted sedimentation for the complex agrees nicely with the results of Tubes 2 and 3, suggesting that complex formation may have occurred in the presence of UDP-gal or UDP-gal and glucose. The S value of the A-protein also seemed to be consistent with the predicted S value since the sedimentation of A-protein (about 30%) was as predicted on the basis of time and speed of centrifugation using data reported by Martin and Ames (22) for other proteins under similar conditions to calculate time required

for sedimentation.

By these same criteria, the α-LA seemed to sediment faster than expected. Since the S value of α-lactalbumin is 1.75 (117), this observation suggested that the assumed sedimentation coefficient for the A-protein might be in error. If the S value of the A-protein is then calculated in each tube using α-LA as a marker, the results are 2.13 in Tube 1, 2.23 in Tube 2, and 2.97 in Tube 3, all considerably lower then expected. Based on these values an A: α-LA complex would then have a sedimentation coefficient of about 4. The A activity peak corresponding to A: α-LA complex would then be expected to sediment 51% down the tube. No such fast sedimenting peak was observed, indicating no detectable complex formation if the A-protein indeed has the lower S_{20} of 2.2. Hence the results of this first experiment can be interpreted as showing complex formation or not, depending on which sedimentation coefficient is correct for the A-protein.

Additional experiments were required in order to better resolve the A-protein from α-LA, to clearly define the A-protein sedimentation coefficient, and to increase resolution of A-protein and any A: α-LA expected.

A second experiment under identical conditions used 200 units of A-protein and 400 µg α-LA in the 0.1 ml sample layers. Under these conditions the A-protein alone sedimented to 31% as shown in Tube 1 of Figure 2, α-lactalbumin alone in Tube 2 sedimented 23%, and A-protein and α-LA together in the absence of substrates (Tube 3) sedimented to 29% and 22% respectively. These results are in good agreement with the results for the A-protein of Tube 1, Figure 1,

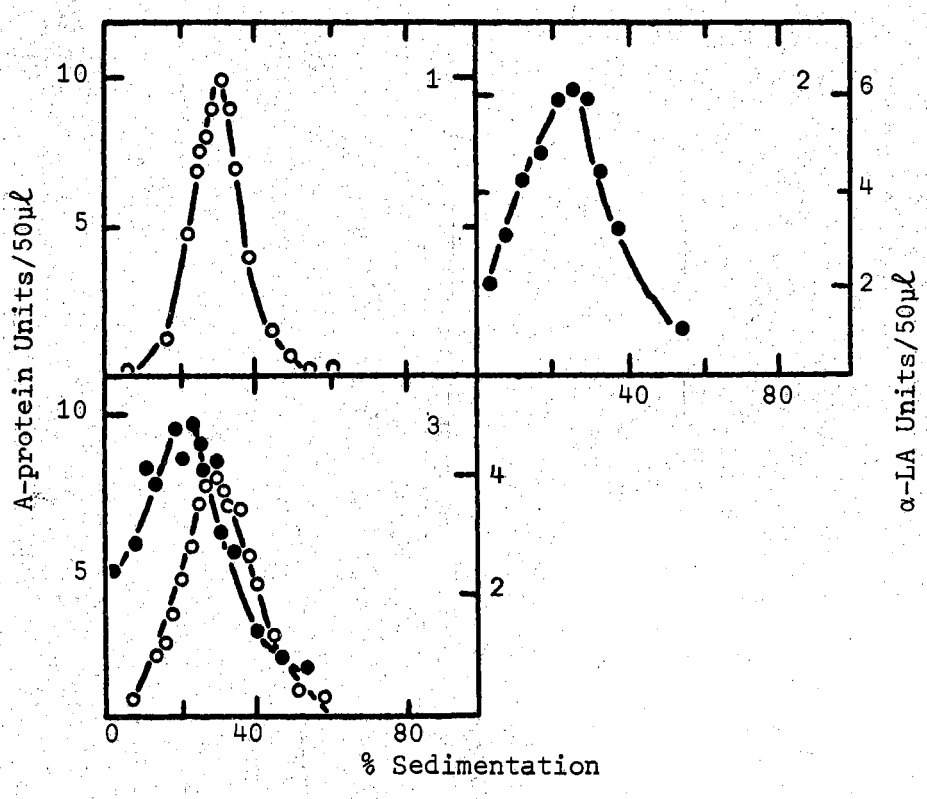


Figure 2. Sedimentation Patterns of the A-Protein (200 Units) and α-LA (400 µg) in the Absence of Substrates.

5-20% sucrose gradients containing 20 mM Tris, pH 7.4, and 5 mM $MnCl_2$ were centrifuged at 35,000 RPM, 2°, for 15 hours. Total fractions collected: Tube 1 = 55, Tube 2 = 43, Tube 3 = 45.
Tube 1: 200 units A; % Sed = 31%.
Tube 2: 400 µg α-LA; % Sed = 23%.
Tube 3: 200 units A, 400 µg α-LA
% Sed.; α-LA = 22%, A = 29%.
O, A-protein activity; ●, α-LA activity

and showed the individual proteins to sediment the same as those combined but in the absence of substrates.

Figure 3 shows the results from a centrifugation at 35,000 RPM for twenty hours using 5-15% sucrose gradients instead of 5-20% in an attempt to increase resolution of the protein peaks. Tube 1 contained UDP-gal and glucose while Tubes 2 and 3 contained no substrates. Sample layers of 0.1 ml contained 200 U A-protein, 400 µg α-LA, UDP-gal, and glucose for Tube 1; 200 U A and 400 µg α-LA, no substrates for Tube 2; and 200 U A-protein alone, no substrates for Tube 3. Figure 3 shows improved resolution of the A and α-LA peaks with no significant difference in sedimentation of the A peak in the presence and absence of substrates or α-lactalbumin, suggesting that no stable complex is formed under these conditions.

From Figures 2 and 3, no complex formation was observed. The S value of the A-protein was much lower (2.6S) than the expected value of about 5. The faster sedimentation of A-protein in Tubes 2 and 3 of Figure 1 is probably due to experimental error. This seems likely since centrifugation and fraction collecting techniques were not as refined as in experiments of Figure 3 and later. Only 30 to 45 fractions were collected in Figures 1 and 2, respectively, while 60 to 70 fractions were routinely collected in experiments from Figure 3 on. The increased number of fractions would make peak location more reliable. Another factor contributing to assymetrical peaks is the enzymatic assay itself. The assays were sometimes erratic resulting in displaced peaks or multiply-peaked protein bands.

The sedimentation patterns for the A-protein shown in Figure 4 are the results of experiments using 2000 µg α-LA and 50 units A-

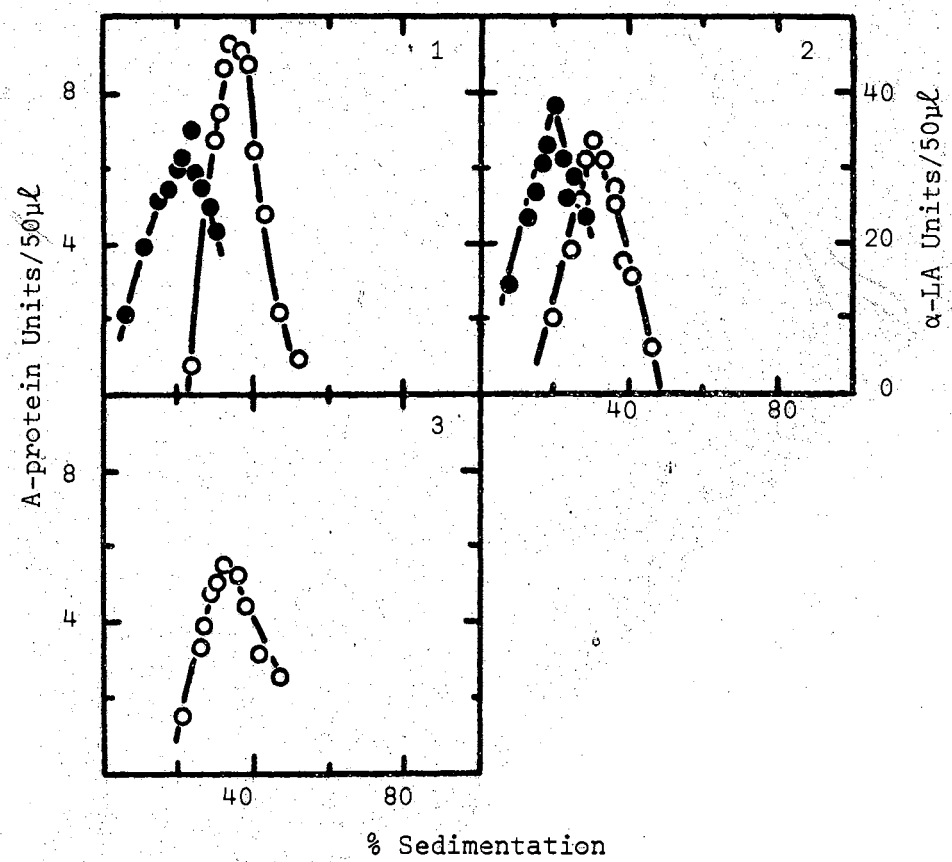


Figure 3. Sedimentation Patterns of the A-Protein (200 Units) and α-LA (400 µg) in 5-15% Sucrose Gradients With and Without Substrates.

5-15% gradients containing 20 mM Tris, pH 7.4, and 5 mM $MnCl_2$ were centrifuged at 35,000 RPM, 2°, for 15 hours. Total fractions collected: Tube 1 = 64, Tube 2 = 62, Tube 3 = 63.

Tube 1: 200 units A, 400 µg α-LA, UDP-galactose, glucose
% Sed.; α-LA = 23%, A = 34%.

Tube 2: 200 units A, 400 µg α-LA, no substrates
% Sed.; α-LA = 21%, A = 31%.

Tube 3: 200 units A, no α-LA, no substrates
% Sed. of A = 32%.

O, A-protein activity; ●, α-LA activity.

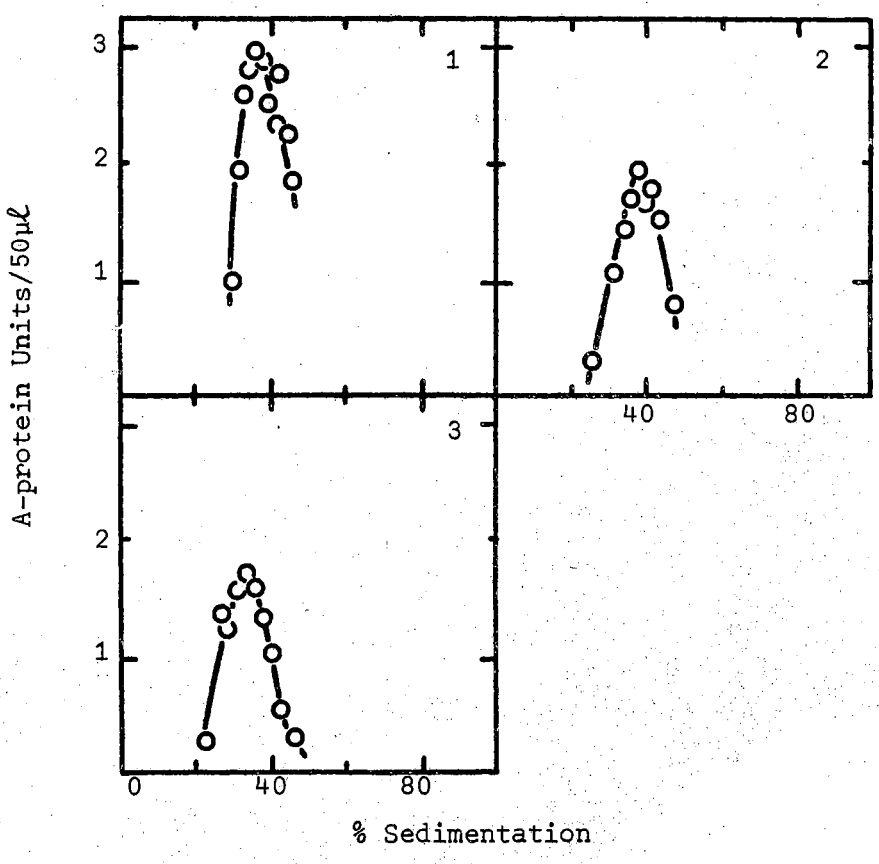


Figure 4. Sedimentation Patterns of the A-Protein (50 Units) ± α-LA (2000 µg) and Substrates in 5-15% Sucrose Gradients.

5-15% gradients containing 20 mM Tris, pH 7.4, and 5 mM $MnCl_2$ were centrifuged at 35,000 RPM, 2°, for 20 hours. 64 fractions were collected from each tube. Only the sedimentation of the A-protein was determined.

Tube 1: 50 units A, 2000 µg α-LA, UDP-galactose, glucose % Sed. of A = 36%.

Tube 2: 50 units A, 2000 µg α-LA, UDP-galactose % Sed. of A = 37%.

Tube 3: 50 units A, % Sed. of A = 33%.

protein in 0.1 ml samples. This experiment used a high ratio of α-LA to A-protein in an attempt to drive the A-protein into complex formation. Experimental conditions were the same as in Figure 3. No significant difference was noted for the sedimentation of the A-protein whether in the presence or absence of α-LA and/or substrates. Tube 1 contained A-protein, α-LA, UDP-gal, and glucose (sedimentation of A-protein was 37%); Tube 2 contained A-protein, α-LA, and UDP-gal (sedimentation of A-protein = 37%); Tube 3 contained A-protein alone which sedimented 33% down the tube. Only A-protein activity was determined in this experiment. The sedimentation patterns are somewhat erratic since the levels of A-protein, as determined by enzymatic assay, show a considerable amount of variation. Some points were suggestive of skewed or split peaks which, if real, could be indicative of incomplete complex formation.

A shallower sucrose density gradient of 5-10% was used in Figure 5 in attempts to increase resolution of the sucrose density gradient system and perhaps thereby determine whether the A-peaks are split into two peaks due to complex formation or are simply erratic assay results. The A-protein sedimentation was 40% in Tube 1 (containing 50 units A, 2000 μg α-LA, UDP-gal, and glucose), 39% in Tube 2 (containing 50 units A, 2000 μg α-LA, and UDP-gal), and 36% in Tube 3 (containing only 50 units A-protein). However, Tube 2 contained an apparent second A-protein peak which sedimented at 45%. The expected sedimentation of an A: α-LA complex would be approximately 61% if the A-protein has an S_{20} of approximately 3.1 (based on α-LA as a marker). Therefore the second A-protein peak seems not to be due to complex formation but instead is probably due to erratic assays

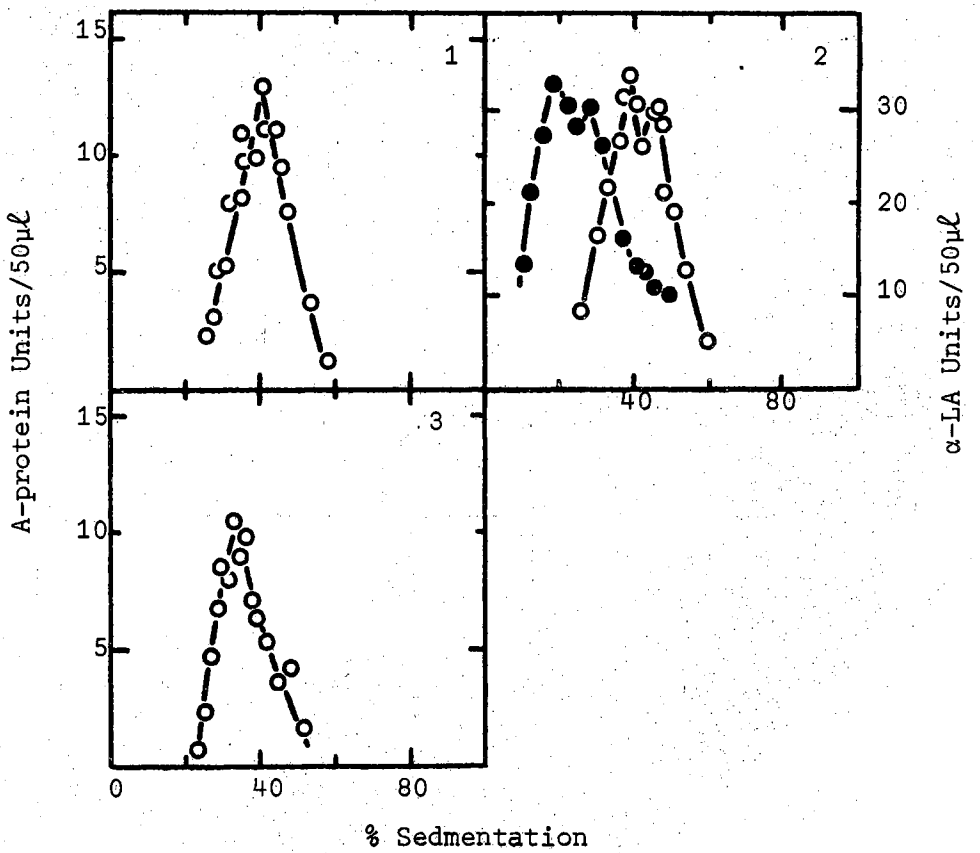


Figure 5. Sedimentation Pattern of the A-Protein (50 Units) ± α-LA (2000µg) and Substrates in 5-10% Sucrose Gradients.

The 5-10% sucrose gradients containing 20 mM Tris, pH 7.4, and 5 mM $MnCl_2$ were centrifuged at 35,000 RPM, 2°, for 20 hours. Total fractions collected: Tube 1 = 62, Tube 2 = 64, Tube 3 = 65.

Tube 1: 50 units A, 2000 µg α-LA, UDP-galactose, glucose % Sed. of A = 40%.

Tube 2: 50 units A, 2000 µg α-LA, UDP-galactose % Sed.; α-LA = 19%, A = 39 and 45%.

Tube 3: 50 units A
% Sed. of A = 33%.

0, A-protein activity; ●, α-LA activity

since it did not appear so clearly in any other experiments and is comsiderably above the expected sedimentation of the complex. However, one cannot dismiss the possibility that this second A-peak is a second molecular form of the enzyme.

A separate experiment used 5-10% sucrose gradients in which 110 units of A-protein and 2000 µg α-LA was centrifuged at 65000 RPM for 5 hours at 2° in the SW 65 rotor. Under these conditions the A-protein sedimented 18% in the absence of α-LA and substrates and 21% in the presence of α-LA, UDP-gal, and glucose. Neither of the A peaks showed any tendency to separate into two peaks. The SW 65 rotor and centrifugation for shorter time periods at 65000 RPM appeared to give more reliable results. The occasional splitting of an A-peak or tendency to split may be due to erratic assays.

The results from these experiments led to the conclusion that there is no detectable complex of A-protein and α-lactalbumin under any of these conditions. The detection of any stable complex suffers from the reliability and sensitivity of the ensymatic assay for each of the two proteins. Consequently, the reliability of some experimental results can be questioned. Hence, a more reliable and sensitive means of assaying for one of the proteins was desired.

Iodination of α-Lactalbumin

α-Lactalbumin was iodinated in order that a more sensitive means of assaying for α-lactalbumin could be used in additional experiments to detect complex formation.

Iodination was by the procedure of Greenwood and Hunter (45) in a conical centrifuge tube with slight modifications as described below.

A typical iodination reaction was done at room temperature and used one millicurie of ^{125}I (fresh, carrier free as $Na^{125}I$ in 7 µl H_2O solution) added to 25 µl of 0.4 M phosphate buffer, pH 7.5, and 200 µg α-LA in 40 µl. These solutions were thoroughly and rapidly added in order and mixed. The iodination reaction was begun by rapidly injecting 50 µl of chloramine-T solution (containing 176 µg chloramine-T in 50 µl H_2O) for a final reaction volume of 122 µl. Experience proved that high iodination efficiency could be obtained only if the chloramine-T was injected rapidly as a "jet" into the solution containing α-LA and $Na^{125}I$. Efficiency and specific activity were higher if the final reaction volume is kept to a minimum (0.1-0.2 ml) and if fresh ^{125}I solution is used. The reaction proceeded for one minute with agitation and was then stopped by the rapid addition of 0.2 ml of sodium metabisulfite solution (2.4 mg/ml H_2O). The reaction mixture was agitated for about thirty seconds before the addition of 0.2 ml of a 10% potassium iodide solution. The latter addition provided cold carrier iodide which helped to free α-lactalbumin from any non-covalently bound ^{125}I.

The entire reaction mixture (0.522 ml) was loaded on a Bio-Gel P-30 column (1 cm x 53 cm). The reaction vessel was rinsed with another 0.2 ml of 10% KI solution and this rinse was also loaded onto the P-30 column with the reaction mixture. The column was then eluted with 20 mM Tris, pH 7.5, containing 5 mM $MgCl_2$. Fractions of 0.8 ml were collected. Aliquots of 50 µl were counted in 10 ml of Bray's liquid scintillation fluid (118) for one to ten minutes to find ^{125}I peaks. Counting efficiency was not determined since no ^{125}I standard was available, therefore a counting efficiency of 50% was assumed for

the purposes of estimation of ^{125}I. α-Lactalbumin was located by A_{280} readings. The elution profile of the Bio-Gel P-30 column is shown in Figure 6. Note the A_{280} profile shows α-LA to elute between fractions 23 and 30. The void volume of this column came at fraction 17, indicating that α-LA-^{125}I did not dimerize. The first ^{125}I peak superimposes on the α-LA peak, indicating ^{125}I is bound to the α-lactalbumin. The second ^{125}I peak corresponds to unbound ^{125}I present as iodide in the reaction mixture after the addition of metabisulfite which reduces I_2 to Iodide. The α-LA-^{125}I peak has little contamination by free ^{125}I from the second peak because of the return to near background between the two peaks.

Fractions 23 through 30 were pooled and the tubes rinsed for a final pool volume of 10.3 ml. Recovery of α-LA was 202.5 µg or essentially 100% as determined by A_{280} reading on the pooled samples. The pooled samples were evaporated on a rotary evaporator in vacuo at room temperature to a final volume of 1.0 ml. The specific activity of the α-lactalbumin was 0.69 microcuries per µg α-LA based on an assumed counting efficiency of 50%. The α-LA concentration in the pool was 200µg/ml.

The activity of the ^{125}I-labeled α-LA of the pool in the enzymatic assay for α-lactalbumin was determined by comparing activities of 1.5 µg unlabeled and labeled α-LA. The unlabeled α-LA solution was the same one used for iodination and activity of it was determined by making a standard curve from 0.2 to 1.5 µg α-LA in the lactose synthetase assay for α-lactalbumin. By these criteria, the iodinated α-lactalbumin retained 97.5% of its activity in the lactose synthetase assay. This is in agreement with earlier findings on the

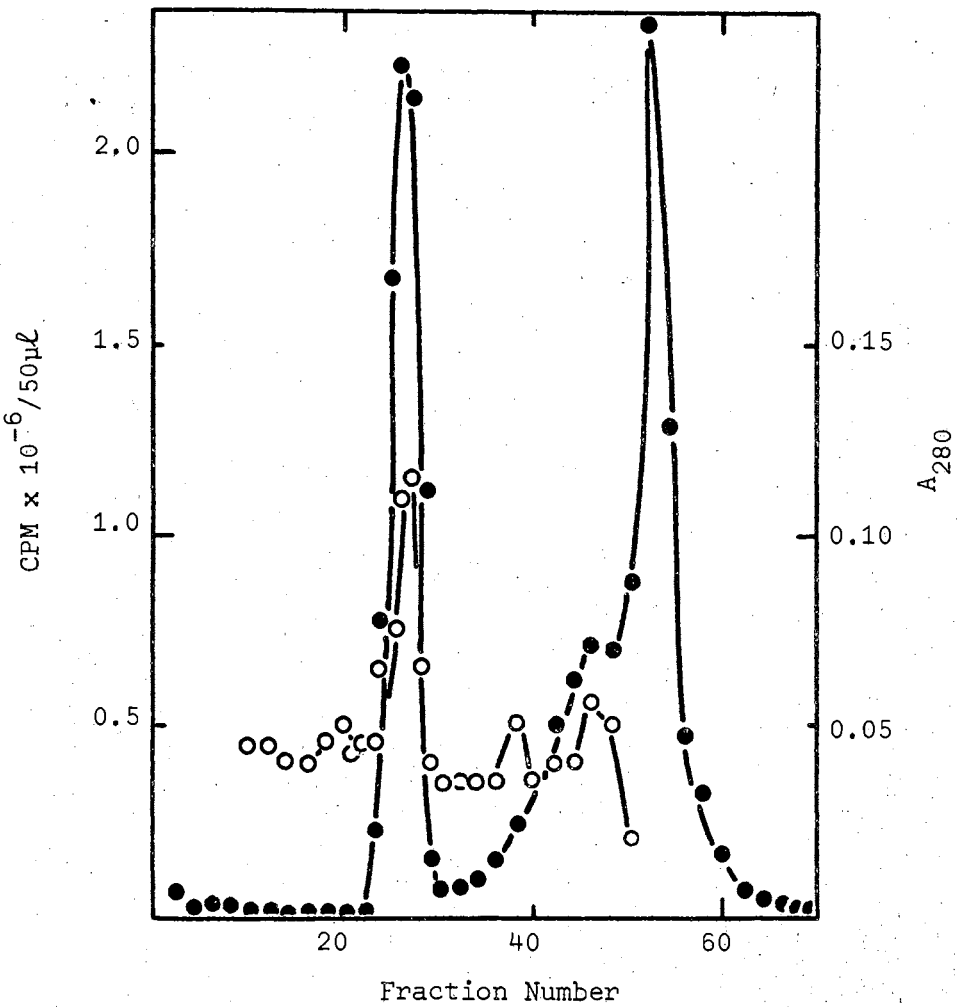


Figure 6. Gel Filtration of the α-LA Iodination Reaction Mixture on Bio-Gel P-30.

The iodination reaction mixture (0.522 ml) plus rinse (0.2 ml) was loaded on a Bio-Gel P-30 column (1 cm x 53 cm) and eluted with 20 mM Tris, pH 7.4, 5 mM $MgCl_2$. Fractions (0.8 ml) 23-30 were pooled for recovery of α-LA-^{125}I. ●, CPM for ^{125}I; 0, A_{280} for α-LA.

iodination of α-LA using the procedure of Covelli and Wolf (43) which is reported to fully iodinate proteins using a KI_3 solution. With this method, fully iodinated α-LA was 80% biologically active (119). Thus iodination appears to be a relatively mild modification reaction which, at low levels, does not destroy biological activity of α-LA. This is supported by nitration studies of α-LA which report little loss in activity upon addition of one nitro group per mole of α-lactalbumin (52), indicating that the susceptible tyrosine residue is not absolutely required for biological activity.

The product obtained by the above described procedure could have a maximum of 1 to 2% of the α-LA molecules iodinated on the basis of moles ^{125}I in 1 mc carrier free ^{125}I per mole of α-LA in the reaction mixture. It would be expected that the labeled α-lactalbumin would then contain predominately monoiodotyrosine. This was substantiated by pronase digestion of α-LA-^{125}I.

Unlabeled carrier α-LA (200 µg) was added to 4 µg α-LA-^{125}I from the 1.0 ml pooled sample. This was digested with 4 mg pronase (Sigma) in 0.2 M $NaHCO_3$, pH 7.5, and 0.05 ml $Na_2S_2O_3$ (2.4 ml/ml) in a final volume of 0.5 ml at 37° for 18 hours (120,121). Aliquots of 1 and 5 µl from the digestion mixture, along with standards of mono- and di-iodotyrosine, KI, and undigested α-LA-^{125}I were spotted on a sheet of Eastman Chromagram 6065 cellulose prepared thin layer sheets. The chromatogram was developed descending for 13 hours with n-butanol: 0.5N NH_4OH: abs. ethanol (5:2:1) (122) at room temperature to resolve mono- and di-iodotyrosine from the other amino acids. The chromatogram was then dried and sprayed with ninhydrin solution in collidine to visualize the amino acids.

An autoradiogram was made of the developed thin layer chromatogram by placing x-ray film (GAF Medical X-Ray products, Supreme Monopak) next to the chromatogram. Exposure for 36 hours showed no ^{125}I as free iodide in the undigested α-LA-^{125}I sample. The digested samples showed ^{125}I only in monoiodotyrosine and in undigested material remaining at the origin. Exposure for three weeks showed a trace of diiodotyrosine and a trace of free $^{125}I^-$.

Thus the iodinated αlactalbumin contained ^{125}I bound mostly as monoidotyrosine. Iodination of α-LA by similar procedures at other times gave results similar to those reported here although specific activities were sometimes slightly less. It also seems likely that ^{125}I- labeled α-lactalbumin is enzymatically active. Either the α-LA-^{125}I preparation described above or similar preparations were used in experiments involving α-LA-^{125}I for detection of complex.

Centrifugation Studies With α-LA-^{125}I

The results of the first sucrose density gradient centrifugation experiment using α-LA-^{125}I are shown in Figure 7. A 5-20% sucrose gradient in 50 mM glycylglycine, pH 8.0, and 5 mM $MnCl_2$, was used with centrifugation at 65,000 RPM for 17 hours in the SW 65 rotor. Samples contained 1.2 μg α-LA-^{125}I (about 5000 cpm) and 90 units A-protein. Tube 1 contained no substrates, Tube 2 contained UDP-gal, and Tube 3 contained both UDP-gal and glucose. Conditions in this experiment were identical to those which give maximum activity when assaying for α-LA in the lactose synthetase assay. Therefore, it would be expected that this would be near optimum conditions for complex formation. Also, since α-LA-^{125}I is being monitored, the problem of resolution of

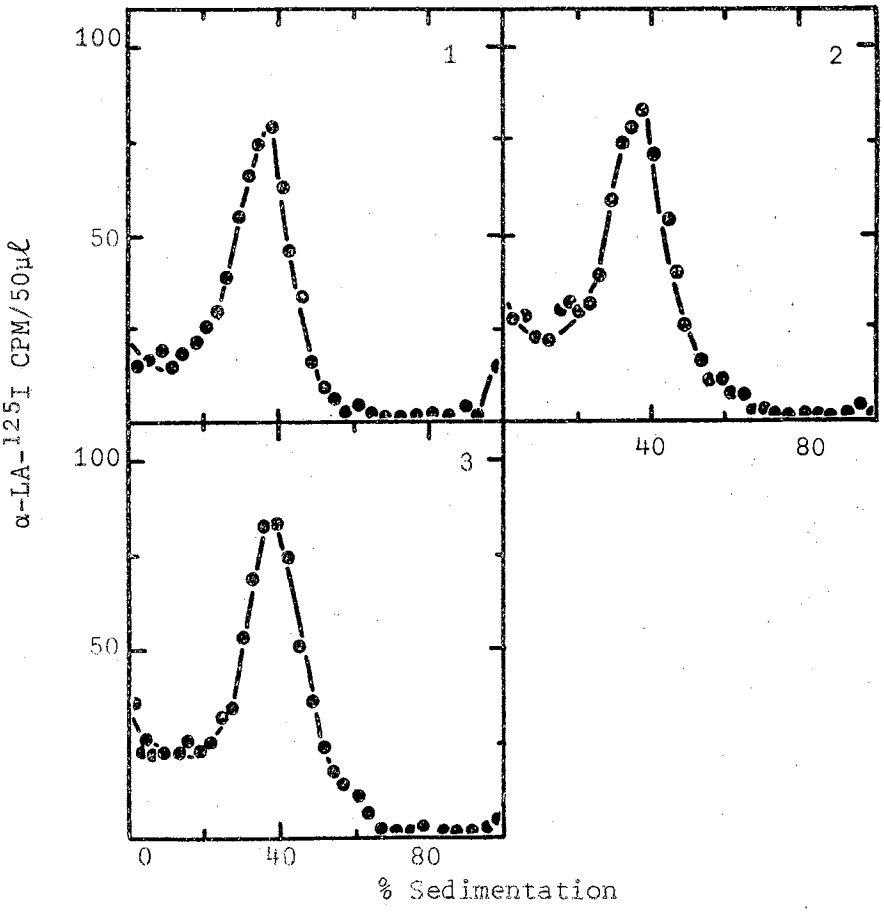


Figure 7. Sedimentation Patterns of α-LA-^{125}I (1.2 μg) With A-Protein (90 Units) ± Substrates in 5-20% Sucrose Gradients.

Sucrose gradients (5-20%) containing 50 mM glycylglycine, pH 8.0, and 5 mM $MnCl_2$ were centrifuged at 65,000 RPM, 2°, for 17 hours. α-LA-^{125}I was assayed by liquid scintillation counting of 50 μℓ aliquots. Total fractions collected; Tube 1 = 69, Tube 2 = 69, Tube 3 = 67.

Tube 1: 90 units A, 1.2 μg α-LA-^{125}I, no substrates; α-LA-^{125}I sed. = 37%.

Tube 2: 90 units A, 1.2 μg α-LA-^{125}I, UDP-galactose; α-LA-^{125}I sed. = 37%.

Tube 3: 90 units A, 1.2 μg α-LA-^{125}I, UDP-galactose, glucose; α-LA-^{125}I sed. = 37%.

ambiguous minor A-protein peak sedimentation differences is not present. If α-LA-^{125}I is involved in a detectable complex, one would observe a major shift of α-LA-^{125}I from the normal α-LA sedimentation position at the top of the tube to near the bottom of the tube. Since the conditions used in this experiment are also optimum for α-lactalbumin assay, one might expect the entire α-LA-^{125}I peak to be involved in complex, thereby shifting the entire ^{125}I peak to near the bottom of the tube.

As shown in Figure 7, there is no significant difference in the sedimentation pattern of α-LA-^{125}I in the presence or absence of substrates. Sedimentation of α-LA-^{125}I in Tube 1 was 37% (no substrates); Tube 2 - 37% (containing UDP-gal); and Tube 3- 37% (containing UDP-gal and glucose). Counting was done for 10 minutes on 50 µl aliquots from each fraction.

The conclusion from this experiment is that no detectable complex was formed under conditions of relatively high levels A-protein and very low levels of α-LA-^{125}I in the presence of UDP-gal and glucose or UDP-gal.

Another experiment, under identical conditions as those used in Figure 7, used 4.4 µg α-LA-^{125}I (24,000 cpm total) and 90 units A-protein. Tube 1 contained only α-LA-^{125}I (no A-protein, no substrates), Tube 2 contained α-LA-^{125}I plus A-protein with glucose (no UDP-gal), Tube 3 contained α-LA-^{125}I, A-protein, UDP-gal, and glucose. Sedimentation of α-LA-^{125}I, shown in Figure 8, was 35%, 36%, and 40% for Tubes 1, 2, and 3, respectively. Again, there is no appreciable difference in the sedimentation of α-LA-^{125}I when alone and then in the presence of A-protein and substrates. Importantly,

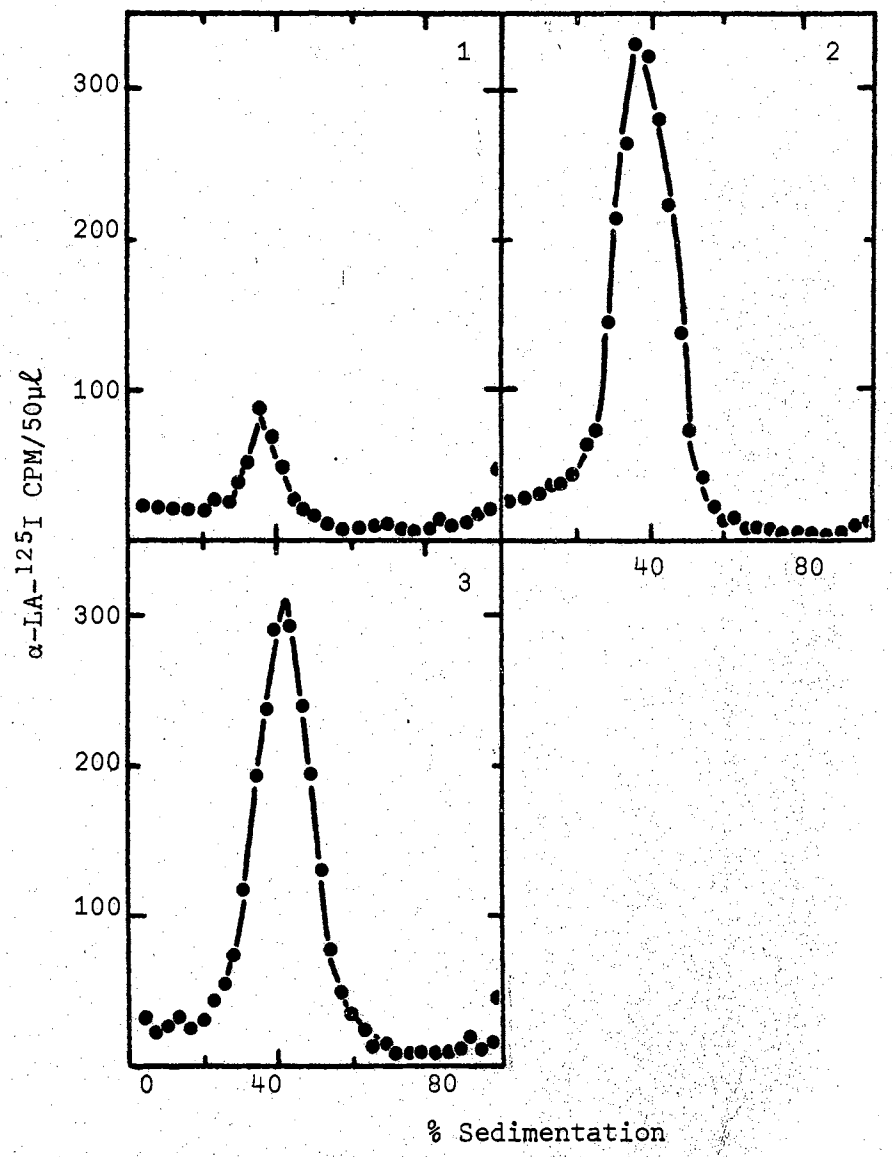


Figure 8. Sedimentation Patterns of α-LA-^{125}I (4.4 μg) ± A-Protein (90 Units) and Substrates.

5-20% sucrose gradients containing 50 mM glycylglycine, pH 8.0, and 5 mM $MnCl_2$ were centrifuged at 65,000 RPM, 2°, for 17 hours. Total fractions collected: Tube 1 = 65, Tube 2 = 67, Tube 3 = 69.

Tube 1: 4.4 μg α-LA-^{125}I, no substrates; α-LA-^{125}I sed. = 35%.

Tube 2: 90 units A, 4.4 μg α-LA-^{125}I, glucose; α-LA-^{125}I sed. = 36%.

Tube 3: 90 units A, 4.4 μg α-LA-^{125}I, UDP-galactose, glucose; α-LA-^{125}I sed. = 40%.

α-LA-^{125}I in the presence of A-protein and glucose showed no change in sedimentation. The effect of glucose alone on complex formation had not been tested before.

A different appraoch was tried. Higher temperatures of around 20° usually favor protein-protein associations due to hydrophobic interactions while lower temperatures of around 2° usually favor disruption of hydrophobic interactions (20). Hence, centrifugation at 20° instead of the previously used 2° might favor demonstration of complex formation if it were due to hydrophobic interactions. It was also apparent that during the time of the centrifugation experiment any complex formed might dissociate and the two proteins be separated if the rate of association-dissociation is sufficiently fast. Therefore α-LA (unlabeled) was used throughout a gradient in an effort to centrifuge A-protein in the constant presence of α-LA.

The results are shown in Figure 9. Sucrose gradients (5-20%) were centrifuged at 65,000 RPM for 15 hours at 20°. Tube 1 contained no A-protein and no substrates. A sample of 4.4 μg α-LA-^{125}I (24000 cpm) in 0.1 ml was layered on Tube 1. Tube 2 contained 4.4 μg α-LA-^{125}I, 89 units A-protein, UDP-gal, and glucose. Tube 3 was identical to Tube 2 except that unlabeled α-LA was present throughout the gradient at a concentration of 100 μg/ml. Sedimentation of the α-LA-^{125}I peaks was 55%, 55%, and 58% in Tubes 1, 2, and 3 respectively. The sedimentation of α-LA-^{125}I was greater at the higher temperature than in earlier experiments at 2° but no difference in α-LA-^{125}I sedimentation occurred in the presence and absence of A-protein and substrates. Likewise, cold α-LA in the gradient had no effect on the sedimentation of α-LA-^{125}I. The reason for the very low

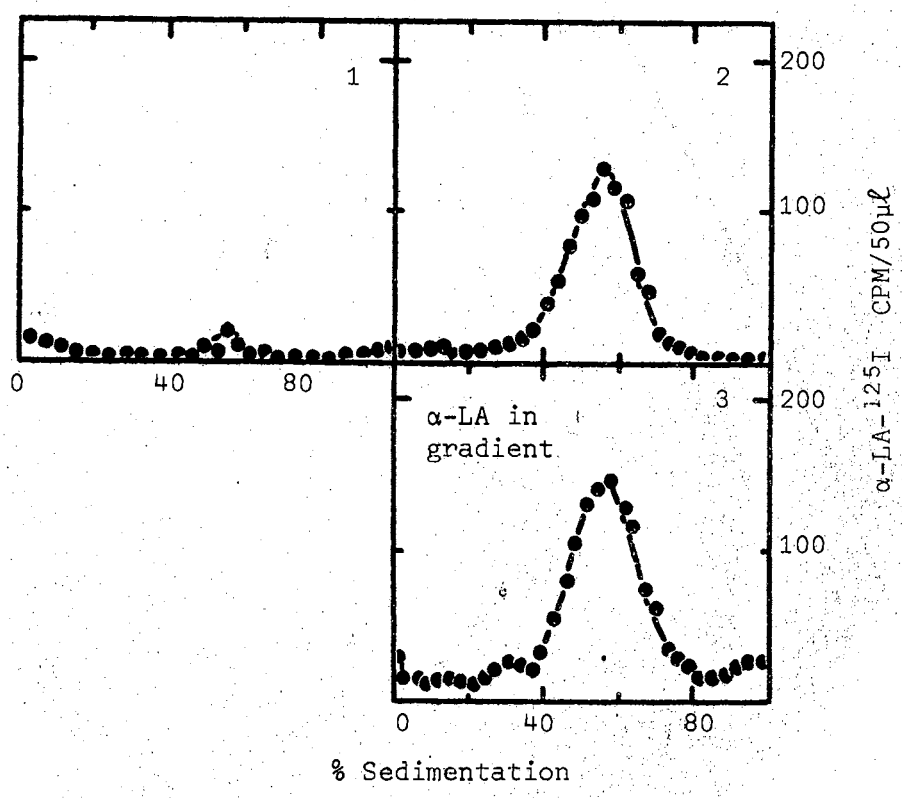


Figure 9. Sedimentation Patterns of α-LA-^{125}I (4.4 μg) ± A-Protein (90 Units) and Substrates at 20°.

5-20% sucrose gradients containing 50 mM glycylcine, pH 8.0 and 5 mM $MnCl_2$ were centrifuged at 65,000 RPM for 15 hours at 20°. Aliquots (50 μℓ) were counted for α-LA-^{125}I. Tube 3 contained unlabeled α-LA (200 μg/ml) throughout the gradient. Total fractions collected: Tube 1 = 63, Tube 2 = 67, Tube 3 = 66.

Tube 1: 4.4 μg α-LA-^{125}I, no substrates; sed. = 55%.

Tube 2: 89 units A, 4.4 μg α-LA-^{125}I, UDP-galactose, glucose; α-LA-^{125}I sed. = 55%.

Tube 3: 89 units A, 4.4 μg α-LA-^{125}I, UDP-galactose, glucose; plus 200 μg α-LA/ml; α-LA-^{125}I sed. = 56%.

count in Tube 1 was not understood. A possible error in addition of α-LA-^{125}I to the sample layer was indicated, however the end result is unaffected by the α-LA-^{125}I level in Tube 1. Thus there is no demonstrable complex formed under either of the above conditions at 20°.

Two final experiments were done both to establish the sedimentation of the A-protein relative to α-LA-^{125}I as a marker, since none of the above experiments with α-LA-^{125}I were assayed for A-protein, and to determine the reliability of the sucrose density gradient centrifugation method itself in determining sedimentation coefficients of proteins with protein markers present.

Figure 10 shows the sedimentation pattern of α-LA-^{125}I (1 μg) and A-protein (80 U) after centrifugation through 5-20% sucrose gradients at 65,000 RPM for 16 hours in Tube 1. α-LA-^{125}I sedimented 32% while the A-protein sedimented 54%. Using α-LA-^{125}I as a marker (S_{20} = 1.75), the A-protein has an apparent S_{20} of 2.65. This result is in reasonably good agreement with other estimates of the S_{20} for the A-protein based on unlabeled α-LA as a marker. Tube 2 was another experiment, unrelated to those previously discussed. Since the A-protein can catalyze the synthesis of N-acetyllactosamine as well as lactose (3), it was of interest to establish that the two activities were associated with the same enzyme. The A-protein alone in Tube 2 was assayed both for lactose synthetase and N-acetyllactosamine synthetase activity. The two activities are coincident, both sedimenting to 54% as in Tube 1. Hence both activities presumably belong to the same enzyme (or possibly two enzymes with identical sedimentation coefficients).

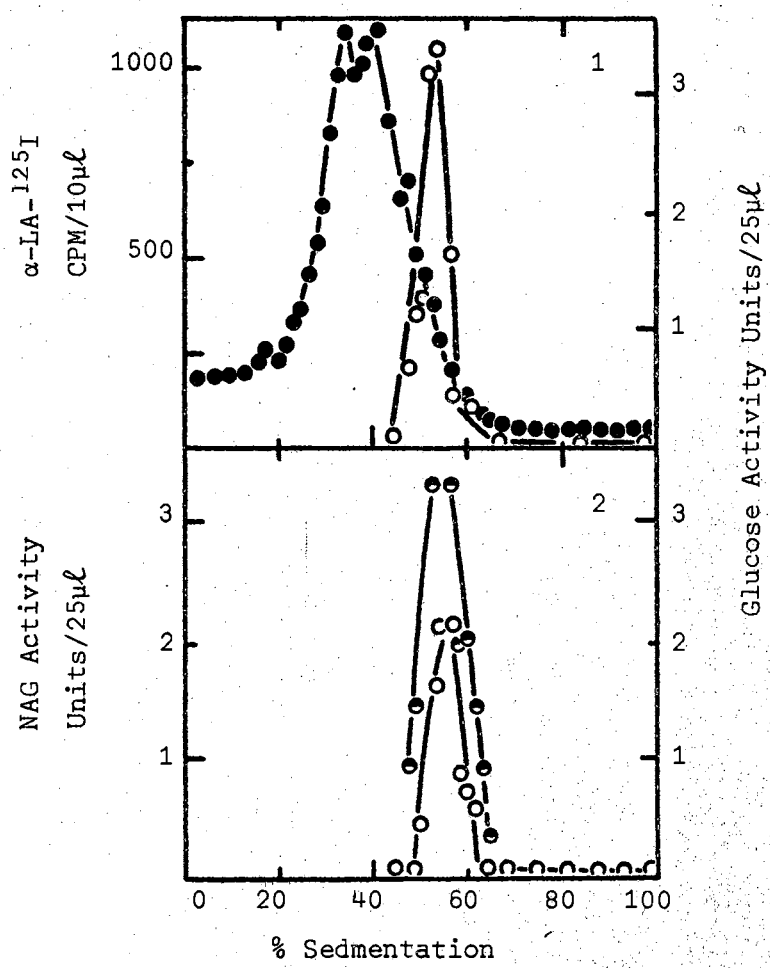


Figure 10. Sedimentation Pattern of α-LA-^{125}I and A-Protein NAG and Glucose Activity in 5-20% Sucrose Gradients.

5-20% sucrose gradients containing 50 mM glycylglycine, pH 8.0, were centrifuged at 65,000 RPM, 2°, for 16 hours. Aliquots (50 μℓ) of every fraction from Tube 1 were counted for α-LA-^{125}I (●). Tube 2 was assayed for both A-protein NAG (⊖) and glucose (○) activity. 61 fractions were collected from both tubes.

Tube 1: 80 units A, 1 μg α-LA-^{125}I; sed. of A-Protein = 54%, α-LA = 36%.

Tube 2: 80 units A; sed. of both NAG and glucose activity was 54%.

Figure 11 shows the results of experiments designed to firmly establish the S_{20} of the A-protein using two marker proteins. Tube 3 contained lyophylized A-protein (70 U) plus 10 µg peroxidase (S_{20} = 3.5); Tube 1 contained A-protein plus peroxidase plus α-LA (100 µg). Tube 2 was identical to Tube 1. Samples were centrifuged in 5-20% sucrose gradients at 2° for 16 hours at 65,000 RPM. The sedimentation coefficients of the A-protein in Tube 1 was 3.14 using peroxidase as a marker of 3.55. Tube 2 and Tube 3 showed sedimentation coefficient of 3.14 and 3.01 S, respectively, using peroxidase as a marker. Again using peroxidase as a marker. α-LA in both Tubes 2 and 3 had an S_{20} = 1.75. These results were in good agreement and clearly showed the technique to be reliable and accurate since the experimental sedimentation coefficient obtained for α-LA of 1.75 is identical to that reported in the literature (117).

To summarize the results of the sucrose density gradient centrifugation studies, no evidence for complex formation was obtained under a wide variety of conditions. These conditions included samples containing; 1) high A and extremely high α-LA concentrations in the sample layers, 2) high A and moderately high α-LA concentrations, 3) relatively low A (50 U) and extremely high α-LA concentrations (2000 µg), 4) assay levels of A (89 U) and α-LA (1-4 µg α-LA-^{125}I) as used in the lactose synthetase assay for α-lactalbumin. The presence or absence of substrates made no difference in sedimentation of either A-protein of α-LA nor did centrifugation at 20° with and without unlabeled α-LA in the gradient instead of 2° have any effect. These results are summarized in Table I.

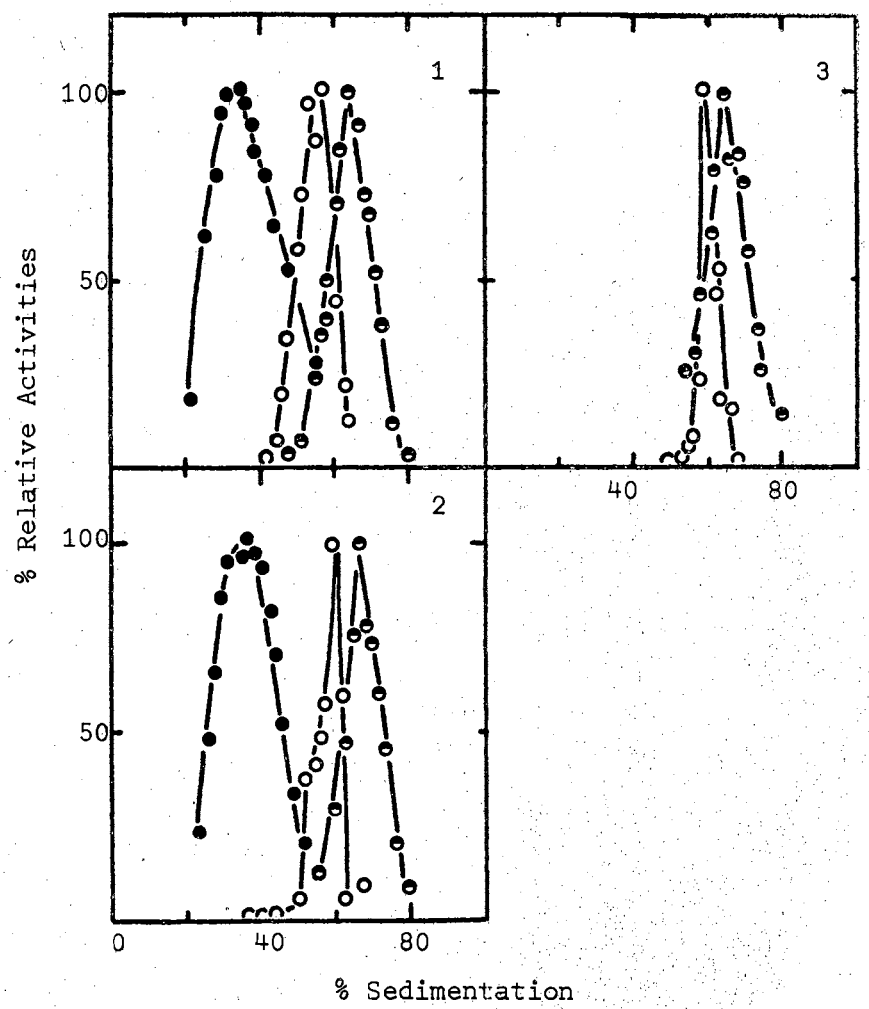


Figure 11. Sedimentation Patterns of α-LA and A-Protein With Peroxidase as a Marker.

5-20% sucrose gradients containing 50 mM glycylglycine, pH 8.0, were centrifuged for 16 hours at 65,000 RPM, 2°.

Tubes 1 and 2: 70 units A, 100 µg α-LA, 10 µg peroxidase; sed. of A = 55 and 59%, α-LA = 32 and 33%, peroxidase = 33% and 34%, respectively.

Tube 3: 70 units A, 10 µg peroxidase; sed. of A = 58%, peroxidase = 63%.

●, α-LA; ○, A-protein; ⊖, peroxidase

TABLE I

SUMMARY OF SUCROSE DENSITY GRADIENT STUDIES

Fig.	% Grad.	RPM	Hrs	°C	Tube No.	Sample Protein Units A	Sample Protein µg α-LA	Substrates* in Samples & Gradients	% Sed. α-LA	% Sed. A
1	5-20	35 K	15	2°	1	200	1000	No S	23	28
					2	200	1000	U	29	37
					3	200	1000	U + G	23	39
2	5-20	35 K	15	2°	1	200	----	No S	--	31
					2	---	400	No S	23	--
					3	200	400	No S	22	29
3	5-15	35 K	20	2°	1	200	400	U + G	23	34
					2	200	400	No S	21	31
					3	200	---	No S	--	32
4	5-15	35 K	20	2°	1	50	2000	U + G	--	36
					2	50	2000	U	--	37
					3	50	----	No S	--	33
5	5-10	35 K	20	2°	1	50	2000	U + G	--	40
					2	50	2000	U	19	39-45
					3	50	----	No S	--	33
7	5-20	65 K	17	2°	1	90	1.2†	No S	37	--
					2	90	1.2†	U	37	--
					3	90	1.2†	U + G	37	--
8	5-20	65 K	17	2°	1	---	4.4†	No S	35	--
					2	90	4.4†	G	36	--
					3	90	4.4†	U + G	40	--
9	5-20	65 K	15	20°	1	--	4.4†	No S	55	--
					2	89	4.4†	U + G	55	--
					3	89	4.4†	U + G + α-LA (200 µg/ml)	56	--
10	5-20	65 K	16	2°	1	80	1	No S	36	54
					2	80	-	No S	--	54
11	5-20	65 K	16	2°	1	70	100	No S + Per▲	32	55
					2	70	100	No S + Per▲	33	59
					3	70	---	No S + Per▲	--	58

* U = UDP-Gal (0.25 mM), G = glucose (20 mM)

† α-LA as α-LA-^{125}I

▲ Per., Peroxidase

Gel Filtration Studies

The demonstration of complex formation between the subunits of tryptophan synthetase was accomplished with both density gradient centrifugation and gel filtration (7). Hence, gel filtration was also tried as a method for demonstrating interaction of the two lactose synthetase proteins.

Gel filtration techniques were carried out as described earlier unless otherwise noted. For columns of 6 mm diameter, a 0.1 ml sample was applied carefully to the top of the gel. Elution was with 20 mM Tris, pH 7.4, containing 5 mM $MgCl_2$ at a flow rate of not more than 20 µl per minute. All experiments were done at room temperature. α-LA-^{125}I was assayed by liquid scintillation counting of an appropriate aliquot from the fractions collected off the column and is reported as counts per minute (CPM).

To stabilize the A-protein the eluting buffers contained 5 mM Mg^{++}. Indeed, after 48 hours at room temperature, 94% of the enzymatic activity of the A-protein remained. Since the duration of a normal gel filtration experiment was usually 18 to 36 hours, there was no appreciable loss in A activity during the experiments. The stability of the A-protein was later proven to be independent of the Mg^{++}, since virtually no activity was lost after 48 hours in the absence of Mg^{++}.

Figure 12 shows the elution profile for chromatography of a sample of A-protein (53 U) and α-LA (250 µg) applied together in a sample to a 6 mm x 65 cm Bio-Gel P-30 (100-200 mesh) column. Fraction volume was 0.2 ml (4 drops). The complete resolution of the A-protein and α-LA on P-30 is almost identical to the results of Brodbeck, et. al. (6).

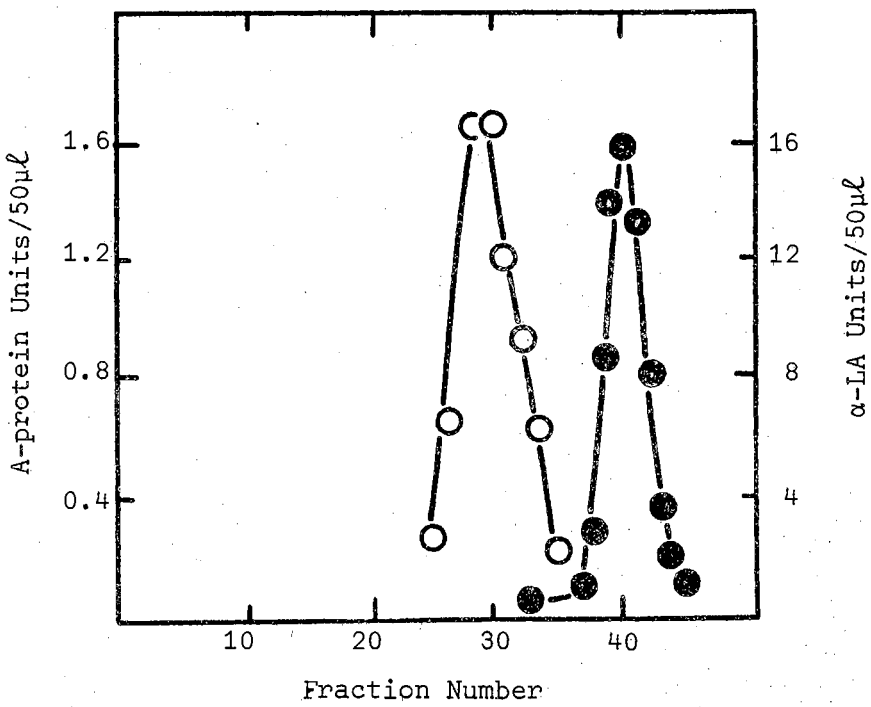


Figure 12. Gel Filtration of A-Protein and α-LA on Bio-Gel P-30.

A-protein (53 units) and α-LA (250 µg) in a 0.1 ml sample were eluted from a Bio-Gel P-30 column (6 mM x 65 cm) with 20 mM Tris, pH 7.4, 5 mM $MgCl_2$. Fraction volume was 0.2 ml.
○, A-protein; ●, α-LA.

To test for binding of small quantities of α-LA to A-protein upon gel filtration, α-LA-^{125}I and A-protein were combined in a sample and chromatographed on a Bio-Gel P-100 (100-200 mesh) column measuring 6 mm x 95 cm. The results are shown in Figure 13. A sample of 100 μg α-LA, 1 μg α-LA-^{125}I (0.7 μc), and 795 units A-protein in 0.1 ml was used in Figure 13-A. Thus, extremely high levels of A and α-LA (unlabelled + α-LA-^{125}I) on the column show no binding of α-LA-^{125}I by A-protein during the gel filtration.

Figure 13-B shows a similar experiment using 795 units A-protein and 1 μg α-LA-^{125}I in 0.1 ml applied to the same column as above. The only difference between Fig. 13-A and Fig. 13-B was the absence of the unlabeled carrier α-LA in Fig. 13-B. There is still no indication of binding of α-LA-^{125}I to the A-protein peak.

A control experiment using bovine serum albumin (1 mg) plus 1 μg α-LA-^{125}I in 0.1 ml was run on the same column. The results, shown in Figure 13-C, indicate that a small amount of binding of α-LA-^{125}I to bovine serum albumin occurred as evidenced by the appearence of a small peak of α-LA-^{125}I eluting ahead of the main α-LA-^{125}I peak and with the bovine serum albumin peak. It was concluded that α-LA probably binds to bovine serum albumin more tightly than to the A-protein.

It was not felt profitable to persue this technique further as a method of demonstrating complex formation. Instead, the gel filtration binding technique described by Hummel and Dreyer (13) appeared more suitable. Although this technique is reported to be satisfactory for demonstrating binding of small molecules to large ones, no reports are known of its use in studying the association of two macromolecules

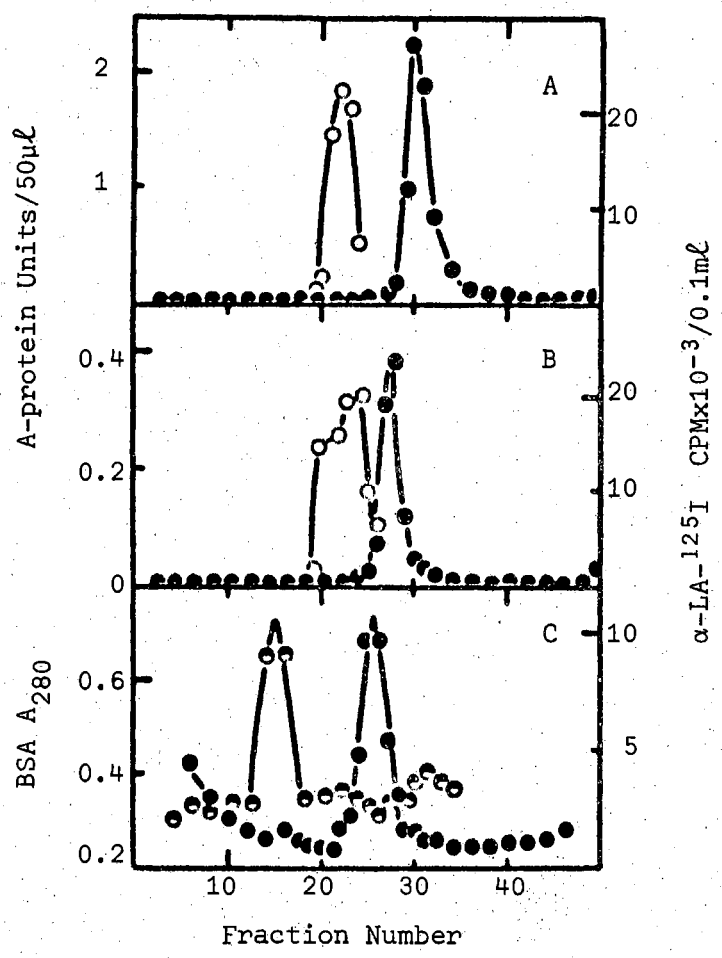


Figure 13. Gel Filtration of A-Protein and Bovine Serum Albumin With α-LA-^{125}I on Bio-Gel P-100.

Samples of α-LA-^{125}I and either A-protein or bovine serum albumin were eluted from a Bio-Gel P-100 column (6 mm x 95 cm) in 0.2 ml fractions. ●, CPM for α-LA-^{125}I; 0, A-protein activity; ⊖, A_{280} for BSA. The 0.1 ml samples contained: A; 100 µg α-LA, 1 µg α-LA-^{125}I (0.7 µc), and 795 units A-protein. B; 1 µg α-LA-^{125}I (0.7 µc), 795 units A-protein. C; 1 µg α-LA-^{125}I (0.7 µc), 1 mg BSA.

such as proteins of different molecular weight. Since the method should apply to macromolecules as well, providing a gel porosity is available which allows one protein to enter the gel while excluding the other, another series of binding experiments used Bio-Gel P-30 which excludes the A-protein but allows α-LA penetrates the gel.

A column of Bio-Gel P-30 (100-200 mesh) which was 6 mm x 65 cm was prepared. The column was equilibrated with two to three bed volumes of buffer (20 mM Tris, pH 7.4) containing α-LA-^{125}I at the level specified for each figure. Any substrates and metals desired were also added to the equilibrating buffer. Substrate and metal concentrations in the equilibrating buffer were 0.25 mM UDP-galactose, 20 mM glucose, 5 mM $MnCl_2$, and 5 mM $MgCl_2$. After equilibrating the column with buffer containing the desired α-LA-^{125}I and substrates, a sample of 0.1 ml was prepared so that its composition was identical to the equilibrating buffer but also contained the A-protein. This sample was incubated fifteen minutes at room temperature and then applied to the P-30 column. Elution of the column was done by a continuation of the equilibrating buffer. The net effect was to elute a zone of A-protein through a P-30 column (from which it is excluded) uniformly equilibrated throughout its length with α-LA-^{125}I (which enters the gel) and substrates, or metals, as desired. Hence, the zone of A-protein in the column is always in the presence of α-LA-^{125}I and any desired substrates and metals. One can thereby duplicate the conditions of the spectrophotometric assay within the column.

Fractions of 0.2 ml (4 drops) were collected and assayed for A-protein to find its elution peak and aliquots of the fractions counted by liquid scintillation to determine the elution profile of

the presumed ligand, α-LA-^{125}I. If no binding of ligand (α-LA-^{125}I) to A-protein occurs, the elution profile of the ligand will be level. However, if binding occurs, the elution profile of the ligand will show a peak coincident with the A-protein peak followed by a depression.

The elution profiles of a number of such experiments are shown in Figure 14. The first profile (14-A) shows the results of an experiment containing α-LA-^{125}I (0.25μg/ml), UDP-galactose, glucose, Mg^{++}, and Mn^{++} in the equilibrating buffer and sample. In addition, the sample contained 40 units A-protein in 0.1 ml of equilibrating buffer. These initial results were encouraging. The peak of α-LA-^{125}I over the A peak seemed to indicate that binding had occurred. However, no "hole" appeared following the peak, contrary to what would be expected in the event of binding. Hence, additional experiments were designed.

The equilibrating buffers used in Figures 14-B, 14-C, and 14-D contained α-LA-^{125}I (20 μg/ml), UDP-galactose, Mn^{++}, and Mg^{++} for 14-B; α-LA-^{125}I (0.25 μg/ml) and Mg^{++} for 14-C; and α-LA-^{125}I (0.25 μg/ml) alone in 14-D. Thus, 14-B had α-LA-^{125}I plus both metals and UDP-galactose, 14-C had α-LA-^{125}I plus Mg^{++} and minus substrates and Mn^{++}, while 14-D had only α-LA-^{125}I. Samples for each contained 50, 40, and 40 units of A-protein for 14-B, C, and D, respectively. The elution profiles in all three are nearly identical with that of 14-A, showing that the observed pattern was not influenced by the presence of substrates or metals.

Figure 14-E shows a control experiment in which equilibrating buffer contained only α-LA-^{125}I (1 μg/ml) and the sample contained

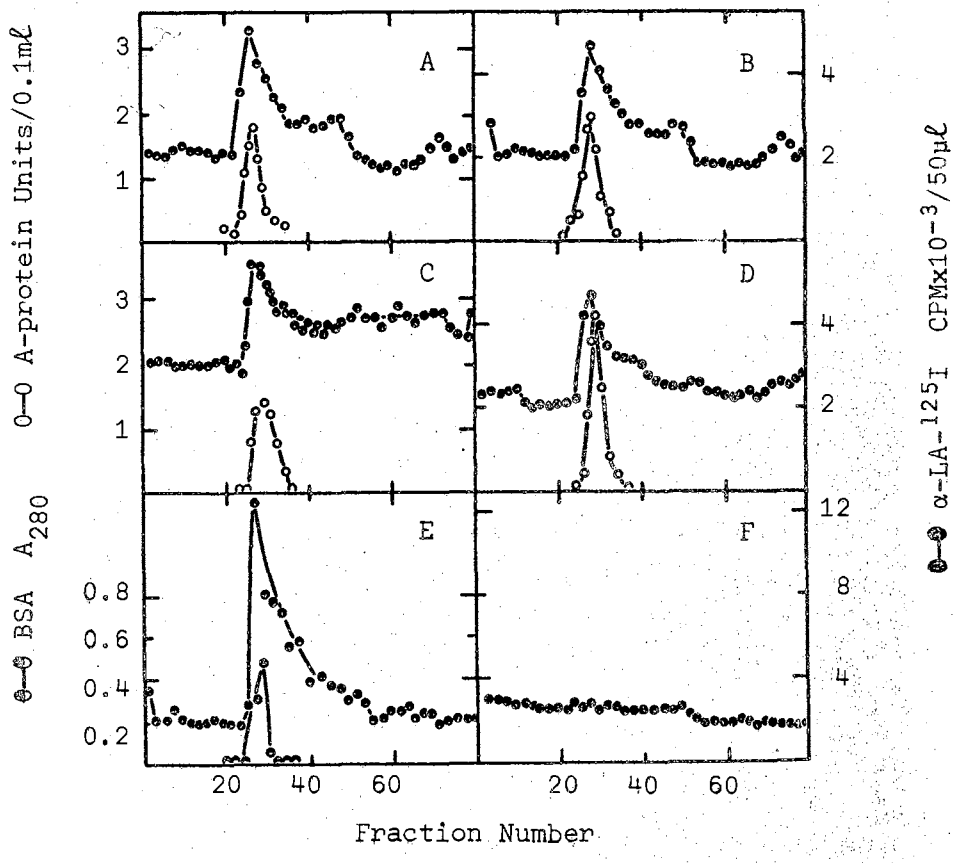


Figure 14. Hummel and Dreyer Type Binding Studies of A-Protein and α-LA-^{125}I on Bio-Gel P-30.

Bio-Gel P-30 columns (6 mm x 65 cm) were equilibrated and eluted with 20 mM Tris, pH 7.4, which also contained: (A) α-LA-^{125}I (0.15 μg/ml), 0.25 mM UDP-galactose, 20 mM glucose, 5 mM $MgCl_2$, 5 mM $MnCl_2$; (B) α-LA-^{125}I (20 μg/ml), 0.25 mM UDP-galactose, 5 mM $MgCl_2$, 5 mM $MnCl_2$; (C) α-LA-^{125}I (0.25 μg/ml), 5 mM $MgCl_2$, 5 mM $MnCl_2$; (D) α-LA-^{125}I (0.25 μg/ml); (E) α-LA-^{125}I (1 μg/ml); (F) α-LA-^{125}I (1 μg/ml). Samples (0.1 ml of equilibrating buffer) also contained 40, 50, 40, and 40 units of A-protein for A, B, C, and D, respectively; E contained 1 mg BSA; F was 0.1 ml 20 mM Tris alone.

additional bovine serum albumin (1 mg per 0.1 ml equilibrating buffer). Essentially the same elution profile was obtained. This was unexpected and therefore another experiment was done to check the system itself. Since a peak is always observed but never the "hole" after it, it was questioned whether or not the hole could be seen if present. To answer this question, the column was again equilibrated with α-LA-^{125}I (1 μg/ml) and a sample (0.1 ml) of buffer alone containing no α-LA-^{125}I was loaded and eluted. Clearly, this should produce a "hole" in the elution profile of α-LA-^{125}I. Figure 14-F shows these results. There was no "hole" observed, suggesting that if one were present in the other experiments it was not resolved in the elution profiles.

These results are best explained with the aid of an observation made while equilibrating the P-30 columns with α-LA-^{125}I. Although an equilibrium was reached in the column effluant, it was always lower than the α-LA-^{125}I level being applied to the column. This suggested that α-LA might be adsorbed in the column. This is particularly likely since only low ionic strength buffer was used in order to duplicate the assay conditions. The appearance of a peak in the elution profile might then be best explained if the A-protein caused a non-specific desorption of bound α-LA-^{125}I so that the α-LA-^{125}I removed from the column would elute as a tailed peak with the A-protein. This explanation seems attractive since bovine serum albumin gave nearly identical results to the A-protein and the appearance of the peak was not affected by the presence or absence of substrates or metals.

It seems highly unlikely that these results could then represent binding of α-LA-^{125}I to A-protein. It can be concluded that this

method conducted under conditions similar to those used in the assay is not suited for these association studies since it would be extremely difficult to distinguish a specific association from a non-specific interaction. However, future studies using a higher ionic strength might overcome this problem although conditions would not be the same as used in the assay.

Equilibrium Dialysis Studies

Equilibrium dialysis was briefly investigated as a potential method for demonstrating an A: α-LA complex. The success of this method depended on the availability of a dialysis membrane which would be permeable to α-LA but impermeable to A-protein. Dialysis tubing was unsuitable since it is routinely used for dialysis of α-LA for prolonged time periods with no detectable loss of α-LA. Several commercial ultrafiltration membranes were therefore investigated.

A dialysis cell (from Chemical Rubber Co.) with 1.0 ml capacity on each side of the membrane was used. Membranes were washed and soaked in buffer for 30 minutes to 2 hours prior to use. All experiments were done at room temperature. Appropriate aliquots were removed at different times from each side for assay.

Figure 15 shows the equilibrium dialysis of α-LA-^{125}I (15-A) and A-protein (15-B) through a Gelman Metricel type GA-10 membrane having a large pore size of 0.05 microns. Both experiments were done in 20 mM Tris, pH 7.4. In figure 15-A, side 1 of the dialysis cell contained 100 µg α-LA-^{125}I (30,000 cpm) per ml of buffer while side 2 contained only buffer. In figure 15-B, side 1 contained 130 units A-protein (exact A-protein concentration unknown but the A preparation

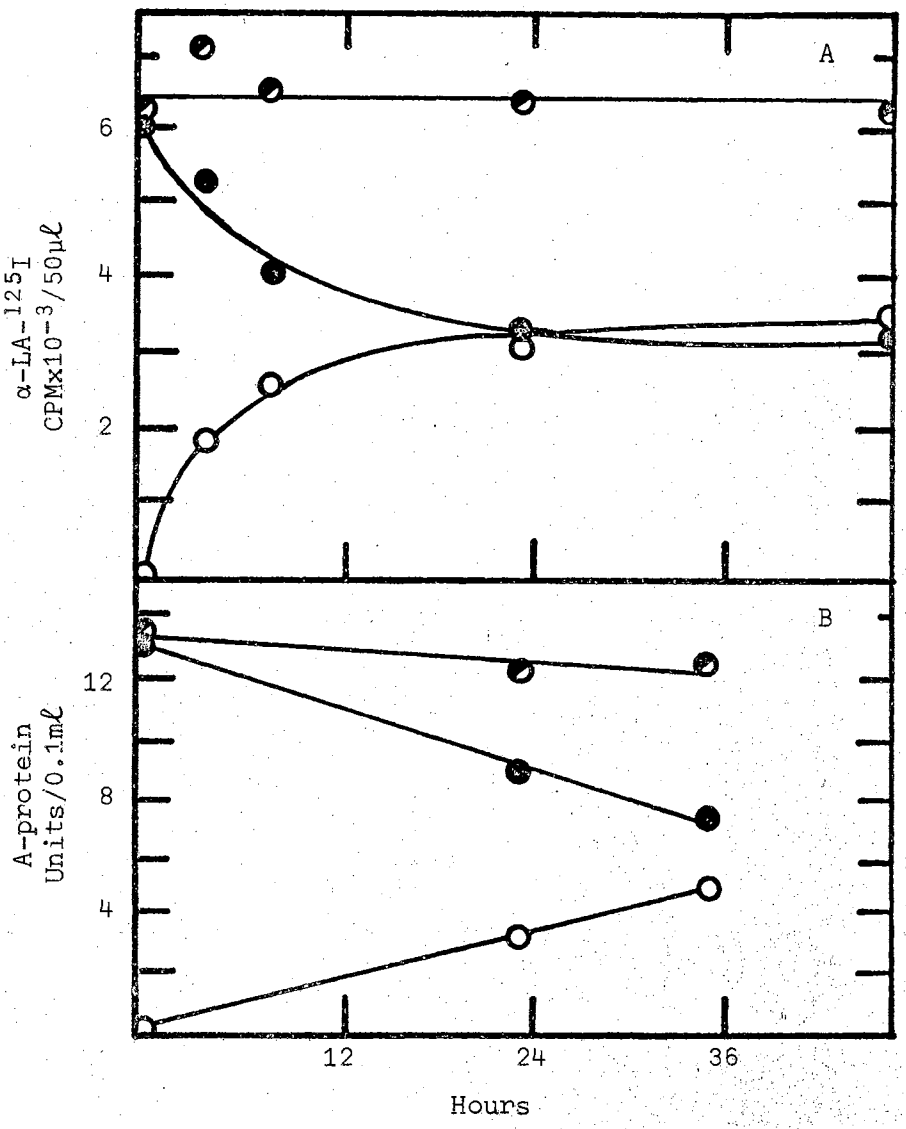


Figure 15. Equilibrium Dialysis of α-LA-^{125}I and A-Protein With a Membrane of 0.05 Micron Pore Size.

The dialysis membrane was a Gelman Metrical GA-10 with 0.05 micron pore size. Aliquots were removed from each side as indicated. Dialysis was at 23°.
A: Side 1 (●) contained 100 μg α-LA-^{125}I (30,000 CPM) /ml.
B: Side 1 (●) contained 130 units A-protein.
Side 2 (○) contained only buffer in both A and B. The sum of side 1 and side 2 (◐) is also shown.

used had a high total protein concentration and also contained about 30% ammonium sulfate) while side 2 contained only buffer. Each of the figures also shows the sum of sides 1 and 2. Clearly this membrane could not be used since it is readily permeable to both proteins.

Membranes having a smaller pore size were investigated. Results of dialysis of the A-protein using a Sartorius No. S11733 cellulose acetate membrane having a pore size range of 0.01-0.02 microns are shown in Figure 16. Side 1 contained 260 units A-protein in 1.0 ml 20 mM Tris, pH 7.4, 5 mM Mg^{++}. Side 2 contained only 1.0 ml of buffer. The zero time assay on side 1 was lost, however it is clear that the A-protein was lost from side 1 with time. This could not be due to loss in A-activity (unless due to some property of the membrane) since the A-protein lost only 4% of its activity under identical conditions in a separate experiment on the stability of the A-protein. Since no A-protein appeared in side 2, the membrane is impermeable to the A. The A-protein was probably adsorbed by the membrane resulting in the loss in activity. This membrane was therefore not suitable.

The only other dialysis membrane investigated was a Gelman metrical type PEM with pore size of 75 Å (Figure 17). Side 1 contained 2.5 µg α-LA-^{125}I in buffer, Side 2 contained only buffer. The very low α-LA-^{125}I concentration of 2.5 µg/ml was chosen so as to maximize the relative change in α-LA concentration upon binding to an excess of A-protein in later experiments if this membrane was suitable. Again, the membrane appeared to absorb α-LA-^{125}I from side 1 and was impermeable to α-LA-^{125}I since there was virtually no increase in its concentration in side 2 during the 18 hours of incubation.

Further attempts to study the A: α-LA association using

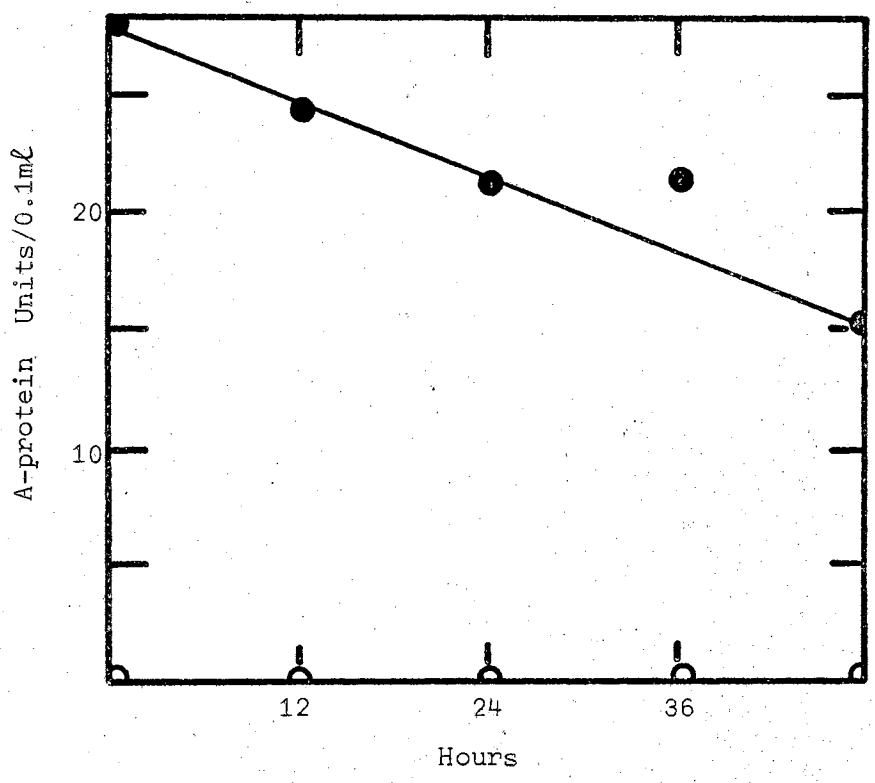


Figure 16. Equilibrium Dialysis of A-Protein Through a Cellulose Acetate Membrane.

●, Side 1 contained 260 units A-protein in 1.0 ml of 20 mM Tris, pH 7.4. ○, Side 2 contained 1.0 ml of 20 mM Tris, pH 7.4. A Sartorius Cellulose Acetate membrane with a 0.01 - 0.02 micron pore size was used. Aliquots of 0.1 ml were assayed. Dialysis was at 23°.

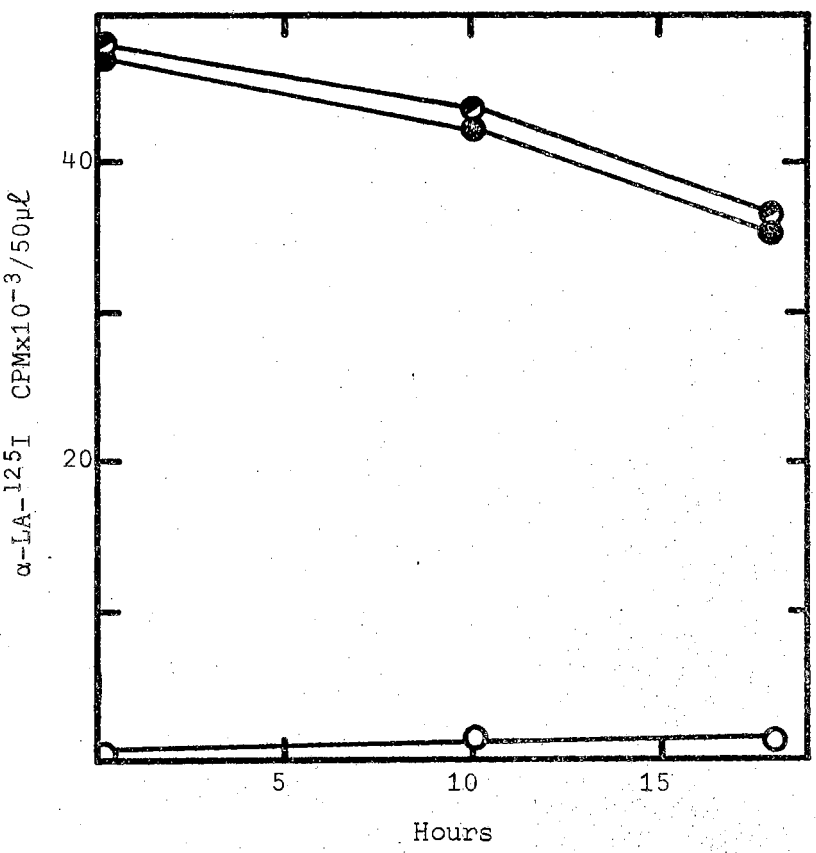


Figure 17. Equilibrium Dialysis of α-LA-^{125}I Through A Membrane With A 75 Å Pore Size.

●, Side 1 contained 2.5 μg α-LA-^{125}I (2μc)/1.0 ml 20 mM Tris, pH 7.4. ○, Side 2 contained 1.0 ml 20 mM Tris, pH 7.4. ⊕, Sum of Side 1 and Side 2. A Gelman Metrical Type PEM membrane with a 75 Å pore size was used. Aliquots (50 μℓ) were removed from each side at the time intervals shown and counted. Temperature was 23°.

equilibrium dialysis were abandoned for lack of a suitably selective dialysis membrane.

Fluorescence Studies

The only remaining method used to study the interaction of A-protein with α-lactalbumin was fluorimetry. The procedure was as previously described in the methods section earlier.

The changes in the fluorescence spectrum of α-LA upon pH and temperature perturbation have been reported by Kronman (24). Significant changes in both intensity and λmax of the emission spectrum occurs with decrease in pH or temperature. It therefore seemed likely that the fluorescence spectrum of α-LA might also be influenced by the substrates for lactose synthetase, UDP-galactose or glucose or both, if they bind to α-LA. Another possibility would seem to be that one might see a change in the spectrum of α-LA upon its association with the A-protein.

The excitation and emission spectrum of α-LA (10 μg/ml) in 50 mM glycylglycine, pH 8.0, is shown in Figure 18. The excitation and emission λmax are 287 and 342 mμ, respectively. This is in good agreement with the values of Kronman (24). The λmax for tryptophan and lysozyme fluorescence excitation and emission spectra under these same conditions were 282 and 362 (tryptophan) and 288 and 353 (lysozyme) respectively, and are reported in Table II for comparative purposes. Except for the λmax for each of the spectra, they are indistinguishable in general shape.

The assessment of the influence of substrates on the fluorescence spectrum of α-LA is summarized in Table III. The values reported

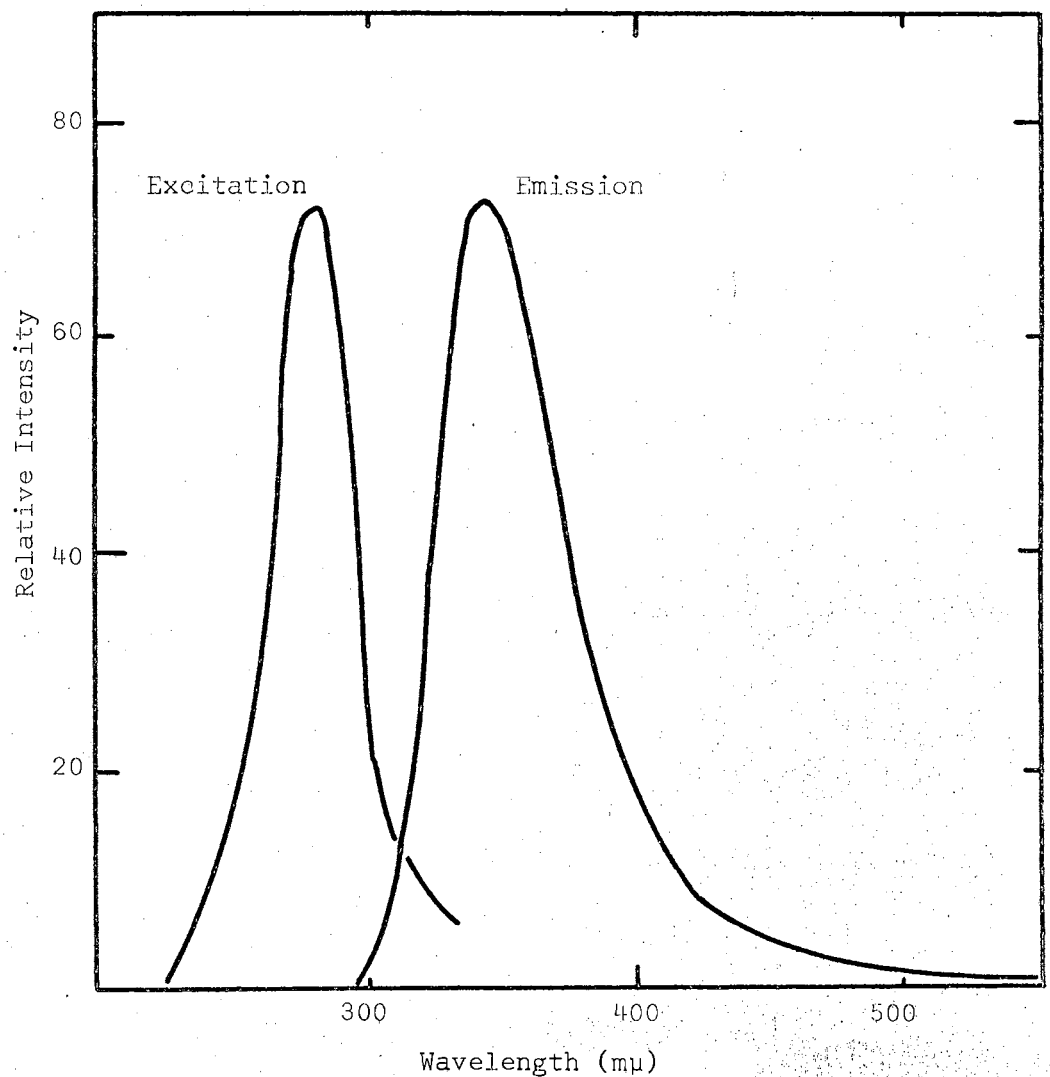


Figure 18. Fluorescence Excitation and Emission Spectrum of α-LA.

A solution containing 10 μg α-LA/1.0 ml 50 mM glycylglycine, pH 8.0, was used for the fluorescence spectra. The excitation spectrum was obtained with the emission wavelength set at 342 mμ and the emission spectrum was obtained with the excitation wavelength set at 287 mμ.

TABLE II

FLUORESCENCE EXCITATION AND EMISSION MAXIMA FOR TRYPTOPHAN, LYSOZYME, AND α-LA

Compound	Excitation λmax	Emission λmax
Tryptophan	282	362
Lysozyme	288	353
α-LA	287	342

TABLE III

EFFECT OF GLUCOSE AND UDP-GALACTOSE ON THE INTENSITY OF THE α-LA EMISSION SPECTRUM

Cuvette Contents	Relative Intensity*
α-LA	73
α-LA + 20 mM Glucose	74
α-LA + 0.25 mM UDP-galactose	63
α-LA + UDP-galactose + glucose	62

* Excitation at 286 mμ, emission at 345 mμ

are the relative intensities for the emission spectra of α-LA (10μg/ml) in 50 mM glycylglycine. The same concentration of glucose (20 mM) was added to the cuvette as is used in the lactose synthetase spectrophotometric assay with no effect on the intensity or λmax of the spectrum. Other experiments confirmed the lack of influence of glucose on the α-LA emission spectrum at concentrations up to 0.1 M. The addition of UDP-galactose (0.25 mM) to the cuvette containing α-LA caused a marked quench in the α-LA fluorescence. This at first suggested binding of UDP-galactose to α-LA. Additional experiments showed the quench to be proportional to the amount of UDP-galactose added to the α-LA. However, this quenching could be duplicated by the addition of several other compounds such as UDP, UMP, uracil, GDP, CDP, cytidine, and thymidine to the cuvette containing α-LA. Also UDP-galactose, UDP, UMP, or uracil caused a marked quench in the fluorescence of both lysozyme and tryptophan as well as α-lactalbumin. These results indicated that the quenching of the α-LA fluorescence spectrum by UDP-galactose (or other purine or pyrimidine containing compounds) was due not to a specific binding of the UDP-galactose to α-LA but instead due to internal absorption of the excitation light beam to produce quenching.

The quenching of α-LA fluorescence by UDP-galactose was unchanged by glucose when both UDP-galactose and glucose were added (Table III). Thus there is no effect of the combined or separate substrates on α-LA beyond the primary internal quenching by UDP-galactose.

Attempts to obtain fluorescence spectra of the A-protein were unsuccessful due to a high amount of fluorescence in the A-protein preparation. During isolation and purification of the A-protein it

was noticed that the A-protein seemed to have an unusually low extinction coefficient at 280 mμ which often made it necessary to use A_{220} readings to monitor purification columns. Since UV fluorescence in most proteins is due to their aromatic amino acid content, it seemed doubtful that the high fluorescence shown by A-protein preparations could arise from a protein having an apparently low extinction coefficient at 280 mμ. It seemed more likely that the fluorescence is due to some contaminant in the protein preparation.

Dansylation of α-Lactalbumin

Another attempt to use fluorescence to study the interaction of lactose synthetase components with α-lactalbumin was the use of dansylated α-lactalbumin. The dansyl group is a fluorescent reporter molecule which attaches to the ε-NH_2 of lysine in most proteins. The dansylated protein then shows a dansyl fluorescence spectrum which is often sensitive to conformational changes in the protein (28).

The preparation of dansylated α-lactalbumin was carried out in the cold according to the general procedure of Horton and Koshland (28). A solution of α-LA (10 mg/ 4.0 ml H_2O) was adjusted to pH 8.5 with NaOH; 0.5 ml of 1.0 M $NaHCO_3$, pH 8.5, was added followed by 0.25 ml H_2O for a final volume of 4.75 ml. A 0.25 ml aliquot of a solution of dansylchloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) in acetone (2.0 mg/ml) was added rapidly and stirred vigorously to produce a fine suspension of dansylchloride. The solution was allowed to stir for approximately 15 hours in the cold. The reaction mixture was centrifuged in a clinical centrifuge for 20 minutes at maximum speed to remove unreacted dansyl chloride. The supernatant liquid

was then evaporated to dryness on a rotary evaporator. The protein precipitate adhering to the walls of the tube was extracted three times each with 2 ml acetone while in a dry ice-acetone bath to remove non-covalently bound dansyl. The dansylated α-LA was redissolved in 1.0 ml 20 mM Tris, pH 7.4, and chromatographed on a Bio-Gel P-30 column (1.2 cm x 47 cm). Dansylated α-LA was located by A_{340} reading as was the unreacted free dansyl salt peak; α-LA was located by A_{280} reading of 0.8 ml fractions. The P-30 elution profile showed two adjacent α-LA protein peaks, both containing dansyl groups. Using extinction coefficients of 3.3 A_{340}/mM/cm for the dansyl group (28) and 2.0 A_{280}/mg/ml for α-LA, the extent of dansylation of α-LA in each of the two protein peaks was calculated as 0.053 and 0.018 moles dansyl per mole of α-LA for the first and second peaks, respectively. The elution position of the second peak was the same as for native α-LA, however the first peak eluted as a higher molecular weight species of α-LA. The first peak was inactive in the lactose synthetase assay while the second peak retained activity. A separate experiment showed that the aggregated α-LA in the first peak was not due to the acetone extraction procedure nor to reaction conditions in the absence of dansylchloride.

The fluorescence spectrum of dansylated α-LA (50 µg/ml 20 mM Tris, pH 7.4) is shown in Figure 19. The intensity of the fluorescence emission spectrum of dansylated α-LA (50 µg/ml) was unchanged after the addition of A-protein (approximately 100 units), or A-protein + UDP-galactose, glucose, and Mn^{++} at assay concentrations. Thus, since there was no perceptible influence of the A-protein or substrates on the fluorescence of dansylated α-LA, there was

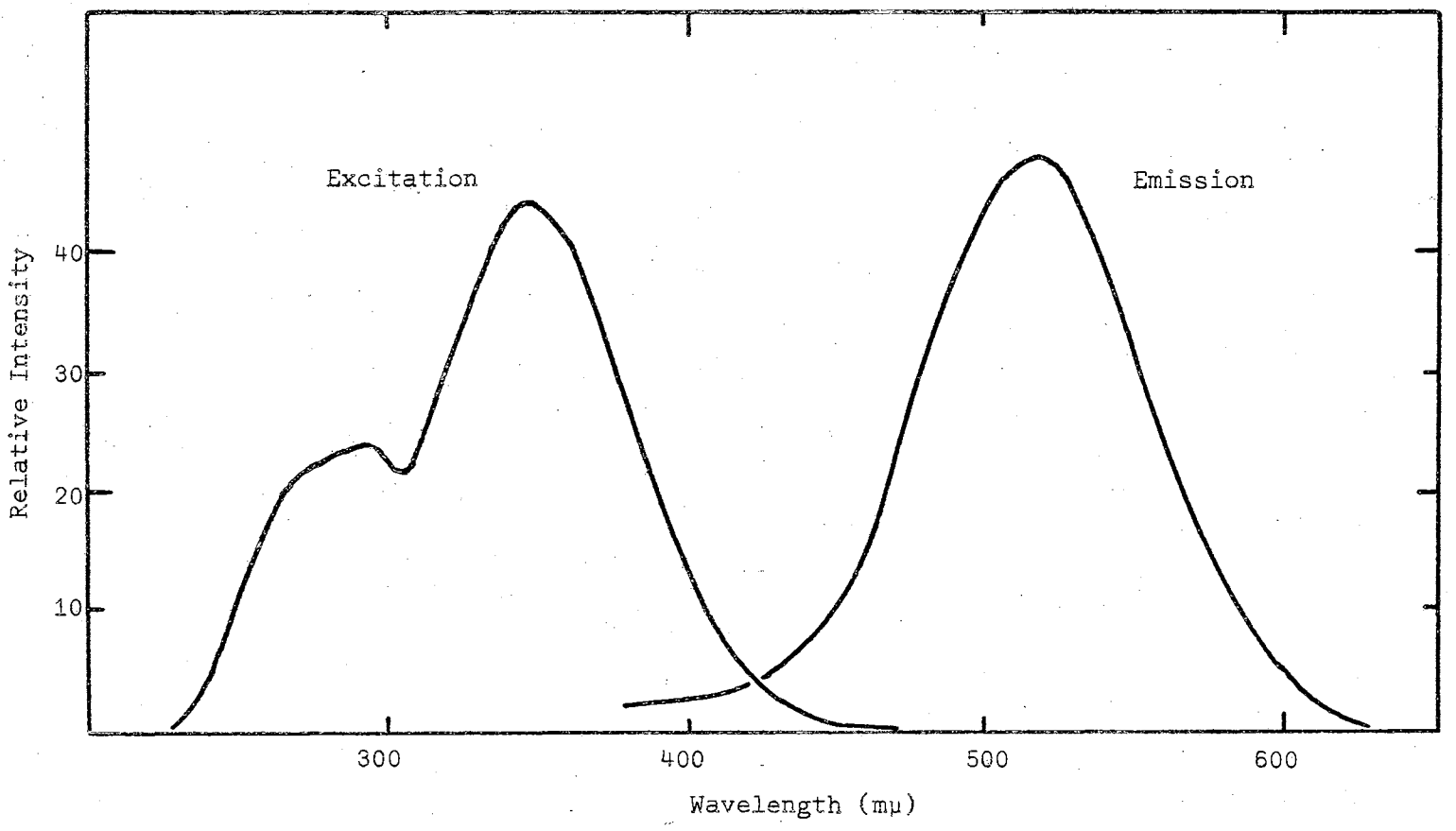


Figure 19. Fluorescence Excitation and Emission Spectrum of Dansylated α-LA.

A solution containing 50 μg dansylated α-LA/1.0 ml 50 mM glycylglycine, pH 8.0, was used for the fluorescence spectra. For the excitation spectrum, the emission wavelength was set at 515 mμ. For the emission spectrum, the excitation wavelength was 346 mμ.

apparently no strong interaction of the components with α-LA, providing the dansyl group was in a region of α-LA which was environmentally or conformationally sensitive and dansylation did not affect activity.

There was no detectable complex formed between α-LA and the A-protein by any of the above described methods. From the kinetic data of Ebner, et. al. (10), it is clear that a physical interaction is required for maximum lactose synthetase activity. The inability to demonstrate an active complex would most likely be due to the equilibrium constant and rate constant of the association-dissociation reaction. If the association constant were sufficiently low, an insufficient amount of complex would be formed for detection by any of the above methods. Even if the association constant were relatively high, a high rate constant would allow rapid association-dissociation to occur during transport, making detection using transport experiments unlikely.

It is worthy to note that, of the naturally occurring subunit enzymes for which a subunit complex has been shown, very high association constants of 10^7 to 10^8 were found (7, 12,10). The case of glutamate mutase (85) also gives precedence to the inability to demonstrate a physical complex between two subunits previously shown to interact in kinetic studies.

Other techniques such as analytical ultracentrifugation are available which may demonstrate a rapidly equilibrating complex between α-LA and A-protein but these would require large quantities of both proteins. Since the A-protein is available only in small quantities at present, these methods are now infeasible but may deserve investigation in the future if large quantities of A-protein should

become available. Under the present limitation of small quantities of A-protein, the available methods were duly investigated with negative results.

CHAPTER IV

STUDIES ON THE CARBOHYDRATE ACCEPTOR SPECIFICITY OF THE GALACTOSYL TRANSFERASE ACTIVITY OF THE LACTOSE SYNTHETASE A-PROTEIN

Experimental Procedure

Materials and Reagents

Glucosamine, melibiose, gentiobiose, cellobiose, β-methyl-D-glucoside, N-acetylmannosamine, N-acetylmuramic acid (NAM), and indoxyl-β-D-glucoside, were purchased from Sigma. Maltose was obtained from Fischer Scientific Co. Mannose, L-fucose, α-methyl-D-glucoside, 2-deoxy-D-glucose and ovalbumin (2x crystallized) were purchased from Mann Biochemicals. Cellobiulose and glucosyl-(β-1,4)-mannose were obtained chromatographically pure as the generous gifts of Dr. J. M. Leatherwood of the Dept. of Animal Science, North Carolina State University, Raleigh, N. C. NAG-NAM was a generous gift from Dr. Nathan Sharon, Weisman Institute, Rehoveth, Israel. The NAG oligomers were gifts from Dr. Patrick Guire of this department. All other chemicals

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Abbreviations: NAG = N-acetyl-D-glucosamine; diNAG = 4-O-β-N-acetyl-D-glucosaminyl-N-acetyl-D-glucosamine(chitobiose); triNAG = chitotriose; tetraNAG = chitotetraose; NAM = N-acetylnuramic acid; NAG-NAM = N-acetylglucosaminyl-(β-1,4)-N-acetylmuramic acid; NAL = N-acetyllactosamine. All sugars are of the D configuration unless otherwise specified.

used were of reagent quality.

Spectrophotometric Assay for A-protein NAL Synthetase Activity

Spectrophotometric assays for NAL synthetase activity were modified from that used for the lactose synthetase assay in Chapter III. KC1(0.1M) in the spectrophotometric assay gave slightly higher assay activities and made the assay much more reproducible and reliable. Hence, all assays used in the NAL synthetase studies reported in Chapter IV used 0.1 M KC1 in the assay. Each 1.0 ml assay contained 0.4 mM UDP-galactose, 0.15 mM NADH, 1.0 mM PEP, 0.05 ml of a 1 to 10 dilution of pyruvate kinase (as described in Chapter III), 50 mM glycylglycine, pH 8.5, 5 mM $MnCl_2$, and 0.1 m KC1 plus 20 mM NAG. For kinetic studies, NAG was varied over the desired concentration range. Any desired α-lactalbumin was added at the needed concentrations. Normally 18 units of lyophylized A-protein were added to each assay.

Spectrophotometric Assay for Acceptor Specificity of the A-Protein Galactosyl Transferase Activity

All assays for A-protein galactosyl transferase acceptor specificity were identical to that used for the NAL synthetase assay described above with the exception of NAG. The acceptor was substituted for NAG at the desired concentration, as was α-lactalbumin. The A-protein was from a large preparation of highly purified lypholized A-protein from the HA_{II} step (114) of purification. The amount of A-protein normally used in an assay was 18 units/ml.

Results

NAG and cellobiose had 25% and 7.5% the activity of glucose,

respectively, with early studies of lactose synthetase (88). Brew, et. al. (3) implicated the A-protein in glycoprotein biosynthesis with the report that NAG and orosomucoid are acceptors. α-LA only partially inhibited A-protein activity with these acceptors.

Several other galactosyl transferases are reported in the literature and some have specificity for a number of acceptors. The most interesting are those described Iyer and Carlson (111), McGuire, et.al. (91), Watkins (107), and Spiro and Spiro (123). In all cases, a wide variety of acceptors were tested. The acceptor specificities of these transferases were similar and since all of these need free NAG as a substrate they appear similar to the lactose synthetase A-protein. However, the concentration of free NAG in tissues is probably not high enough to allow it to be an effective acceptor with these transferases (123), hence it appears unlikely that this reaction is biologically significant. It was therefore of interest to further investigate the acceptor specificity of the galactosyl transferase activity of the lactose synthetase A-protein to better define the biological role of the A-protein *in vivo*.

Acceptor Specificity Studies

The linearity of the rate of the NAL synthetase assay with increasing A-protein concentration is shown in Figure 20. The assay was linear to at least 16 mμmoles UDP/min/ml (0.1 Δ A/min) and most times to 20 mμmoles/min/ml or higher.

The inhibition of the NAL synthetase activity by various levels of α-LA is shown in Figure 21 for both 10 and 20 mM NAG. A maximum inhibition of 82% at 100 μg α-LA/ml was observed with both 10 and

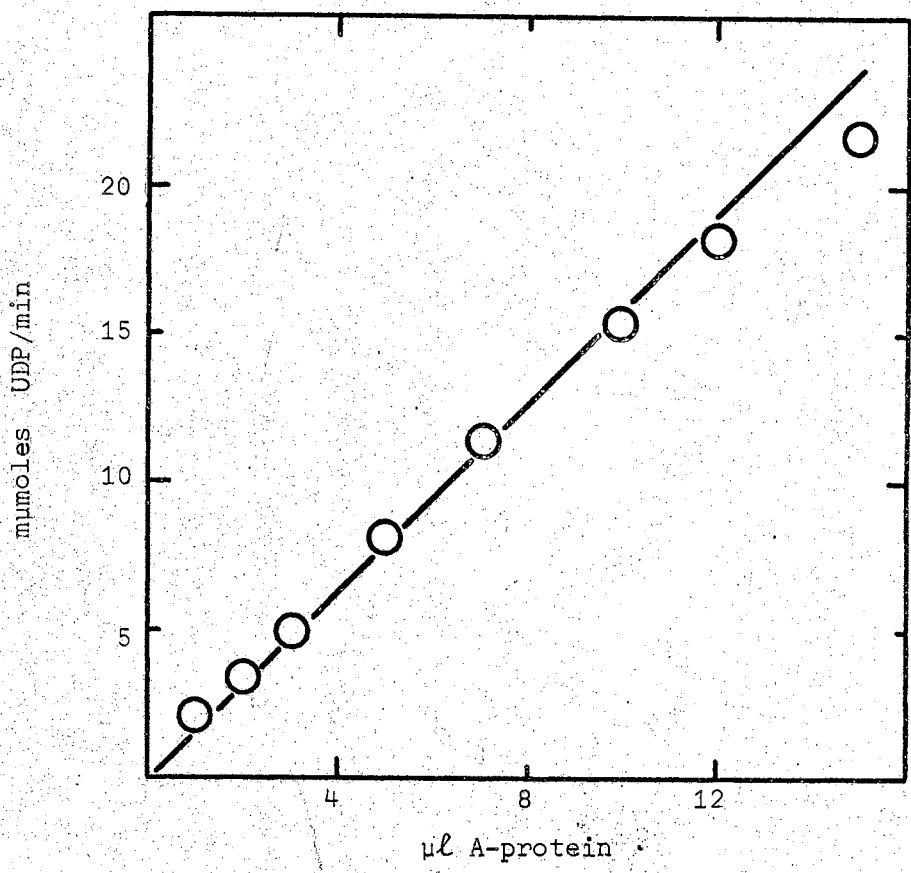

Figure 20. Linearity of Spectrophotometric Assay for NAG Activity With Increasing A-Protein Concentration.

Assay conditions: a stock solution of lyophylized A-protein (3 mg/ml 50 mM glycylglycine, pH 8.5) was used for the enzyme. In addition to the desired amount of A-protein, a 1.0 ml reaction mixture contained: 20 mM NAG, 0.4 mM UDP-galactose, 5 mM $MnCl_2$, 0.1 M KCl, and 50 mM glycylglycine, pH 8.5, plus the coupling system.

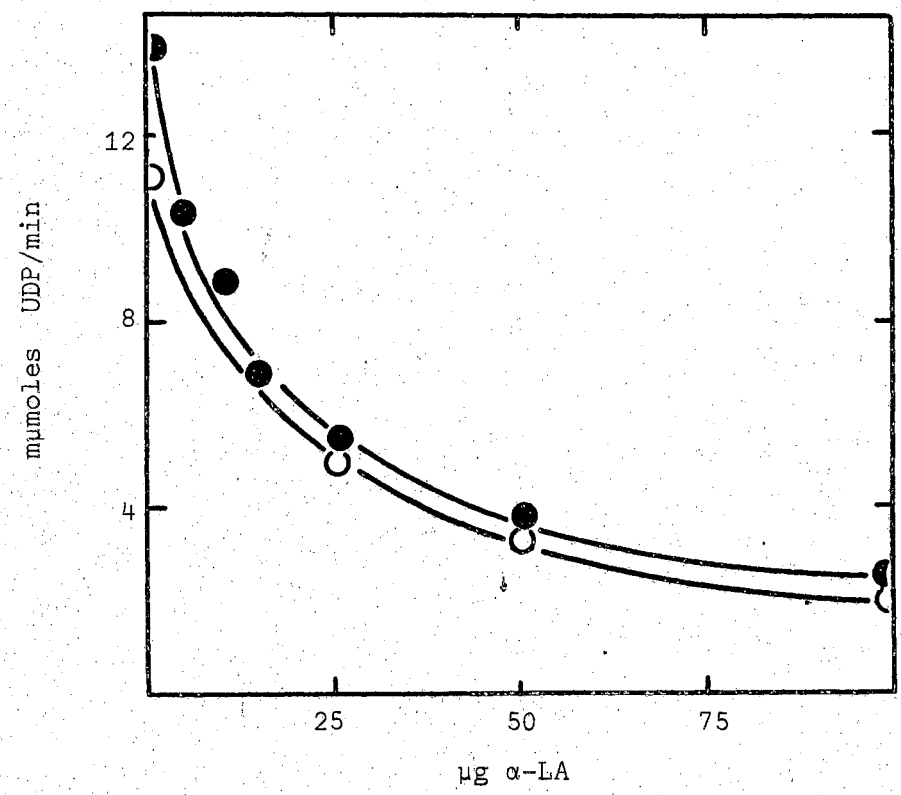

Figure 21. Inhibition of A-Protein NAG Activity by α-LA.

Lyophylized A-protein (14 units) was assayed for inhibition of NAG activity by increasing concentrations of α-LA at both 10 mM (O) and 20 mM (●) NAG in the standard assay.

20 mM NAG.

A reciprocal plot of rate versus mM NAG concentration over a 50-fold concentration range is shown in Figure 22. The K_m for NAG obtained from this plot was approximately 10 mM. A distinguishing feature of Figure 22 is the pronounced change in the slope of the reciprocal plot. The segments of the line before and after the slope change appear linear. The shape of the velocity vs substrate plot showed no sigmoidal character at these low NAG concentrations, and appeared to be a rectangular hyperbola as expected. A similar change in slope was observed in a number of other NAG reciprocal plots. This result suggests the possibility of more than one A-protein NAG site being involved in this reaction. However, an equally likely second possibility is that an inherent property of the coupling system gives lower rates than expected at very low substrate concentrations. The data from a number of experiments could not distinguish between the two possibilities at this point. Further investigation into this aspect of the kinetic studies would seem worthwhile.

The effect of α-lactalbumin on the A-protein K_m and V_{max} values for NAG is shown in Figure 23. The NAG K_m was 8.3 mM in the absence of α-LA, somewhat lower than the K_m reported in the previous figure. The addition of 25 µg α-LA/ml to the assay lowered the K_m for NAG to 1.7 mM. The V_{max} of the reaction was also lowered by the addition of 25 µg α-LA/ml, going from 29 mµmoles with no α-LA to 8.3 mµmoles/min with 25 µg/LA. The reciprocal plots for NAG activity in the presence and absence of α-LA intersect not to the left of the abscissa as in most inhibitor studies but instead interect to the right of the abscissa. Thus α-LA is an inhibitor of the NAL synthetase reaction at

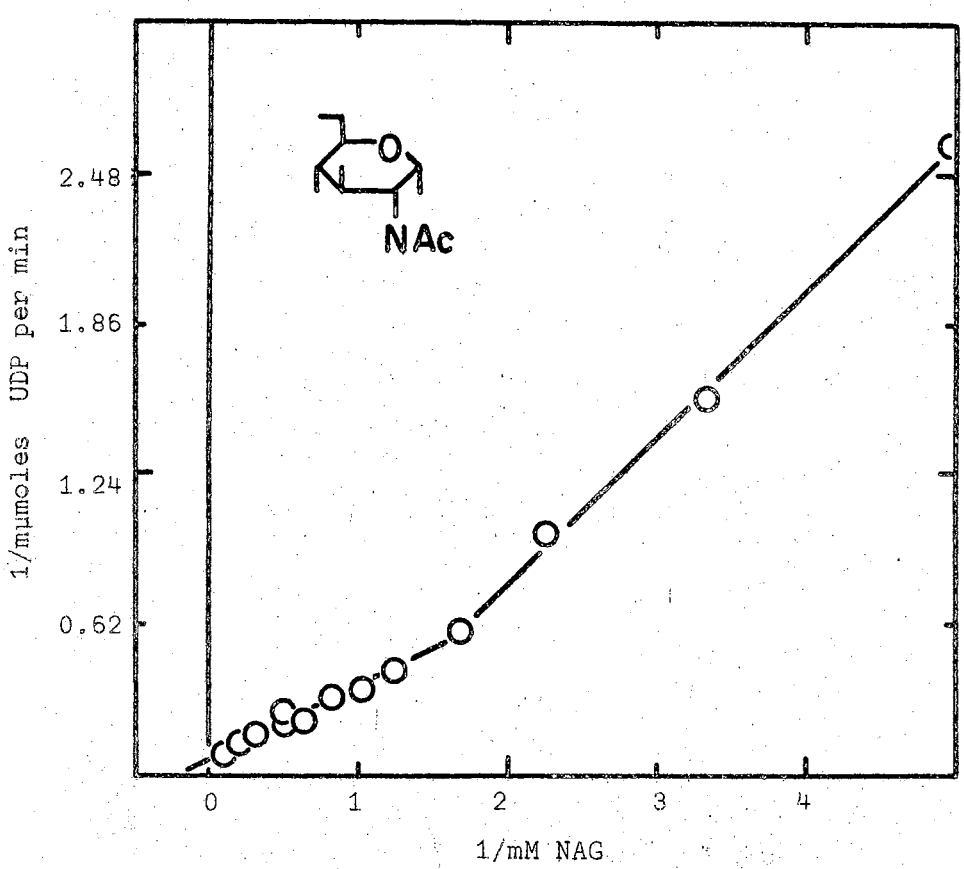

Figure 22. Lineweaver-Burk Plot of A-Protein NAG Activity Over a 50-Fold NAG Concentration Range.

Eighteen units of A-protein were used in the standard assay. The K_m value for NAG = 10 mM.

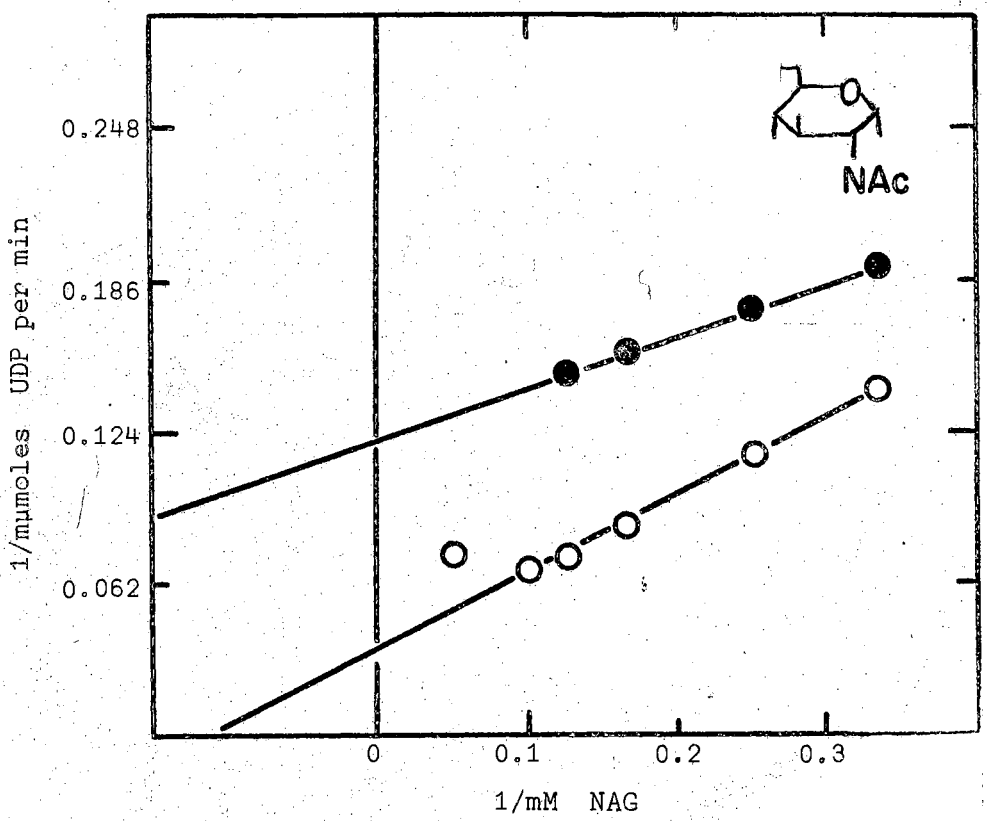

Figure 23. Lineweaver-Burk Plot of Inhibition of A-Protein NAG Activity by 25 μg α-LA/ml.

Eighteen units of A-protein were used in the standard assay with (○) no α-LA and (●) 25 μg α-LA/ml. The K_m value for NAG was 8.3 mM in the absence and 1.7 mM in the presence of 25 μg α-LA/ml.

NAG concentrations above the point of intersection (about 1.3 mM) but is an activator of the reaction at concentrations below this NAG level. The results of other experiments showed the point of intersection to vary but was in all cases well to the right of the abscissa. Indeed, Hill (3) reported that α-LA was an inhibitor of the NAL synthetase reaction at NAG concentrations above 3 mM but was an activator at lower concentrations, however, reciprocal plots were not made.

A more extensive study of the effect of α-LA on the NAL synthetase reaction is shown in the Lineweaver-Burke plot of Figure 24. At α-LA levels of 0, 10, 25, and 50 µg/ml the observed NAG K_m's were 12.5, 2.7, 1.2, and 1.0 mM, respectively. Thus, increasing α-LA levels lowered the NAG K_m but also lowered the V_{max} of the reaction, giving inhibition. As in Figure 23, extrapolation of the plots at 0 and 25 µg α-LA showed them to intersect well to the right of the abscissa but at a slightly lower NAG concentration than before. It should be pointed out that the plots with 0, 10, and 25 µg of α-LA do not quite intersect at a common point while the 50 µg plot intersects the zero α-LA plot at a point well beyond the others. It cannot be conclusively determined whether or not a common point of intersection exists. However, the presence of a common intersection point would mean that all levels of α-LA are inhibitory at NAG concentrations above the point of intersection while all levels of α-LA would be activating at NAG concentrations below the point of intersection and might provide insight into the control of NAL synthesis by α-LA. Secondary plots of V_m versus α-LA and K_m versus α-LA were nonlinear. A tertiary plot of 1/Vx-Vo versus 1/α-LA was linear, indicating that the function describing the decrease in V_{max} with increasing α-LA was a rectangular

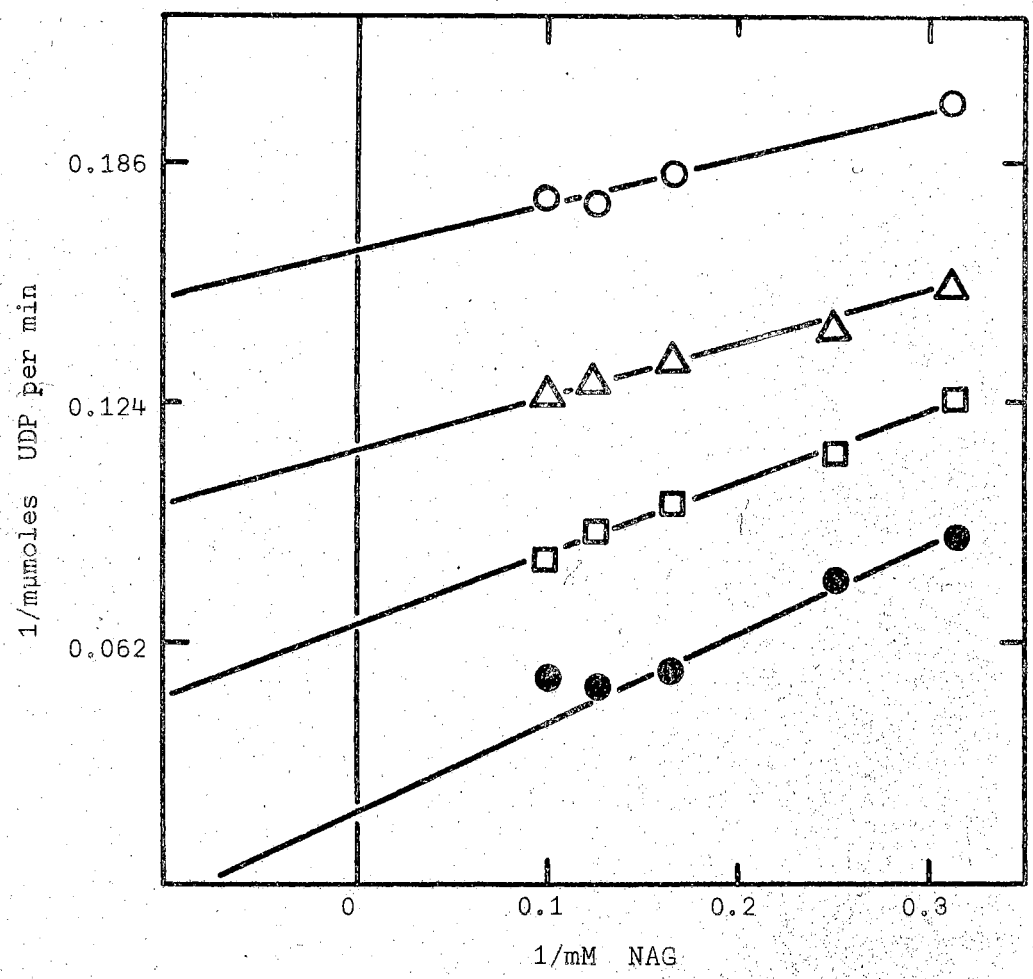

Figure 24. Lineweaver-Burk Plot of Inhibition of A-Protein NAG Activity by 0, 10, 25, and 50 μg α-LA/ml.

Eighteen units of A-protein were used in the standard assay with (●) no α-LA; □, 10 μg; Δ, 25 μg; ○, 50 μg α-LA/ml.

hyperbola.

The availability of NAG oligomers, all β-1,4 linked, prompted an investigation of the specificity of the NAL synthetase reaction as catalyzed by the A-protein. NAG dimer (diNAG) was first investigated as an acceptor. The results of these studies are depicted in the reciprocal plot of Figure 25. The diNAG K_m study was done both in the absence and in the presence of 25 µg α-LA/ml. The K_m's in the presence and absence of α-LA were the same (0.63 mM) while the V_{max} was only slightly decreased from 132 mµmoles UDP/minute to 121 mµmoles/minute. The net result is that the galactosyl transferase reaction using diNAG as an acceptor is virtually unaffected by 25 µg α-LA/ml. Thus the galactosyl transferase activity of the A-protein might not be subject to control by α-LA with certain acceptors.

This conclusion received further support from the studies using triNAG as an acceptor, as shown in Figure 26. The studies were again done in the presence and absence of 25 µg α-LA/ml. The K_m's of the A-protein for triNAG were 1.0 and 1.13 mM in the presence and absence of 25 µg α-LA/ml, showing virtually no change upon the addition of α-LA. The V_{max}'s for the reaction with triNAG were 17.7 mµmoles/min in the absence of α-LA and 19.8 mµmoles/min in the presence of α-LA.

To test the possibility that higher levels of α-LA might have a greater influence on the ability of triNAG to serve as an acceptor with the A-protein, various levels of α-LA up to 1 mg/ml were tested in a normal assay having 2.5 mM triNAG as the acceptor. TriNAG was chosen as an acceptor since it seemed most likely to be free from any NAG contamination based on examination of the triNAG purification data. Results of these studies (Figure 27) showed that the activity with

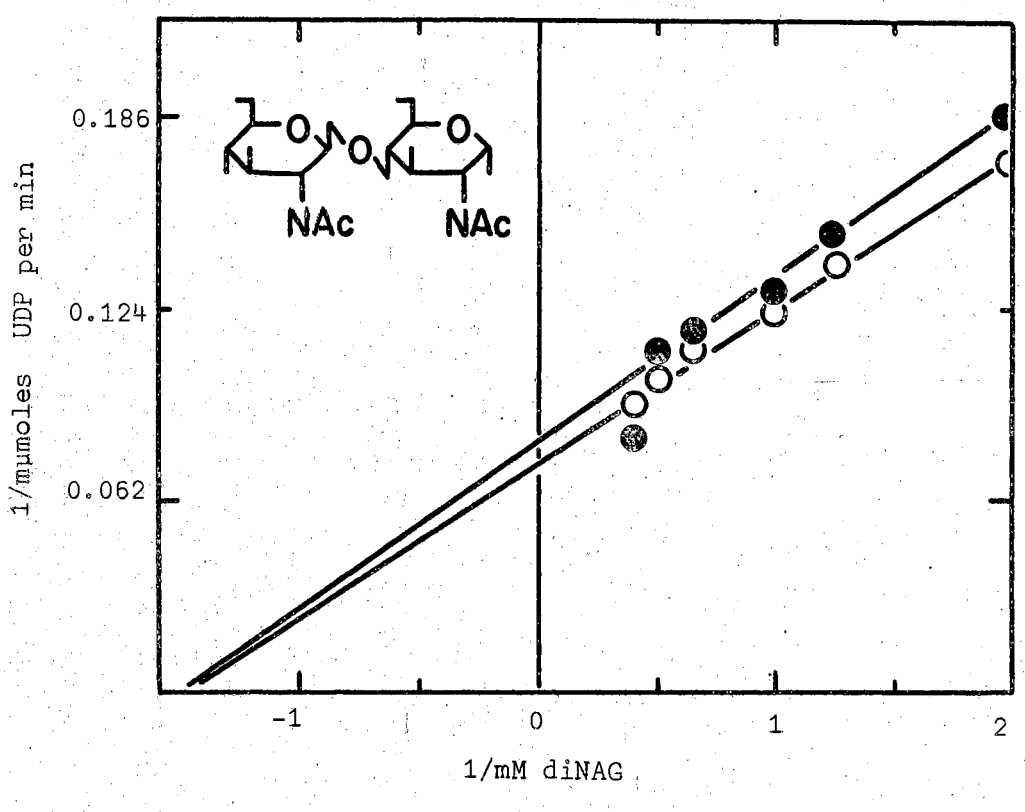

Figure 25. Lineweaver-Burk Plot of A-Protein diNAG Acceptor Activity ± α-LA.

○, No α-LA; ●, 25 μg α-LA/ml. Eighteen units of A-protein were used in the standard assay. The diNAG K_m value was 0.63 mM.

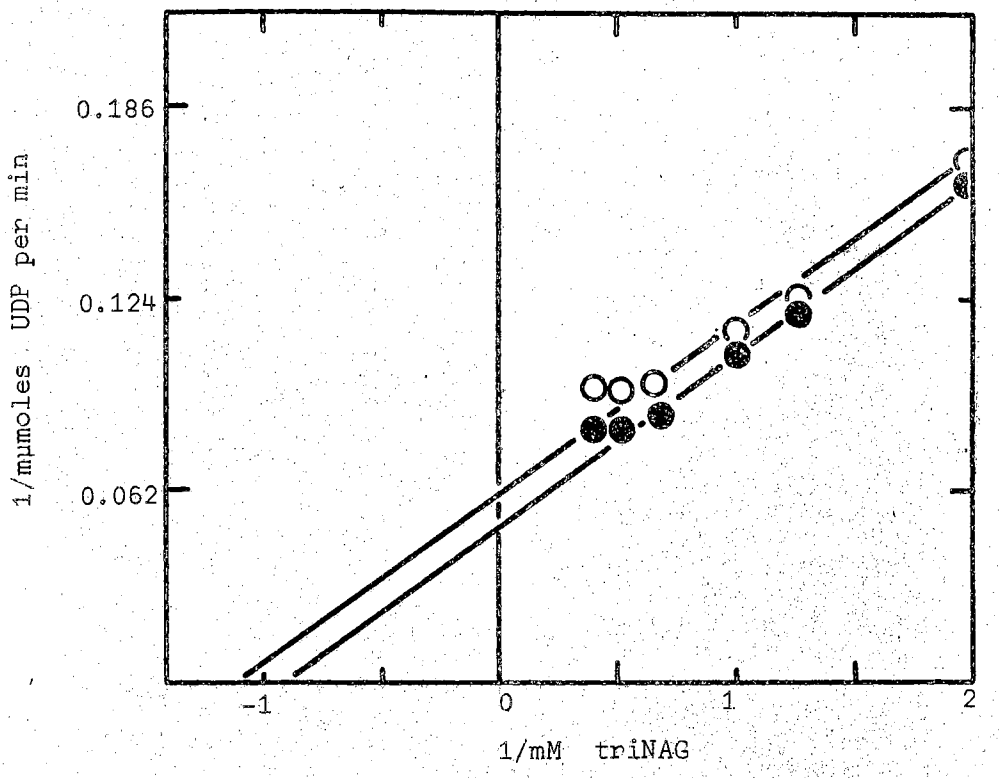

Figure 26. Lineweaver-Burk Plot of A-Protein triNAG Acceptor Activity ± α-LA.

○, no α-LA; ●, 25 μg α-LA/ml. Each assay contained eighteen units A-protein. The K_m value for triNAG was 0.9 mM in the absence and 1.11mM in the presence of 25 μg α-LA/ml.

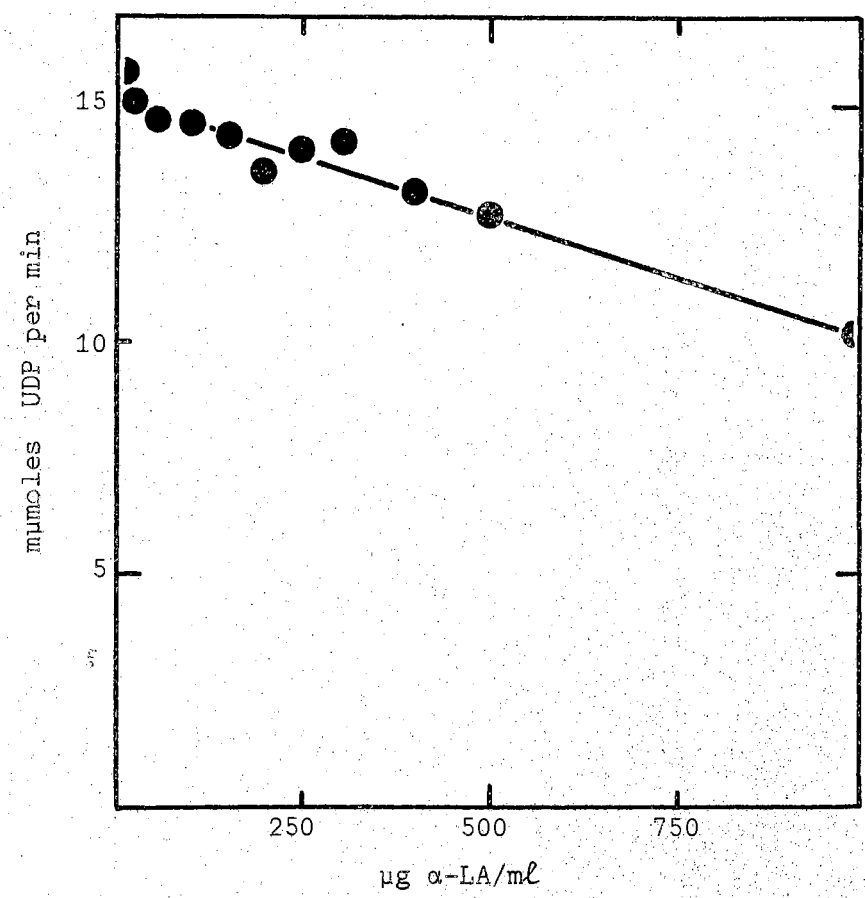

Figure 27. Inhibition of A-Protein triNAG Activity by Increasing α-LA Concentrations.

Assays contained 18 units A-protein per mℓ and 2.5 mM triNAG under standard assay conditions.

2.5 mM triNAG was inhibited only 32% with 1 mg α-LA/ml. The decrease in rate was linear over α-LA concentrations from zero to 1 mg/ml. These results show that rates with triNAG are affected little, if any, by the 25 µg α-LA used in the preceding K_m study of triNAG. Particularly interesting was the finding that α-LA concentrations as high as 1 mg/ml had only moderate effects on the reaction rate. The significance of this finding seems even greater since α-LA is present in milk at levels of about 1 mg/ml (14). Thus one would expect little control of the triNAG reaction by α-LA if such an acceptor were present in vivo.

Another NAG oligomer, tetraNAG, was also tested as an acceptor in the A-protein galactosyl transferase reaction. The studies were conducted in the presence and absence of 25 µg α-LA/ml. The results, shown in the reciprocal plot of Figure 28, again show that α-LA has little effect on the galactosyl transferase reaction using a NAG oligomer (tetraNAG). The K_m's for tetraNAG were 1.4 and 1.2 mM in the presence and absence of α-LA while the V_{max} was unchanged.

The results of the studies using NAG oligomers as acceptors in the A-protein galactosyl transferase reaction show that the presence of more than one NAG residue in the NAG oligomer had little effect on the reaction, although diNAG is slightly more active than the other oligomers and appears to be the best substrate. All oligomers tested were better substrates than NAG itself, having both lower K_m's and as high or higher maximum velocities. A plot of K_m and V_{max} versus the number of NAG residues in the NAG oligomers used as an acceptor is shown in Figure 29.

A number of other oligosaccharides were tested as galactosyl

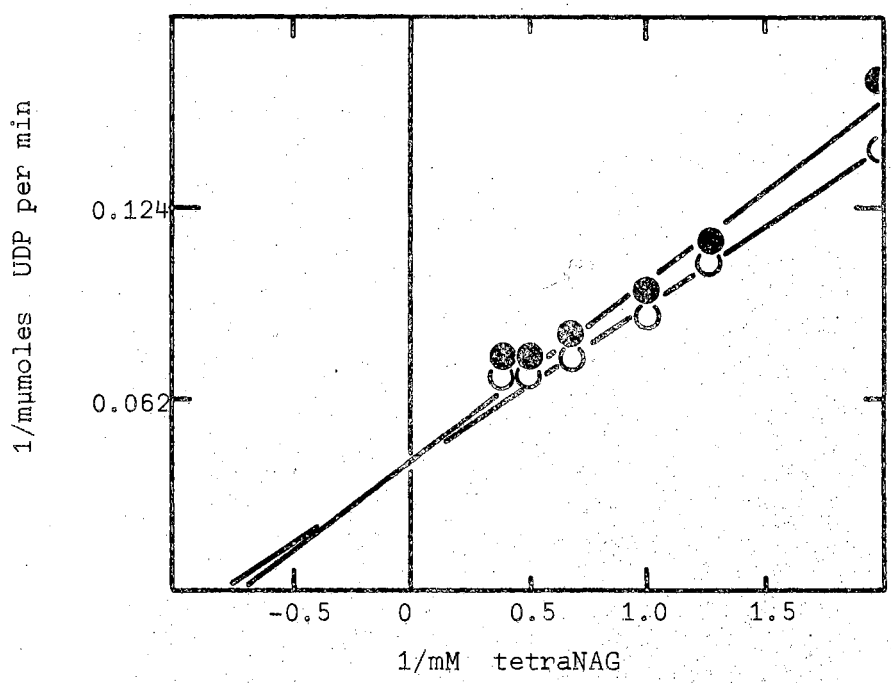

Figure 28. Lineweaver-Burk Plot of A-Protein tetraNAG Acceptor Activity ± α-LA.

○, no α-LA; ●, 25 μg α-LA/ml. Eighteen units of A-protein was used for the standard spectrophotometric assay. The K_m value for tetraNAG is 1.2 mM in the absence and 1.4 mM in the presence of 25 μg α-LA/ml.

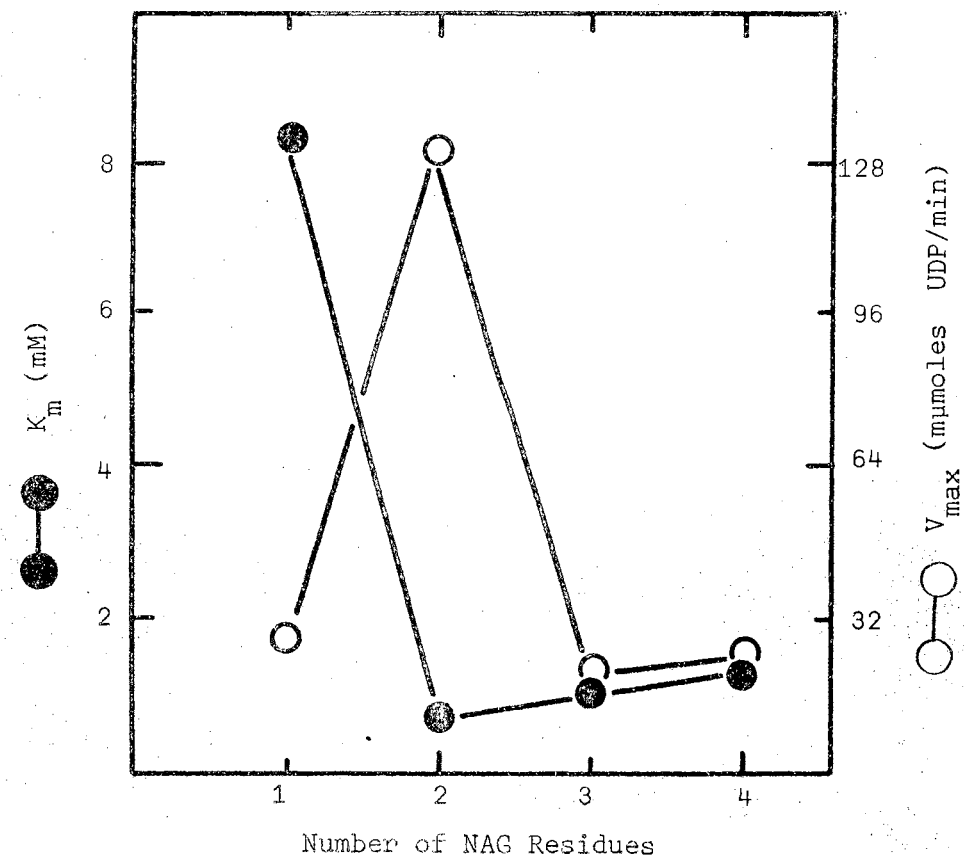

Figure 29. A-Protein K_m and V_{max} Values vs the Number of Residues in the NAG Oligomers.

acceptors with the A-protein. NAG-NAM differs from diNAG in that the carbohydrate moiety on the reducing end is N-acetylmuramic acid instead of NAG and was therefore a test of the specificity of the A-protein for the second residue in an oligomer having a terminal non-reducing β-linked NAG residue. A reciprocal plot of NAG-NAM activities with and without 100 µg α-LA/ml is shown in Figure 30. The K_m for NAG-NAM was 1.6 mM in the absence and 2.2 in the presence of 100 µg α-LA per ml. The V_{max} was unaffected by α-LA. A pronounced change in slope is noted in the reciprocal plot of NAG-NAM with α-LA present, similar to that observed with NAG in Figure 22. The significance of this marked change in slope is not understood at this time.

Cellobiose was 7.5% as good an acceptor as glucose with the lactose synthetase studied by Babad and Hassid (89). Cellobiose was also an acceptor with the A-protein as shown in the reciprocal plots of Figure 31. The K_m and V_{max} were unchanged by the presence or absence of 100 µg α-LA per ml. The K_m was very high (0.833 M) indicating that cellobiose is a poor acceptor. The interesting conclusion drawn from this study is that either the β-1, 4 linkage between the two glucosyl residues in cellobiose or the presence of the second glucose residue decreases the specificity of the A-protein for the N-acetyl group of NAG and also abolishes any apparent control by α-LA, thereby allowing the non-reducing glucose residue to be an acceptor. Glucose alone is a very poor acceptor (K_m 1.5M) in the absence of α-LA (92).

Figure 32 shows that maltose (glycosyl-α-1,4-glucose) is not an acceptor in the absence of α-LA. However, in the presence of 100 µg α-LA/ml, maltose was a poor acceptor (K_m=5M). Thus the β-1,4 linkage of cellobiose rather than the second glucose residue allows the non-

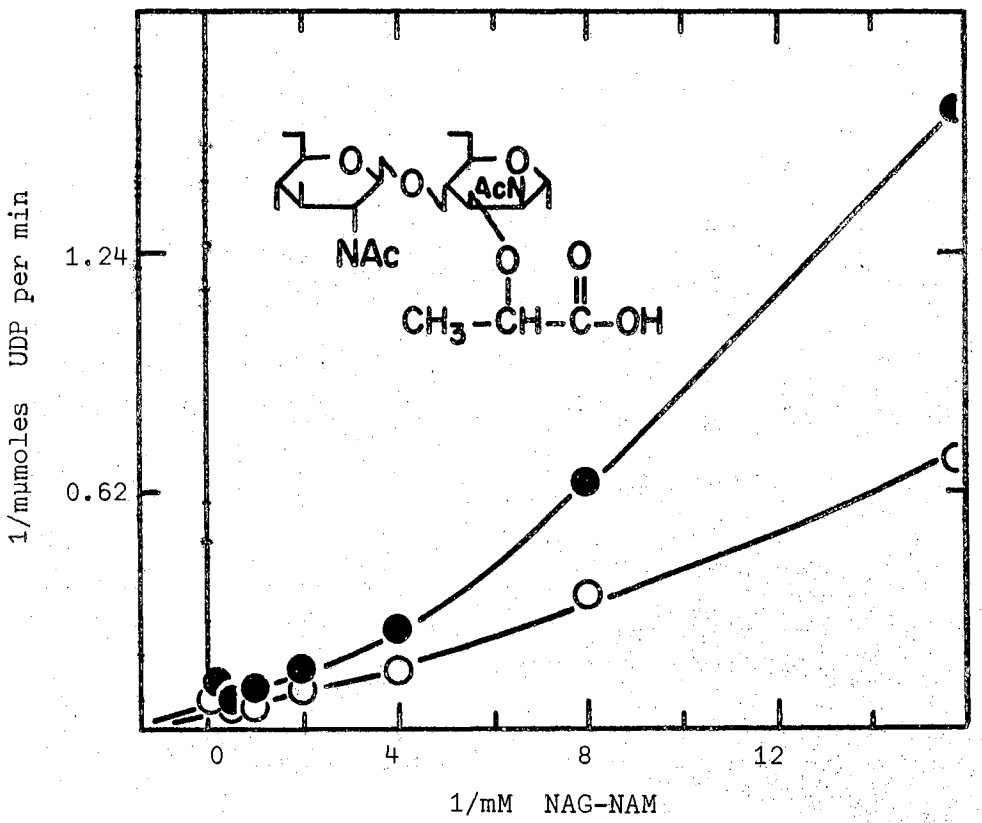

Figure 30. Lineweaver-Burk Plot of A-Protein NAG-NAM Activity in the Presence and Absence of 100μg α-LA/ml.

○, no α-LA; ●, 100 μg α-LA/ml. Activities are for eighteen units of A-protein. K_m values for NAG-NAM are 1.6 mM in the absence and 2.2 mM in the presence of 100 μg α-LA/ml.

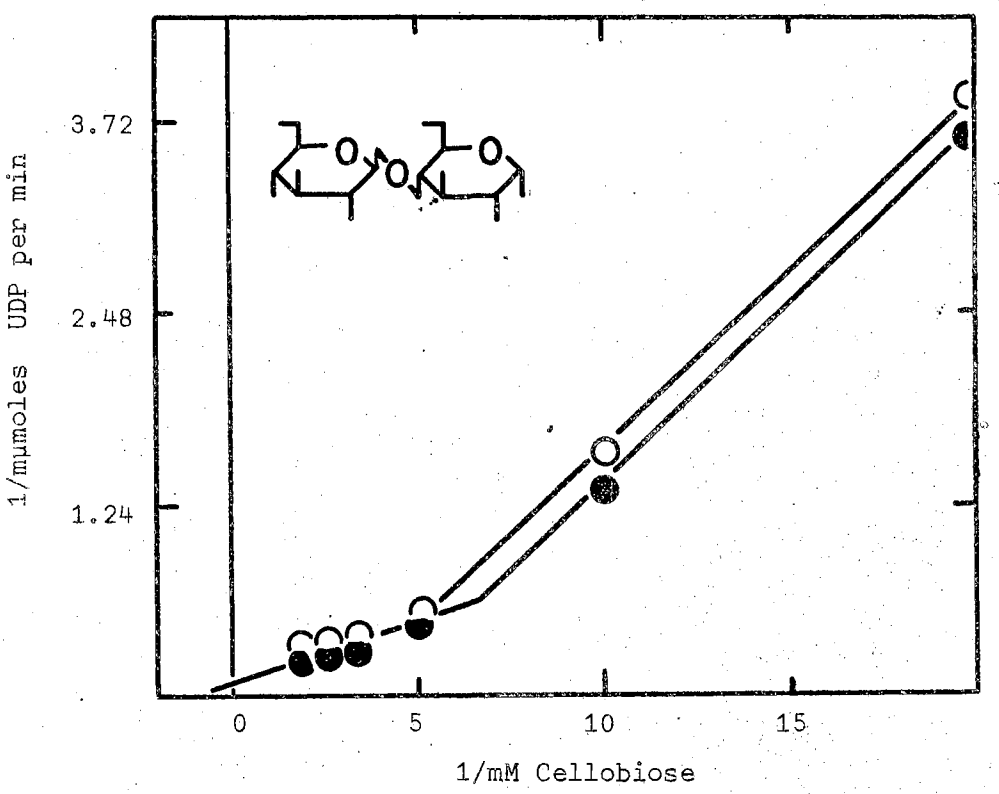

Figure 31. Lineweaver-Burk Plot of A-Protein Cellobiose Activity ± α-LA.

○, no α-LA; ●, 100 μg α-LA/ml. Assays contained eighteen units of A-protein. The K_m value for cellobiose was 0.83 M.

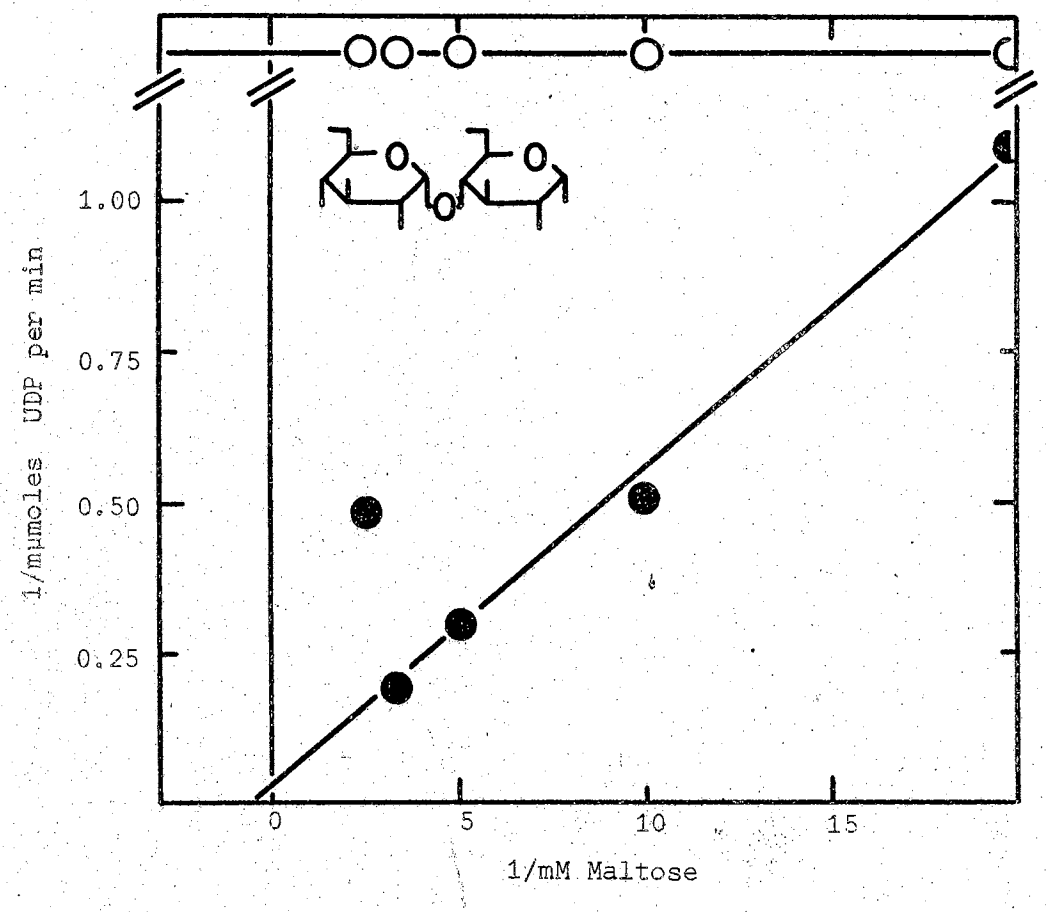

Figure 32. Lineweaver-Burk Plot of A-Protein Maltose Activity ± α-LA.

○, activity of 18 units A-protein using maltose as the acceptor in the absence of α-LA; ●, activity of 18 units A-protein using maltose as the acceptor in the presence of 100 μg α-LA/ml. The K_m value for maltose is 5 M.

reducing glucose of cellobiose to act as an acceptor in the presence or absence of α-LA. The α-linkage of maltose does not allow it to be an acceptor in the absence of α-LA.

The use of ovalbumin as an acceptor was prompted by the report of McGuire, et.al., (91) that ovalbumin as well as NAG was an acceptor with the galactosyl transferase isolated from goat colostrum. Ovalbumin has a single carbohydrate chain per molecule (124) which terminates in a NAG residue β-1,4 linked to mannose. It is one of the few glycoproteins which has its carbohydrate chain terminated by NAG. With the A-protein, ovalbumin was as good an acceptor as NAG in the spectrophotometric assay (Figure 33). The apparent K_m for ovalbumin was 1.66 mM and was unaffected by the presence or absence of 100 µg α-LA/ml, however the reaction was slightly inhibited by α-LA. A separate experiment with 0.4 mM ovalbumin showed that 600 µg α-LA/ml inhibited thereaction 48%.

Table IV summarizes the K_m and V_{max} results obtained for the NAG oligomers, NAG-NAM, cellobiose, maltose, and ovalbumin. Of these, only NAG, maltose, and glucose were appreciably affected by the presence of 100 µg or less of α-LA, suggesting that the other acceptors are not subject to stringent control by α-LA.

A number of other carbohydrates were tested as acceptors at fixed concentrations in the presence and absence of α-LA with the A-protein (Table V). Mannose, L-fucose, and melibiose (galactosyl-α-1,6-glucose) were not acceptors. UDP-NAG was not an acceptor at 0.4 mM, a concentration equal to that of UDP-galactose in the assay. 2-Deoxyglucose, maltose, and α-methylglucose were acceptors only in the presence of α-LA. The activity with maltose and α-methylglucose only in the

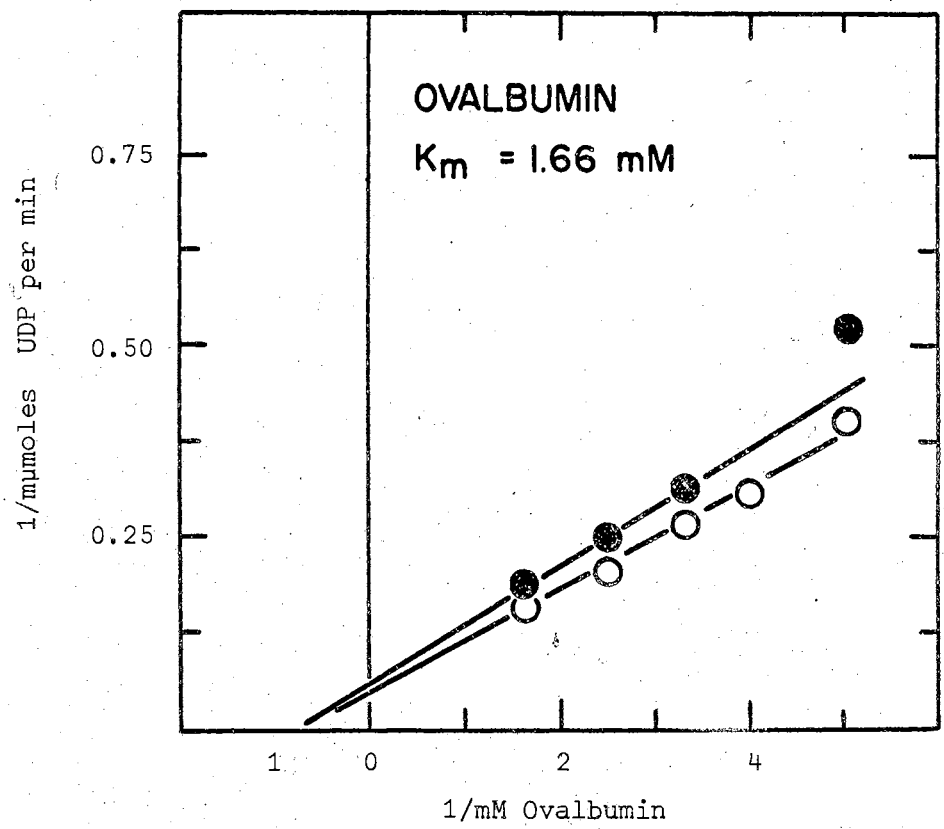

Figure 33. Lineweaver-Burk Plot of A-Protein Activity With Ovalbumin as the Acceptor ± α-LA.

O, activity of 18 units A-protein using ovalbumin as the acceptor in the absence of α-LA; ●, activity of 18 units A-protein using ovalbumin as the acceptor in the presence of 100 μg α-LA/ml.

TABLE IV

A-PROTEIN K_m AND V_{max} VALUES FOR OLIGOMER ACCEPTORS IN THE PRESENCE AND ABSENCE OF α-LA

ACCEPTOR	K_m (mM)		V_m (mμmoles UDP/min		μg α-LA per ml
	-α-LA	+α-LA	-α-LA	+α-LA	
NAG	8.3	1.7	26.9	82.7	25
diNAG	0.62	0.62	131.1	120.3	25
triNAG	1.0	1.13	17.6	19.7	25
tetraNAG	1.2	1.4	23.7	23.7	25
NAG-NAM	1.6	2.2	20.2	20.2	100
Cellobiose	833	833	124.2	124.2	100
Maltose	∞	5000	0	129.0	100
Glucose*	1500	5	-----	19.4	100
Ovalbumin	1.66	1.66	26.9	20.2	100

*Glucose data from D. K. Fitzgerald, Ph.D. Thesis (127).

TABLE V

ACCEPTOR ACTIVITIES WITH 18 UNITS OF A-PROTEIN IN THE PRESENCE AND ABSENCE OF α-LA

Acceptor	Anomeric Bond	Conc. (M)	Activity mμmoles UDP/min No α-LA	Activity mμmoles UDP/min 100μg α-LA/ml
NAG		0.02	18.7	3.15
Glucose		0.02	0	17.4
Glucose		0.25	0	18.0
2-deoxy-D-glucose		0.25	0	1.72
Mannose		0.25	0	0
L-fucose		0.25	0	0
Glucosamine		0.25	0.13	0.55
N-acetylmannosamine		0.25	0.19	0.66
N-acetylmuramic acid		0.119	8.26	6.85
α-methylglucose	α-1	0.25	0	1.12
β-methylglucose	β-1	0.25	1.54	1.62
β-indoxylglucose	β-1	0.02	4.34	3.24
Maltose	α-1,4	0.25	0	4.23
Cellobiose	β-1,4	0.25	2.17	2.17
Cellobiulose*	β-1,4	0.25	1.90	1.80
Glucosylmannose*	β-1,4	0.25	0.57	0.65
Gentiobiose	β-1,6	0.25	1.04	6.7
Melibiose (gal-glu)	β-1,6	0.25	0	0
UDP-NAG	α-1	0.4mM	0	0

*Activity actually determined at 0.1 M and then calculated for 0.25 M, assuming linearity in rates.

presence of α-LA suggested that α-glucosides are probably not acceptors with the A-protein alone.

A variety of β-linked glucose derivatives were tested as acceptors in the presence and absence of α-LA including β-methylglucose, β-indoxylglucose, cellobiose, cellobiulose, glucosyl mannose, and gentiobiose. All were acceptors though none were as good as NAG or NAG oligomers. β-Indoxylglucose was the best β-glucoside acceptor. β-Methylglucose, cellobiulose, and glucosyl mannose were comparable to cellobiose in activity and were unaffected by α-LA. Gentiobiose was comparable to cellobiose in the absence of α-LA but its acceptor activity increased 6.5 fold with 100 μg α-LA/ml.

These results indicate that the A-protein can use as acceptors most heterosaccharides having a terminal non-reducing NAG or glucose residue β-linked to a wide variety of compounds. However, a terminal NAG residue is a much better acceptor than a terminal glucose residue. The effectiveness of the NAG oligomers as acceptors was little affected by the number of NAG residues in the oligomer nor by the substitution of N-acetylmuramic acid (as in NAG-NAM) for the reducing NAG residue of diNAG. Since UDP-NAG was not an acceptor and its NAG residue is α-linked, one might conclude that the terminal α-linked NAG is not an acceptor (although specificity for the UDP moiety might also be important in this case) while the terminal β-linked NAG is an acceptor, regardless of the nature of the succeeding sugar residue. This conclusion is supported by the activity with ovalbumin as an acceptor which has a single carbohydrate chain terminating in a β-linked NAG residue (124).

The specificity of the A-protein about the 2-position of the NAG

or glucose was of interest since the only difference between glucose and NAG is at this position. Table IV shows that mannose was not an acceptor in the presence or absence of α-LA while glucosamine and N-acetylmannosamine were very poor acceptors. N-Acetylmuramic acid was a surprisingly good acceptor at a concentration of 0.119 M. 2-Deoxy-glucose was an acceptor only with α-LA present. These results suggest that the active acceptor compounds must have the 2'-OH in the glucose configuration or it must be absent. The substitution of an NH_2 for the 2'-OH of glucose allows low activity as an acceptor but the N-acetyl derivative (NAG) is far superior. Epimerization of the 2'-N-acetyl group from the NAG to the N-acetylmannosamine configuration greatly reduced the activity as an acceptor. The surprisingly high activity observed with NAM suggests that the bulky 3'-O-lactate group attached to N-acetylmannosamine greatly reduces the specificity for NAG configuration about the 2'position.

Another interesting aspect of the steric specificity of the A-protein for acceptor was the observation that L-fucose (6-deoxy-L-galactose) and melibiose (galactosyl-β-1,6-glucose) were not acceptors while gentiobiose (glucosyl-β-1,6-glucose) was. Thus a specificity for the glucose rather than galactose configuration in the 4'-OH of the terminal non-reducing residue existed.

In summary, the galactosyl transferase activity of the A-protein has a rather broad acceptor specificity for any β-linked NAG or glucose, although NAG glycosides were by far the best glycoside acceptors tested. This β-glycoside specificity does not appear subject to strict control by α-LA in contrast to the marked influence of α-LA on the specificity for NAG or glucose alone. The observation that ovalbumin

is a good acceptor both supports the specificity studies done on glycosides and clearly implicates the A-protein in glycoprotein biosynthesis. Again, however, the specificity of A-protein for ovalbumin does not appear subject to strict control by moderate levels of α-LA (100 μg/ml) although it is somewhat inhibited by higher α-LA levels. Whether or not this reaction can occur *in vivo* is clearly a question of cellular α-LA levels, "compartmentalization", and time involved. The involvement of the A-protein in glycoprotein biosynthesis seems highly likely since it synthesizes the sequence gal-β-1,4-NAG which is the most common sequence found in glycoproteins (125). The role of α-LA as a "specifier protein" in regard to NAG and glucose activity is clear (3). Whether or not it also functions as a "specifier protein" between lactose and glycoprotein biosynthesis is still questionable as sufficient literature or experimental data is not available for one to decide at this time. However, the relatively poor inhibition of activity with ovalbumin by α-LA suggests that α-LA may not function as a "specifier protein" but instead as a "permitter protein" since its presence (at 100 μg/ml) gives 100% activity with glucose but has little effect on ovalbumin activity. Therefore, a new specificity (glucose) would be "permitted" to coexist with the original specificity (ovalbumin) in the presence of α-LA. The A-protein could then carry out both reactions in the presence of α-LA.

CHAPTER V

DISCUSSION

Kinetic studies of the lactose synthetase reaction show that the assay for either of the two required proteins can be performed by using saturating amounts of the other proteins (9). The mutual saturation of each protein by the other implied that the active form of the enzyme may consist of an association complex between the two proteins. Any such interaction could be intimately involved in the control of the enzymatic reaction *in vivo* since the association-dissociation reaction of an interacting enzyme system is related to metabolic control with other enzymes (11). The nature of the interaction of α-lactalbumin with the lactose synthetase A-protein was investigated to better understand the control of lactose biosynthesis. The kinetic data for lactose synthetase (6) suggested an interaction similar to that of the tryptophan synthetase system (7,8) which involves the formation of a subunit complex as the enzymatically active species.

Initial experiments with the lactose synthetase system were designed to demonstrate, by means of transport experiments, any detectable stable complex which might be formed between α-lactalbumin and the A-protein. Since the tryptophan synthetase system required substrate and cofactor (serine and pyridoxal phosphate) for complex formation, the two lactose synthetase proteins might similarly require a combination of its substrates for complex formation. This seemed

particularly likely since the A-protein and α-lactalbumin are easily separated by gel filtration on Bio-Gel P-30 in the absence of substrates. Hence, the transport experiments were performed in the presence of the desired substrates at the same concentrations as used in the lactose synthetase spectrophotometric assay. These substrate concentrations were chosen primarily because they give maximum enzymatic activity in the spectrophotometric assay and therefore must interact with the active form of the enzyme. This choice of concentrations seemed particularly reasonable for the lactose synthetase system since the analagous tryptophan synthetase subunits form a complex in the presence of 0.15 mM serine and 0.02 mM pyridoxal phosphate, concentrations well below those used in the tryptophan synthetase assay (60 mM and 0.04 mM, respectively) (7).

The studies on the interaction of α-LA with the A-protein are discussed in some detail in Chapter III. The results of the density gradient centrifugation studies (Figures 1 through 9) are summarized in Table I. In Figures 1 through 5, an attempt was made to demonstrate any stable complex formed as a shift in the sedimentation of the A-protein peak upon complex formation. The conditions were varied to include high ratios of α-LA to A-protein in the presence and absence of substrates. No significant shift in the sedimentation of the A-protein peak was observed, regardless of the presence or absence of substrates.

These experiments suffered from the insensitivity and variability of the enzymatic assay for the individual proteins, as can be seen in the sedimentation patterns shown in Figures 1, 2, and 3. The high excess of α-LA as used in Figure 1 might be expected to drive the

equilibrium reaction of the association complex toward association and thereby cause a complete conversion of A-protein into complex to produce a major shift in A-protein sedimentation. However, should incomplete complex formation occur, only a portion of the A-peak would appear in a peak representative of complex. Therefore, the limit of detection of any minor amount of complex formed would be dependent on the sensitivity of the assay system. Thus small amounts of complex formation could not be reliably detected by the enzymatic assay of the fractions.

To increase sensitivity, ^{125}I labeled α-LA was used as the limiting protein with high levels of A-protein in an attempt to drive the reaction toward association. The radioactively labeled α-LA would provide a sensitive means of assaying for α-LA, thereby allowing the sensitive and reliable detection of very small amounts of complex formation. Also, the expected major shift in the position of the α-LA-^{125}I peak from the top of the centrifuge tube for free α-LA-^{125}I (1.75 S) to the lower portion of the tube upon formation of an A: α-LA-^{125}I complex (4.85 S) would be unambiguous when detected by counting the α-LA-^{125}I. This would overcome the ambiguities of smaller shifts in the A-protein position, from 3.1 S for the free A-protein to 4.85 for the complex, which were difficult to accurately detect due to the variability in the enzymatic assay. The results of experiments using α-LA-^{125}I are reported in Figures 7, 8, and 9.

There was no significant shift in the α-LA-^{125}I peak in any of these three density gradient centrifugation experiments regardless of the presence or absence of substrates. The A-protein levels were as high or higher than that required to give maximum activity when

assaying for α-LA using excess A-protein, hence this level of A-protein should be adequate for complex formation in the centrifuge experiments. It is significant that there was a distinct absence of skewing or displacement of the α-LA-^{125}I peak nor did a second minor peak appear below the major peak as might be expected if incomplete complex formation had occurred.

The experiment reported in Figure 9, which was performed at 20° instead of the usual 2°, again showed no shift of the α-LA-^{125}I peak in the presence of A-protein and substrates, indicating no detectable complex formation. This higher temperature of 20° is reported to favor association reactions which occur as a result of hydrophobic interactions (20). It therefore seems unlikely that a temperature effect due to hydrophobic interactions between the A-protein and α-LA could be the cause of the inability to demonstrate complex formation.

The second approach in the study of the A-protein: α-LA interaction involved gel filtration. The technique of gel filtration has been widely used to study both ligand interactions with macromolecules and protein-protein interactions (35,36,37). The use of α-LA-^{125}I in gel filtration experiments affords a sensitive means of detecting minute amounts of α-LA-^{125}I bound to A-protein during gel filtration experiments. Hence, this method might be expected to show binding of small amounts of α-LA-^{125}I to the A-protein in the event of incomplete complex formation. The results of these experiments showed no detectable binding of α-LA-^{125}I to the A-protein upon gel filtration on Bio-Gel P-30 (Figure 13). Indeed, Figure 13 unexpectedly showed a small but significant peak of α-LA-^{125}I to co-migrate with bovine serum albumin, suggesting a higher affinity of α-LA for serum albumin than

for A-protein. However, it is not altogether surprising that no binding of α-LA-^{125}I to A-protein occurred upon gel filtration of the two since this procedure is routinely used to separate them during purification. This experiment, however, does seem to rule out the possibility that the purified A-protein might still contain small amounts of α-LA associated with it and therefore give the endogenous lactose synthetase activity in the absence of exogenous α-LA (91). This is in agreement with the earlier findings that incubation of A-protein with α-LA antisera did not change the endogenous lactose synthetase activity of the A-protein, indicating that the endogenous activity is not due to contaminating or bound α-LA in the A-protein preparation (127). Should the A-protein preparation contain contaminating bound α-LA, one might expect an exchange of bound α-LA with the α-LA-^{125}I of the chromatography experiment in Figure 13 to produce an α-LA-^{125}I peak co-migrating with the A-protein. No such co-migration was observed.

The Hummel and Dreyer (13) type gel filtration experiments are more widely used than conventional gel filtration for demonstration of binding phenomena. However, there are no reports in the literature of this method being used to study interaction of macromolecules although the suggestion was made that it should be applicable. Upon using this method to study α-LA: A-protein interactions, it was observed that α-LA-^{125}I appeared to strongly adsorb to the polyacrylamide Bio-Gel P-30 beads used to pack the columns of Figure 14. The application of another protein sample caused desorption of the α-LA-^{125}I bound to the gel, giving a false α-LA-^{125}I peak. It was therefore difficult to distinguish between true specific protein-protein interactions, such as would occur with complex formation, and non-specific adsorption

phenomena, making interpretation of the results shown in Figure 14 extremely difficult. However, had a stable complex been formed, an α-LA-^{125}I peak superimposed over the A-protein peak would be expected. This initial α-LA-^{125}I peak should be followed by a depression of the α-LA-^{125}I base line (a "hole") equal in area to the peak. The peak was present in all cases, even when a sample of bovine serum albumin was applied instead of the A-protein. However, in no case was a "hole" observed following the α-LA-^{125}I peak. These results suggested that the peak was most likely due to displacement of adsorbed α-LA-^{125}I rather than to complex formation. It should be pointed out, however, that it would be difficult to rule out incomplete complex formation due to weak interactions on the basis of these results.

Attempts to find a suitable equilibrium dialysis system to study the binding of α-LA-^{125}I to the A-protein were unsuccessful. Fluorescence studies also gave no evidence of interaction of dansylated α-LA with the A-protein in the presence or absence of substrates.

The conclusion drawn from the results reported in Chapter III is that no demonstrable complex was formed under any of the conditions used. The lack of a demonstrable complex between the lactose synthetase A-protein and α-LA is not unique among the enzymes known to require two proteins for activity. Delmer and Mills (81) failed to demonstrate complex formation between the subunits of tryptophan synthetase isolated from Nicotiana tabacum, even though this same complex is easily shown for the E. coli enzyme (7,8). This suggested that minor variations in the enzyme, such as its source, can greatly alter the ability of the enzyme subunits to undergo a detectable association to form complex. The association reaction itself must then be highly

sensitive to variations of the protein in addition to variations in the environment. Another enzyme, the glutamate mutase reported by Switzer and Barker (86), has clearly been shown in kinetic studies to require interaction of two proteins in order to have enzymatic activity, yet they were also unable to demonstrate an active complex between the subunits.

The successful demonstration of complex formation between the interacting proteins of an enzyme depends on the properties of the enzyme and the nature of its association-dissociation reaction. The only successful demonstrations of complex formation using transport experiments have been in systems such as tryptophan synthetase (7,8) and ribonucleoside diphosphate reductase (12) which have high association constants and are slow to re-equilibrate. Freiden (11) has shown that the demonstration in transport processes of a stable complex in an interacting system is unlikely if the system has either too low an association constant or if it has a sufficiently high association constant but is in rapid equilibrium. One must, therefore, interpret negative transport experiments with caution for even though there is no demonstrable complex in a system studied by such methods, one cannot rule out the possibility of a very weak association and hence it is subject to resolution by the transport process or an association which re-equilibrates faster than the time required for transport and resolution and would again be resolved.

The finding that the A-protein and α-LA form no demonstrable complex in any of the transport experiments used in Chapter III but distinctly interact in the assay as shown by kinetic studies (9), suggests that the interaction of the A-protein with α-LA either has a

low association constant and hence interaction is poor, or that once an association occurs, even if the association constant is quite high, it rapidly re-equilibrates under the conditions of a transport experiment and is thereby resolved. In this case, demonstration of an association complex would be extremely difficult. A long-lived stable complex would not seem necessary for maximum catalytic activity and a rapidly equilibrating complex affords a rapid and sensitive means of control of the reaction.

The suggestion that the A-protein and α-LA are a rapidly equilibrating association-dissociation system led to the question of control of the lactose synthetase reaction and the role of each of the two proteins in the reaction. It is informative in this respect to compare several other enzymes which require two proteins in regard to the role of the participating proteins in catalysis of the desired reaction. For the tryptophan synthetase system the α-subunit is proposed to control the path of the reaction by controlling the breakdown of the reaction intermediate or the β_2-subunit. Hence, the α-subunit has been termed a "reaction specifier" (78). Both trehalose phosphate synthetase (65,66) and sucrose synthetase (64) are subject to control by macromolecules, termed specifier molecules, which alter the specificity of the enzymes for the nucleotide moiety of the nucleotide diphosphate sugar substrate. The phosphoenolpyruvate-dependent formation of fructose-1-phosphate also has its substrate specificity altered by the addition of a "fructose specifier protein" (63).

A different type of control is found with DNA-dependent RNA polymerase. The presence of the δ-factor (initiating factor), which appears to be a protein subunit, allows the replicase to initiate

synthesis of RNA. However, once the reaction is initiated the S-factor is no longer required as the reaction will continue without it (67,68).

Acetyl CoA carboxylase (57,58) and oxalacetic acid transcarboxylase (60) both require a biotin containing protein for activity. This biotin-protein apparently serves as a carboxyl-carrier protein and is a substrate for the catalytic subunits, hence the interesting mechanism of one "subunit" serving as the substrate of another.

These cases are examples of four types of protein interaction to provide control of the final reaction. The first type was the "reaction specifier" subunit of tryptophan synthetase which controls the desired breakdown of a reaction intermediate. The second was modification of the substrate specificity in the presence of a "specifier protein". The third is the requirement of an initiation factor by Qβ-replicase to begin a reaction but not to continue it. The fourth and most interesting type is that one subunit is a carrier-protein and substrate of another. In attempting to answer the question of the control of lactose synthetase and the role of the A-protein and α-LA in catalysis, it is interesting to note the brief comment by Hill, et. al. (4) that there is no detectable partial reaction catalyzed by the individual proteins such that the final reaction would be a sum of the two. It is also interesting that the A-protein alone catalyzes the synthesis of N-acetyllactosamine. The substrate specificity of the A-protein in the presence of α-LA is for glucose while in the absence of α-LA it is for N-acetylglucosamine. Hence α-LA has been termed a specifier protein in that it appeared to control the substrate specificity and thereby the final product of the A-protein catalysis,

perhaps in a manner similar to other two-protein enzymes.

The studies on the carbohydrate acceptor specificity of the A-protein galactosyl transferase activity reported in Chapter IV were designed to provide insight into the biological role of the A-protein and whether or not α-LA is indeed a specifier protein which controls the acceptor specificity of the A-protein. In addition to catalyzing the synthesis of lactose in the presence of α-LA, the A-protein appears to be involved in the biosynthesis of other oligosaccharides and most likely in glycoprotein biosynthesis.

The results reported in Chapter IV show that the A-protein is a potent galactosyl transferase which transfers galactose from UDP-galactose to a wide variety of acceptors. The A-protein can use as an acceptor most any heterosaccharide having a terminal non-reducing NAG or glucose residue β-linked to a wide variety of compounds. α-Linked glucosides, eg., maltose and α-methylglucose, are not acceptors in the absence of α-LA (Table V). Although the A-protein used compounds with both β-glucosyl and β-N-acetylglucosaminyl end groups as acceptors, the compounds having a β-N-acetylglucosaminyl end group were far better acceptors (Table II). This suggested a specificity for the N-acetyl group at the NAG C-2 position in the absence of α-LA. However, the finding that N-acetylmuramic acid was a relatively good acceptor was an exception to this generalization.

The pentultimate residue in an oligomer had little influence on the activity of the compound as an acceptor. Nor did a β-1,6 bond in a disaccharide (as in gentiobiose) instead of a β-1,4 bond (as in cellobiose) have any major effect on the activity. In fact, β-methyl glucose was essentially equal to cellobiose and gentiobiose as an

acceptor, indicating that only the anomeric linkage of the terminal nonreducing saccharide moiety and not the identity of the pentultimate moiety was specified by the A-protein. Again, however, the generalization just expressed has an apparent exception in the case of β-indoxylglucose (Table V) which is the best glucoside acceptor, subbesting that the indoxyl group may somehow enhance its activity.

It is interesting to compare the acceptor specificity of the A-protein with that of other galactosyl transferases described in the literature. The galactosyl transferase activity isolated from goat colostrum by McGuire, et. al. (91) was active with a variety of β-linked NAG acceptors, including NAG, β-methyl NAG, diNAG, and triNAG. Glucose was not an acceptor. The enzyme also exhibited transferase activity toward a number of glycoproteins which had a β-N-acetylglucosaminyl end group, including native ovalbumin and sialidase and β-galactosidase treated orosomucoid.

The galactosyl transferase described by Iyer and Carlson (111) was also isolated from goat colostrum and used both NAG and sialidase treated ovarian cyst glycoprotein (which has a β-N-acetylglucosaminyl end group after treatment) as acceptors. The galactosyl transferase studied by Ziderman, Gompertz, Smith, and Watkins (108) exhibited activity toward β-N-acetylglucosaminyl residues, including NAG, diNAG, and β-methylNAG. The galactosyl transferase purified from calf thyroid particles by Spiro and Spiro (123), thought to be involved in the assembly of the oligosaccharide chains of the carbohydrate units of thyroglobulin, exhibited activity toward free NAG as well as thyroglobulin glycopeptides which contained terminal NAG residues (123).

The early studies on the acceptor specificity of lactose

synthetase (which probably were done in the presence of unknown amounts of α-LA) reported by Babad and Hassid (87) clearly show that NAG and cellobiose as well as glucose are acceptors for the galactosyl transferase activity which is in agreement with the results reported in Chapter IV of this dissertation. Hill, et. al. (4) report that the A-protein will transfer galactose to sialidase and β-galactosidase treated orosomucoid (which has a β-NAG end group available after such treatment) and that this activity is inhibited little (17%) by α-LA at a concentration of 1 mg/ml. These reports, together with the results shown in Chapter IV, indicate that the A-protein can use glycoproteins as acceptors. It also seems likely that the A-protein is identical to the galactosyl transferases reported by Iyer and Carlson (112), and McGuire, et. al. (91). The β-galactosyl transferase activity described by Ziderman, et. al. (107) appears closely similar to the bovine-milk A-protein.

The primary biological function of the A-protein has been considered to be the catalysis of lactose synthesis in the presence of α-LA. However, the A-protein utilizes NAG instead of glucose as an acceptor in the absence of α-LA for the synthesis of NAL. The presence of α-LA greatly inhibits NAL synthesis. In contrast to the marked influence of α-LA on the specificity of the A-protein toward NAG and glucose, the specificity of the A-protein for β-linked NAG or glucose residues does not appear to be under the strict control of α-LA. The synthesis of N-acetyllactosamine *in vivo* as a result of the specificity of the A-protein for NAG would seem unlikely since NAG does not occur in high enough concentrations in most tissues to serve as an acceptor (123), hence this reaction appears to be of little biological significance.

However, the specificity of the A-protein for β-NAG end groups in oligosaccharides together with its activity toward ovalbumin strongly suggest that the A-protein is involved in glycoprotein biosynthesis. This suggestion is strengthened by the observation of Sprio (125) that the bond synthesized by the A-protein, the galactosyl-β-1,4-N-acetyl-glucosamine bond, is the most common sequence found in glycoprotein carbohydrate chains. Also, the other galactosyl transferases discussed above exhibit activity toward a variety of glycoprotein acceptors and are presumably engaged in glycoprotein biosynthesis in vivo. Since the A-protein is so similar to these other transferases, it too must be involved in glycoprotein biosynthesis.

The interesting aspect of the involvement of the A-protein in glycoprotein biosynthesis is that of its possible control by α-LA. The acceptor specificity studies on the NAG oligomers and ovalbumin show them to have closely similar K_m values. It is noteworthy that α-LA has no appreciable inhibition on the A-protein activity with these acceptors. It would therefore appear that if the A-protein is responsible for the β-galactosyl transferase activity required for glycoprotein biosynthesis, it is not inhibited by α-LA at 34 μg/ml as is found in bovine mammary tissue (126). This is supported by the observation of Hill, et. al. (4) which showed that the A-protein transferase activity toward appro priately treated orosomucoid was inhibited only 17% by 1 mg α-LA/ml, an α-LA concentration comparable to that in milk. However, the biosynthesis of glycoproteins is known to occur inthe particulate cell fraction (124). Coffey and Reithel (94,95) report the isolation of intact particles containing lactose synthetase activity. This suggests that assay concentrations of α-LA at which one shows

inhibition or lack of inhibition may not be meaningful in a cell "microsomal" membrane environment, particularly if the two proteins coexist in the cell as a single particle.

If indeed the A-protein transferase activity with glycoprotein acceptors is uninhibited by in vivo α-LA levels, then the A-protein is in a unique metabolic situation of having a new acceptor specificity granted it by the presence of α-LA for the synthesis of lactose while the original specificity for glycoproteins remains. It would have one principal acceptor (glycoprotein) in the absence of α-LA and two concomittant acceptors (glycoprotein and glucose) in the presence of α-LA. It would therefore appear that α-LA can initiate lactose synthesis but cannot appreciably inhibit glycoprotein biosynthesis. This makes α-LA a rather unusual "specifier protein" and conflicts with the previous proposal for simultaneous reciprocal control of lactose and glycoprotein biosynthesis by α-LA (4).

The requirement for α-LA by the A-protein in order to catalyze appreciable lactose biosynthesis cannot be refuted (6). Since the two proteins obviously interact as shown in kinetic studies of the lactose synthetase reaction (6) but appear to form no demonstrable complex as shown by the results reported in Chapter III, it would appear that the two proteins function in a rapidly equilibrating association reaction.

There are at least two possible mechanisms which may explain the mechanism of α-LA influence on the specificity of the A-protein. The most likely mechanism would require that α-LA be a modifier of the A-protein and upon binding induces a conformational change in the A-protein which is reflected in the specificity change. This mechanism

would require that α-LA bind to and dissociate from the A-protein very rapidly, probably once for each reaction catalyzed, in order to be characteristic of a rapidly equilibrating association-dissociation system which is consistent with the transport experiments where no detectable complex was observed.

A second probable mechanism would seem to be a distinct possibility in explaining the role of α-LA. Since the normal acceptor for the A-protein alone appears to be an appropriate heteropolysaccharide moiety of a glycoprotein, it is not unreasonable that lactose synthesis could occur if α-LA were to bind glucose to form an α-LA; glucose complex. This complex could then bind to the A-protein to simulate the normal glycoprotein substrate and thereby allow galactosyl transfer to occur to glucose instead of the usual NAG residue. The mechanism finds precedent in the biotin-protein described in the acetyl CoA-carboxylase studies of Vagelos, et. al. (58) which served as both a carboxyl carrier protein and a substrate of the other two subunits of the enzyme. It would also seem likely that if one protein served as the substrate of another, such as is proposed for α-LA, the substrate-enzyme interaction might occur more rapidly than most conventional protein-protein association interactions studied to date. Hence, a rapidly-equilibrating association-dissociation system for α-LA and the A-protein might be expected as the data in Chapter III suggests.

Either of these two mechanisms would explain the apparent ability of the A-protein to catalyze both lactose and glycoprotein biosynthesis in the presence of α-LA. The reciprocal relationship between the α-LA and glucose concentrations required to obtain maximum lactose synthetase activity in the assay (127) and the reciprocal relationship

between the K_m values for α-LA and glucose, as observed with human lactose synthetase by Andrews (93), could be interpreted to support either mechanism. A strong point against the α-LA: glucose substrate mechanism is the brief comment by Hill (4) that binding studies showed no detectable binding of glucose by α-LA, however, no data was given.

Perhaps the strongest point in favor of the conformational change mechanism appears to be the studies on the inhibition of NAG activity by α-LA as shown in Figure 24. If the lactose synthetase reaction were to occur via the α-LA: glucose substrate mechanism, one might expect this complex to react at the same site as NAG and other oligosaccharides or glycoproteins. Hence, the inhibition of NAG activity by α-LA would be expected to be competitive. The data of Figure 24 clearly show this inhibition not to be simple linear competitive but is instead much more complex. This would appear to favor the mechanism of α-LA inducing a conformational change in the A-protein rather than α-LA being a substrate of the A-protein.

Thus it appears that the definition of the <u>in vivo</u> role of both the A-protein and α-LA as well as the elucidation of the mechanism of the lactose synthetase reaction must await further studies.

SUMMARY

Studies on the interaction of native and iodinated α-lactalbumin with the lactose synthetase A-protein using sucrose density gradient centrifugation and gel filtration techniques showed no demonstrable α-lactalbumin: A-protein complex, regardless of the presence or absence of substrate(s) at concentrations and conditions similar to those used in the lactose synthetase assay. Attempts to find an equilibrium dialysis membrane suitable for A-protein: α-lactalbumin binding studies were unsuccessful. Fluorescence studies showed no evidence of interaction of native or dansylated α-LA with lactose synthetase substrates or A-protein. It was concluded that the α-LA: A-protein interaction is a rapidly equilibrating association-dissociation system.

The acceptor specificity of the A-protein galactosyl transferase activity was investigated. α-LA was inhibitory using NAG as an acceptor but other glycoside and oligosaccharide acceptors with terminal β-glucosyl or β-N-acetylglucosaminyl residues as well as N-acetylmuramic acid were not inhibited by α-LA. α-Linked glucosides (maltose and α-methylglucose) and 2-deoxyglucose were acceptors only in the presence of α-LA. β-NAG oligomers and NAG-NAM, were the best acceptors. Ovalbumin was a good acceptor which was inhibited little by α-LA. Mannose, fucose, melibiose, and UDP-NAG were not acceptors, regardless of the presence or absence of α-LA. α-LA is neither inhibitory nor required for activity with β-linked glycosides of NAG or glucose or with ovalbumin as acceptors but α-glucosides are acceptors

only in the presence of α-LA. Thus, lactose synthesis and certain glycoprotein synthesis are compatible in the presence of α-LA. It is proposed that α-LA serves not as a specifier of the A-protein acceptor specificity but instead as a protein which permits lactose biosynthesis to occur concomittantly with glycoprotein biosynthesis.

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