THE SUBUNITS OF LACTOSE SYNTHETASE

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THE SUBUNITS OF LACTOSE SYNTHETASE

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CHAPTER I

INTRODUCTION

The Biosynthesis of Lactose

Lactose, the predominant sugar of bovine milk, was discovered and isolated by Fabritius Bartolettus in 1628 (1) and its structure was definitely established by Hayworth in 1927 (2) as 4(-β-D-galactopyranosyl)-D-glucose. Early studies on the biosynthesis of lactose from D-glucose showed that a number of enzymes were involved in the formation of lactose. Terner (3) and Kittinger and Reithel (4) have reported hexokinase (EC 2.7.1.1) activity in the lactating glands of the guinea pig. Phosphoglucomutase (EC 2.7.5.1) activity in bovine mammary homogenates was demonstrated by Kittinger and Reithel (4) and by Gander et al. (5). UDPG-pyrophosphorylase* (EC 2.7.7.9) activity in the mammary glands of lactating rats was observed by Smith and Mills (6) and by Maxwell, Kalckar and Burton (7) in 1955. Earlier, Caputto and Trucco (8) observed the presence of UDPGal-4-epimerase (EC 5.1.3.2) in mammary tissue. Gander et al. (5, 9) observed galactosyl transferase (EC 2.4.1) activity in extracts from the mammary gland of lactating cows which formed lactose-1-phosphate from UDPGal and G-1-P. The conversion of lactose-1-phosphate to lactose could not be accounted for, and was

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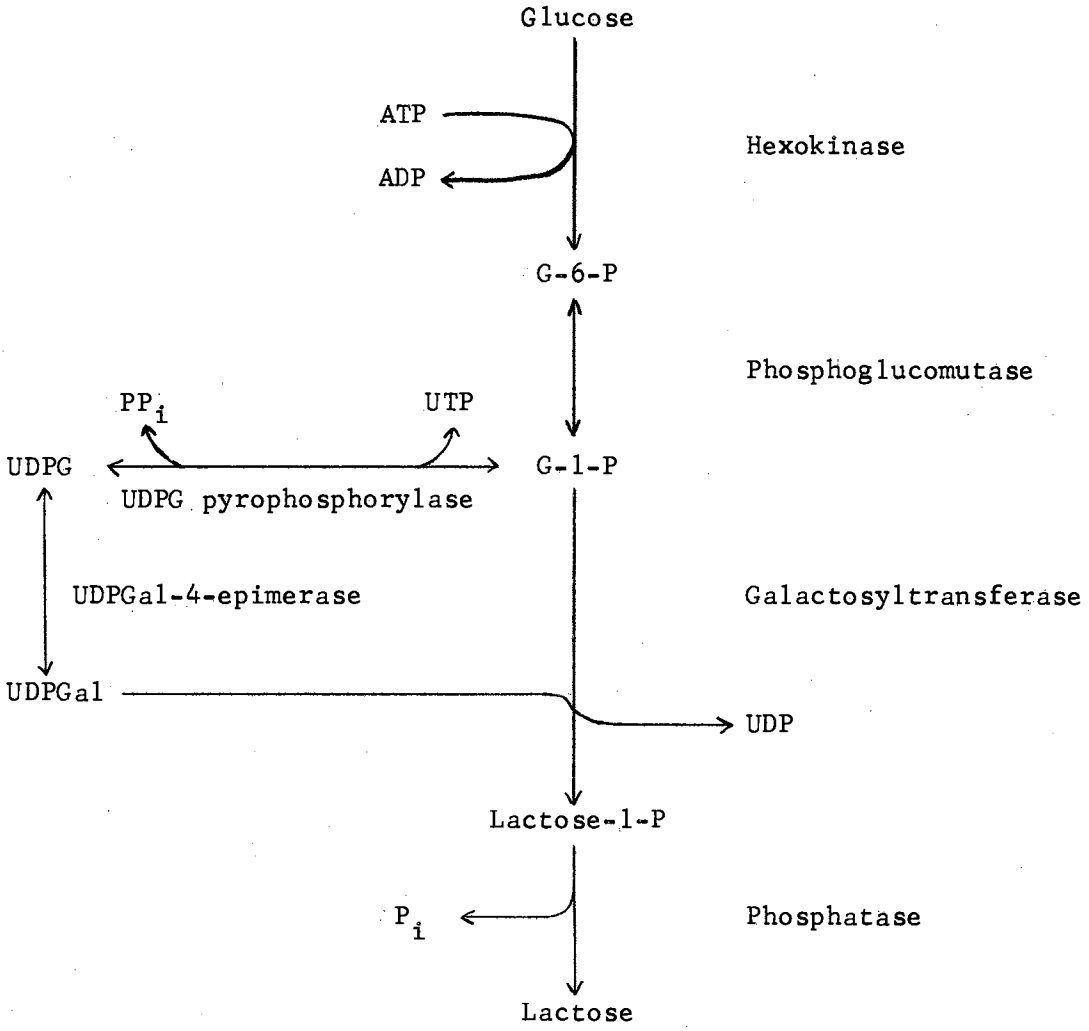
*Abbreviations are in accordance with the IUPAC-IUB Combined Commission on Biochemical Nomenclature, J. Biol. Chem., 241, 527 (1966).

assumed to be due to a nonspecific phosphatase (9). These results led Gander et al. to propose the following pathway of lactose synthesis from glucose:



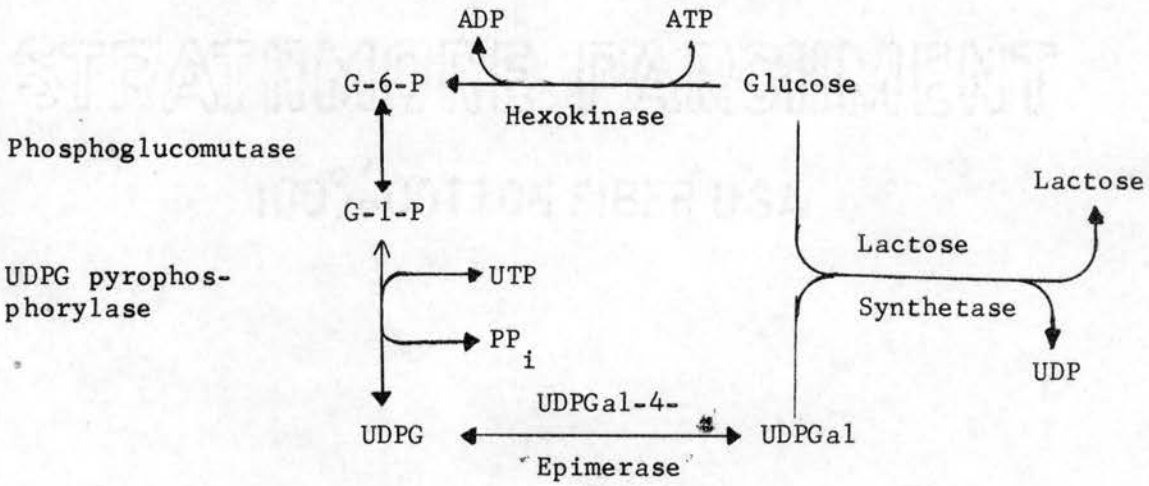
The final proof for this scheme is lacking since a specific phosphatase for the hydrolysis of lactose-1-P was never demonstrated in mammary tissue.

On the other hand incorporation studies with various possible lactose precursors were consistent with view that lactose is formed directly from UDPGal and glucose. When acetate-1-^{14}C was injected

intravenously into the bovine, the specific activities of the glucose and galactose moieties in lactose were always equal, due to the fact that all ^{14}C activity had passed through the plasma glucose pool (10). Significant differences, however, were observed by Schambye et al. (11) who completely degraded the individual hexoses in lactose, following intravenous injection of acetate-1-^{14}C. The greatest activity was in carbon atoms 3 and 4 of both hexoses, while there was more activity in carbons 1 and 2 of galactose than in the corresponding atoms of glucose. Using the perfused udder technique, Wood, Schambye and Peeters (12) investigated the mammary gland metabolism of 1-^{14}C acetate. In another experiment Wood, Siu and Schambye (13) injected labeled acetate intra-arterially into one side of the cow udder. In the perfusion experiment practically all the radioactivity in lactose was found in galactose; very little was found in glucose. This labelling pattern was in sharp contrast to the results obtained from intravenous injection but was confirmed by the intra-arterial injection studies. These authors concluded that the pathway of galactose synthesis from acetate and its subsequent incorporation into lactose, does not coincide with the pathway of incorporation into glucose. Studies in vitro by Reithel et al. (14) showed that lactose may be formed directly from glucose in homogenates of guinea pig mammary tissue under anaerobic conditions. Later, Kittinger and Reithel (15), working with guinea pigs, obtained lactose from both ^{14}C-glucose and ^{14}C starch with approximately equal labelling in the hexose moieties of lactose. As a result of these and other experiments, the authors were led to the view that the galactose moiety of lactose arises from G-1-P and the glucose moiety from glycogen.

Hansen et al. (16) presented additional evidence for free glucose

as the galactosyl acceptor in the intact cell. When the isolated bovine udder was perfused simultaneously with glucose 2-^{14}C and glycerol-1,3-^{14}C, the glucose moiety of lactose was labelled almost exclusively in C-2 and the galactose moiety was labelled predominantly in C-2, C-4, and C-6, and also in C-1 and C-3, but little in C-5. Glycerol alone, on the other hand, labelled the galactose predominantly in C-4 and C-6. If G-1-P were the galactosyl acceptor, both the glucose and galactose moieties of lactose should have had the same labelling pattern. More recently, Carubelli et al. (17) isolated lactose from lactating rat mammary gland slices incubated with glucose-U-^{14}C for 1 min. The fact that lactose was labelled exclusively in the glucose moiety was taken as further evidence that lactose is formed from glucose and not from G-1-P. The following biosynthetic pathway was adopted:



Recently, evidence for this pathway was furnished by Bartley, Abraham and Chaikoff (18). The incorporation of various isotopes of D-glucose, uniformly labelled D-galactose-^{14}C and D-mannose-1-^{14}C into lactose was studied in slices from lactating mammary glands. Three findings suggested that D-glucose and not α-D-G-1-P, was involved in lactose

biosynthesis:

a) The extent of incorporation of radioactivity from variously labelled forms of glucose into lactose was the same regardless of the site or kind of the label ^{14}C or ^{3}H.

b) The addition of unlabelled glucose was required for incorporation of ^{14}C from D-mannose-1-^{14}C into lactose.

c) Lactose formed from D-mannose-1-^{14}C and unlabelled glucose was labelled exclusively in the galactose portion.

In 1962 Watkins and Hassid (19) were able to demonstrate that lactose biosynthesis occurred in a particulate preparation from lactating guinea pig or cow mammary glands and that the substrates which gave the most rapid synthesis of lactose were UDP-D-Gal and D-glucose. No evidence was obtained with either particulate or cell-free preparations for the formation of lactose-1-P.

The enzyme catalyzes the following reaction:

$$\text{UDP-D-Gal} + \text{D-glucose} \longrightarrow \text{Lactose} + \text{UDP}$$

and is called UDP-D-galactose: D-glucose-galactosyltransferase (EC 2.4.1.C) or more commonly: lactose synthetase which is the name that will be used throughout this dissertation.

A similar enzyme was isolated and purified 70 fold from bovine milk by Babad and Hassid (20, 21). The milk enzyme appeared to be specific for UDP-D-Gal and no other galactose containing dinucleotide (guanosine, adenosine, cytidine, thymidine) could serve as substrate for lactose synthetase. PP_i, ITP, UTP, UDP, P_i, UMP, TTP, α-D-Gal-1-P, lactose, uridine and dUMP were found to inhibit the enzyme (listed in order of their decreasing inhibition). No inhibition could be shown with ATP,

CTP or D-galactose.

α-D-G-1-P, α-D-Gal-1-P, L-glucose, D-xylose, maltose or α-D-methyl-D-glucoside would not act as an acceptor for the D-galactose moiety of UDPGal. However, N-acetyl-D-glucosamine was 25 percent as effective as D-glucose (21). The lactose synthetase reaction was found to be irreversible; it showed maximum activation by 40 mM Mn^{++} and was inhibited by EDTA and Hg^{++}. The K_m for UDP-D-Gal was 5.0×10^{-4} M and for glucose was 2.5×10^{-2} M. The enzyme had a temperature optimum of 42° and pH optimum of 7.5 (21).

Recently, we have shown (22) that the soluble enzyme from milk has two protein components, called A and B. Individually neither protein exhibits any catalytic activity. A working hypothesis is that the A and B-Proteins are naturally occurring subunits of lactose synthetase and activity is dependent upon some type of AB complex.

The purpose of the research described in this dissertation was to purify and study the properties of the enzyme responsible for lactose synthesis in the bovine. The first part presents evidence that lactose synthetase requires the two proteins, A and B, for activity. The purification, crystallization and some properties of the B-Protein will be described as well as the partial purification of the A-Protein. The second part is concerned with the particulate lactose synthetase from mammary glands, its subcellular distribution and solubilization and some properties of the microsomal A and B-Proteins.

CHAPTER II

LITERATURE REVIEW

It was demonstrated during the course of this investigation that lactose synthetase was isolated as two protein fractions, both of which are necessary for catalytic activity. The protein fractions have been designated as A and B and are considered to be naturally occurring subunits of lactose synthetase. Similar types of observations have been made with a number of other enzymes which were isolated from bacterial systems. In this literature review an attempt will be made to summarize the properties of the enzymes that have naturally occurring subunits. To date such observations have been made with the following four enzymes:

1. Tryptophan Synthetase (EC 4.2.1.20)
2. Glutamate Mutase
3. (+)-Citramalate Hydro-lyase (Hydro-lyase EC 4.2.1)
4. Glycine Decarboxylase

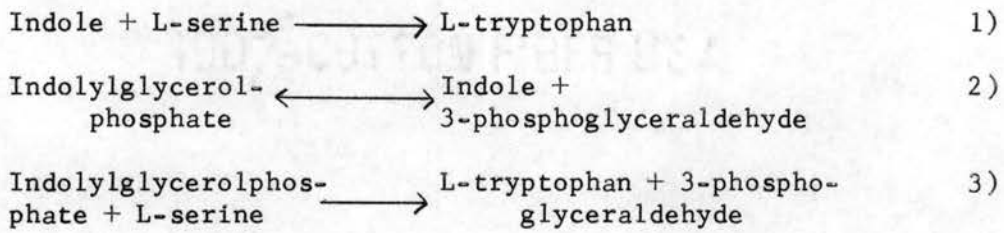
The term "subunit" is somewhat nebulous and in this dissertation will refer to smaller protein fragments of the native protein. Subunits may result from a) the chemical cleavage of the protein, b) the interaction of allosteric modifiers with certain regulatory enzymes or c) natural occurrence. The subunits may be isolated separately and catalysis is dependent upon their re-combination.

The occurrence of subunits in many proteins and enzymes has become apparent and the prevalence of such structures has suggested that there

may be some biological significance to this phenomena (23). There is also evidence to indicate that subunit structure is involved in the control of enzymatic activity (24). The uniqueness of lactose synthetase as well as the four enzymes listed above is that they apparently exist in nature largely as subunits and that the regulation of the enzymatic activities involves the control of the interaction of the subunits to yield the active enzymatic complex.

Tryptophan Synthetase

Over the past 10 years, Yanofsky and his coworkers have investigated the properties of tryptophan synthetase (EC 4.2.1.20, L-serine hydrolyase (adding indole)). This enzyme was isolated from E. coli and catalyzes the final step in tryptophan synthesis (25). The enzyme consists of two proteins designated as A and B, and the AB complex can catalyze the following three reactions (25, 26, 27):



The B subunit can catalyze Reaction 1 even in the absence of subunit A. The A subunit can catalyze Reaction 2 in the absence of the B subunit. Both, Reaction 1 and Reaction 2, proceed best when catalyzed by the complex formed from the two subunits. Reaction 3 takes place only in the presence of the AB complex (25).

The small molecular weight A protein was purified about 80 fold and crystallized from ammonium sulfate at pH 6.2 (28). The ultra-centrifugal

and the electrophoretic pattern showed no impurities in the A protein. The molecular weight of the A protein was determined by a modified Archibald method and calculated to be 29,500 $\pm$ 500. Dinitrophenol end group analysis of the A protein indicated that the A protein consisted of a single peptide chain having an amino terminal methionine residue (29). When A was degraded by a modified Edman paper strip method, the terminal sequence was:

$$H_2N\text{-Met-Glu-}NH_2\text{-Arg-Tyr}$$

Hydrazinolysis of the A protein showed only serine in significant amounts indicating the presence of one single COOH-terminal end group which provided further evidence that the A protein is a single peptide chain (30). The failure of carboxypeptidase A to digest the protein suggested the presence of either a lysine, arginine or proline residue near the carboxyl terminus. On the basis of the release of free amino acids upon addition of carboxypeptidase B and from the results of peptide composition studies, it was concluded that the COOH-terminal sequence of the A protein of tryptophan synthetase was:

Ala-Ala-Thr-Arg-Ser-COOH

To obtain evidence relating to the active site of the A protein, amino acid substitutions in mutants of E. coli were investigated. The amino acid substitutions associated with three types of tryptophan synthetase A gene mutants were located at two adjacent residues of a single peptide chain (31, 32). With each mutational event only one amino acid change could be detected which was associated with mutational changes in the A gene. Thus mutations in the A gene had been shown to result in

the formation of altered A proteins, which are enzymatically inactive. In addition, revertant strains which have a wild type enzymatic behavior also revealed single amino acid differences from the inactive mutant enzyme (32, 33, 34). Initial studies aimed at detecting the amino acid residues at or near the active site or sites of the A protein indicated a requirement for one or two intact cysteine residues for maximum enzymatic activity. Also the presence of a histidine and methionine residue at or near the site of substrate binding was suggested (35).

Wilson and Crawford (36) reported on the purification and properties of the B-protein of tryptophan synthetase. Treatment of a crude extract of E. coli with heat, protamine sulfate, precipitation with ammonium sulfate, chromatography on DEAE-Sephadex and a second heat treatment resulted in approximately 100 fold purified B-protein. The final product obtained by this purification appeared homogeneous in starch gel electrophoresis but showed minor quantities of a slower moving component when analyzed in the ultracentrifuge. The sedimentation coefficient ($S_{20,W}$) of the major component was 5.0 S and did not change with protein concentration in the range of 1.1 to 10.0 mg per ml. The average molecular weight for the B-protein, according to Archibald's method, was 108,000 $\pm$ 5 percent.

The combining ratio of the A and B subunits was determined by the method of Whitaker (37) on a column of Sephadex G-200. Since the molecular weight of the A subunit was calculated to be 29,500 (28), a molecular weight of 137,500 for the AB complex would indicate equimolar combination of subunits whereas a molecular weight of 167,000 would indicate that two A subunits combined with one B subunit. The elution volume observed yielded an estimated molecular weight of 163,000 for the

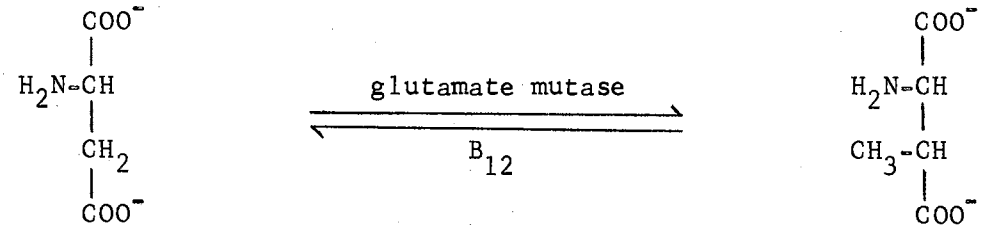
complex which indicated that the complex contained two A and one B subunits. This combining ratio was also indicated from stoichiometric calculation. For example 10^6 units of A activity in reaction 1) represented 7.2 μmoles of A protein whereas 10^6 units of B activity represent only 3.4 μmoles of B protein.

More recent results suggest that the B protein may itself be composed of two subunits (38). The A protein is a single polypeptide chain (29, 30) and therefore the fully formed complex should consist of four chains: two A protein molecules and one (dimeric) B protein molecule. These results were confirmed by Creighton and Yanofsky (39) by means of sucrose gradient centrifugation, Sephadex gel filtration and enzymatic activity measurements. Creighton and Yanofsky (39) designated the fully associated tryptophan synthetase as $\alpha_2\beta_2$, in which α is equal to the monomeric A protein and β_2 is equal to the B protein consisting of two identical polypeptide chains with two bound molecules of pyridoxal-P (36). β catalyzes Reaction 1, α catalyzes Reaction 2. Reaction 3 is catalyzed by the $\alpha_2\beta_2$ complex.

Pyridoxal-P and serine together markedly increase the association of the two subunits. Expressions for apparent association and dissociation constants were derived; the apparent association constants for the subunits were found to range from 4×10^6 to 2.6×10^9 M^{-1} under various conditions of incubation. The apparent rate constants for the association were estimated to be 2×10^4 to 6×10^5 sec^{-1} M^{-1}. Likewise, the dissociation rate constants determined were 4.8×10^{-3} to 1.8×10^{-4} sec^{-1} (39). Thus, the tryptophan synthetase complex is readily dissociable; the half life of the complex, however, may be increased by the presence of pyridoxal-P, serine and NaCl.

Glutamate Mutase

The first step in the fermentation of L-glutamate to pyruvate and acetate by extracts of Clostridium tetanomorphum is the conversion of L-glutamate to L-threo-β-methylaspartate (40, 41). This cobamide coenzyme-dependent glutamate mutase system (threo-3-methyl-L-aspartate carboxy-aminomethylmutase, EC 5.4.99.1) catalyzes the reaction:



L-glutamate L-threo-B-methylaspartate

Suzuki and Barker (42) isolated and purified the glutamate mutase system from extracts of C. tetanomorphum. Fractionation of a crude extract with calcium phosphate gel yielded two fractions, a gel supernatant fraction (designated by S) and a gel eluate fraction (designated by E) which appeared to be much more active when assayed combined than when tested separately. The S protein was purified by fractional precipitation at various pH values and by fractional ammonium sulfate precipitation (41). For the determination of mutase activities, two assay systems were used: an aerobic spectrophotometric assay and a static anaerobic assay. At first, it appeared that only the aerobic assay method seemed to be dependent on the S protein (40); however, more highly purified mutase preparations showed a requirement for the S protein in the anaerobic as well as the spectrophotometric assay (41).

The glutamate mutase reaction depended on cobamide coenzyme and

relatively high concentrations of sulfhydryl compounds (up to 40 mM mercaptoethanol were used). Maximal enzymatic activity was observed at pH 8.5 and at or below 40°.

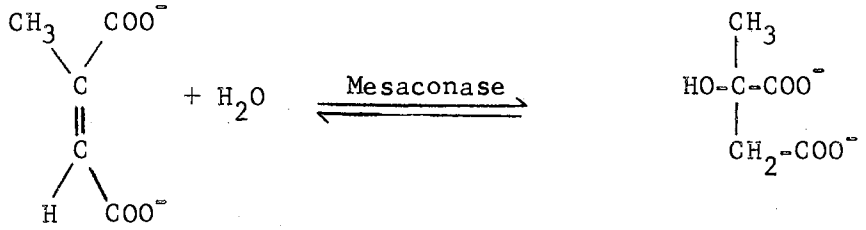
Extensive purification of component E led to a 75 to 80 percent pure protein (42) as judged by ionophoresis and ultracentrifucation. Charcoal and protamine sulfate treatment of a cell-free extract of C. tetanomorphum, ammonium sulfate fractionation and chromatography on DEAE-cellulose, CMC-cellulose and brushite columns resulted in a 180 fold purified component E which contained less than one percent of S-activity. Sedimentation analyses for the E component gave an $S_{20,W}$ value of 6.90, corresponding to a molecular weight of approximately 128,000. Component E was irreversibly inactivated by 50 mM Tris-HCl buffer, pH 8.0. However, this inactivation could be prevented completely by 0.3 M sodium glutamate, largely by 50 mM glutamate or less effectively by 50 mM methylaspartate. These results indicated that glutamate and methylaspartate had a special affinity for the E component.

As mentioned earlier both purified component E and partially purified component S were essential for glutamate mutase activity under either aerobic or anaerobic assay conditions. Determination of the influence of the relative amounts of the E and S components on mutase activity revealed that the optimal amount of component S clearly depended upon the absolute amount of component E. Likewise, the optimal amount of component S increased with the amount of component E in the assay system. At superoptimal levels of S, considerable inhibition occurred. Since, however, the component S preparation was not pure, the inhibition could have been caused either by an excess of component S per se or by an impurity in the preparation. When the E-protein was titrated against

saturating amounts of the S-protein, no inhibition at high concentration of E could be observed. In addition to inhibition by high levels of component S, the interaction between the two components was markedly influenced by the concentration of mercaptoethanol and depended, presumably, upon the extent of reduction of the system. However, it was not demonstrated whether one or both components needed to be reduced to obtain maximal mutase activity.

(+)-Citramalate Hydro-lyase

The enzymatic system (+)-citramalate hydro-lyase (hydro-lyase EC 4.2.1) is also involved in the fermentative degradation of L-glutamate to pyruvate and acetate. More commonly called "mesaconase" this enzyme catalyzes the reversible conversion of mesaconate to citramalate:



Mesaconate Citramalate

Blair and Barker (43) isolated and partially purified this enzyme from extracts of Clostridium tetanomorphum. When chromatographed on a DEAE cellulose column the enzyme could be resolved into two apparent protein fractions, called component I and II. The separation, however, was not absolute: after separation on DEAE component I was still contaminated with component II and vice versa. Selective extraction of an ammonium sulfate precipitate of component I and gel filtration on Sephadex G-100 resulted in an 80 fold purification of component I. Component II was

further purified by precipitation with ammonium sulfate and column chromatography on hydroxylapatite. The over-all increase in specific activity of component II was approximately 100 fold.

The molecular weights of the two components were estimated by gel filtration on Sephadex G-100. Component I was believed to have a molecular weight between 20,000 and 40,000 and component II above 10^5. In addition to their molecular weights, the components differed in ionic properties (which permitted an easy separation of DEAE) and in the sensitivity to inactivation by exposure to oxygen. Component II was rapidly and completely inactivated by exposure to oxygen, whereas component I was not affected by oxygen. To activate component II incubation with sulfhydryl reagents (cysteine, mercaptoethanol) and Fe^{++} was required prior to the assay. The proportionality between the rate of reaction and the amount of limiting component I or II was found to be linear between 0 and 25 µg protein of component I and between 0 and 30 µg protein of component II.

Though no active complex of this enzyme system has been demonstrated the authors assumed that components I and II were subunits of mesaconase. Mammalian aconitase (44) catalyzes a reaction similar to mesaconase but unlike the Clostridia enzyme it appears to contain no easily separable subunits. It is noteworthy that mesaconase is the second enzymatic system in C. tetanomorphum involved in the degradation of glutamate that consists of two protein components.

Glycine Decarboxylase

Peptococcus glycinophilus is an aerobic bacterium which ferments glycine to acetate, CO_2 and NH_3 by a pathway in which both carbons of

acetate are derived from the α carbon of glycine, and in which CO_2 is derived from the carboxyl group (45, 46). The initial step in this pathway involves the labilization of the glycine carboxyl group by the glycine decarboxylase system (47, 48). The exchange of bicarbonate with the carboxyl group of glycine:

$$\begin{array}{l} CH_2\text{-}{}^{14}COO^- \\ | \\ NH_3^+ \end{array} + HCO_3^- \underset{\text{pyridoxal-P}}{\overset{\text{glycine decarboxylase}}{\rightleftharpoons}} \begin{array}{l} CH_2\text{-}COO^- \\ | \\ NH_3^+ \end{array} + H{}^{14}CO_3^-$$

may be followed by measuring the incorporation of ^{14}C into $H^{14}CO_3^-$. Klein and Sager (47) resolved the glycine carboxyl-CO_2 exchange system into two protein components. Chromatography of a partially purified extract from P. glycinophilus on Sephadex G-100 resulted in two protein fractions, P_1 and P_2, both of which were necessary for the labilization of the carboxyl group. The high molecular weight protein P_1 was further purified by chromatography on DEAE-Sephadex; the over-all purification was approximately 60 fold. Extensive purification of the heat stable P_2 protein, the lower molecular weight fraction, resulted in a 220 fold purification. When P_2 was chromatographed on a hydroxylapatite column (the final step in the purification) it was resolved into two peaks both of which had P_2 activity of the glycine decarboxylase system. Since combination of these two peaks did not yield higher activity than the additive activity of each, it was suggested that P_2 existed in at least two states of molecular aggregation (47).

P_1 contained tightly bound pyridoxal-P which could be removed from the enzyme by treatment with cysteine, followed by dialysis. Incubation with low levels of pyridoxal-P fully restored the glycine decarboxylase

activity. The Michaelis constants for pyridoxal-P_5, glycine and bicarbonate were determined to be 4.6×10^{-6} M, 0.032 M and 0.031 M, respectively (48). When P_2 was titrated against saturating amounts of P_1 a rectangular hyperbola was obtained suggesting that the P_1, P_2 interaction followed Michaelis-Menton kinetics. Since the molecular weight of P_2 had not been determined, Klein and Sagers expressed the K_m value (1.3) of the P_2 protein as mg/ml rather than in molarity.

To date, four enzymes all isolated from microbial sources appear to exist as naturally occurring subunits and the combination of the subunits then forms the active catalytic protein complex. Lactose synthetase appears to be the first mammalian enzyme shown to exist as naturally occurring subunits. No doubt, other examples will appear in the very near future and will give further support to the view that many regulatory proteins consist of subunits and that certain types of metabolic control involves direct interaction of diversified metabolites with subunit proteins.

CHAPTER III

THE SOLUBLE LACTOSE SYNTHETASE FROM MILK

Experimental Procedure

Chemicals

UDP-D-Gal-1-^{14}C was synthesized enzymatically by the procedure described by Anderson et al. (49). The product was tested for purity by ascending chromatography in 7.5 volumes 95 percent ethanol plus 3.0 volumes 1 M ammonium acetate pH 7.5 (50), and by radioautography on No Screen film (Eastman Kodak) for nine days. The product was further checked by enzymatic conversion to UDPGlcUA by UDPGal-4-epimerase and by UDP-dehydrogenase (EC 1.1.1.22) and appeared to be pure by the above criteria. The over-all yield of the conversion of UDPG to UDPGal based on the enzymatic assay for UDPGal was 24.6 μmoles of UDPGal (37 ± three percent). More recently UDPGal-^{14}C (gal-^{14}C (U.L.)) (120 millicuries/millimole) was purchased from New England Nuclear Corp., Boston, Mass. Non-radioactive UDPGal was synthesized according to the method of Moffatt and Khorana (51, 52). The reaction mixture contained 1.5 mmole UMP and 2.0 mmoles Gal-1-P. UDPGal was separated from the reaction mixture by eluting from a DEAE-cellulose column (5 x 35 cm) with a linear gradient from 0 to 250 mM triethylamine-acetate pH 5.5, two liters each. UDPGal, 0.48 mmoles (32 percent based on UMP), were obtained when UDPGal was isolated according to the method of Moffatt (53).

Other special chemicals were obtained from the following sources: ATP, UTP, P-enolpyruvate, NADH and pyruvate kinase (type 1, crude) from Sigma; Bio Gel P-30 and Bio Rad AG 11A8 from Bio Rad Laboratories; Cellex D from Calbiochem and Biodryex from Lövdalens Industry Aktiebolag, Centralpalatset, Tegelbacken 2, Stockholm, Sweden. All other chemicals were reagent grade.

Unpasteurized bovine skim or whole milk was purchased from the Department of Dairy Science, Oklahoma State University. Fresh milk from the sheep and the goat were kindly supplied by Dr. Noble of the Animal Husbandry Department. The donor of the generous gift of human milk wishes to remain anonymous.

Methods

Unless otherwise stated all procedures were carried out between 0° and 4° C. Protein was usually determined by the method of Lowry et al. (54) with bovine serum albumin as standard (Mann Type V). However the concentration of the B-Protein after its separation on Bio Gel P-30 was estimated at 280 mμ. The value for $\mathcal{E}_{280}^{1\%}$ of the purified lyophilized B-protein was determined to be 17.1 and this value was used to estimate the concentration of the B-Protein. Because the B-Protein has more tryptophan residues than does the average protein, the method of Lowry et al. always gives too high a protein concentration.

Enzymatic Assay for Lactose Synthetase Subunits. Enzymatic activity was determined either by measuring the incorporation of UDPGal-^{14}C into lactose-^{14}C or by determining spectrophotometrically the amount of UDP formed (55). Kinetic experiments were consistent with the view that the

A and B-Proteins interact to form an enzymatically active AB complex, since A may be saturated by B and vice versa. Thus A or B activity could be estimated in the presence of saturating amounts of the counterpart. This type of assay was similar to the one used for estimating the enzyme subunits of tryptophan synthetase (56).

Spectrophotometric Assay. The spectrophotometric determination of UDP permitted a convenient assay for the enzyme (55). The amount of UDP formed during lactose synthesis was determined in the presence of P-enolpyruvate, pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27) and NADH which led to the formation of lactate and NAD as shown in the following equations:

$$\text{UDPGal} + \text{G} \xrightarrow[\text{(EC 2.4.1.c)}]{\text{Lactose Synthetase}} \text{UDP} + \text{Lactose}$$

$$\text{UDP} + \text{P-enolpyruvate} \xleftrightarrow[\text{(EC 2.7.1.40)}]{\text{Pyruvate Kinase}} \text{UTP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^{+} \xleftrightarrow[\text{(EC 1.1.1.27)}]{\text{Lactate Dehydrogenase}} \text{Lactate} + \text{NAD}^{+}$$

Pyruvate kinase contained sufficient lactate dehydrogenase necessary for the spectrophotometric determination of UDP. The rate of conversion of NADH to NAD^{+} was followed at 340 mμ on a Cary model 14 recording spectrophotometer.

The validity of the lactose synthetase assay depended on a reliable estimation of the UDP formed during lactose synthesis. A titration experiment showed that the change in absorbance at 340 mu was directly proportional to the amount of UDP added, e.g. 0.1 μmole of UDP gave a change in absorbance of 0.61 at 340 mμ which was equivalent to 0.1 μmole NADH

oxidized or lactose formed. Figure 1 shows the titration of UDP as a linear function of the change in absorbance at 340 mμ.

The standard assay mixture for lactose synthetase activity contained 1 μmole of P-enolpyruvate, 0.15 μmole of NADH, 50 μmoles of Tris-HCl, pH 7.4, 40 μmoles of $MnCl_2$, 0.084 μmole of UDP-D-Gal, 20 μmoles of D-glucose, 0.36 mg of pyruvate kinase, varying amounts of lactose synthetase, and water in a total volume of 1 ml. A NADH oxidase present in the soluble fraction of milk interfered with this assay, but it was completely inhibited by 40 mM $MnCl_2$. To correct for endogenous activity, the reference cuvette contained all of the reagents except the substrates for the lactose synthetase reaction. A unit of enzyme is defined as the amount of enzyme causing the formation of 1 mμmole of UDP per min.

The proportionality between the rate of reaction and the amount of the limiting protein fraction was determined with the A and B-Protein from skim milk, purified through the Bio Gel P-30 column. The proportionality between the initial rate of reaction and the amount of the limiting protein is shown in Figure 2 for the A-Protein and in Figure 3 for the B-Protein.

Incorporation Assay. Since the spectrophotometric assay was not sufficiently sensitive to assay for lactose synthetase in skim milk, an incorporation assay was used during the first steps of the purification procedure. A typical incorporation mixture contained the following components in a final volume of 0.1 ml: 5 μmoles of Tris-HCl, pH 7.4; 4 μmoles of $MnCl_2$; 2 μmoles of D-glucose; 6.3×10^{-2} μmole of UDP-D-Gal-1-^{14}C (3420 cpm); and varying amounts of enzyme. For routine analysis, Tris, $MnCl_2$ and glucose were added as a freshly prepared

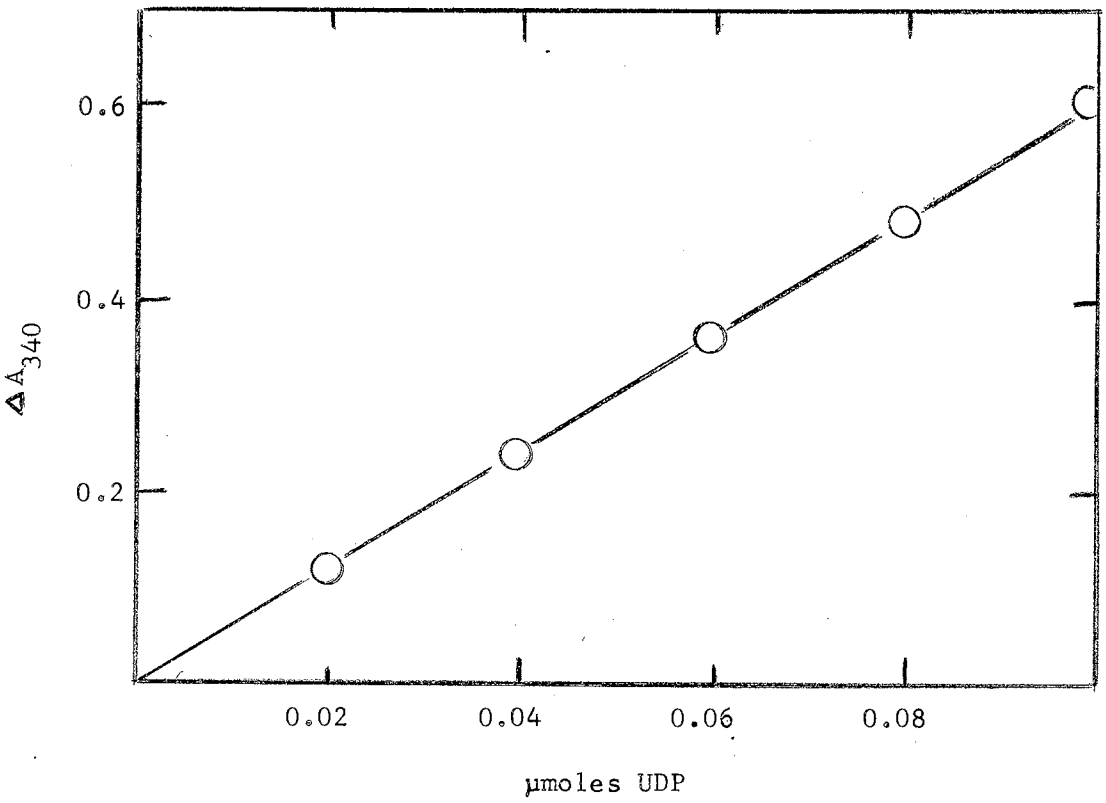


Figure 1. Enzymatic Titration of UDP

UDP was estimated as described in the text using the standard spectrophotometric assay for lactose synthetase activity. The total change in A_{340} is shown as a function of the number of μmoles of UDP added to an assay mixture.

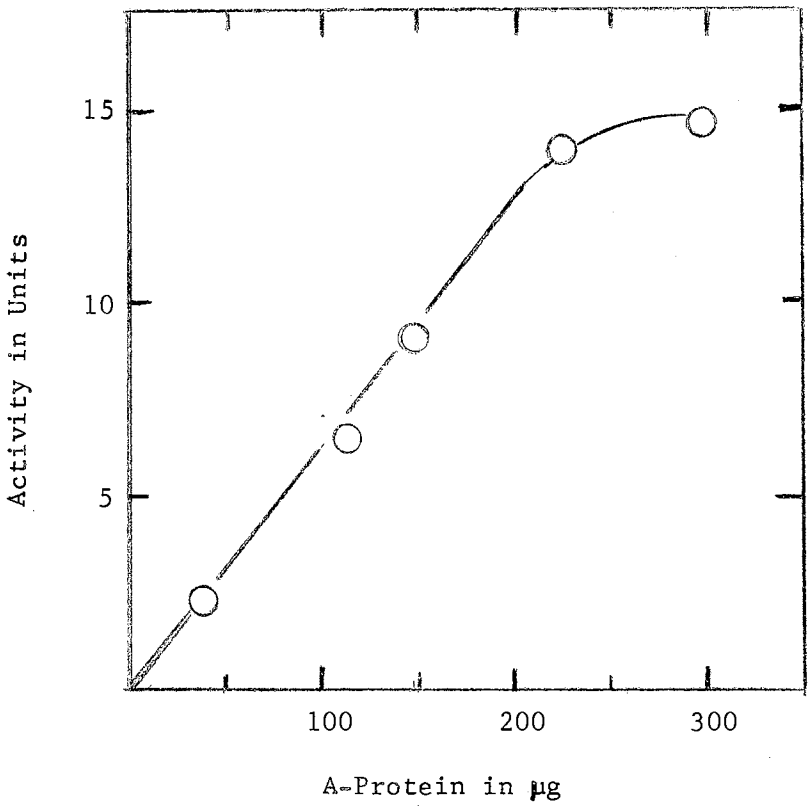


Figure 2. Spectrophotometric Assay for the A-Protein

The assay mixture contained 15 units of the B-Protein (18.8 µg of B) and varying amounts of the A-Protein. All other additions to the assay mixture were similar to those described in the text. Activity is plotted against micrograms of the A-Protein added to the assay mixture. Components A and B were obtained by separation of lactose synthetase on a Bio Gel P-30 column (3 x 160 cm).

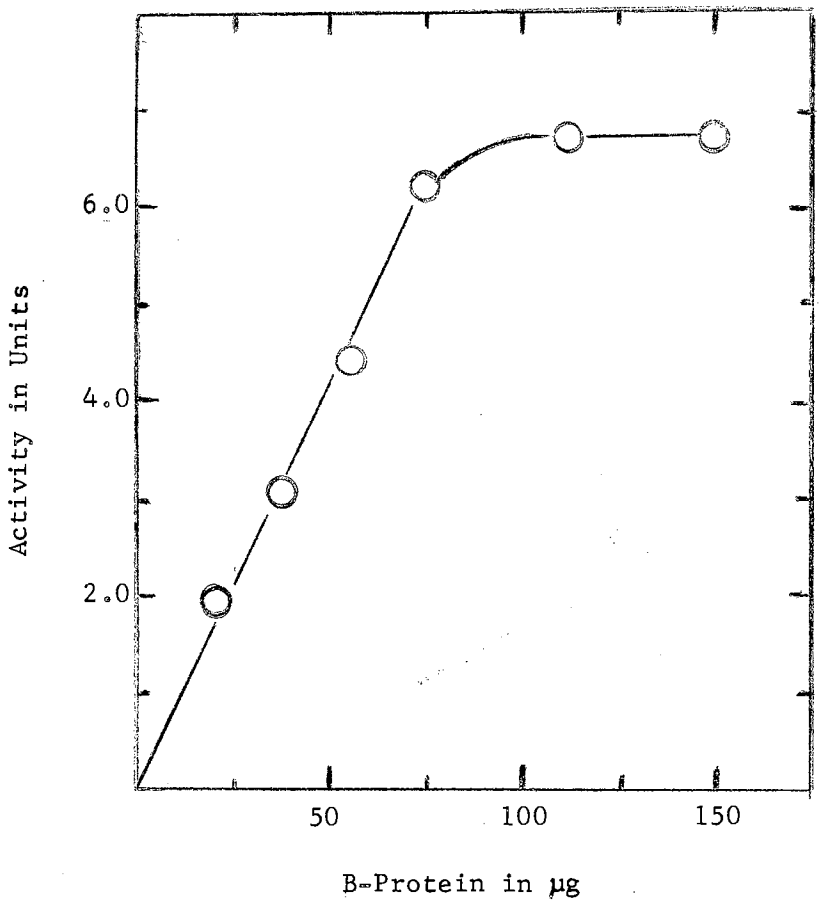


Figure 3. Spectrophotometric Assay for the B-Protein.

The assay mixture contained 6.8 units of the A-Protein (37.5 µg of A) and varying amounts of the B-Protein (see Figure 2). All other additions to the assay mixture were similar to those described in the text. Lactose synthetase activity is plotted against micrograms of the B-Protein added to the assay mixture.

mixture. Prolonged storage of this mixture caused the solution to turn brown due to oxidation of $MnCl_2$. After 5 to 30 min. of incubation at 37°, the reaction was stopped by adding 0.2 ml of 0.3 N $Ba(OH)_2$ to the ice-cooled mixture. This was neutralized with 1.5 volumes of a five percent solution of $ZnSO_4 \cdot 7H_2O$, and the precipitate was removed by centrifugation. The precipitate was suspended in 0.4 ml of H_2O by shaking the centrifuge tube vigorously with a Vortex mixer and the suspension was recentrifuged. The combined clear supernatant solution was passed through a column (0.5 x 8 cm) (disposable Pasteur pipette) containing Bio-Rad AG 11A8 and then through a Dowex 1-formate column (0.5 x 8 cm) placed directly below the Bio-Rad column. Both columns were equilibrated and eluted with 10 mM lactose. The Bio-Rad column removed salts which interfered with the quantitative adsorption of UDPGal-1-^{14}C on the Dowex-1 column. Four 2 ml fractions were collected and counted in the Tri-Carb liquid scintillation counter with Bray's scintillation mixture (57). An assay mixture containing no glucose was used to correct for small quantities of D-galactose-1-^{14}C formed during the incubation, whereas an incubation mixture containing ^{14}C-lactose was used to correct for the lactose adsorption on the columns as well as in the $BaSO_4$ precipitate. The average recovery of lactose was 95 percent. The rate of lactose synthesis was proportional to enzyme concentration up to approximately 60 percent incorporation of UDP-D-Gal-1-^{14}C into lactose, as shown in Figure 4.

A unit of enzyme is defined as the amount of enzyme that causes the formation of 1 mμmole of lactose-^{14}C per min. of incubation under the standard conditions of the assay. Specific activity is expressed as units per mg of protein. In both the spectrophotometric and incorporation

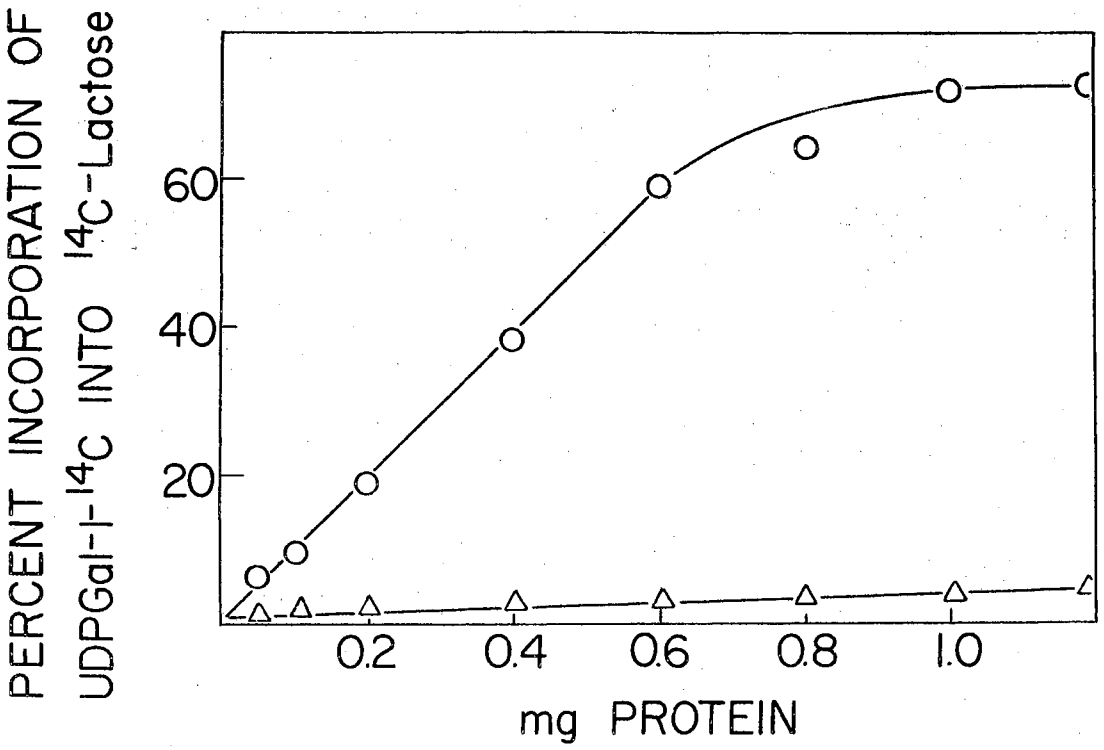


Figure 4. Percent Incorporation of UDPGal-1-^{14}C Into ^{14}C-Lactose

A partially purified soluble enzyme from bovine skim milk (65 percent ammonium sulfate precipitate), not separated into the subunits, was used as the source of the enzyme. O, rate of lactose formation; Δ, rate of endogenous radioactivity obtained in the absence of glucose.

assays it is very important to preincubate the reaction mixture at room temperature for three to five minutes. In the case of the spectrophotometric assay, all components were present in the cuvette during the incubation. The incorporation assay was started after a five minute preincubation period by the addition of UDP-galactose-^{14}C.

Since the incorporation assay is run at a temperature 12° C higher than the spectrophotometric assay, a correction had to be made to account for the differences in total units due to the temperature variation. Assuming a $Q_{10} = 2$ the two assays compared favorably and were interconvertible.

Other Methods. DEAE-cellulose was prepared and regenerated by washing batchwise with 0.5 N NaOH for 15 minutes, H_2O until neutral, 0.5 N HCl for 15 minutes, H_2O until pH 4 to 5, 0.5 N NaOH for 15 minutes and finally with H_2O until neutral. To equilibrate, the resin was washed with 1 M of the appropriate buffer at the desired pH and to ensure a correct pH, the pH was measured by placing the electrode directly into the suspension of the resin. Before use the cellulose was washed with the appropriate buffer until the conductivity of the effluent solution was equal to that of the buffer. The conductivity was measured with a Radiometer Model CDM 2 Conductivity Meter equipped with a flow through cell. Before packing a column care was taken to ensure that the pH and the ionic strength were at their desired values.

Regeneration of Biodryex--After using, the wet Biodryex was stored in 95 percent ethanol. To regenerate, the Biodryex was blended with two to three volumes of absolute ethanol for three minutes in a Waring Blendor. The resin was filtered on a Buchner Funnel and the above treatment was

repeated two more times. Then the Biodryex was washed with ether while on the Buchner funnel and was spread out on paper to dry at ambient temperature. The regenerated dry powder was stored in an airtight bag in the coldroom.

Preparation of Dowex-1 in the Formate Form--Used Dow-1(chloride) was recycled batchwise three times with 1 N HCl, H_2O and 1 N NaOH; the final wash was with 1 N HCl followed by H_2O. After recycling the Dow-1 (chloride) was washed in a large column (6 x 80 cm) with a solution of 4 N formic acid; 1 N ammonium formate until chloride ion could not be detected. Since the formate ion also precipitates with Ag^+ the following procedure was used to detect Cl^- in presence of formate ions. A few drops of the Dow-1 eluate were diluted 10 to 20 fold, acidified with three drops of 1 N HNO_3 and two to three drops of a solution of 1 N $AgNO_3$ were added. A blank, free of chloride ions, ensured that formate did not give any precipitation. After the resin was chloride ion free, two to three bed volumes of a solution of 80 percent formic acid were passed through the Dow-1 resin which then was washed until the pH of the effluent solution was between 5 and 6.

Regeneration of Bio Rad AG 11A8--This resin was regenerated by the procedure outlined in Calbiochem Technical Bulletin 113.

Results

Isolation of Lactose Synthetase From Skim Milk

Lactose synthetase is presumed to consist of two naturally occurring subunits A and B. After the separation of the two subunits different isolation procedures were employed to purify the A and the B-Protein

(Figure 5). The incorporation assay was used throughout the purification of the B-Protein except for the Bio Gel P-30 and the DEAE-column steps where the spectrophotometric assay was used. For the detection of the A-Protein activity the incorporation assay was used in the steps prior to the first ammonium sulfate precipitate. The other steps were assayed with the spectrophotometric assay.

All of the fractionation procedures were carried out between 0° and 4° C. The centrifugations were carried out at 2° to 4° at 15,000 x g for 20 minutes. Fresh unpasteurized bovine skim milk was cooled to 4° and the pH was adjusted to 4.6 by the addition of 2 N HCl dropwise over a period of 15 to 20 minutes. The precipitated casein was removed by centrifugation. After filtering through glass wool, the supernatant fluid was adjusted to pH 7.4 with 1 N Tris and then made 0.03 M in $MnCl_2$ by the addition of 1 M $MnCl_2$. After centrifugation, solid ammonium sulfate (243 g per liter) was added to the supernatant solution to bring the concentration to 40 percent saturation and the precipitate was discarded. The supernatant solution was brought to 65 percent saturation (168 g per liter) centrifuged and the precipitate was dissolved in a minimum volume of 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4. In a typical preparation, 1,200 ml of skim milk yielded 25 ml of a solution containing 95 mg protein per ml. To remove the ammonium sulfate, the solution was passed through a Bio Gel P-30 column (2 x 23 cm) equilibrated with 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4. Ammonium sulfate was detected with a conductivity meter and lactose synthetase activity was determined spectrophotometrically. The over-all purification of the combined eluates was about 20-fold; however the elution profile for lactose synthetase activity did not correspond to the protein elution pattern. The first

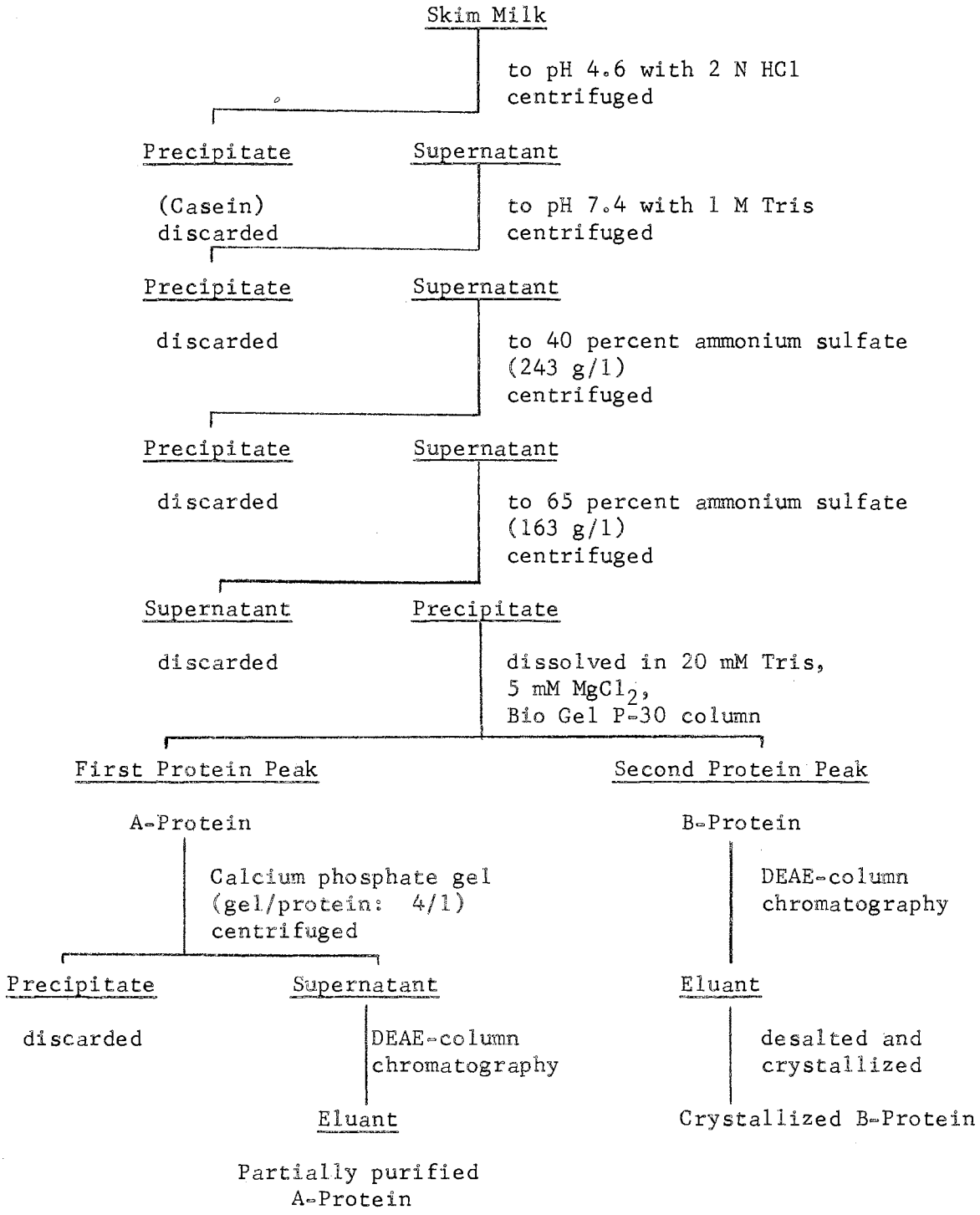


Figure 5. Summary of the Purification Procedures for the A and B-Proteins.

four to six fractions though high in protein, did not contain any lactose synthetase activity. Since these first few fractions exhibited strong NADH oxidase activity it was originally believed that upon chromatography on Bio Gel P-30 lactose synthetase was partially separated from the NADH oxidase. Chromatography of the enzyme on a Sephadex G-50 column (1.8 x 55 cm) in 20 mM Tris-HCl, 5 mM $MgCl_2$ resulted in a major and a minor protein peak but no lactose synthetase activity could be detected in either peak. This intriguing result led to a re-examination of the results obtained from the Bio Gel P-30 column.

Rechromatography of the enzyme on Bio-Gel P-30 resulted in an unsymmetrical protein peak with inactive protein on either side of the lactose synthetase activity curve. The fractions that contained lactose synthetase were combined. However the recovery of total units of enzymatic activity was very poor. When the remaining fractions from either side of the active enzyme were combined, lactose synthetase activity was observed, the total units obtained fully account for the apparent initial loss of activity. It therefore appeared that upon chromatography on Bio Gel P-30, lactose synthetase was separated into two fractions of different molecular weights. The fraction with the higher molecular weight was designated as A; the fraction with the lower molecular weight was designated as B.

Resolution of Lactose Synthetase Into Two Protein Fractions

To completely separate the two proteins, approximately 900 mg of protein from the 65 percent saturated ammonium sulfate precipitate dissolved in 10 ml of 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4, were applied to a column (3 x 160 cm) of Bio Gel P-30. The column was equilibrated and

eluted with the above buffer. Fractions, 3.5 ml each, were collected after the first 300 ml passed through the column. Lactose synthetase activity was determined spectrophotometrically for both the A and the B-Proteins. Fraction A was assayed in presence of material from the peak tube of fraction B and fraction B was assayed in presence of material from the peak tube of fraction A. Similar resolutions into the two fractions, A and B, were obtained from the skim milk of the sheep, goat and human. Figure 6 shows the distribution of protein (A_{280}) as well as lactose synthetase activity when fraction A was assayed in the presence of fraction B and when fraction B was assayed in presence of fraction A. With the spectrophotometric assay no lactose synthetase activity could be shown when either fraction was assayed individually. However some enzymatic activity could be observed in the A peak with the incorporation assay suggesting that (to some extent) lactose synthetase was present in the A peak as the AB complex. AB activity also could have been due to incomplete separation of the two subunits; however, this was ruled out by immunological studies which will be discussed later. Thus, by chromatography on Bio Gel P-30 the soluble lactose synthetase from the skim milk of the bovine, sheep, goat and human was completely resolved into two protein fractions.

It was of interest to compare the activities of the B-Protein isolated from these four sources when assayed in the presence of the A-Proteins from the other animals. The peak tubes (Figure 6) containing either the A or the B-Protein were assayed spectrophotometrically for lactose synthetase activity. In the presence of limiting amounts of bovine A-Protein, the addition of B-Protein from the sheep, goat and human gave approximately the same rates as did the bovine B-Protein.

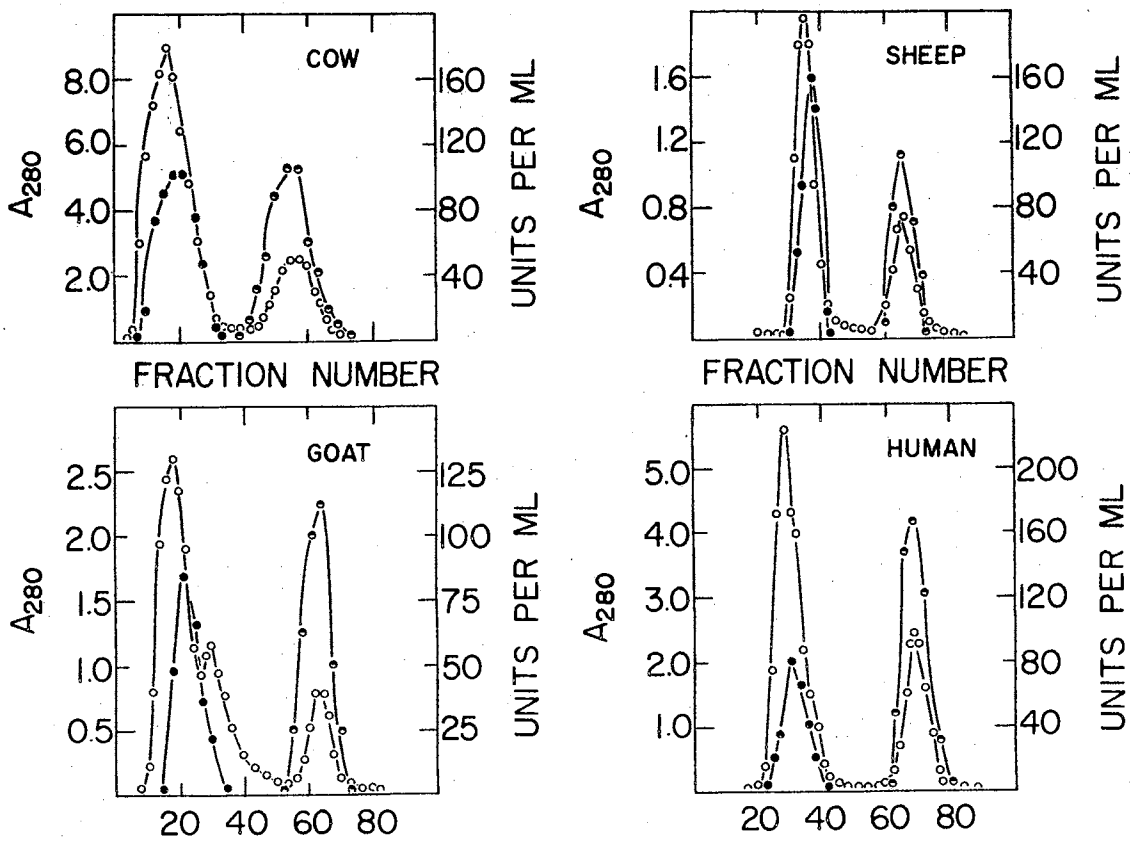


Figure 6. Gel Filtration of Partially Purified Lactose Synthetase From the Cow, Sheep, Goat and the Human on Bio Gel P-30.

O, protein distribution in eluate fractions (A_{280}); ●, lactose synthetase activity of the A-Protein in presence of 0.2 ml of the B-Protein obtained from the peak tubes of the B-Protein; ⊖, lactose synthetase activity of the B-Protein assayed in presence of 0.2 ml of the A-Protein obtained from the tubes with maximum A activity.

Furthermore, the A-Protein of the sheep formed an active AB complex with the B-Proteins from the bovine and the goat. Similar observations were made with the A-Protein of the goat and the B-Protein from the bovine and sheep.

These results suggested that the subunits of lactose synthetase from different species have the ability to form an enzymatically active complex, regardless of origin. It will be of interest to determine if the structures of the A and B-Proteins isolated from the various species are identical. Such studies would give some insight of the enzymatic mechanism.

The possibility existed that lactose synthetase may be separated into the two fractions due to treatment with ammonium sulfate or low pH, but this was ruled out by the following experiment. Skim milk, 400 ml, was centrifuged for four hours at 78,000 x g to remove the majority of the casein. The supernatant solution was concentrated to 30 ml by the use of Biodryex and 15 ml of this solution were passed through the Bio Gel P-30 column (3 x 160 cm) as previously described. The results obtained are shown in Figure 7 and were similar to those in Figure 6. They indicate that lactose synthetase exists as the two fractions, A and B, in skim milk.

Further Purification of the A-Protein

The A-Protein of lactose synthetase was further purified by negative adsorption on well-aged calcium phosphate gel and by chromatography on DEAE-cellulose. However to date no further studies on the purification of the A-Protein have been undertaken.

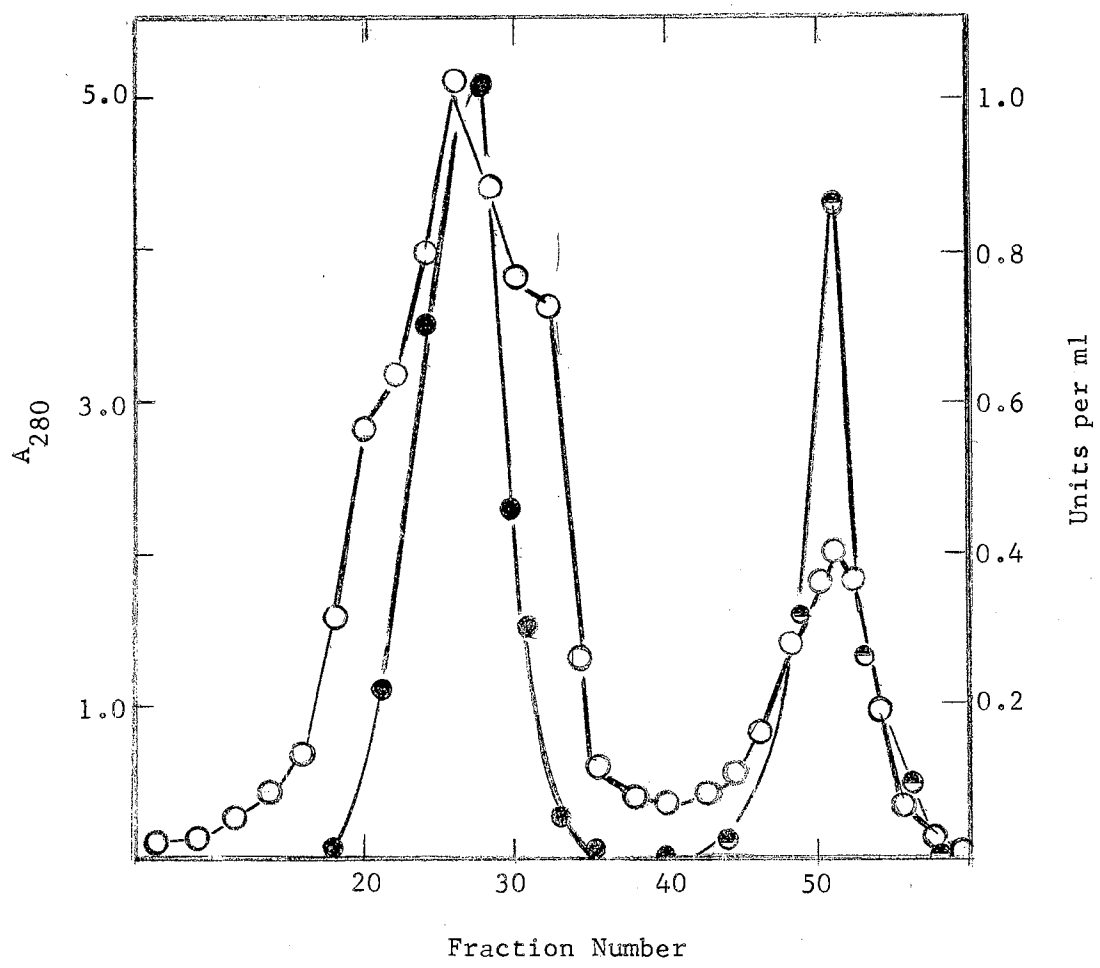


Figure 7. Gel Filtration of a Skim Milk Concentrate on Bio Gel P-30.

○, protein distribution in eluted fractions (A_{280}); ●, lactose synthetase activity of the A-Protein when assayed in presence of 0.2 ml of the B-Protein obtained from tubes 50 and 51; ⊖, lactose synthetase activity of the B-Protein assayed with 0.2 ml of the A-Protein obtained from tubes 28 and 29.

Calcium Phosphate Gel. After the separation of the subunits on Bio Gel P-30 the fractions containing A activity for lactose synthetase were pooled. To 100 ml of this solution, containing 3.7 mg protein per ml, a suspension of calcium phosphate gel (33.4 mg per ml, aged over two years) was added over a period of 15 to 20 minutes to give a final concentration of 4 mg gel per mg protein. After stirring for 20 minutes, the gel was removed by centrifugation and discarded. Almost all the A-Protein remained in the supernatant fraction but was adsorbed up to 30 percent when additional calcium phosphate gel was added to give a gel to protein ratio of two to one. Solid ammonium sulfate (516 g per liter) was added to 120 ml of the gel supernatant solution to concentrate the protein and after centrifugation the precipitate was dissolved in 6 ml of 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4. It appeared that the negative adsorption of the A-Protein on the calcium phosphate gel depended entirely on the age of the gel. Freshly prepared gel (58) proved to be less selective and only a three to four fold purification could be obtained, whereas a ten fold purification was obtained with the aged gel.

DEAE Column Chromatography. The solution from the previous step was passed through a column (2.5 x 30 cm) of Sephadex G-25 equilibrated and eluted with a solution of 1 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 8.0. The effluent was passed through the conductivity meter directly onto the DEAE column (2.5 x 11 cm) previously equilibrated with 1 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 8.0. When the conductivity increased sharply the Sephadex G-25 column was disconnected and the DEAE column was washed with 86 ml of the above buffer. A linear gradient between zero and 200 mM KCl (200 ml each) in the same buffer was used to elute the enzyme.

Fractions, 5 ml each, were collected and assayed spectrophotometrically for A activity. Figure 8 shows the protein distribution (A_{280}) as well as lactose synthetase activity of the A-Protein when assayed in the presence of saturating amounts of the B-Protein. The fractions containing A activity were pooled and concentrated with Biodryex.

The results of the purification of the A-Protein are summarized in Table I. In this scheme two different preparations of lactose synthetase are listed: preparation one was purified through the Bio Gel P-30 column giving a 15.6 fold purification. For the further purification of the enzyme another preparation was used and is listed in Table I as preparation 2. Starting from the P-30 column eluate, preparation 2 could be purified 27.4 fold, which then would give a 427 fold over-all purification of the A-Protein from skim milk through the DEAE column.

Further Purification and Crystallization of the B-Protein

DEAE-Column Chromatography. The B-Protein of lactose synthetase was further purified by chromatography on DEAE-cellulose. The fractions from the Bio Gel P-30 column containing the B-Protein were pooled and the solution (195 ml containing 1.75 mg protein per ml) was adjusted to pH 7.8 with 1 M Tris and passed through a DEAE column (5 x 25 cm) previously equilibrated with a solution of 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.8. After washing with the same buffer, the B-Protein was eluted with a linear gradient from 20 to 250 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.8 (300 ml in each chamber). Figure 9 shows the protein distribution (A_{280}) and the activity of the B-Protein when assayed spectrophotometrically in the presence of the A-Protein. The B-Protein was eluted as a nearly symmetrical peak suggesting that it was, at this stage, relatively pure

TABLE I

PURIFICATION OF THE A-PROTEIN OF LACTOSE SYNTHETASE

Step	Procedure	Prepa-ration	Volume	U/ml	Total Units x 10^{-3}	Protein	Total Protein	Specific Activity	% Recovery	X-Fold-Pur
			ml	$\frac{m\mu moles}{min \cdot ml}$	$\frac{m\mu moles}{min}$	mg/ml	mg	$\frac{m\mu moles}{min \cdot mg}$		
0	Initial Skim Milk	1	1000.0	56.2	56.20	33.700	33700.0	1.67		
1	Supernatant After Milk Addition		915.0	49.9	45.60	9.480	8650.0	5.26	81	3.2
2	Ammonium Sulfate Precipitate		18.4	1210.0	22.30	53.500	985.0	22.60	40	13.5
3	Pooled Tubes Off P-30		129.0	148.0	19.10	5.750	744.0	26.00	34	15.6
3	Pooled Tubes Off P-30		100.0	21.8	2.18	3.700	370.0	5.90		
4	Calcium Phosphate Gel Supernatant	2	120.0	13.4	1.61	0.145	17.4	92.5	74	15.7
5	Pooled Tubes Off DEAE		140.0	5.65	0.79	0.035	4.9	162.0	36	27.4

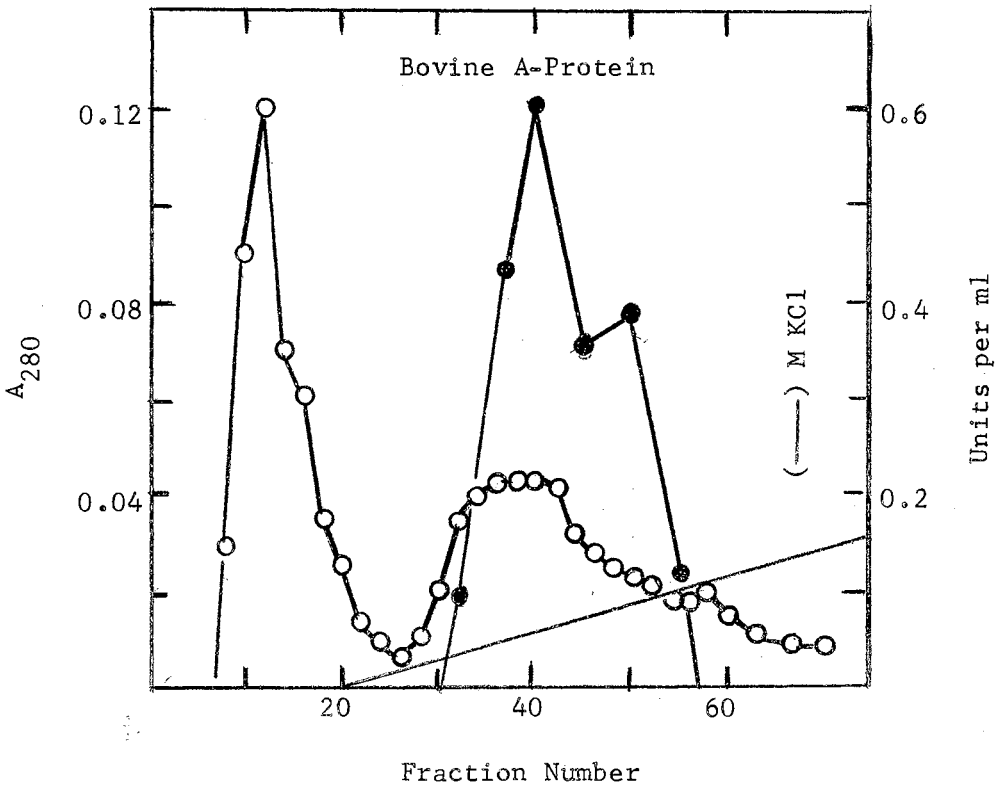


Figure 8. DEAE-Cellulose Chromatography of the A-Protein.

The ammonium sulfate precipitate (17.5 mg of protein) of the calcium phosphate supernatant solution was desalted and passed through a DEAE column (2.5 x 11 cm) previously equilibrated with 1 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 8.0. After washing the DEAE column with 86 ml of the above buffer the enzyme was eluted with a linear gradient (———) from zero to 200 mM KCl (200 ml each). Fractions, 5 ml each, were collected and assayed spectrophotometrically for A-activity. O, protein distribution in eluate fraction (A_{280}); ●, lactose synthetase activity of the A-Protein when assayed in presence of saturating amounts of the B-Protein (purified through the DEAE column).

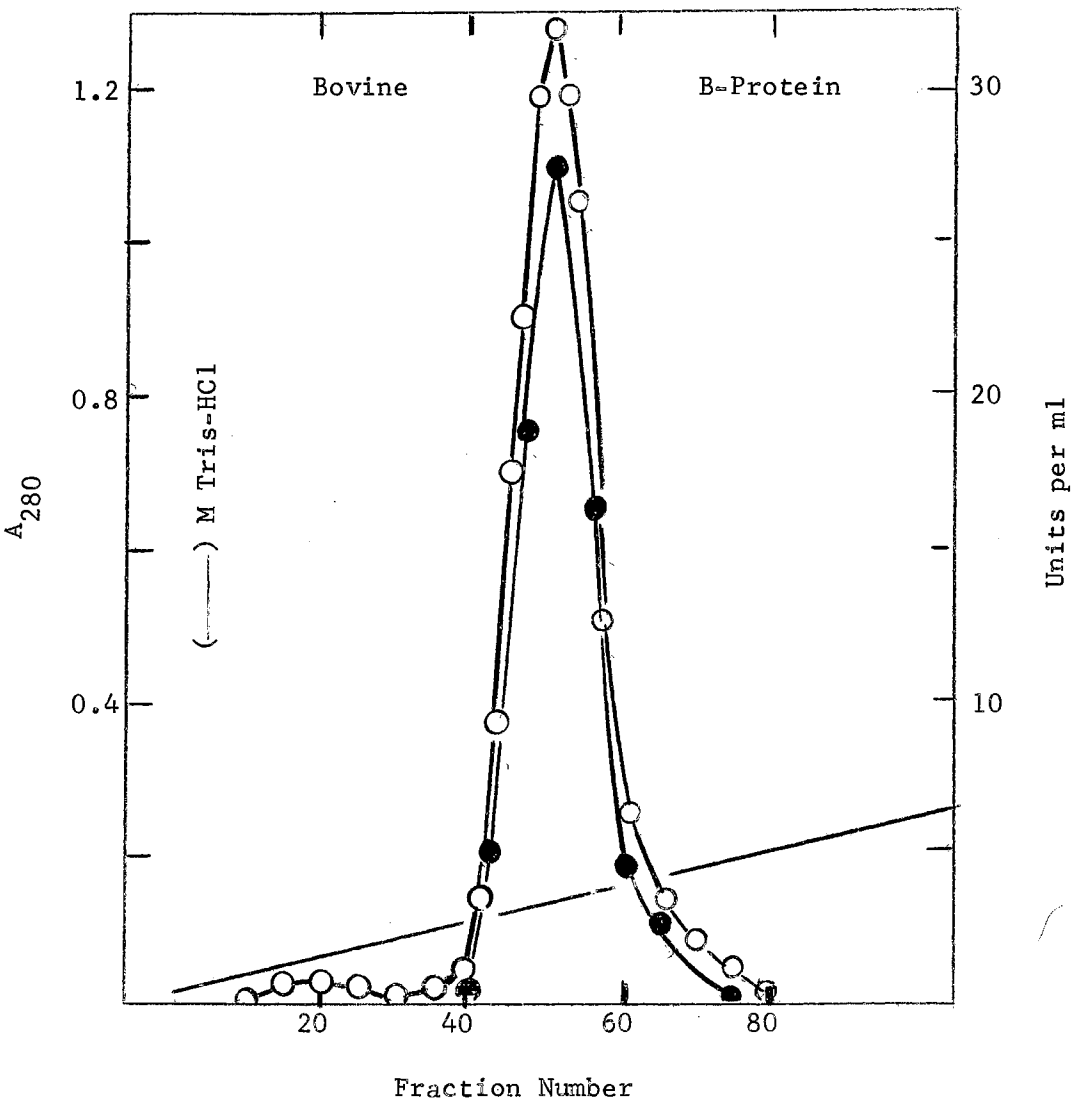


Figure 9. DEAE Column Chromatography of the B-Protein.

The solution containing the B-Protein from the Bio Gel P-30 column was brought to pH 7.8 with 1 M Tris and passed through a DEAE column (5 x 25 cm) previously equilibrated with 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.8. After washing the column with 100 ml of the same buffer, the B-Protein was eluted with a linear gradient (——) from 20 to 250 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.8 (300 ml in each chamber). O, protein distribution in eluate fractions (A_{280}) and ●, lactose synthetase activity of the B-Protein when assayed spectrophotometrically in the presence of the A-Protein (purified through calcium phosphate step).

with respect to charge. Table II summarizes the results of the purification of the B-Protein.

Crystallization of the B-Protein. The fractions from the DEAE column eluate containing B activity were pooled and solid ammonium sulfate (516 g per liter) was added to precipitate the B-Protein. After centrifugation the precipitate was dissolved in deionized water and the solution was passed through a Sephadex G-25 column equilibrated and eluted with water. The pooled protein fractions, free of any endogenous salt as checked by conductivity measurements, were lyophilized. The lyophilized product was a white amorphous powder. This material was divided into three parts and dissolved in water to a final concentration of approximately 10.7 mg protein per ml. Solutions of saturated ammonium sulfate of varying pH's, as determined with a pH-meter upon 1/50 dilution with H_2O, were added to the solutions containing the B-Protein. At pH 5.73 no formation of crystals could be observed. However crystallization occurred within 14 days after 1.10 ml of a saturated solution of ammonium sulfate, pH 6.6, were added in small portions to 1 ml of the solution of the B-Protein. Faster crystallization was observed at pH 7.54; however more amorphous material was found in this preparation than in the crystals obtained at pH 6.6. The final concentration of ammonium sulfate was 52 percent saturation at pH 6.6 and 54 percent saturation at pH 7.54. Figure 10 shows a photomicrograph of some of the crystals obtained at pH 6.6. The photograph was taken with a phase contrast microscope by Dr. Eric Noller, Department of Microbiology. The magnification was 515 diameters.

TABLE II

PURIFICATION OF THE B-PROTEIN OF LACTOSE SYNTHETASE

STEP	PROCEDURE	VOL.	U/ml	TOTAL UNITS $x10^{-3}$	PROTEIN	TOT. PROT.	SPEC. ACT.	% RECOV.	X-FOLD-PUR
		ml	$\frac{m\mu moles}{min \cdot ml}$	$\frac{m\mu moles}{min}$	mg/ml	mg	$\frac{m\mu moles}{min \cdot mg}$		
0	Initial Skim Milk	4000	80.5	322	33.7	134800	2.39	100	
1	Supernatant After $MnCl_2$ Addition	3660	68.1	249	9.48	34700	7.18	77.3	3.0
2	Ammonium Sulfate Precipitate	73.5	2648	194	53.5	3930	49.5	60.2	20.7
3	Pooled Tubes Off P-30	195	845	164	1.75	341	483	50.9	202
4	Pooled Tubes Off DEAE	240	697.4	167.4	1.16	278	601	52.0	251

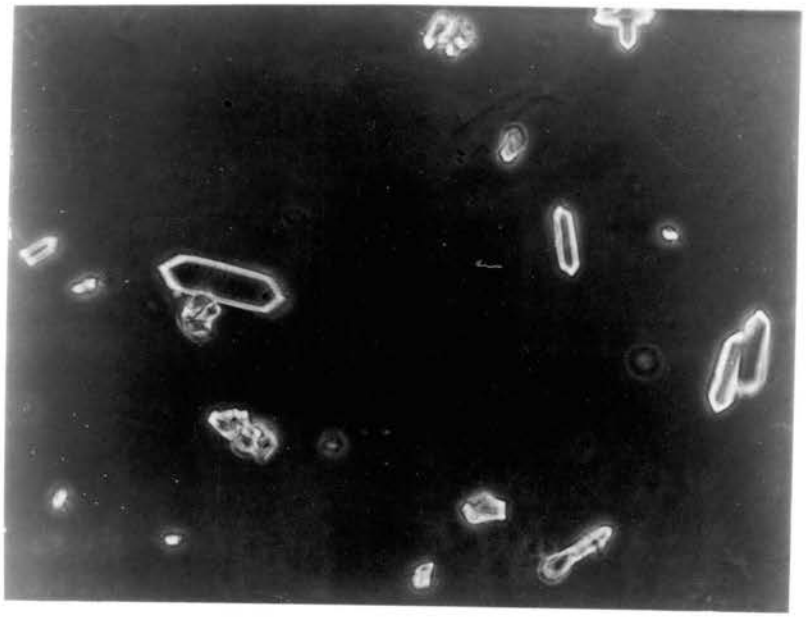


Figure 10. Photomicrograph of Crystalline B-Protein.

The crystals were photographed by a Zeiss phase contrast microscope equipped with a Leitz photographic head. Magnification: 515 diameters.

Properties of the A-Protein

No extensive experiments were done on the determination of the properties of the A-Protein because the major effort has been the purification and characterization of the B-Protein. However observations made during the partial purification indicate some general features of the A-Protein. Incubation at 55° C for two minutes resulted in a loss of 60 percent of the total activity, whereas there was a complete loss of activity when incubation was one minute at 65° C. When stored at 4° C or at -5° C at pH 7.4, activity was lost over a period of 15 to 30 days. However, when stored at 4° C in the presence of 10 mM $MnCl_2$, at pH 7.4, 85 percent of the original activity could be recovered after 50 days. The A-Protein also could be lyophilized from a solution containing 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4 and reconstituted by the addition of water, without significant loss of activity. Therefore it appears that divalent ions (Mg^{++} or Mn^{++}) not only activate the enzyme but also aid its stabilization.

Fractionation of the A-Protein with acetone at -5° to -10° C led to considerable loss in activity. The A-Protein precipitated between 33 and 45 percent acetone but only 47 percent of the total units were recovered and the increase in specific activity was less than two fold. The molecular weight of the A-Protein has not been determined, but the elution pattern obtained on Bio Gel P-30 indicates that it is above 30,000.

Properties of the B-Protein

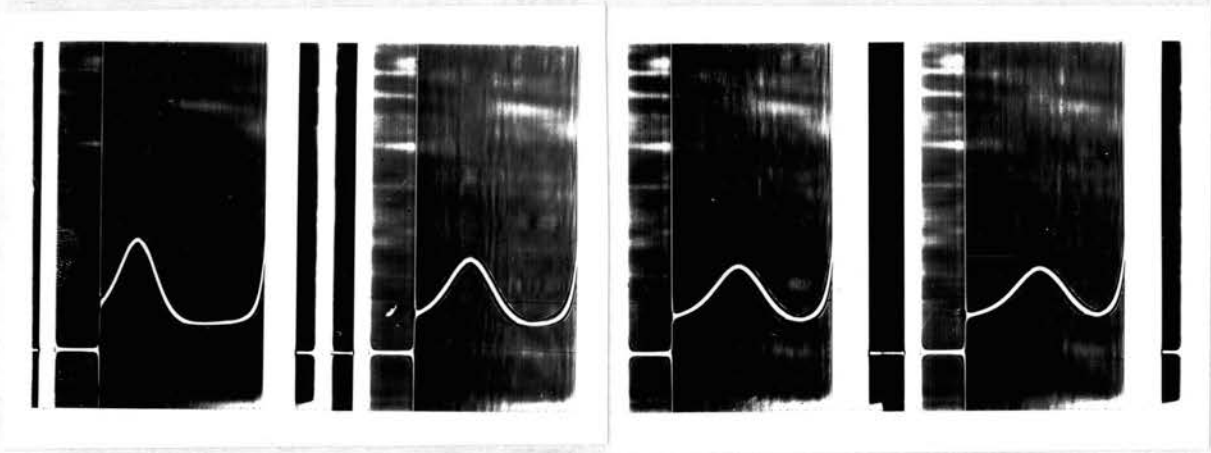
The B-Protein was easy to purify since it had a high resistance

towards heat at neutral pH. Initial attempts to separate the two subunits of lactose synthetase by heat denaturation of the A-Protein and other inert proteins, thus omitting the Bio Gel P-30 column, were unsuccessful since the B-Protein coprecipitated with the other proteins. The best separation of the A and B-Proteins was achieved by chromatography of lactose synthetase on Bio Gel P-30 at 4° C.

The B-Protein could be precipitated with an equal volume of 10 percent trichloroacetic acid. After centrifugation the precipitate was dissolved in 0.25 M Tris-HCl, pH 7.4; full B activity for lactose synthetase was retained. In the purified form the B-Protein (1.16 mg/ml) withstood boiling for 20 minutes at pH 7.4 without losing activity. However, digestion with papain at 40° C for 24 hours at pH 5.2, followed by destruction of the papain as described by Hill and Schmidt (59) resulted in complete loss of B activity.

The B-Protein, eluted from the DEAE column, was concentrated with Biodryex to 21 mg protein per ml in 20 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4, and analyzed in the Beckman Model E ultracentrifuge. Figure 11 shows a series of Schlieren patterns obtained from the B-Protein; the patterns were photographed at 105, 150, 180 and 200 minutes after the centrifuge reached its full speed at 59,780 rpm. The B-Protein sedimented as a single symmetrical peak indicating its homogeneity in the ultracentrifuge. The $S_{20,W}$ for the B-Protein was calculated to be 1.70 assuming a partial specific volume of 0.735 (60).

Early observations on the spectral properties of the B-Protein showed that the ultraviolet spectrum of the B-Protein differed from most proteins by having a distinct shoulder at 290 mμ and an unsymmetrical peak between 285 and 270 mμ. Figure 12 shows a typical ultraviolet



105 150 180 200

Time in minutes

Figure 11. Schlieren Pattern of the B-Protein

The sedimentation pattern of the B-Protein (purified through DEAE, 21 mg protein per ml) in 75 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.8, were photographed at the time indicated after the centrifuge reached its full speed of 59,780 rpm. Camera enlargement ratio is 2.1083; bar angle is 75°; and the temperature is 20° C.

spectrum of the bovine B-Protein obtained after purification through the DEAE column, and a difference spectrum measured against the B-Protein dissolved in 0.1 N NaOH. The normal ultraviolet spectrum resembles a tryptophan spectrum and suggests that there are exposed tryptophanyl residues present in the B-Protein. The difference spectrum is typical for a mixture of tryptophan and tyrosine residues. The extinction coefficient of a one percent solution of desalted lyophilized B-Protein was 17.1. The tyrosine to tryptophan ratio was 1.06 as calculated by the method of Goodwin and Morton (61). Figure 13 shows the ultraviolet spectra of the B-Proteins (from P-30) isolated from the milk of the goat, sheep and the human, and the spectrum of prolactin. This relatively small protein also showed a distinct shoulder at 290 mμ but it was not as intense as in the case of the B-Protein.

These apparent spectral similarities of prolactin and the B-Protein first suggested that prolactin might be identical to or at least related to the B-Protein. However, prolactin (NIH-P-S_4, Endocrinology Study Section, Natl. Inst. Health (NIAMD)) at the same or higher concentrations as the B-Protein did not substitute for the B-Protein in the spectrophotometric assay for lactose synthetase activity. Also the concentration of the B-Protein in milk was relatively high (above 100 mg per liter of skim milk). These findings indicated that the B-Protein was not prolactin. Dr. Bruce Larson (University of Illinois) pointed out that the properties of the B-Protein closely resembled those of α-lactalbumin including the molecular weight which was estimated to be about 15,000 from the elution off the Bio Gel P-30 column. In fact, all the characteristics listed above for the B-Protein agree quite closely with those for the α-lactalbumin.

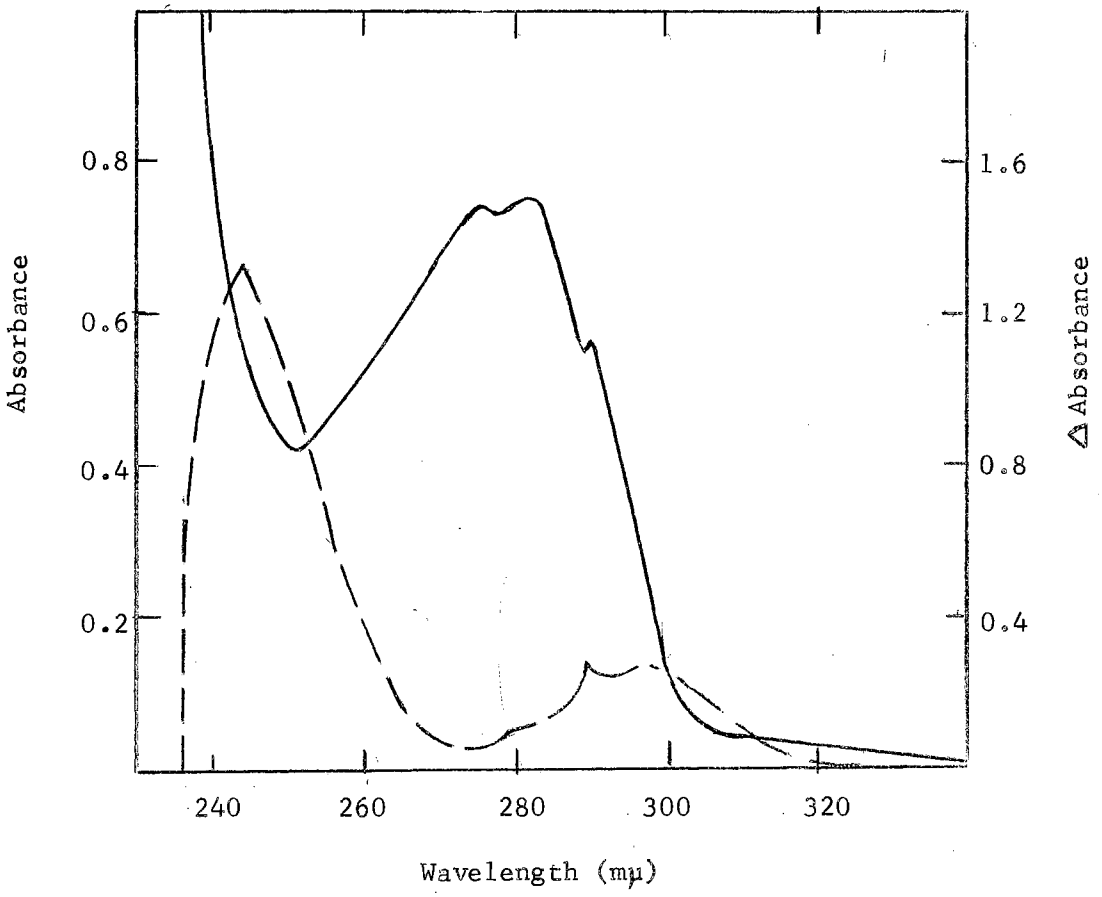


Figure 12. Ultraviolet Spectra of the B-Protein From Bovine Milk.

(———), Spectrum of the B-Protein (0.44 mg per ml of water) obtained after lyophilization of the desalted solution of purified B-Protein after DEAE column chromatography. (- - - - -), Difference spectrum of the B-Protein in water against B-Protein in 0.1 N NaOH.

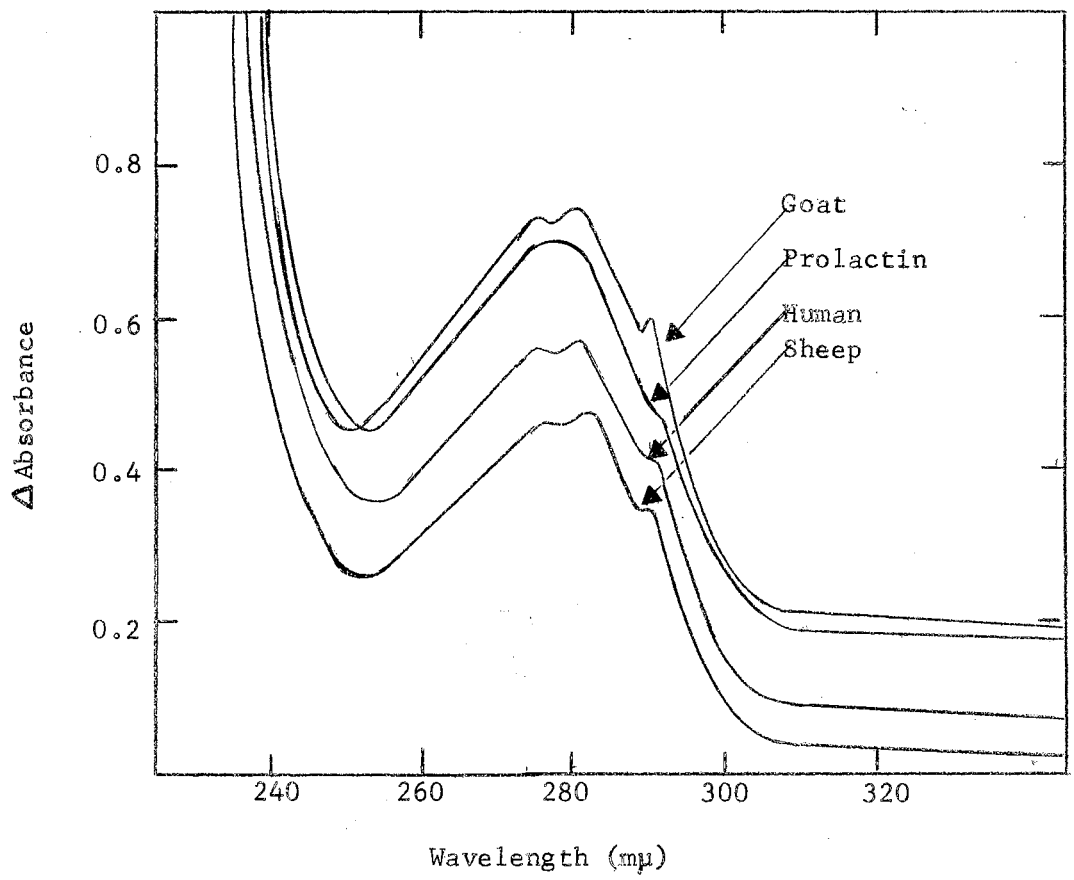


Figure 13. Ultraviolet Spectra of the B-Proteins From the Milk of Sheep, Goat and Human and of Prolactin.

The B-Proteins were obtained from the Bio Gel P-30 columns in 20 mM Tris, 10 mM $MgCl_2$, pH 7.4. Prolactin (0.5 mg per ml) was dissolved in the same buffer.

Accordingly the ability of 2x, 3x and 5x crystallized α-lactalbumin to substitute for the B-Protein was determined. The B-Protein preparation used for the comparison to α-lactalbumin was crystalline material obtained from the DEAE column eluate. The precipitate obtained between 50 and 65 percent ammonium sulfate at pH 6.6 was passed through a G-25 column in water and lyophilized. The preparation is actually amorphous but corresponds to crystalline α-lactalbumin which is a similar type of amorphous preparation (62). The specific activities of the crystallized B-Protein, 2x, 3x and 5x crystallized α-lactalbumin were, when assayed spectrophotometrically at saturating levels of A-Protein (84 ug), 79.6, 81.0, 80.5 and 81.6 units per mg, respectively.

The correspondence of α-lactalbumin to the B-Protein was determined by titration of the A-Protein against varying levels of the B-Protein and α-lactalbumin. Figure 14 shows the saturation curve when 84 μg of the A-Protein (purified through the calcium phosphate step, approximately 200 fold) were titrated against varying but identical protein levels of the B-Protein and α-lactalbumin. Figure 15 shows the reverse titration in which varying amounts of the A-Protein were titrated against a constant amount of the B-Protein (17.8 ug) and α-lactalbumin (18.0 ug). Figure 16 shows the relationship between lactose-^{14}C formation and varying amounts of a mixture of A-Protein and α-lactalbumin or A and B-Proteins, with the B-Protein and α-lactalbumin at essentially the same concentration. Both the B-Protein and α-lactalbumin were saturated by the A-Protein in the mixture.

Other data also strongly indicate that the B-Protein of lactose synthetase is α-lactalbumin. Immunological assays of the B-Protein were carried out by Dr. B. Larson using the Oudin technique as previously

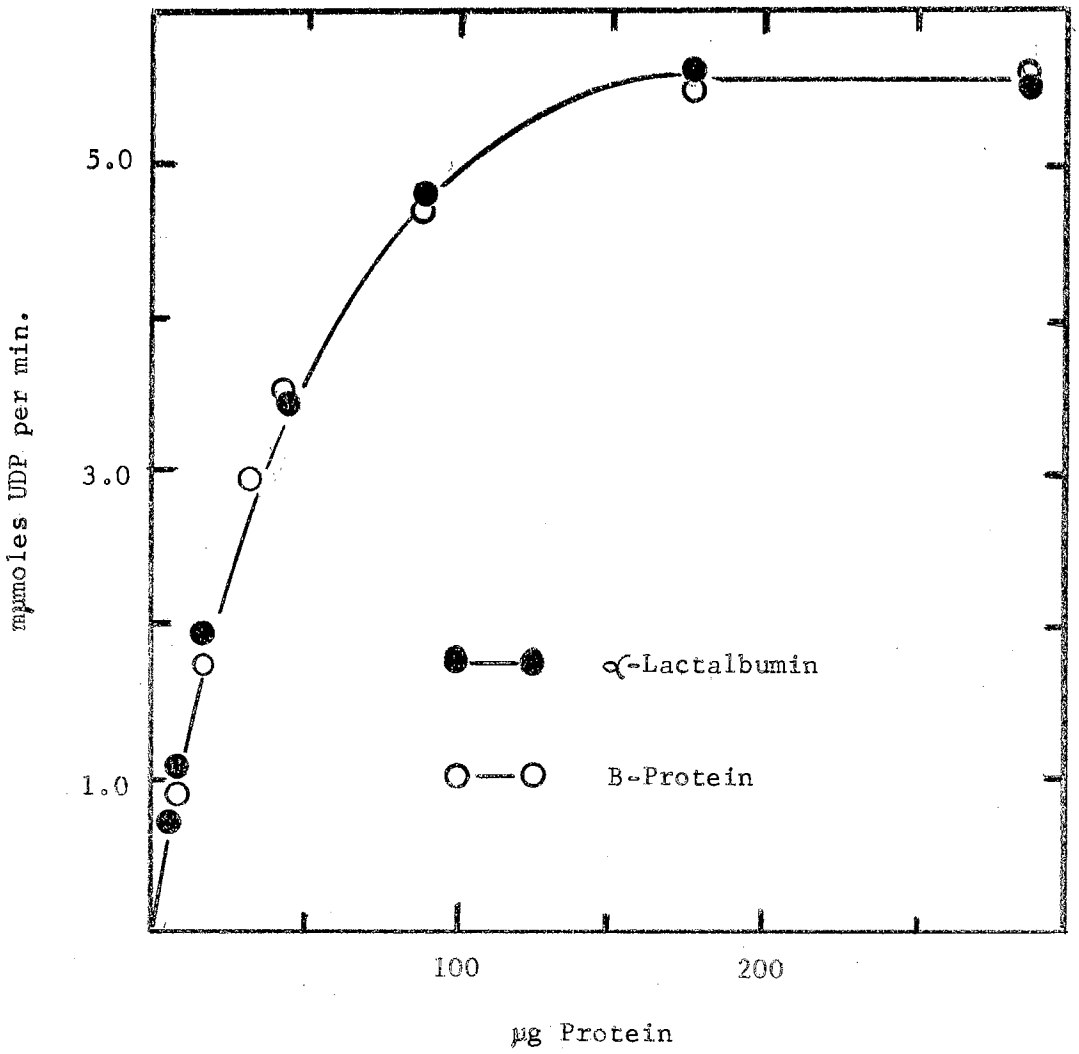


Figure 14. Titration of the B-Protein and α-Lactalbumin by the A-Protein.

The rate of UDP formation is plotted against varying amounts of the B-Protein or 3x crystallized α-lactalbumin when assayed spectrophotometrically in the presence of a constant amount (84 μg) of the A-Protein.

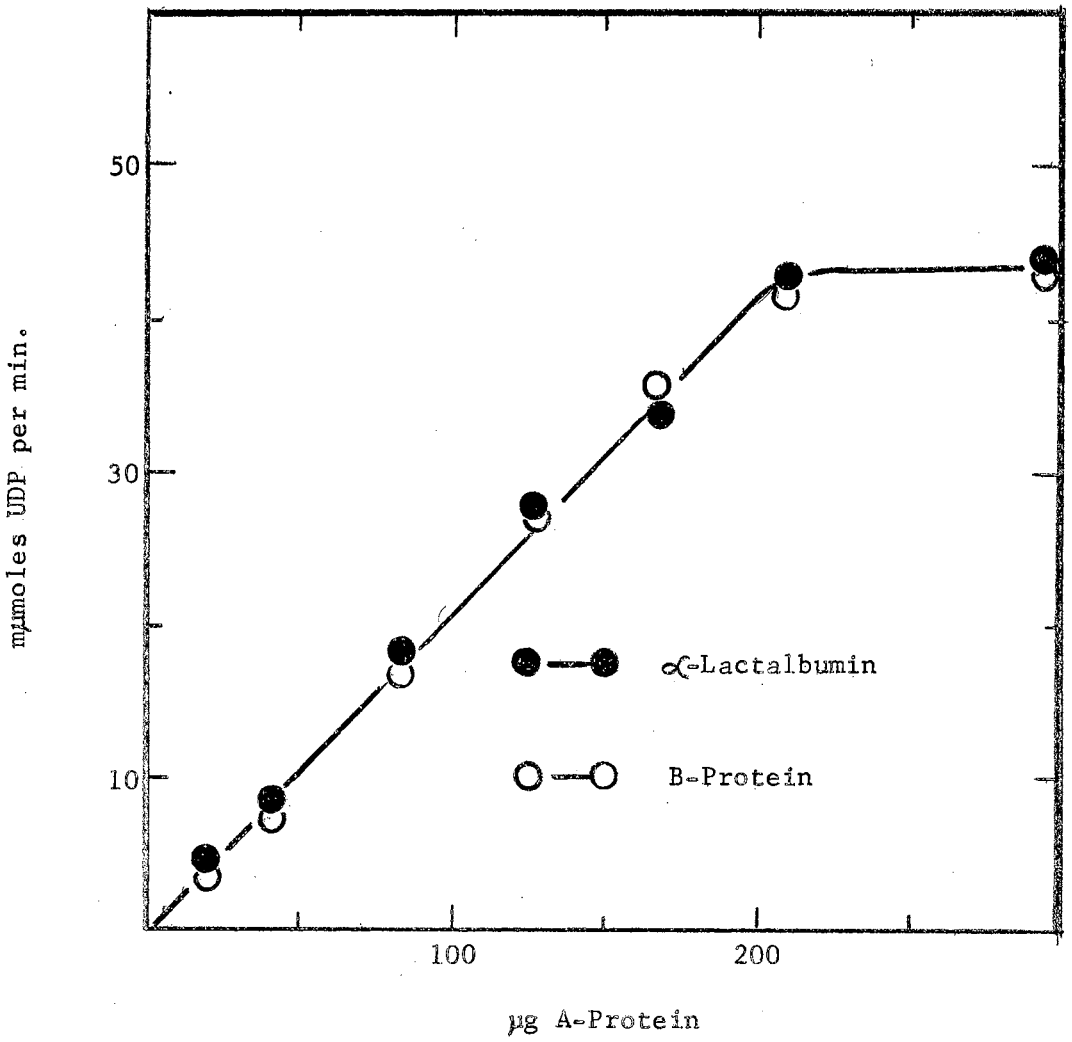


Figure 15. Titration of the A-Protein by the B-Protein or α-Lactalbumin.

The rate of UDP formation is plotted against varying amounts of the A-Protein when assayed spectrophotometrically in the presence of a constant amount of 3x crystallized α-lactalbumin (18.0 μg) or B-Protein (17.8 μg).

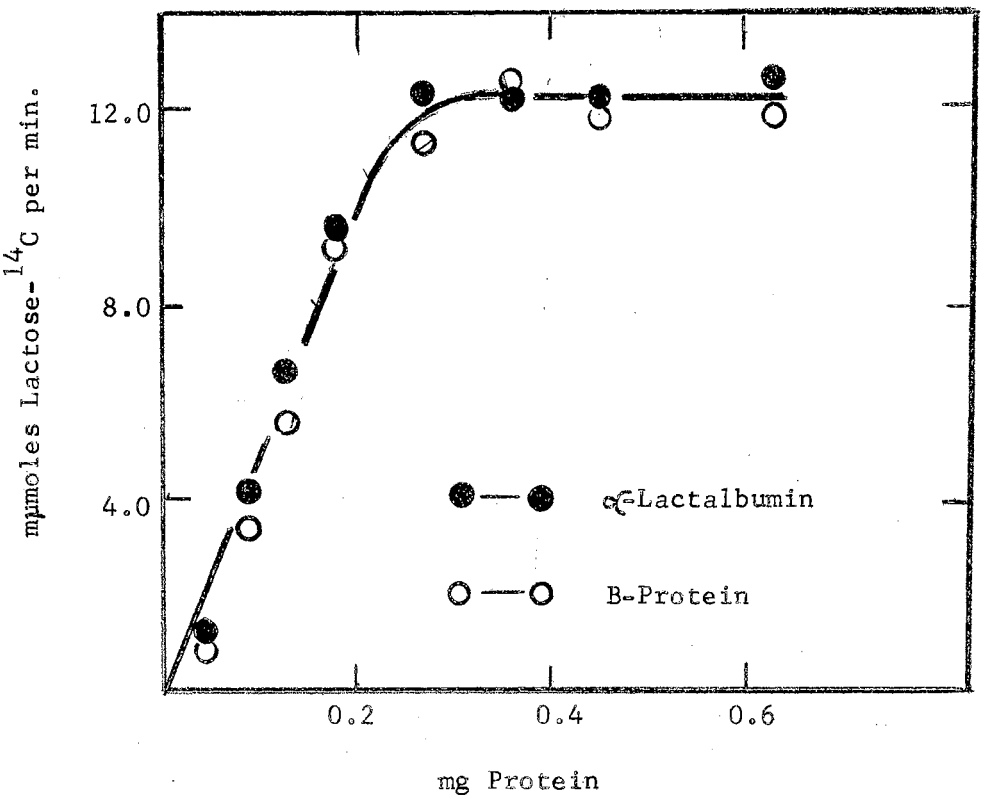


Figure 16. The Rate of ^{14}C-Lactose Formation When the B-Protein or α-Lactalbumin was Saturated with the A-Protein.

A mixture was formed from one volume of B-Protein or α-lactalbumin (each at 0.88 mg per ml) and one volume of A-Protein (17 mg per ml) in 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4. Under these conditions the B-Protein and α-lactalbumin were saturated by the A-Protein. O, mixture containing A and B-Protein; ●, mixture containing A-Protein and α-lactalbumin. The mg protein refers to the protein content of the mixture.

described for the determination of α-lactalbumin in complex mixtures (63). Antibodies were prepared from five times crystallized α-lactalbumin isolated from bovine milk. Immunological analysis of the B-Protein showed it to be a minimum of 91 percent α-lactalbumin, which is equivalent to about 3x crystallized α-lactalbumin.

Spectral data give additional evidence that the B-Protein of lactose synthetase is α-lactalbumin. For example, the ultraviolet spectra are identical and the ratio of A_{280}/A_{290} is 1.31 for the B-Protein in good agreement with 1.32 for α-lactalbumin as reported by Wetlaufer (62). The crystal forms of the B-Protein are similar to those reported by Aschaffenburg and Drewry (64) for α-lactalbumin, which was shown to crystallize as elongated parallelepipeds occasionally arranged in rosettes (65).

Reexamination of the extinction coefficient of the B-Protein gave a value of $E_{280}^{1\%} = 22.3$. This value is somewhat higher than the one reported by Wetlaufer (62) for α-lactalbumin.

All the present data agree with the view that the B-Protein is identical to α-lactalbumin. However, the possibility still does exist, that both the B-Protein and α-lactalbumin contain a small amount of contaminating protein which might account for the activity. On the other hand such a possibility is highly unlikely since the amount of the contaminant would have to be equal in all preparations used. α-Lactalbumin is usually prepared from the whey proteins of milk by ammonium sulfate fractionation and isoelectric precipitation (66). The B-Protein has been prepared from the whey proteins of milk by chromatography on Bio Gel P-30, DEAE chromatography and subsequent crystallization. It is highly unlikely that a minor contaminant would appear at the same

concentration in preparations obtained by different procedures.

α-Lactalbumin is a major constituent of the whey proteins of milk and has been recognized for some twenty years though no biological function other than a nutritional one could be ascribed for this protein. The above results show that α-lactalbumin can substitute for the B-Protein of lactose synthetase in the rate and incorporation assays at equal protein concentrations and that the B-Protein as isolated is immunologically identical to α-lactalbumin. These and other chemical and physical similarities support the conclusion that α-lactalbumin is identical to the B-Protein, one of the subunits of lactose synthetase.

CHAPTER IV

LACTOSE SYNTHETASE FROM MAMMARY TISSUE

Experimental Procedure

Chemicals

Sodium cholate and Tween 80 were obtained from Mann Biochemicals; sodium lauryl sulfate, digitonin, the venom of Trimeresurus flavoviridis and UTP from Sigma; and Linde 3A molecular sieves from Linde (Union Carbide). All other chemicals used were the same as described in Chapter III. Mammary tissue from lactating cows was obtained from the Wilson Packing Plant, Oklahoma City, and in some cases from Dr. B. L. Larson, University of Illinois.

Methods

Protein was determined by the method of Lowry et al. (54). Unless otherwise stated, all procedures were carried out between 0° and 4° C. The mammary tissue from freshly slaughtered cows or rats was freed from excessive fat and connective tissue quickly frozen in dry-ice and stored at -15° C.

Preparation of an Active Particulate Fraction From Bovine Mammary Tissue. Watkins and Hassid (19) reported that lactose synthetase is associated with a particulate fraction which contains mitochondria and microsomes.

A preliminary subcellular distribution study showed that the majority of the enzymatic activity was associated with the microsomal fraction. As a result of these experiments the procedure of Watkins and Hassid was modified so that larger amounts of particles could be prepared.

Frozen mammary tissue was thawed, cut into small pieces and passed three times through a mechanical meat grinder. One hundred g portions were homogenized at 0° C for 20 seconds in 250 ml of 0.25 M sucrose with a Virtis overhead homogenizer at a variac setting of 80. The mixture was centrifuged at 15,000 x g for 20 minutes and the supernatant solution, containing large amounts of fat, was filtered through glass wool. To increase the yield of microsomes, the precipitate was resuspended in 0.25 M sucrose (approximately 1/4 of the original amount used), homogenized and recentrifuged as indicated above. The combined supernatant solutions were centrifuged at 50,000 x g for 90 minutes, the microsomal pellet was washed twice in 0.25 M sucrose and resuspended to give a concentration of approximately 40 mg protein per ml in 0.25 M sucrose. The amount of microsomal protein thus obtained varied between 70 and 340 mg per 100 g of mammary tissue depending upon the quality and age of the tissue. Less microsomes were obtained from tissue that had been stored frozen for a long time. The microsomes could be stored in 0.25 M sucrose at 4° C for up to 14 days without significant loss of enzymatic activity.

Enzymatic Assay for Lactose Synthetase From Mammary Tissue. Enzymatic activity in all preparations from mammary tissue was determined by measuring the incorporation of UDPGal-^{14}C into lactose-^{14}C as described in Chapter III except for the addition of UTP. Watkins and Hassid (19)

reported the presence of a phosphatase in extracts of guinea pig and bovine mammary tissue which interfered with the incorporation of UDPGal-^{14}C into lactose-^{14}C. The phosphatases hydrolyzed UDPGal-^{14}C faster than it was used by lactose synthetase thus decreasing the actual amount of substrate for the lactose synthetase reaction. To overcome this difficulty, Watkins and Hassid added UTP to make the molar ratio of UTP to UDPGal equal to 7. Thus, a typical incorporation mixture contained the following in a final volume of 0.1 ml: 5 μmoles Tris-HCl, pH 7.4; 4 μmoles $MnCl_2$; 2 μmoles D-glucose; 0.5 μmoles UTP; 7.0×10^{-2} μmoles UDPGal-^{14}C (2880 cpm); and varying amounts of enzyme. Instead of UTP, ATP could be used in such crude systems. After 30 to 60 minutes incubation at 37° C, the reaction mixture was treated as described in Chapter III. In the assay for the A-Protein, 0.09 to 0.23 mg of purified B-Protein (eluate of the DEAE column) were added whereas in the assays for the B-Protein 0.14 mg of the A-Protein (eluate of the Bio Gel P-30 column) were added to the assay mixture.

Results

Initial Attempts to Solubilize Lactose Synthetase

Before working on the soluble lactose synthetase from bovine skim milk many unsuccessful attempts were made to solubilize the microsomal lactose synthetase. These included: extraction of acetone and butanol powders of whole tissue or microsomes; treatment of particles with detergents; treatment of particles with snake venom (67); treatment of particles with molecular sieves (68); and sonic oscillation of particles.

a) Acetone and butanol powders from mammary tissue and particulate

fractions were prepared (69) and extracted with one percent and five percent lauryl sulfate and one percent and five percent sodium cholate in 0.1 M phosphate or 0.25 M Tris-HCl, pH 7.4. No activity was obtained in any of the extracts though untreated particles showed 21 percent incorporation of galactose-1-^{14}C from UDPGal-1-^{14}C into ^{14}C-lactose within a two hour period of incubation.

b) Several non-ionic and anionic detergents were tested for their ability to solubilize the enzyme from the particles. The anionic detergents, 0.5 percent sodium cholate and 0.4 percent lauryl sulfate in 0.1 M phosphate at pH 7.4, were more effective in solubilizing protein (39.2 and 45.2 percent, respectively) than were the non-ionic detergents 0.2 percent Tween 80 and 0.5 percent digitonin which solubilized 21.6 and 28.0 percent of the particulate protein. No lactose synthetase activity was present in the soluble fraction or in the microsomal residues after treatment with the anionic detergents. In contrast, the particulate residues remaining after treatment with non-ionic detergents had at least full activity and in some cases showed a slight increase in activity.

c) Heat deactivated snake venom (67) from 20 mg of Trimeresurus flavoviridis in 5 µl of 5 mM Tris-HCl, pH 7.4, was incubated with 78 µg of microsomal protein in 5 µl of 0.1 M KCl, pH 9.0. The final pH was 9.0 and the mixture was incubated at 4° C for 16 hours. No lactose synthetase activity could be detected in the soluble or particulate fraction.

d) Person and Zipper (68) reported on the solubilization of cytochrome c from liver mitochondria by treating them with a Zeolite molecular sieve (Linde 3A molecular sieve). This procedure was adapted for the solubilization of microsomal protein. One gram of powdered 3A

molecular sieve was ground in a cold mortar at -10° C together with 10 mg of microsomal protein. The powder was extracted with 10 ml of 0.25 M sucrose and centrifuged at 1,000 x g for 15 minutes and the supernatant solution was centrifuged for one hour at 105,000 x g. About 80 to 90 percent of the microsomal protein could be solubilized but only 25 percent could be recovered from the 3A sieve since it is also a cationic exchanger. Remaining microsomes after Zeolite treatment were still active but only slight activity (5.2 percent of the total activity) could be found in the soluble portion.

e) Particles oscillated at 0° C in a Ratheon sonic oscillator for five minutes liberated 23 percent of the protein into the soluble fraction. Thirteen percent of the original microsomal lactose synthetase was liberated into the soluble fraction and 36 percent was recovered in the microsomal pellet. The remaining 51 percent could not be recovered. Oscillation for 30 min. solubilized 12 percent of the original microsomal activity but this time only 15 percent could be recovered in the particulate fraction. Similar results were obtained with a "Branson Sonic Power Oscillator."

Solubilization of Microsomal Lactose Synthetase

Despite these systematic studies lactose synthetase could not be solubilized to an appreciable extent. It was at first thought that this enzyme must be very unstable when released from the microsomes. After lactose synthetase was resolved into the two subunits it became apparent that the solubilized enzyme was dependent on the addition of the B-Protein to obtain maximum activity. Moreover the addition of the B-Protein obtained from bovine skim milk stimulated lactose synthetase activity in

intact microsomes approximately two fold, suggesting that B may be limiting in microsomes. Lactose synthetase activity in extracts obtained from the repeated sonic oscillation of microsomes were greatly stimulated by the addition of the B-Protein as shown in Table III. Lactose synthetase activity in extracts prepared from microsomes treated with detergents or from a bovine mammary acetone powder was also stimulated by the addition of the B-Protein but to a lesser extent than that in extracts obtained by sonic oscillation. Table IV shows the effect of various extracting agents on the solubilization of microsomal lactose synthetase when assayed in presence of the B-Protein. To compare the efficiency of solubilization of the enzyme by the different methods used, the total units of lactose synthetase activity released into the soluble fraction were divided by the total microsomal protein. Sonic oscillation of a microsomal suspension in 0.25 M sucrose or 0.25 M Tris-HCL, 20 mM $MnCl_2$, pH 7.2, proved to be the most efficient way to solubilize the enzyme. Extraction of a bovine mammary acetone powder with 0.2 M Tris-HCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4, or 0.25 M Tris-HCl, 20 mM $MgCl_2$, pH 7.4, or 0.25 M sucrose was only 35 percent as efficient as sonic oscillation. These results demonstrate that microsomal lactose synthetase is readily solubilized, although maximum enzymatic activity is dependent upon the addition of B-Protein. Because further experiments showed that the B-Protein was not destroyed by sonic oscillation under similar conditions, it appears that fraction B is limiting in microsomes.

Subcellular Distribution of Lactose Synthetase Subunits

As mentioned above lactose synthetase activity in intact microsomes was stimulated two fold by the addition of the B-Protein obtained from

TABLE III

SOLUBILIZATION OF MICROSOMAL LACTOSE SYNTHETASE FROM BOVINE MAMMARY TISSUE AND ITS DEPENDENCE ON FRACTION B OF LACTOSE SYNTHETASE FROM BOVINE SKIM MILK

Preparation	Total Volume	Total Protein	Absence (-) of Presence (+) of B in Assay	Total Enzyme	Specific Activity
	ml	mg		units	units/mg protein
Intact microsomes	135	2,960	-	726	0.25
			+	1,332	0.45
Supernatant after first oscillation	135	1,715	-	354	0.21
			+	3,630	2.12
Supernatant after second oscillation	65	845	-	24	0.03
			+	1,420	1.68
Supernatant after third oscillation	39	265	+	226	1.01

TABLE IV

COMPARISON OF METHODS FOR SOLUBILIZATION OF MICROSOMAL LACTOSE SYNTHETASE

EXPERIMENTAL METHOD				SOLUBLE FRACTION			mg Microsomal Protein Used	Efficiency $\frac{\text{Total U}}{\text{mg Protein Used}}$
Treatment	Extracting Media	Temp.	Time	U/ml	Total Units	Specific Activity		
		°C	min.	$\frac{\text{mμmoles}}{\text{min} \cdot \text{ml}}$	$\frac{\text{mμmoles}}{\text{min}}$	$\frac{\text{mμmoles}}{\text{min} \cdot \text{mg}}$		
Butanol Extraction of Tissue	20 mM Tris-HCl, 20 mM $MgCl_2$, pH 8.1	25°	45	8.84	18.5	4.42	103	0.18
	20 mM Tris-HCl, 20 mM $MgCl_2$, pH 8.1	0°	45	7.84	16.2	3.92	103	0.16
Acetone Powder Extraction	0.25 M Sucrose	0°	60	8.80	13.3	0.52	252	0.53
	0.25 M Tris-HCl, 20mM $MgCl_2$, pH 7.4	0°	60	4.4	3.4	0.44	76	0.46
	0.20 M Tris-HCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4	0°	180	7.0	43.5	0.46	94.5	0.46
Extraction of Microsomes with Detergents	0.1 M Tris-HCl, 20 mM $MgCl_2$, pH 7.4 0.2% Tween 80	0°	60	1.68	2.8	0.56	7.3	0.38
Sonic Oscillation of Microsomes	0.25 M Sucrose	0°	5	15.7	12.5	6.68		1.08
			10	21.4	17.0	7.86	11.6	1.47
			30	27.6	22.2	17.2		1.90
	0.25 M Tris-HCl, 20 mM $MgCl_2$, pH 7.4	0°	5	13.1	10.6	5.14		0.91
			10	20.8	16.7	6.12	11.6	1.44
			30	27.0	21.7	16.8		1.87

skim milk and the solubilized lactose synthetase was greatly stimulated (10 fold or more) by the addition of the B-Protein. Also, evidence was obtained which showed that the soluble portion of rat or bovine mammary tissue stimulated the activity of microsomal lactose synthetase. These data suggested that there may be an unequal distribution of the A and B-Proteins within mammary cells and accordingly the subcellular distribution of the A and B-Proteins of the enzyme was investigated following a modified method of Schneider and Hogeboom (70).

Frozen mammary tissue from lactating cows or rats was cut into 3 to 5 mm slices and washed three times in 0.25 M sucrose at 4° C. After each wash, the tissue was gently blotted between several layers of filter paper to remove the majority of the residual milk present in the tissue. Removal of residual milk is essential since the enzyme is soluble in milk and would appear in the 105,000 x g supernatant fraction thus giving a false distribution of the microsomal enzyme.

After washing the slices, the bovine mammary tissue was passed three times through a mechanical meat grinder. This material (10 g) was homogenized for 30 seconds with 50 ml of 0.25 M sucrose with the Virtis overhead homogenizer (Variac setting at 80). The resulting suspension was centrifuged at 700 x g for 10 minutes. The precipitate was washed with 20 ml of 0.25 M sucrose and recentrifuged at 700 x g. The precipitate obtained (nuclear fraction) was suspended in 0.25 M sucrose and freed from large pieces of connective tissue by filtering the suspension through four layers of cheesecloth. The combined 700 x g supernatant solutions were centrifuged for 10 min. at 5,000 x g. The precipitate, after washing in 0.25 M sucrose, yielded the mitochondrial fraction. The 5,000 x g supernatant solution was centrifuged

for 60 min. at 105,000 x g. The microsomal pellet was suspended in 0.25 M sucrose and the microsomal suspension and the soluble fraction were re-centrifuged for 120 min. at 105,000 x g. In a similar way, 9.5 g of lactating rat mammary tissue were subjected to differential centrifugation except that the rat mammary tissue was not passed through the meat grinder before the homogenization.

Table V shows the subcellular distribution of the A and B-Proteins from bovine and rat mammary tissue. In both species, the A-Protein is associated primarily with the microsomal fraction, whereas, the B-Protein is found in the microsomal and the soluble fractions of the cell. This distribution pattern agrees with the earlier observation that the B-Protein is limiting in microsomes.

Gel Filtration Studies

It was of interest to compare the elution pattern on Bio Gel P-30 of the A-Protein isolated from microsomes and the B-Protein isolated from the soluble fraction to that of the A and B-Proteins isolated from bovine skim milk. A bovine microsomal suspension (100 ml containing 8.4 mg protein per ml of 0.25 M sucrose) was oscillated in a Ratheon sonic power oscillator for 30 minutes at 0° and then centrifuged for 90 minutes at 105,000 x g. The microsomal pellet was suspended in 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4, reoscillated and centrifuged as indicated above. Solid ammonium sulphate (516 g per liter) was added to the combined supernatant solution (190 ml) and after centrifugation the precipitate was suspended in 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4 (total volume, 15.6 ml). Eight ml of this solution were passed through a 3 x 160 cm Bio Gel P-30 column as described in Chapter III. Figure 17 shows the

TABLE V

SUBCELLULAR DISTRIBUTION OF THE A AND B PROTEINS FROM BOVINE AND RAT MAMMARY TISSUE

	RAT Mammary Tissue								BOVINE Mammary Tissue							
			A-Protein			B-Protein					A-Protein			B-Protein		
Fraction	Vol.	Total Protein	Total Units	% Distribution	Specific Activity	Total Units	% Distribution	Specific Activity	Vol.	Total Protein	Total Units	% Distribution	Specific Activity	Total Units	% Distribution	Specific Activity
	ml	mg	mμmoles / min.		mμmoles / min-mg	mμmoles / min.		mμmoles / min-mg	ml	mg	mμmoles / min.		mμmoles / min-mg	mμmoles / min.		mμmoles / min-mg
Nuclear	1.5	14.6	2.7	9.4	0.19	4.1	10.7	0.28	1.5	20.8	4.1	9.0	0.20	0.9	3.7	0.04
Mitochondrial	2.0	15.6	2.8	9.8	0.18	1.6	4.1	0.10	2.0	13.4	7.8	17.1	0.58	0.5	2.7	0.04
Microsomal	4.5	93.5	21.2	73.8	0.22	11.9	31.0	0.13	3.8	26.8	31.5	69.0	1.17	10.6	42.6	0.34
Soluble	25.0	130.	2.0	7.1	0.02	20.8	54.0	0.16	26.5	74.4	2.2	4.8	0.03	12.9	51.8	0.17

protein elution pattern (A_{280}) as well as lactose synthetase activity of the A-Protein when assayed in presence of saturating amounts of B-Protein obtained from bovine skim milk. The A-Protein eluted with the majority of the high molecular proteins and its elution volume corresponded to the A-Protein isolated from soluble lactose synthetase of skim milk.

In a similar manner, the soluble B-Protein was demonstrated to be associated with a low molecular weight fraction from the P-30 column. The soluble fraction of a bovine mammary extract was made 20 mM in Tris-HCl, 30 mM $MgCl_2$, pH 7.4, and the precipitate was discarded. Solid ammonium sulfate (390 g per liter) was added to obtain 60 percent saturation in ammonium sulfate and the precipitate was discarded. The supernatant solution was brought to 75 percent saturation in ammonium sulfate (106 g per liter) and the precipitate was dissolved in 0.25 M Tris-HCl, pH 7.4. Eight ml of this solution (23 mg protein per ml) were passed through the 3 x 160 cm Bio Gel P-30 column. Figure 18 shows the protein elution pattern (A_{280}), and lactose synthetase activity of the B-Protein when assayed in the presence of saturating amounts of A-Protein obtained from bovine skim milk. The elution pattern of the B-Protein from the soluble fraction of bovine mammary tissue corresponds to the elution pattern of B-Protein obtained from the soluble milk enzyme.

Fractions 31 to 49 were pooled (Figure 18) and concentrated with Biodryex to approximately 1/10 of the original volume. This solution was heated at 100° for two minutes and the precipitate was removed by centrifugation and discarded. An ultraviolet spectrum of this partially purified material resembled the spectrum obtained from the purified B-Protein from skim milk. No further attempts were made to characterize the B-Protein from the soluble mammary tissue extract since it was

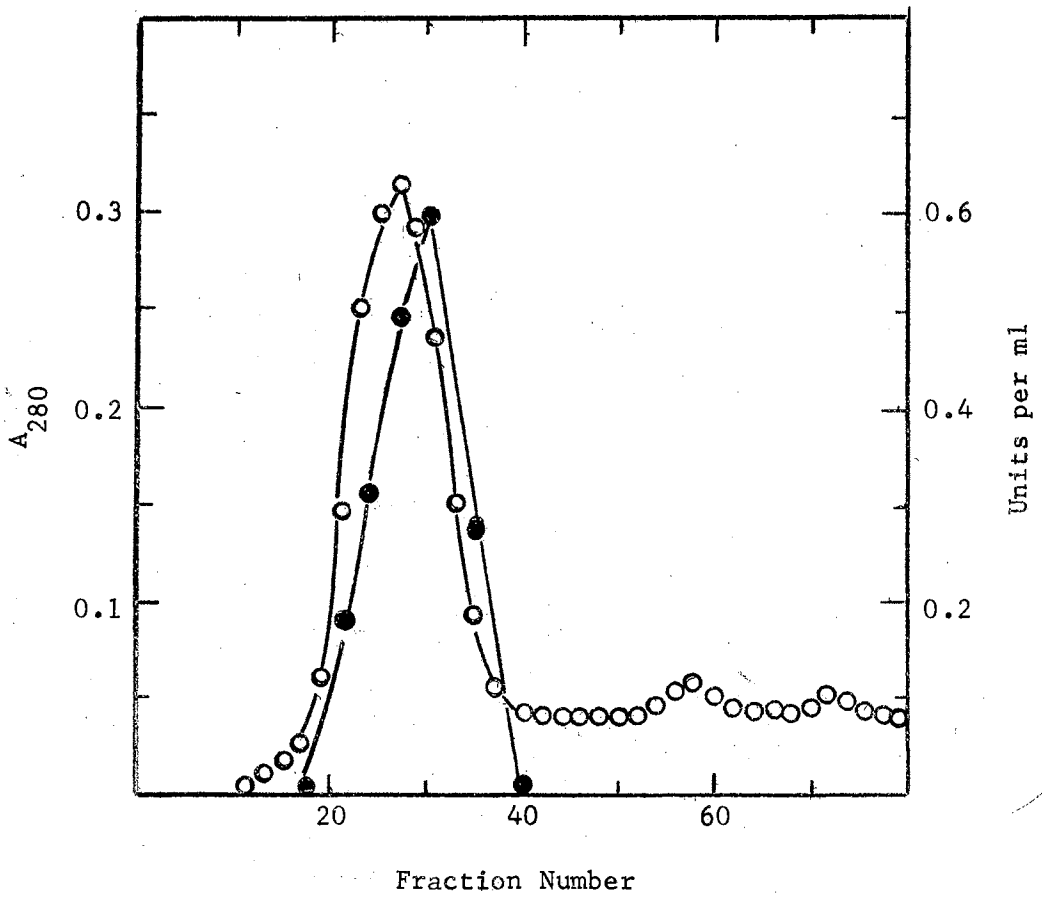


Figure 17. Gel Filtration of the A-Protein of Solubilized Lactose Synthetase From Bovine Mammary Microsomes.

The solubilization, fractionation and gel filtration are described in the text. Fractions, 5.0 ml each, were collected after the first 210 ml passed through the column. O, protein distribution in elute fractions (A_{280}); ●, lactose synthetase activity of the A-Protein in presence of 17.5 units of the B-Protein (22.5 µg of B). The incorporation assays were carried out as described in Methods.

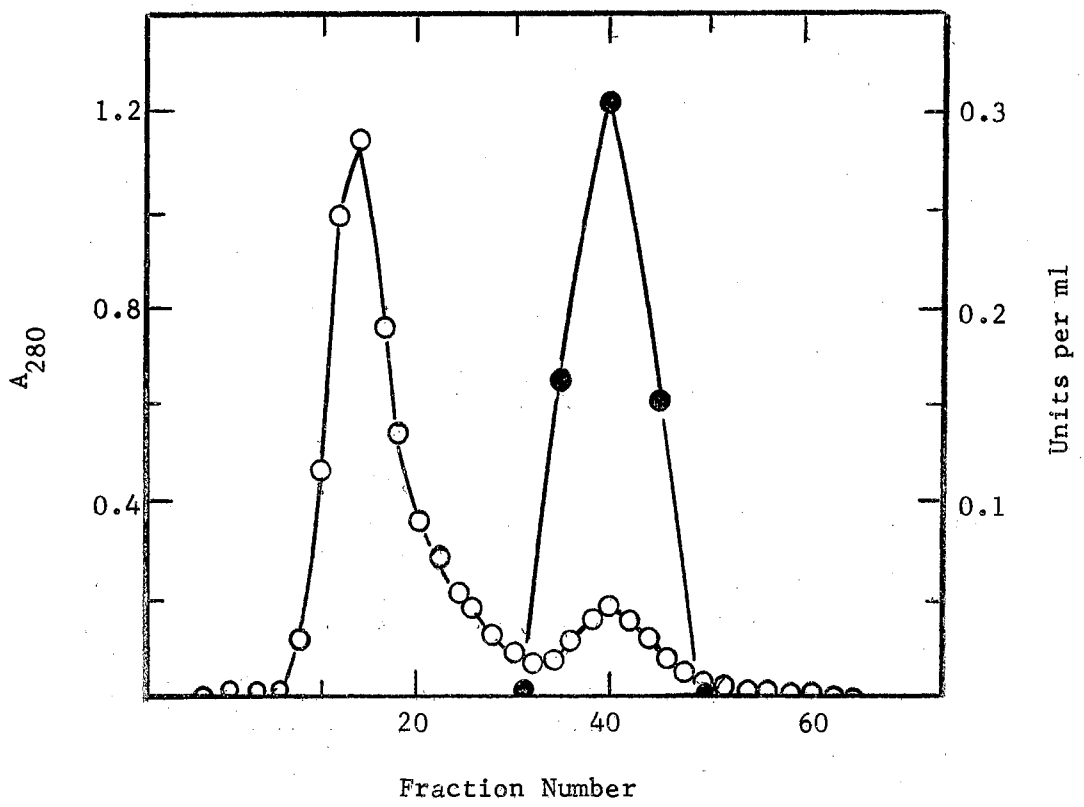


Figure 18. Gel Filtration of the B-Protein of Lactose Synthetase From Bovine Mammary Tissue.

The fractionation and gel filtration are described in the text. Fractions, 4.3 ml each, were collected after the first 300 ml passed through the column. O, protein distribution in eluate fractions (A_{280}); ●, lactose synthetase activity of the B-Protein in presence of 2.37 units of the A-Protein (15.0 µg of A). The incorporation assays were carried out as described in Methods.

isolated in only small amounts from this tissue.

Dissociation of Microsomal Lactose Synthetase

As a result of the subcellular distribution study, it was apparent that the A and B-Proteins necessary for lactose synthetase were present in microsomes. Since the majority of the B-Protein was found in the soluble portion of the cell, B seemed to be associated only weakly with the microsomal protein. If this is true, it should be possible to find conditions whereby the microsomal B-Protein is more specifically released into the soluble fraction than is the A-Protein. Attempts were made to dissociate the A and B-Proteins of microsomal lactose synthetase under a variety of mild conditions.

Bovine mammary microsomes were suspended in 0.25 M sucrose or in 20 mM Tris-HCl, pH 7.4, and incubated for one hour at 0° in the presence of EDTA, acetone, KCl and ammonium sulfate. The incubation mixture was then centrifuged for 90 minutes at 105,000 x g, the remaining microsomal pellet was washed with 20 mM Tris-HCl, pH 7.4, and finally dissolved in the same buffer. The combined 105,000 x g supernatant solutions were dialyzed overnight against 500 ml of 0.25 M sucrose or 20 mM Tris-HCl, pH 7.4, (changed three times) and then concentrated to approximately 2 ml with Biodryex.

Both the microsomal and soluble fractions were assayed for the A and B-Protein of lactose synthetase. Incubation blanks containing no glucose showed that in the solubilized fractions even in the absence of UTP only small amounts of neutral sugars were formed from endogenous pyrophosphatase activity. In all these assays, the addition of UTP could be omitted. Table VI shows the effect of EDTA, acetone, KCl and

ammonium sulfate on the release of the A and B-Proteins of microsomal lactose synthetase when microsomes were incubated in either 0.25 M sucrose or 20 mM Tris-HCl, pH 7.4. Comparison of the percent distribution of the A and B-Proteins between the remaining microsomes and the solubilized material revealed that under identical experimental conditions the B-Protein was released into the soluble fraction to a much greater extent than the A-Protein. When incubated in 0.25 M sucrose, essentially no A activity was found in the soluble fraction even in presence of EDTA, acetone, KCl or combinations thereof, whereas up to 62 percent of the total microsomal B activity was released. On the other hand 34 percent of the total microsomal A activity was solubilized when the microsomes were incubated in presence of 20 mM Tris-HCl, pH 7.4, suggesting that under condition of low osmotic pressure, microsomal protein may be partially solubilized. The addition of EDTA, acetone, and ammonium sulfate resulted only in a slight increase in solubilized A activity whereas up to 95 percent of the total microsomal B activity was released under identical conditions. These findings are consistent with all earlier observation that the B-Protein is limiting in microsomes.

The fact that the B-Protein could be almost completely solubilized when 20 percent acetone was in the incubation mixture suggested that the binding of B to the microsomes was partly hydrophobic in nature. Twenty mM KCl also caused some release of the B-Protein from the microsomes. However, 10 mM EDTA was more than twice as effective as 20 mM KCl indicating that the B-Protein was solubilized partially due to an increased ionic strength and partially due to chelation of divalent ions (Mn^{++}, Mg^{++}). High levels of Mg^{++} or Mn^{++} (20) are required for lactose

TABLE VI

DISSOCIATION OF MICROSOMAL LACTOSE SYNTHETASE FROM BOVINE MAMMARY TISSUE

Incubation Mixtures	A - PROTEIN							B - PROTEIN						
	MICROSOMES		SOLUBLE FRACTION		Σ	% DISTRIBUTION		MICROSOMES		SOLUBLE FRACTION		Σ	% DISTRIBUTION	
	Total Units	Specific Activity	Total Units	Specific Activity	Total Units	Micro Somes	Soluble Fraction	Total Units	Specific Activity	Total Units	Specific Activity	Total Units	Micro Somes	Soluble Fraction
	mμmoles min.	mμmoles min-mg	mμmoles min.	mμmoles min-mg	mμmoles min.			mμmoles min.	mμmoles min-mg	mμmoles min.	mμmoles min-mg	mμmoles min.		
0.25 M Sucrose	150	2.2	0.9	0.1	151	99	1	103	1.5			103	100	
0.25 M Sucrose 10 mM EDTA	135	1.7	0.1	0.1	135	99	1	67	0.8	30	5.5	97	69	31
0.25 M Sucrose 20% Acetone	128	2.5	0.4	0.1	128	99	1	78	1.5	23	3.5	101	77	23
0.25 M Sucrose 10 mM EDTA 20% Acetone	125	2.3	2.0	0.1	127	99	1	40	0.8	64	10	104	38	62
0.25 M Sucrose 20 mM KCl	129	1.7	1.7	0.1	131	99	1	85	1.3	14	1.1	99	86	14
0.25 M Sucrose 10 mM EDTA 20 mM KCl	128	1.8	0.1	0.1	128	99	1	64	0.6	38	3.3	102	62	38
20 mM Tris-HCl, pH 7.4	33.5	1.89	19.1	1.14	52.6	63.6	34.3	11.8	0.60	18.7	1.12	30.5	38.6	61.4
20 mM Tris-HCl, pH 7.4 10 mM EDTA-Tris, pH 7.4	24.6	1.79	19.7	1.25	44.3	55.5	44.5	6.9	0.50	22.4	1.42	29.3	23.6	76.4
20 mM Tris-HCl, pH 7.4 10 mM EDTA-HCl, pH 7.4 20% Acetone	19.2	1.22	23.4	1.38	42.6	45.0	55.0	1.43	0.09	25.4	1.50	26.8	5.3	94.7
20 mM Tris-HCl, pH 7.4 10 mM EDTA-HCl, pH 7.4 20% Acetone 0.2 M Ammonium Sulfate	17.0	1.30	22.4	1.40	39.4	43.2	56.8	1.54	0.12	29.2	1.81	30.7	4.9	95.1

synthetase activity which may indicate that divalent cations aid in the formation of the active AB complex.

Lactose Synthetase in Microsomes Isolated From Bovine Milk

As previously indicated, the A and B-Proteins of lactose synthetase exist in a soluble form in bovine skim milk whereas in mammary tissue, lactose synthetase activity is associated mainly with the microsomal fraction. Only small amounts of lactose synthetase activity were found in the soluble fraction of bovine or rat mammary tissue.

Milk contains microsomes which are associated mainly with the cream fraction. These microsomes were examined for their lactose synthetase activity as was the distribution of the A and B-Proteins in whole milk.

Bovine milk microsomes were isolated according to a procedure by Morton (71). Fresh whole milk (100 ml) was cooled to 4° and centrifuged at 2,500 x g for 30 minutes. The cream was carefully removed with a spatula and washed by suspending in 50 ml of H_2O. After centrifugation for 20 minutes at 2,500 x g the cream was again suspended in 50 ml of H_2O and churned by shaking for 20 minutes at room temperature. The buttermilk was decanted off and centrifuged together with the washings at 78,000 x g for 90 minutes. The microsomal precipitate was washed in 20 mM Tris-HCl, 5 mM, $MgCl_2$, pH 7.4, recentrifuged at 78,000 x g for 90 minutes and suspended in the same buffer. The skim milk was centrifuged at 16,000 x g for 30 minutes and the precipitate (heavy casein) was discarded. The supernatant solution was centrifuged two more times at 78,000 x g and the precipitate (light casein) was discarded. Table VII shows the distribution of the A and B-Protein of lactose synthetase in bovine whole milk. Although the soluble fraction contains approximately

TABLE VII

DISTRIBUTION OF THE A AND B-PROTEIN OF LACTOSE SYNTHETASE IN BOVINE WHOLE MILK

Fraction	Volume	Total Protein	A-Protein			B-Protein		
			Total Units	% Distribution	Specific Activity	Total Units	% Distribution	Specific Activity
	ml	mg	mμmoles / min		mμmoles / min·mg	mμmoles / min		mμmoles / min·mg
Microsomal	7.0	191.8	63.5	1.1	0.33	46.4	0.9	0.24
Soluble	103.0	762.0	5640	98.9	7.40	5510	99.1	7.25

99 percent of the activity of the two proteins of lactose synthetase, the microsomes isolated from cream had the ability to incorporate the galactose moiety of UDP-D-^{14}C-galactose into ^{14}C-lactose. The specific activities of the A and B-Proteins of milk microsomes were similar to those of the particulate mammary enzyme. Due to coprecipitation, some lactose synthetase activity was found in the light and heavy casein fractions. However, after washing these precipitates, the activity was found in the soluble fraction.

These results suggest that milk microsomes are similar to tissue microsomes, an observation previously made by Morton (71). Morton found that the lipoprotein particles from milk had chemical and enzymatic properties similar to those of cytoplasmic microsomes and called them "milk microsomes." He concluded that the microsomes of milk are derived directly from the secretory cells of the mammary gland. On the basis of these observations and the results presented here it is to be assumed that lactose synthetase from mammary tissue is identical to the enzyme from milk. The exact manner by which lactose synthetase becomes solubilized in milk, however, is not clear at the present time.

CHAPTER V

DISCUSSION

The results reported in this dissertation led to the conclusion that lactose synthetase is the first mammalian enzyme which can be isolated in the form of two naturally occurring subunits. The larger molecular weight subunit was designated as the A-Protein and the smaller molecular weight subunit was designated as the B-Protein. The B-Protein was shown to be identical to α-lactalbumin based on its ability to substitute in the incorporation and spectrophotometric assays and its similarity in molecular weight, ultraviolet spectrum and tyrosine to tryptophan ratio.

The active form of the enzyme is assumed to be an AB complex even though concrete evidence for such a complex is lacking at the present time. An indication for its existence was observed when the A-Protein peak from the Bio Gel P-30 column was assayed by the incorporation assay; there was a slight amount of activity in the absence of added B-Protein. Immunological assays have shown that there was no α-lactalbumin in the A peak indicating that the separation of free A and B-Protein was complete.

Some Aspects on the Control of Lactose Biosynthesis

To date only four enzymes, isolated from bacterial systems, are known to exist as naturally occurring subunits. The properties of these

enzymes: tryptophan synthetase, (+)-citramalate hydro-lyase, glycine decarboxylase and glutamate mutase were discussed in some detail in the literature review.

The results of this investigation suggest that lactose synthetase may be similar in many respects to tryptophan synthetase, that is the A and B-Proteins are subunits of an AB complex which is largely dissociated in milk and to a lesser extent in mammary tissue. Lactose synthetase is an enzyme that is undoubtedly under hormonal regulation since milk secretion is under strict hormonal control. It is conceivable that the cell could regulate lactose biosynthesis by controlling the association of the two subunits of lactose synthetase. However, this enzyme catalyzes the terminal step in the lactose biosynthetic sequence and other types of control such as feedback inhibition or repression might be more significant in the control of lactose biosynthesis. It is of interest to consider whether association of the subunits of lactose synthetase could be of any physiological significance.

Creighton and Yanofsky (39) have suggested with tryptophan synthetase that the role of pyridoxal-P and serine in the association of the α_2 and β_2 subunits is similar to that of certain cofactors and substrates which act as allosteric effectors in other systems. For example, Gerhardt and Schachman (24) have shown that aspartate transcarbamylase (EC 2.1.3.2) was composed of two subunits: a regulatory subunit that bound the allosteric modifier and a catalytic subunit that catalyzed the enzymatic reaction. The catalytic subunit was not influenced by the allosteric modifier. The activity of this enzyme is controlled by feedback inhibition by CTP, the end-product of the biosynthetic pyrimidine pathway. Yet, aspartate transcarbamylase is distinctly different from

lactose synthetase since the subunits of aspartate transcarbamylase dissociate only in presence of mercurials whereas lactose synthetase apparently exists in nature principally in the form of subunits. The fact that lactose synthetase exists in nature as subunits does not rule out the possibility that the association of the subunits to form an AB complex will require the presence of substrates or cofactors.

Another possibility for the control of lactose biosynthesis would be the regulation of the amount of enzyme formed. In the presence of an inducer (most frequently the substrate of the enzyme or a related small molecular substance) induction may take place which results in an increase of the amount of enzyme formed. The converse of induction is repression, which is a specific inhibition of the formation of an enzyme or a series of enzymes by the accumulation of a product of a biosynthetic sequence of reactions. At present it is not known if the biosynthesis of lactose is regulated by either one of these mechanisms. Since the secretion of milk is under strict hormonal control induction of one enzyme or sequential induction of a group of metabolically related enzymes could be envisioned. The hormone might affect the production of a specific inducer or act as the inducer itself.

At the genetic level the synthesis of an enzyme is controlled by another factor: a given enzyme can only be formed if the corresponding gene is present in the cell. Beadle (72) postulated the necessity of a separate gene for the formation of every enzyme and his "one-gene-one-enzyme" theory was widely held. The enzymes whose genes are situated in one group are all induced or repressed to the same extent thus giving rise to coordinate induction or repression (73, 74).

Weinhouse and his coworkers (75) determined the enzymatic activities

of hexokinase, UDPG-pyrophosphorylase and UDPGal-4-epimerase in rat mammary glands during the entire pregnancy and lactational cycle. Hexokinase, pyrophosphorylase and epimerase activities were very low in the unstimulated glands but gradually increased throughout pregnancy and reached high levels during lactation. The over-all increase of hexokinase, pyrophosphorylase and epimerase was 4.2; >100; >300 fold, respectively. On weaning, the enzymatic activities dropped rapidly to essentially the levels of the unstimulated gland. These observations may be taken as suggestive evidence that lactose biosynthesis during lactation is controlled by coordinate induction of the enzymes involved in the biosynthesis of lactose.

Since Beadle proposed his "one-gene-one-enzyme" theory in 1945 it became apparent that many enzymes are composed of a finite number of subunits. Monod, Wyman and Changeux (76), in their model on allosteric transitions, called a protein, containing a finite number of identical subunits (protomers), an oligomeric protein. Examples of such proteins are β-lactoglobulin or L-lactate dehydrogenase, M_4 (77). No specific nomenclature was given to the association of two or more different proteins as is the case with enzymes consisting of naturally occurring subunits. It soon became obvious that Beadle's "one-gene-one-enzyme" hypothesis had to be modified to the "one-gene-one-subunit" theory. To date, only incomplete evidence for such a proposal is available.

In mapping out the tryptophan operon in chromosomes from E. coli, Yanofsky (78) was able to show that two different genes, 1 and 2, are involved with tryptophan synthetase. Region 1 corresponded to the A(α) protein and region 2 to the B(β) protein (79). Yanofsky and his coworkers (31, 32) also obtained mutants of E. coli which synthesized only

the α-subunit or the β-subunits of tryptophan synthetase suggesting that there are indeed two genes responsible for the synthesis of the subunits of tryptophan synthetase.

In a similar way, one might predict that the lactose operon in mammalian systems should contain different genes for each of the enzymes involved in the biosynthesis of lactose, including two different genes for lactose synthetase.

Forces Involved in the Association of Lactose Synthetase Subunits

Lactose synthetase appears to be unique in that the A and B-Proteins in milk are both soluble whereas in mammary tissue the A-Protein is principally associated with the microsomes and the B-Protein is distributed between the microsomes and the soluble portion of the cell. The distribution observed in mammary tissue may be misleading since the B-Protein may have been dissociated from the AB complex in microsomes during the isolation procedure. Studies on the dissociation of microsomal B showed that it was bound weakly to microsomal A. The A-Protein was demonstrated by gel filtration to be present in solubilized microsomes from bovine mammary tissue and the B-Protein was shown to be present in partially purified soluble extract of bovine mammary tissue. It would appear that the A and B-Proteins in whole tissue were similar to the A and B-Proteins present in milk.

In order to get an understanding of the forces holding the lactose synthetase subunits together, the microsomal enzyme was subjected to a variety of mild chemical treatments. The results obtained from these incubation experiments were discussed in some detail in Chapter IV. Incubation in the presence of 10 mM EDTA·2 Na released 31 percent of

the B-Protein into the soluble fraction whereas 20 mM KCl (having the same ionic strength as 10 mM EDTA·2 Na) only released 14 percent. These results suggested that, to some extent, the B-Protein is bound in the microsomes to the A-Protein by Mg^{++} or Mn^{++} since these two cations were shown to activate lactose synthetase (20). The importance of metallic ions in binding together protein subunits has long been recognized. Kägi and Vallee (80) reported that o-phenantroline will cause the dissociation of yeast alcohol dehydrogenase (EC 1.1.1.1) by forming a chelate complex with Zn^{++}. Amylase from B. subtilis dissociated upon removal of Zn^{++} and dimerization ensued when the Zn^{++} was replaced. Ca^{++} has also been implicated in association (81). Casein in milk exists as four fractions: α_s-casein (25 percent), β-casein (35 percent), K-casein (15 percent) and m-casein. At low temperature, α_s-casein exists as a monomer and β-casein as a polymer, the latter being due to homoassociation or association of identical particles (82). As the temperature rises, α_s-casein associates with β-casein to give heteroassociation. Another heteroassociation involved both α_s- and K-casein. Ca^{++} is believed to provide bridging links which aid in the stabilization of the complex at 37° C. There is also evidence that trypsin dissociates in absence of Ca^{++} (83).

In many enzymes having a mono- or diphosphate ester as substrate, the divalent cations also are required for the binding of the substrate to the enzyme. Thus in lactose synthetase, Mg^{++} or Mn^{++} may fulfill a dual role. They may function as structural component of the active enzyme and are also required as participants in the formation of the enzyme-substrate complex. Such a dual role has been demonstrated in the case of Mg^{++} in yeast enolase by Brewer and Weber (84) and in rabbit

muscle enolase by Winstead and Wold (85).

Significant dissociation of the microsomal lactose synthetase also was observed in presence of 20 percent acetone. In 0.25 M sucrose and 20 percent acetone the microsomal B-Protein was released to 23 percent into the soluble fraction. The addition of 10 mM EDTA to the acetone containing incubation mixture caused an increase in solubilization up to 62 percent. In 20 mM Tris-HCl, pH 7.4, the B-Protein was released into the soluble fraction up to 76 percent and 95 percent under similar conditions of incubation. The addition of an organic solvent such as acetone seemed to have a marked effect on the dissociation of lactose synthetase subunits.

Winstead and Wold (85) observed dissociation of rabbit muscle enolase in the presence of 10 mM EDTA and 20 percent aqueous dioxane or 20 percent aqueous acetone. Upon dialysis, the native enzyme could be reformed. Nearly all proteins contain a relatively high proportion of amino acids with non-polar side chains which tend to adhere to one another in aqueous environments by forming hydrophobic bonds (86). These bonds are probably one of the more important factors involved in maintaining the tertiary structure of proteins. In his work with alkaline phosphatase, Schlesinger (87) obtained indirect evidence that hydrophobic bonds are formed in the dimerization process of this enzyme. Tanford (88) has dealt with the dissociation of subunits on a theoretical basis and pointed out that the interaction of ethanol and other neutral organic agents with large non-polar groups would favor dissociation if most of the newly exposed groups are hydrophobic in nature.

Kauzman (86) pointed out that hydrophobic bonds involving aliphatic side chains are more stable at room temperature than they are at 0° C

because of the endothermicity of the transfer of non-polar groups from water to non-polar solvent. Measurements of the thermodynamic changes accompanying hydrophobic bonding in protein aggregation and binding reactions frequently showed small enthalpy and large entropy changes (86). In many cases such as dimerization of insulin, antigen-antibody interaction or binding of aromatic dyes to the hydrophobic side chains in protein, the association reaction is favored because of their entropy changes which at room temperature greatly outweighed the enthalpy effects.

There is another piece of evidence which suggests that hydrophobic forces may be partially responsible for the formation of an AB complex of lactose synthetase. Initial attempts to separate the two subunits by heat denaturation of the A-Protein were unsuccessful since the B-Protein coprecipitated with other proteins. Such results suggested that increased temperature promoted the association of the A and B-Proteins to form the AB complex. Similar observation indicating hydrophobic interaction was made by Creighton and Yanofsky (39) with the subunits of tryptophan synthetase where association of the subunits was observed at 37° C but not at 0° C. Another possible explanation would be that the B-Protein itself forms aggregates at elevated temperature which also would be due to hydrophobic binding. Larson and Rolleri (89) observed denaturation of α-lactalbumin when the albumin fraction of milk was heated. At a concentration of 0.54 g serum protein per 100 ml of skim milk, 50 percent of the α-lactalbumin was denatured after treatment for 30 minutes at 77° C. Later Wetlaufer (62) observed that α-lactalbumin formed aggregates upon heating a four percent solution at pH 6.6 for ten minutes in a boiling water bath. When ammonium sulfate was added to 50 percent saturation a heavy amorphous precipitate formed in the tubes containing the heated α-lactalbumin whereas

only slight cloudiness was observed in the unheated controls.

The results discussed above and earlier in Chapter IV lead to the conclusion that the AB complex of lactose synthetase is readily dissociable *in vitro* at low temperatures. Under *in vivo* conditions a higher degree of association would be expected since hydrophobic interaction between the A and the B-Protein was shown to be one of the forces holding the subunits together. In addition, divalent cations (Mg^{++} and Mn^{++}) seem to be involved in the complex formation. However one always has to keep in mind that there is no single force responsible for a definite protein structure. Subunit association as well as protein structure in general is a cooperative phenomenon in which covalent and non-covalent forces are always involved. It is by no means certain that an unequivocal identification of one particulate force as being most important in a given instant can always be made (88).

The Relationship Between the B-Protein of Lactose Synthetase and α-Lactalbumin

The results presented in Chapter III showed that α-lactalbumin could substitute for the B-Protein of lactose synthetase in the rate and incorporation assay. The conclusion was made that α-lactalbumin was the small molecular weight subunit of lactose synthetase. α-Lactalbumin, a major constituent of the whey proteins of milk, has been recognized for some twenty years though no biological function other than a nutritional one could be ascribed to this protein.

The name "lactalbumin" has been applied to various impure crystalline protein preparations derived from milk (90, 91). Pedersen (92) found several components in bovine whey and called the slowest moving

component in the ultracentrifuge α, the next β, etc. The term α-lactalbumin for the slowest moving component was first used by Svedberg (93). Sørensen and Sørensen (94) published a procedure for isolating a "crystalline insoluble substance" from bovine whey and showed that it was not β-lactoglobulin. Gordon and his coworkers (60, 66, 95) modified the procedures of Sørensen and Sørensen and suggested the name α-lactalbumin for the crystalline preparation.

Since α-lactalbumin occurs in large quantities in whey (0.7 to 1.5 g per liter have been reported (96)) and since it is readily crystallized this protein has been characterized to some extent. Gordon and Ziegler (97) determined the amino acid composition of crystalline α-lactalbumin. High contents of aspartic acid, lysine and tryptophan and a low content of arginine, methionine and proline were found: 23 acidic groups were in excess over the basic groups. The existence of α-lactalbumin as an anionic molecule at pH 7 is in agreement with the fact that α-lactalbumin crystallizes as the ammonium salt. Completely deionized, α-lactalbumin will not dissolve in water but will dissolve as the ammonium salt. The presence of excessive acidic groups agrees with the view that divalent cations are involved in the complex formation of α-lactalbumin or the B-Protein with the A-Protein of lactose synthetase.

Wetlaufer (62) documented additional data on α-lactalbumin. The $S_{20,W}$ of α-lactalbumin was found to be concentration dependent and was 1.87 when extrapolated to zero protein concentration. The sedimentation velocity of the B-Protein is 1.70 at a concentration of 21 mg protein per ml. When extrapolated to zero protein concentration, using Wetlaufer's data on the concentration dependence, the $S_{20,W}$ is 1.84 which is in good agreement with Wetlaufer's data.

α-Lactalbumin substituted for the B-Protein of lactose synthetase at identical protein concentrations in both the spectrophotometric and the incorporation assays. The specific activities of the B-Protein and 2x, 3x and 5x recrystallized α-lactalbumin were essentially the same. However, α-lactalbumin was reported to be heterogenous when its homogeneity was studied by electrophoresis. Klostergaard and Pasternack (98) found electrophoretic heterogeneity in α-lactalbumin prepared by the method of Aschaffenburg and Drewry (64) in lactate, pH 3.3; phosphate, pH 7.5; Tris, pH 7.4; and barbitol, pH 8.5. Such results were interpreted to be partially due to strong reversible interactions with the buffer (64).

The possibility could exist that both the B-Protein and α-lactalbumin have small amounts of contaminating proteins which could account for the activity. However such a possibility is highly unlikely since the amount of the contaminant would have to be equal in all the preparations used. α-Lactalbumin is usually prepared from the whey proteins of milk by the method of Gordon and Ziegler (66) by ammonium sulfate fractionation and isoelectric precipitation. The B-Protein was prepared from the whey proteins of the milk by chromatography on Bio Gel P-30 and on DEAE-cellulose. It is unlikely that a minor contaminant would appear at the same concentration in the product of different preparation procedures.

Furthermore, starch gel electrophoresis of the B-Protein and 5x α-lactalbumin in 8 mM aluminum lactate, 3 M urea pH 3.3 ($\mu = 0.05$), showed that the 5x α-lactalbumin migrated as a single band whereas the B-Protein contained three minor bands in addition to the major

band.[1] These results essentially rule out the possibility that a minor protein contaminant is responsible for B-Protein activity since the 5x preparation was as effective as the 3x α-lactalbumin and the B-Protein in the enzymatic assays.

Kronman and Andreotti (99) reported heterogeneity in α-lactalbumin prepared from dairy cows of widely different genetic history and suggested that the majority of heterogeneity of α-lactalbumin could be accounted for on the basis of protein-protein or ion-protein interactions but the possibility of structural differences within α-lactalbumin (similar to the isozyme of lactate dehydrogenase) could not be ruled out. Blumberg and Tombs (100) found that two variants of α-lactalbumin were produced by humped Zebu cows; one of the variants, α-lactalbumin A, migrated electrophoretically faster than the form found in Western breeds. Aschaffenburg (101) concluded that these two variants of α-lactalbumin could be ascribed to the existence of two alleles of an autosomal gene resulting in a polymorphism which in the case of α-lactalbumin was restricted to Zebu cattle.

The heterogeneity in α-lactalbumin is certainly open to question though the majority of evidence indicates that some type of protein-protein or ion-protein interaction may be responsible. Cann (102) has shown that multiple bands observed on the electrophoresis of bovine serum albumin were due to ion-protein interaction and suggested that due caution must be used in drawing conclusions from electrophoretic data. Barlow and Margoliash (103) observed that electrophoretic inhomogeneity resulted from the binding of buffer constituents to pure

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[1]Dr. K. E. Ebner, personal communication.

crystalline monomeric cytochrome c from ten species. The electrophoretic patterns depended on the particular cytochrome c used as well as the nature of the anion of the buffer. The fact that α-lactalbumin is highly charged and can form salts would indicate that ion-protein interactions are significant.

The B-Protein isolated from the milk of the bovine, sheep, goat and the human were comparable in the spectrophotometric assay for lactose synthetase activity. It would be of interest to see if α-lactalbumin A, the variant occurring in Zebu cattle, would substitute for the B-Protein of lactose synthetase isolated from the whey of Western cattle and if the B-Protein from the milk of the sheep, goat and human are immunologically different from bovine α-lactalbumin. If this were the case a situation similar to lactate dehydrogenase would arise where subunits of genetically different origins can combine to yield the active enzyme (104).

SUMMARY

Lactose synthetase from the milk of the cow, goat, sheep and human was resolved by chromatography on Bio Gel P-30 into two protein fractions, A and B, which individually did not show any catalytic activity. Recombination of the A and B-Protein, however, restored full lactose synthetase activity. The A-Protein from bovine skim milk was further purified by negative adsorption on calcium phosphate gel and chromatography on DEAE-cellulose; the over-all purification of the A-Protein was 340 fold. The B-Protein was further purified by chromatography on DEAE-cellulose and crystallized from ammonium sulfate at pH 6.6. The over-all purification of the B-Protein was 250 fold. The B-Protein of lactose synthetase was shown to be identical to α-lactalbumin. Microsomal lactose synthetase from bovine mammary tissue was solubilized by sonic oscillation of microsomes; however, lactose synthetase activity was dependent on the addition of the B-Protein from milk. The A-Protein was found to be mainly in the microsomes whereas the B-Protein was about evenly distributed between the microsomes and the soluble fraction of the cell. Some aspects of the control of lactose biosynthesis, the forces involved in the association of the AB complex and the relation between the B-Protein and α-lactalbumin were discussed.

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