The Isolation and Identification of the B Protein of Lactose Synthetase as α-Lactalbumin*

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SUMMARY

The B protein, a subunit of lactose synthetase (EC 2.4.1.6), was crystallized from bovine skim milk and bovine mammary tissue. The B protein was identified as α-lactalbumin, based on the following criteria: substitution in the enzymic rate assays, spectra, immunological titrations, amino acid composition, mobility on starch gel electrophoresis, molecular weight, and cochromatography on diethylaminoethyl cellulose and Sephadex G-100. Thus the biological function of α-lactalbumin is as a natural occurring subunit of lactose synthetase.

Lactose synthetase (UDP-galactose:α-glucose 1-galactosyl transferase EC 2.4.1.6) catalyzes the following reaction:

\[ \text{UDP-α-galactose} + \alpha-\text{d-glucose} \rightarrow \text{lactose} + \text{UDP} \]

Lactose synthetase is a microsomal enzyme in mammary glands of lactating cows or guinea pigs (1) and a soluble enzyme in bovine milk (2, 3). The soluble enzyme from bovine milk has been partially purified although the overall recovery was low (3). Recently, the soluble enzyme from milk was shown to require the presence of two proteins, called A and B, for activity, and it was found that individually these proteins did not exhibit any catalytic activity (4). The present view is that the A and B proteins are naturally occurring subunits and that enzymic activity is dependent upon the formation of an AB complex.

The B protein has been crystallized and has properties similar to α-lactalbumin. α-Lactalbumin substituted for the B protein of lactose synthetase at identical protein concentrations in both the spectrophotometric and incorporation rate assays (5, 6), suggesting that they were identical.

The present communication describes the purification and crystallization of the B protein of the soluble lactose synthetase isolated from bovine skin milk. Evidence is presented to show that the B protein of lactose synthetase is α-lactalbumin and thus α-lactalbumin is one of the subunits of lactose synthetase.

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EXPERIMENTAL PROCEDURE

Materials

The source of chemicals and special reagents has been previously described (7). Mammary tissue from lactating cows was obtained from the Wilson Packing Company, Oklahoma City, and handled as previously described (7). Other chemicals were obtained from the following sources: DEAE-cellulose from Brown Company (Selectacel) or from Whatman (DE 32), Bio-Gel P-30 was obtained from Bio-Rad, starch for gel electrophoresis was from Connaught Laboratory (Toronto), and Sephadex G-100 and blue dextran were from Pharmacia. Five times crystallized α-lactalbumin, three times crystallized β-lactoglobulin, and antisera to five times crystallized α-lactalbumin were gifts from Dr. B. L. Larson, University of Illinois. Cytochrome c1, type III, was from Sigma, and serum albumin, type V, was from Mann.

Methods

Protein was determined by the method of Lowry et al. (8) with bovine serum albumin as the standard (Mann, type V). Ultraviolet and difference spectra were determined on a Cary model 14 Spectrophotometer at 25°. Conductivity was measured with a Radiometer model CDM 2 conductivity meter. The standard curve for the immunological determination of α-lactalbumin was established by the procedure described by Larson and Hageman (9). The schlieren pattern of the B protein was obtained on a Beckman model E ultracentrifuge, and amino acid analyses were determined on a Beckman-Spinco model 120 amino acid analyzer. The protein samples were hydrolyzed in 6 N triple distilled HCl at 110° (10). Starch gel electrophoresis was conducted on thin gels as described by Abbott and Johnson (11) and the gels were stained in 0.01% Nigrosin (Fisher) in 2% trichloracetic acid for 12 hours and washed with 5% acetic acid. The molecular weight of the B protein and α-lactalbumin was determined from Sephadex G-100 at 4° as described by Andrews (12) with cytochrome c1, β-lactoglobulin, and bovine serum albumin as standards. Blue dextran was used for determining the void volume.

Enzymic Assays

Lactose synthetase activity was determined by measuring UDP formation (spectrophotometric assay) or by measuring the
The absence of glucose.

of lactose formation in the complete system; A, rate obtained in skim milk (75% ammonium sulfate precipitate), not separated into the subunits, was used as the source of the enzyme. O, rate obtained in the absence of glucose.

The subunits of lactose synthetase (A or B protein) may be assayed in the presence of saturating amounts of the counterpart protein and assays for the A and B protein have been described (4). The percentage incorporation of UDP-\(^{14}\)C into lactose-\(^{14}\)C (incorporation assay). The details of the spectrophotometric and incorporation assays have been previously described (4). The rate of incorporation of UDP-d-galactose-\(^{14}\)C into lactose-\(^{14}\)C (incorporation assay). The details of the spectrophotometric and incorporation assays have been previously described (4). The percentage incorporation of UDP-galactose-\(^{14}\)C into lactose-\(^{14}\)C as a function of protein concentration (not separated into subunits) is shown in Fig. 1.

The subunits of lactose synthetase (A or B protein) may be assayed in the presence of saturating amounts of the counterpart protein and assays for the A and B protein have been described with the use of the spectrophotometric assay (7). Similar assays for the A and B protein may be performed by the incorporation assay. However, in both assays, care must be taken to ensure that the level of the saturating protein is high enough so that the protein under assay is all in the form of the AB complex. The incorporation assay is generally used in crude systems (prior to chromatography on Bio-Gel P-30), whereas the spectrophotometric assay is used in more purified preparations (4). The spectrophotometric assay was used to assay for the B protein in skim milk.

**Purification of B Protein from Skim Milk**

All centrifugations were for 20 min at 12,000 \(\times g\) at 4°.

**Fraction 1: Skim Milk**—Fresh unpasteurized bovine skim 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 human milk wishes to remain anonymous.

**Fraction 2: MnCl\(_2\) Supernatant Solution**—Bovine skim milk (4000 ml) was cooled at 4° and the pH was adjusted to 4.6 by the addition of 2 N HCl dropwise over a period of 15 to 20 min. The precipitated casein was removed by centrifugation. After filtration through glass wool, the supernatant solution was adjusted to pH 7.4 with 1 M Tris and then made 0.04 M in MnCl\(_2\) by the addition of 1 M MnCl\(_2\) and centrifuged.

**Fraction 3: Ammonium Sulfate Precipitate**—Solid ammonium sulfate (209 g per liter) was added to the supernatant solution and the precipitate was discarded. The supernatant fluid was brought to 75% saturation (275 g per liter) and centrifuged, and the precipitate was dissolved in a minimum volume of 20 ml Tris-HCl-5 mM MgCl\(_2\) (pH 7.4).

**Fraction 4: Bio-Gel P-30**—To separate completely the A and B proteins of lactose synthetase, the supernatant solution of the 75% ammonium sulfate precipitate was passed in 25-ml portions through a Bio-Gel P-30 column (5 \(\times\) 110 cm) equilibrated and eluted with 20 mM Tris-HCl-5 mM MgCl\(_2\) (pH 7.4). The B proteins from bovine, sheep, goat, and human milk were further purified by chromatography on DEAE-cellulose. The fractions from two Bio-Gel P-30 columns containing the B protein were pooled and the solution (475 ml containing 561 mg of protein per ml) was dissolved in deionized water and the solution was passed through a Bio-Gel P-30 column (5 \(\times\) 25 cm) previously equilibrated with 20 mM Tris-HCl, pH 7.4. After washing with 300 ml of the same buffer, the B protein was eluted with a linear gradient from 20 to 250 mM Tris-HCl, pH 7.8 (300 ml in each chamber).

**Fraction 5: DEAE-cellulose Column Chromatography**—The B protein of lactose synthetase from the bovine was further purified by chromatography on DEAE-cellulose. The fractions from two Bio-Gel P-30 columns containing the B protein were pooled and solid ammonium sulfate (516 g per liter) was added to precipitate the B protein. The precipitate was dissolved in deionized water and the solution was passed through a Bio-Gel P-10 column equilibrated and eluted with water adjusted to pH 6.6 with NH\(_4\)OH. The protein fractions free of ammonium sulfate as checked by conductivity measurements were pooled. The solution was adjusted to 10 to 15 mg of protein per ml (by Assay with \(E_{280}^\text{nm} = 20.1\) ). The solution was allowed to warm to room temperature and the pH was adjusted to 6.8 with 0.1 N NH\(_4\)OH. A saturated solution of ammonium sulfate was added slowly while maintaining the pH at 6.8 until crystallization started. Crystallization usually occurred between
The amount of ammonium sulfate added to initiate precipitation appears to be a function of protein concentration. For example, a solution containing 50 mg per ml of protein required about 45% ammonium sulfate to initiate precipitation.

The slightly turbid solution was allowed to stand for 30 min at room temperature and was centrifuged at 20,000 × g for 30 min. The supernatant solution was allowed to sit at room temperature and crystallization was usually completed within 12 to 24 hours. The crystals were centrifuged at 10,000 × g and were dissolved in a minimum amount of water to which a drop of 0.1 N NH₄OH had been added. The B protein was readily recrystallized by the above procedure. A summary of the purification scheme based on 2 liters of skim milk is presented in Table I. If desired, the B protein may be stored as a lyophilized powder. The crystals were dissolved in water (adjusted to pH 8.6 with NH₄OH) and deionized by passing through a Bio-Gel P-10 column equilibrated in pH 8.6 water. The contents of the tubes containing the B protein were pooled and lyophilized. Dialysis against deionized water is to be avoided since the B protein can readily pass through the dialysis bag (Visking Corporation, 1-inch width). For example, 25 to 100% of the B protein was lost in 48 hours when dialyzed against water. The loss was less when rehydrated (soaked in 10% glycerol for 12 hours) or when new dialysis tubing was used.

The crystal form is dependent upon the mode of crystallization and initial protein concentration. Rapid crystallization from solutions low in protein results in small crystals (Fig. 4, top), whereas the crystallization procedure described results in large club-like crystals (Fig. 4, bottom).

### Isolation of B Protein from Bovine Mammary Tissue

The B protein was isolated from bovine mammary tissue by a procedure similar to the one used for isolation from skim milk. Bovine mammary tissue, 500 g (from late lactation), containing a minimum of milk was ground three times in a mechanical meat grinder. Portions of the minced tissue, 100 g, were homogenized

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total units</th>
<th>Recovery of activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Skim milk</td>
<td>2,000</td>
<td>61,400</td>
<td>400</td>
<td>100</td>
<td>6.5</td>
</tr>
<tr>
<td>2. MnCl₂ supernatant solution</td>
<td>1,920</td>
<td>18,880</td>
<td>373</td>
<td>93</td>
<td>19.8</td>
</tr>
<tr>
<td>3. Ammonium sulfate precipitate</td>
<td>138</td>
<td>6,800</td>
<td>356</td>
<td>89</td>
<td>52.4</td>
</tr>
<tr>
<td>4. Bio-Gel P-30</td>
<td>1,310</td>
<td>1,548</td>
<td>348</td>
<td>87</td>
<td>225.0</td>
</tr>
<tr>
<td>5. DEAE-cellulose column chromatography</td>
<td>530</td>
<td>1,222</td>
<td>289</td>
<td>72</td>
<td>236.0</td>
</tr>
<tr>
<td>6. First crystallization</td>
<td>43.6</td>
<td>814</td>
<td>197</td>
<td>49</td>
<td>242.0</td>
</tr>
<tr>
<td>7. Second crystallization</td>
<td>22.1</td>
<td>694</td>
<td>166</td>
<td>42</td>
<td>243.0</td>
</tr>
</tbody>
</table>

In 20 mM Tris-5 mM MgCl₂, pH 7.4, in a Virtis homogenizer with a variac setting of 80. The combined solutions were centrifuged at 15,000 × g for 15 min and the precipitate was resuspended (250 ml of the above buffer per 100 g of tissue), rehomogenized, and centrifuged as before.

The combined supernatant solutions were oscillated in portions in a Raytheon 10-kc sonic oscillator (maximum setting) for 15 min at 0°. The combined solution was made 50 mM in MnCl₂ by the addition of 1 mM MnCl₂ and then centrifuged at 15,000 × g. The supernatant solution was made 50% in ammonium sulfate (313 g per liter) and the precipitate was discarded. The supernatant solution was brought to 75% saturation in ammonium sulfate (516 g per liter) and after centrifugation the precipitate was dissolved in a minimum volume of 20 mM Tris-5 mM MgCl₂.

### Fig. 3. DEAE-cellulose 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 mM Tris and passed through a DEAE-cellulose column (5 × 25 cm) previously equilibrated with 20 mM Tris-HCl-5 mM MgCl₂, pH 7.8. After washing the column with 300 ml of the same buffer, the B protein was eluted with a linear gradient (----) from 20 to 250 mM Tris-HCl-5 mM MgCl₂, pH 7.8 (300 ml in each chamber). O, protein distribution in eluate fractions (A₂₈₀); ○, lactose synthetase activity of the B protein when assayed by the spectrophotometric assay in the presence of saturating amounts of the A protein.

### Fig. 4. Photomicrograph of crystalline B protein. Top, crystals obtained from solutions low in protein (less than 10 mg per ml), × 515; bottom, crystals obtained by the procedures described in “Results” at protein concentrations of 10 to 15 mg per ml, × 160.
The procedure was then identical with the one described for the isolation and crystallization of the B protein from milk. About 10 to 20 mg of crystals were obtained from 500 g of tissue.

**Identification of B Protein of Lactose Synthetase as α-Lactalbumin**

Previous experiments had shown that the B protein of lactose synthetase had characteristics similar to those of α-lactalbumin and the suggestion was made that α-lactalbumin was a subunit of lactose synthetase (5). However, further evidence was required to show that the B protein of lactose synthetase was α-lactalbumin. In many of the preparations of α-lactalbumin as reported in the literature, there appears to be a minor protein contaminant associated with α-lactalbumin. The possibility could exist that traces of a protein contaminant could be responsible for the observed activity. The following experiments were performed to show that the B protein of lactose synthetase and α-lactalbumin are identical.

**Rate Assays**—α-Lactalbumin can substitute for the B protein of lactose synthetase at identical protein concentrations in both the spectrophotometric and incorporation rate assays (5). Identical rates were obtained when three times crystallized α-lactalbumin or the B protein was titrated at equal concentrations with varying amounts of the A protein (5). Conversely, similar results were obtained when a constant amount of the A protein was titrated with varying but identical amounts of B protein or α-lactalbumin. The rate of 14C-lactose formation when the equal amounts of B protein or α-lactalbumin were saturated with A protein was linear with protein concentration (5). Also, the specific activities of the B protein and two, three and five times crystallized α-lactalbumin were essentially the same (5).

**Spectral Studies**—The ultraviolet spectra and difference spectrum of five times crystallized α-lactalbumin and three times crystallized B protein are presented in Fig. 5. The difference spectrum of the B protein and α-lactalbumin obtained in 0.05 NaOH showed little difference and these results are presented in Fig. 5. The A280:A260 ratio is 1.31, which is in good agreement with 1.32 as reported for α-lactalbumin by Wetlaufer (13).

**Immunological Titrations**—Equal concentrations of five times crystallized α-lactalbumin and two times crystallized B protein were assayed immunologically by the Oudin technique as described by Larson and Hageman (9) for the assay of α-lactalbumin in a variety of materials. Antisera had been prepared to the five times crystallized α-lactalbumin. At equal protein concentrations, the standard curves obtained for the B protein and α-lactalbumin are similar (Fig. 6).

Further experiments showed that antisera to the five times crystallized α-lactalbumin would completely inhibit lactose synthetase activity. Ten micrograms of five times crystallized α-lactalbumin were incubated for 1 hour at 37° in 0.2 ml of 20 mm Tris-HCl, pH 7.4, with 0 to 50 μl of antisera (9). The mixtures were stored for 18 hours at 4° and, after adding 10 units of A protein, they were assayed for lactose synthetase activity by the spectrophotometric assay. No lactose synthetase activity was detected in the presence of 10 μl of antisera (lowest level used). Without the above incubations, there was a 50-70% loss of activity. Control experiments showed that antisera at comparable levels did not inhibit the indicator enzymes used in the assay. Similar results were obtained with 10 μg of twice crystallized α-lactalbumin.

**Fig. 6. Standard curve for the immunological determination of identical concentrations of five times crystallized α-lactalbumin and twice crystallized B protein by the Oudin technique as described by Larson and Hageman (9).** Antisera had been prepared to the five times crystallized α-lactalbumin.
crystallized B protein. Thus comparable levels of antiserum to α-lactalbumin inhibited lactose synthetase activity when identical levels of B or α-lactalbumin were used in the enzymatic assay.

Amino Acid Composition—The relative amino acid composition of the five times crystallized α-lactalbumin and two times crystallized B protein after hydrolysis for 24 and 48 hours are compared in Table II together with the amino acid composition presented by Gordon and Ziegler (14) for α-lactalbumin.

Electrophoresis on Starch Gel—The electrophoretic patterns of five times crystallized α-lactalbumin and two times crystallized B protein at both pH 3.3 and 8.6 are shown in Fig. 7.

An effort was made to determine whether the heavily stained protein area (Fig. 7) or other areas did indeed have B activity. Two times crystallized α-lactalbumin (containing a minor band just before the major band at pH 3.3) and two times crystallized B protein were subjected to electrophoresis on the thin starch gels at pH 3.3 in the lactate buffer (4-cm slots) and thin vertical strips of gel corresponding to the edge of the slot were removed and stained to locate the protein area. The unstained portion was cut into a series of 1-cm sections and to each section 0.5 ml of water was added. The gel was broken by a glass rod and the mixture was frozen and thawed three times. This mixture was then filtered by suction and each filtrate was assayed for B activity by the spectrophotometric assay. B activity was found only in the area corresponding to the darkly stained protein. Under these conditions, 32% of the B activity was recovered from an initial concentration of 300 μg.

Molecular Weight—The molecular weight estimated by elution from the Bio-Gel P-30 column was about 15,000 (15). The

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>α-Lactalbumin</th>
<th>Five times crystallized B protein</th>
<th>Difference between five times crystallized B protein and five times crystallized α-lactalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>12</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Histidine</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Arginine</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Asparagine</td>
<td>22</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Threonine</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Serine</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Glutamic</td>
<td>14</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Proline</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Glycine</td>
<td>7</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Alanine</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Valine</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Methionine</td>
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<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Leucine</td>
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<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 7. Electrophoretic mobilities of the B protein and α-lactalbumin on starch gel electrophoresis. The starch gel electrophoresis was performed on thin gels on glass plates essentially by the method described by Abbott and Johnson (11). Between 150 and 200 μg of protein were used as samples. The gels were maintained with 0.01% Nigrosin in 2% trichloroacetic acid for 24 hours and then washed with 5% acetic acid. The pH 3.3 buffer consisted of 8 mM aluminum lactate and 3 M urea adjusted to pH 3.3 with lactate acid. The pH 8.6 buffer contained 15 mM Tris, 2.75 mM citric acid, 8.0 mM boric acid, and 0.5 M urea, adjusted to pH 8.6 with NaOH. Left, electrophoretic mobility of once crystallized B, five times crystallized α-lactalbumin, and twice crystallized B protein in aluminum lactate (pH 3.3); right, five times crystallized α-lactalbumin and three times crystallized B protein in Tris-citrate (pH 8.6).

Fig. 8. Schlieren pattern of the B protein. The sedimentation pattern of the B protein (purified through DEAE-cellulose, 21 mg of protein per ml) in 75 mM Tris-HCl-10 mM MgCl₂, pH 7.8, were photographed at 105, 150, 180, and 200 min (from left to right) after the centrifuge reached its full speed of 59,700 rpm. Camera enlargement ratio was 2.1083, bar angle was 75°, and the temperature was 20°. The schlieren pattern of the B protein purified through the DEAE-cellulose column is shown in Fig. 8. The $s_{20,w}$ for the B protein was calculated to be 1.70, assuming a partial specific volume of 0.735 (16). With Wetlaufer's data on the concentration de-
dependence of the $s_{20, w}$ of $\alpha$-lactalbumin, the sedimentation velocity was calculated to be 1.84 which is in good agreement with Wetlaufer's value of 1.87 (13). When a diffusion coefficient of $10.57 \times 10^{-7}$ cm² per sec obtained for $\alpha$-lactalbumin (16) was used, the molecular weight of the B protein was calculated to be 16,000. The molecular weight of the B protein and $\alpha$-lactalbumin determined by chromatography on Sephadex G-100 was 15,500 (Fig. 9).

Cochromatography of B Protein and $\alpha$-Lactalbumin—To show further the correspondence between the B protein of lactose synthetase and $\alpha$-lactalbumin, equal concentrations of two times crystallized B protein and five times crystallized $\alpha$-lactalbumin were mixed together and chromatographed on DEAE-cellulose and Sephadex G-100 as described earlier. The results as shown in Fig. 10 show the presence of only a single species with respect to molecular weight and charge.

Interchange of A and B Proteins in Rat Assay

The data presented in Fig. 2 show that the milk of the cow, sheep, goat, and human may be separated into the A and B proteins by chromatography on Bio-Gel P-30. Furthermore, the A protein (peak tube of the first protein peak, 0.2 ml, Fig. 2) of a given species would combine with the B proteins (peak tube of the second protein peak, 0.1 ml, Fig. 2) from all of the other species to give lactose synthetase activity as measured by the spectrophotometric assay. Conversely, each B protein would combine with all the A proteins to give lactose synthetase activity. Also, the A and B proteins of the rat and bovine are interconvertible in the enzymic assays. The rates obtained by these interconversion experiments were similar. Thus it would appear on a qualitative basis that the A and B proteins of these species are interchangeable.

**DISCUSSION**

The B protein of the soluble lactose synthetase purified from bovine skim milk was crystallized from an ammonium sulfate solution and was shown to be identical with $\alpha$-lactalbumin. Previous experiments (5) have shown that, at identical protein concentrations, five times crystallized $\alpha$-lactalbumin and the B protein gave identical rates in the spectrophotometric and incorporation assays for lactose synthetase. Other evidence to support the conclusion that the B protein of lactose synthetase and $\alpha$-lactalbumin are the same is as follows. Both have the same ultraviolet spectra and there is little difference in the difference spectrum. They have similar $E_{1%}^{1%}$ and $A_{280}/A_{280}$ ratios (5). Both have the same molecular weight and amino acid composition. Mixtures of the B protein and $\alpha$-lactalbumin were inseparable on DEAE-cellulose and Sephadex G-100. The immunological titration curve at identical protein concentrations was the same, indicating that the B protein assayed as 100% $\alpha$-lactalbumin. Both had identical mobility on thin starch gel electrophoresis at pH 3.3 and 8.6.

The amino acid composition of the five times crystallized $\alpha$-lactalbumin and two times crystallized B protein is compared in Table II to the data obtained by Gordon and Ziegler (14) for $\alpha$-lactalbumin. In general, the agreement is quite good except for tyrosine (1 less residue) and proline with nearly 2 additional residues. However, there was little difference in the number of amino acid residues when the five times crystallized $\alpha$-lactalbumin and the two times crystallized B protein were compared under identical analytical conditions. $\alpha$-Lactalbumin was crystallized from bovine milk 10 years ago and is part of the classical "albumin" fractions of the whey proteins (17). A difficulty in many of the preparative procedures has been that $\beta$-lactoglobulin was hard to remove completely from $\alpha$-lactalbumin. The separation of these proteins is based on repeated precipitation of $\alpha$-lactalbumin at pH 4.6, and under these condi-
tions the majority of \( \beta \)-lactoglobulin remains in solution. Hence the absolute removal of \( \beta \)-lactoglobulin by this procedure is difficult. The use of the Bio-Gel P-30 and the DEAE-cellulose step in the present procedure completely separates \( \beta \)-lactoglobulin from \( \alpha \)-lactalbumin. Immunological assays for \( \beta \)-lactoglobulin in the B protein obtained from the DEAE-cellulose column revealed no \( \beta \)-lactoglobulin. No evidence for \( \beta \)-lactoglobulin was found in the starch gel electrophoresis patterns at pH 3.3 and 8.6.

The B protein may be readily isolated from skim milk or whole milk and may also be isolated and crystallized from mammary tissue. The B protein from mammary tissue has the same properties as the B protein isolated from milk. Previous studies on the subcellular distribution of the A and B proteins of lactose synthetase in mammary tissues have shown that the B protein is evenly distributed between the microsomes and the soluble portion and that the B protein may be readily dissociated from the microsomes (7). As measured in terms of activity, bovine skim milk has more B protein than A protein and this may be due to the fact that the B protein is mainly a soluble protein or held loosely to the microsomes and that during the secretory process more B would be lost to milk than A.

Starch gel electrophoresis at pH 3.3 and 8.6 has shown that five times crystallized \( \alpha \)-lactalbumin and the twice crystallized B protein have the same mobilities. In both cases, there is only one major protein band visible. However, at pH 3.3, three times crystallized \( \alpha \)-lactalbumin showed a slower moving minor band just adjacent to the major band. No such band was visible with once crystallized B protein purified by the present procedure and thus it and the twice crystallized B protein appear to be homogeneous at pH 3.3 and 8.6 in thin starch gel electrophoresis. Many previous preparations of \( \alpha \)-lactalbumin appear to be heterogeneous at about pH 3.0 to 3.3 when run in moving boundary or starch gel electrophoresis especially with the use of lactate buffers (17–19). Kronman and Andreotti (20) suggest that the majority of observations on heterogeneity of \( \alpha \)-lactalbumin may be accounted for on the basis of protein-protein or ion-protein interactions. The fact that the B protein and five times crystallized \( \alpha \)-lactalbumin were homogenous in thin starch gel electrophoresis and that activity for B protein could be extracted from the gel at the intense protein stain spots virtually rules out the remote possibility, in light of all the other evidence presented, that the B protein activity is due to a minor protein contaminant.

Babad and Hassid (3) have recently described the partial purification and properties of the soluble lactose synthetase from whole milk. Their preparation was not separated into subunits and apparently contained a mixture of the A and B proteins. The large loss in activity observed in the last purification step probably resulted from the partial separation of the A and B protein by ammonium sulfate and loss of the B protein upon the subsequent dialysis step. The incorporation assay (Fig. 1) gave results similar to those reported by Babad and Hassid (3).

The A and B proteins obtained from the cow, goat, sheep, and human were interchangeable in the rate assays, as were the A and B proteins of the rat and cow. Johke, Hageman, and Larson (21) have reported that \( \alpha \)-lactalbumin from ruminants reacts with antiserum to bovine \( \alpha \)-lactalbumin, but \( \alpha \)-lactalbumin from nonruminants does not react with antiserum to bovine \( \alpha \)-lactalbumin; that is, there is an immunological difference between the \( \alpha \)-lactalbumin of the ruminant and the nonruminant. The present study shows that A and B proteins from ruminants (cows, sheep, and goats) will qualitatively react with the counter-part protein from nonruminants (human and rat), indicating that there may be a distinction between the immunological response and enzymatic activity.

The data show that the B protein of lactose synthetase is \( \alpha \)-lactalbumin and thus a biological function for \( \alpha \)-lactalbumin can be described; that is, \( \alpha \)-lactalbumin is a naturally occurring subunit of lactose synthetase.

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The Isolation and Identification of the B Protein of Lactose Synthetase as $\alpha$-Lactalbumin
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