THE EFFECTS OF pH AND ELECTRODIALYSIS ON THE BINDING OF VITAMIN B_{12} BY β-LACTOGLOBULIN AND ASSOCIATED PEPTIDES

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ABSTRACT

THE EFFECTS OF pH AND ELECTRODIALYSIS ON THE BINDING OF VITAMIN B_{12} BY β-LACTOGLOBULIN AND ASSOCIATED PEPTIDES

By

James Kirk

Milk proteins have been shown to bind vitamin B_{12} in excess of that inherently associated with the milk proteins. Beta-lactoglobulin (β-Lg) was chosen for this work, since past research has shown β-Lg to exhibit the highest vitamin B_{12} binding capacity of the milk proteins.

The β-Lg used in this research was prepared in a crystalline state and its parameters of purity, as determined by chemical and physical analyses, agreed with published values. The vitamin B_{12} binding capacity of the protein was shown to be pH dependent, exhibiting its greatest association with the vitamin in the pH range of 6.6 to 6.8 where approximately 460 μμg of vitamin B_{12} per mg of protein was bound. However, following electrodialysis of β-Lg, no measurable binding of B_{12} by the protein could be detected at pH 6.6 and 6.8.

Polyacrylamide gel electropherograms, sedimentation coefficients and chemical analyses of β-Lg and electrodialyzed β-lactoglobulin [(E)β-Lg] showed no

differences between the two proteins. However, the relative hydrolysis rates of β-Lg and (E)β-Lg by trypsin and chymotrypsin indicated a conformational change in the (E)β-Lg which was believed responsible for the loss in binding capacity.

At pH 9.0 β-Lg exhibited a lower binding capacity than was measured at pH 6.6 and 6.8. This was attributed to a reduction in polar binding sites and a change in conformation resulting from dissociation of the protein to its monomeric state. At pH 9.0 the (E)β-Lg and (E)β-Lg-peptide mixture exhibited binding capacities for the vitamin which varied with the buffer system used in the model system. The binding of vitamin B_{12} by (E)β-Lg may have resulted from the secondary binding to the solvent ions associated with the protein monomers and/or the dissociation of electrodialyzed beta-lactoglobulin.

Polyacrylamide gel electrophoresis of the β-Lg and (E)β-Lg-vitamin B_{12} complexes indicated that the vitamin was only loosely associated with the protein molecules, possibly through electrostatic bonds.

Three peptides were recovered from purified β-Lg by electrodialysis. These peptides were shown to be electrostatically associated to the protein and were arbitrarily designated as "negative," "+2" and "+3," according to their mobility characteristics on thin-layer high-voltage electropherograms. The calculated

molar binding ratios of peptides to β-Lg indicated a random association between the protein and peptides. The molecular weights of the isolated peptides were estimated at 1,500 to 3,600 by gel filtration chromatography, whereas minimum molecular weight calculations from residue weights of the limiting amino acids gave values of 4,000 for the negative peptide and 6,000 for the +2 and +3 peptides. These data may be interpreted to suggest that the peptides may have secondary structures.

Experiments designed to assess the binding of vitamin B_{12} by the peptides in model systems revealed that, under the conditions employed, the peptides did not bind vitamin B_{12}. Therefore, it is believed that the loss of peptides from the protein was not solely responsible for the loss of binding capacity by (E)β-Lg.

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INTRODUCTION

Vitamin B_{12} is unique among the vitamins because it is not synthesized by higher plants and animals. Wherever it is found in nature, its origin can be traced back to bacteria or other micro-organisms, growing in soil, water or in the rumen or intestine of some animals.

Humans are entirely dependent on dietary B_{12}, since their intestinal flora either do not synthesize B_{12}, or it is not released from the cells of synthesizing organisms in the region of the gut from which absorption occurs.

In view of this metabolic deficiency, nature has provided for the initial vitamin B_{12} needs of the mammalian offspring by its presence in the mammary secretions.

In the bovine, the vitamin B_{12} activity of milk is attributed entirely to cobalamin because of a highly selective absorption in the stomach. It is also known that 95% of the naturally occurring vitamin B_{12} in cow's milk is associated with the milk proteins; however, these protein-B_{12} complexes do not exhibit intrinsic factor activity.

Beta-lactoglobulin (β-Lg), the major whey protein in milk, shows the highest vitamin B_{12} content of the milk proteins. In addition to the B_{12} inherently associated to β-Lg, additional B_{12} can be bound by the β-Lg when the two components are equilibrated in model systems.

Previous research indicated that the major factor involved in the binding of vitamin B_{12} by β-Lg was the association of B_{12} with peptides bound to the β-Lg.

It was the intention of this research, with the aid of previously published information, to investigate the mode of vitamin B_{12} binding with the peptides and the peptide carrier protein.

LITERATURE REVIEW

Vitamin B_{12}: History

In 1920 Whipple *et al.* found that feeding liver to dogs made anemic by bleeding accelerated the regeneration of their red blood cells. Minot and Mulphy (1926) followed this discovery with the observation that liver contains a nutritional element required for the cure of pernicious anemia. Investigations were immediately begun to determine the identity of the "anti-pernicious anemia factor" present in liver. Early attempts to purify the anti-pernicious anemia factor were greatly hampered by the minute quantities present in liver, and the consequent need to use pernicious anemia cases in relapse to assay the liver extractions.

In 1948 crystalline vitamin B_{12} (5,6 dimethyl-benzimidazolylcobamide cyanide) was identified as the anti-pernicious anemia factor by Rickles *et al.* (1948), and Smith and Parker (1948).

Proof that cyano B_{12} functions in biological reactions is credited to Barker *et al.* (1958), and Barker *et al.* (1960) following the isolation of deoxy-adenosyl-B_{12}. Vitamin B_{12}, itself, has not been identified as an active cofactor for any known enzymatic reaction. Rather,

a related family of analogs, the B_{12} coenzymes, have been shown to function in this capacity.

Vitamin B_{12}: Physical Properties

Cyanocobalamin crystallizes as dark red needles or prisms with a melting point of 300 C. Whether cyanocobalamin is crystallized from aqueous acetone or from water, a considerable but variable amount of loosely held water (usually 10 to 12 per cent) is associated with the crystals. This can be removed by heating under reduced pressure. Vitamin B_{12} is soluble in water up to 1.25 per cent, relatively soluble in lower alcohols and phenols, but insoluble in most other organic solvents. Aqueous solutions of cyanocobalamin are neutral and show absorption maxima at 278, 361 and 555 nm, which do not shift markedly with changes in pH as shown by Hodgkin et al. (1949). Electrometric titrations and conductivity measurements of Hodgkin et al. (1948) also have shown the absence of any strongly ionizing groups.

Brink et al. (1949) determined the molecular weight of cyanocobalamin, by the boiling point method, to be 1,490 ± 150; these data agree with the molecular weight of 1,360 to 1,575 as reported by Hodgkin et al. (1949) from their x-ray crystallography data. The variance of the molecular weight is attributed to the variable hydration of the B_{12} crystals.

Vitamin B_{12}: Structure

The chemical structure of cyanocobalamin, as it is isolated from the liver, was identified by Hodgkin et al. (1956). The cyanocobalamin molecule can be divided into two major portions: the four reduced pyrrol rings forming the macro "corrin ring" and the nucleotide, which unlike the nucleotides of nucleic acids, contains 5,6 dimethylbenzimidazole as its base with ribose-3-phosphate linked by an α-glycosidic bond. The corrin ring contains trivalent cobalt chelated to the four nitrogen atoms of this ring, a nitrogen atom of the 5,6 dimethylbenzimidazole ring, and a cyanide ion, the latter being an artifact of the isolation procedure (Smith et al., 1952).

Other functional moieties of the cyanocobalamin molecule are: 1-amino-2-propanol to which the 2-OH group of the phosphate is esterified, and propionic acid.

Cobalamin coenzymes, the functional forms of vitamin B_{12}, differ structurally from cyancobalamin in that the cobalt is divalent in nature and the cyanide group is replaced by a molecule of 5-deoxyadenosine.

Vitamin B_{12}: Presence in Milk

The average vitamin B_{12} content of bovine milk has been reported by Collins et al. (1951) and Gregory (1954) to be 6.6 micrograms B_{12} per liter, with a range of 2.0 to 24.0 micrograms B_{12} per liter. Findings of several

workers indicate the vitamin B_{12} content of milk did not vary significantly with breed, season of the year, or stage of lactation (Collins et al., 1951; Collins et al., 1953; Hartman et al., 1956; and Gregory et al., 1958). However, heating does decrease the vitamin B_{12} content in milk, depending upon the amount of heat and the method of processing employed (Ford, 1957; and Kon, 1961).

Gregory (1954) and Gregory and Holdsworth (1955a and 1955b) reported nearly all of the B_{12} present in milk was in the cobalamin form and that in sow's milk the B_{12} is bound to peptides and proteins, thus making it unavailable for test organisms. These data were confirmed by Kim et al. (1965), who reported approximately 95% of the total vitamin B_{12} content of cow's milk associated with the milk proteins. Gizis et al. (1965) determined the binding capacities of the individual milk proteins, in pH 9.0 borate buffer, using Lactobacillus leichmannii and $Co^{60}B_{12}$. They found B_{12} to be ubiquitously distributed among the various milk proteins, with higher concentrations associated with the whey proteins. Gizis et al. (1965) also reported the isolation of two peptides (3,000 and 9,000 M.W.) from dialyzed cow's milk by an application of electrodialysis, which absorbed vitamin B_{12} in the order of 1×10^5 micromicrograms (μμg) B_{12} per mg of protein.

Although milk has been shown to have a considerable vitamin B_{12} binding capacity, Ungley and Childs (1950) have shown that these vitamin-protein complexes do not exhibit intrinsic factor activity.

Characteristics Of Bovine Beta-Lactoglobulin

Beta-Lactoglobulin: Characteristics

β-Lg, the major whey protein of mature bovine milk, is synthesized in the epithelial cells of the mammary gland (Twarog and Larson, 1962) and corresponds to about 7 to 12% of the total milk protein. β-Lg was first isolated and crystallized by Palmer (1934). In 1946 Li indicated the possibility that β-Lg from mixed milk was a heterogeneous protein. Aschaffenberg and Drewry (1955 and 1957) were the first to distinguish genetic variants A and B in β-Lg by their difference in electrophoretic mobilities. Later, Bell (1962) demonstrated the presence of a third genetic variant, β-Lg C, in the milk of Jersey cows. Kiddy et al. (1965) and Townend and Basch (1965) have shown that the presence of β-Lg A, B and C is genetically controlled by codominant autosomal alleles Lg^A, Lg^B and Lg^C. Kiddy et al. (1965) demonstrated that cows can be homozygous or heterozygous in their production of β-Lg, and heterozygous cows give approximately equal amounts of the phenotypic β-Lg. The genetic variance of β-Lg has been shown to

manifest itself in the amino acid composition of the protein. Gordon et al. (1961) and Piez et al. (1961) found β-Lg A to differ from β-Lg B by the substitution of two aspartic and two valine residues for two glycine and two alanine per 35,500 molecular weight. Beta-lactoglobulin C has been differentiated from β-Lg A or B by two additional histidine residues and two less glutamine residues per 36,000 molecular weight (Kalan et al. (1965). Kalan et al. (1965) also determined by partial amino sequencing that the C-terminal (leucine) and N-terminal (isoleucine) residues of the three genetic species were the same. Thus genetic amino acid substitution does not occur at, or near, the terminal portions of the peptide chain.

Values reported for the molecular weight of native β-Lg range from 35,000 to 37,000 (Pedersen, 1936; Bull and Currie, 1946; Ogston and Tilley, 1955; Green and Aschaffenburg, 1959; Wirtz et al., 1964; and Aschaffenburg et al., 1965). The molecular weight generally assigned to native β-Lg is 36,000 at a pH between 5.5 and 7.5 where associative and dissociative reactions are at a minimum. This corresponds to a sedimentation coefficient of 2.85 S.

The first evidence that native β-Lg is a molecular dimer composed of two 18,000 molecular weight polypeptides is attributed to Bull (1946) and Bull and Currie (1946).

Later studies by Timascheff (1964), Townend and Timascheff (1957), Green and Aschaffenburg (1959), Aschaffenburg et al. (1965) and Wirtz et al. (1964), using small-angle x-ray scattering and ultracentrifugation, have shown that native β-Lg at pH 5.7, where minimum aggregation occurs, is composed of two identical spherical subunits (molecular weight 18,000) with a radius of 17.0Å, impinging on each other by 2.3Å at their surface contact. Timascheff (1964) has reported the two subunits form a stable dimer as a result of hydrophobic interactions.

Nozaki et al. (1959) showed the isoionic point of mixed β-Lg in pure water as pH 5.39, while β-Lg A and B exhibited isoionic points of 5.13 and 5.14 respectively. Nozaki et al. (1959) pointed out that these values were lowered as potassium chloride or calcium chloride was added, owing to the binding of potassium and calcium, which could suggest centers of unusually high negative potential on the isoionic β-Lg.

Confirmational studies of β-Lg have been a center of great controversy over the last ten years. Urnes and Doty (1961) and McKenzie (1967) have offered the best interpretation of optical rotary dispersion data, concluding that β-Lg is composed of approximately 0.35 alpha-helix, 0.34 beta-conformation and 0.33 disordered form. Tanford's review (1962) of the titration curves of native β-Lg have shown that the native protein appears to contain

six imidazole groups per 36,000 molecular weight, compared to four for the analytical figure. Investigation of this phenomenon revealed that two carboxyl groups of the native protein are titrated with pH characteristics of imidazole groups because they are buried in the hydrophobic interior of the protein molecule, where dimer attraction forces are known to exist.

Beta-Lactoglobulin: Effect of pH

Below the Isoelectric Point of Beta-Lactoglobulin.--Townend and Timascheff (1957) were the first to show the dissociation of mixed β-Lg into monomeric subunits at pH values below 3.5. Nozaki et al. (1959) have explained the dissociation of β-Lg as nonspecific electrostatic repulsion resulting from a progressive increase in positive charge as the pH is lowered. McKenzie and Sawyer (1966) reported that the extent of protein dissociation is dependent upon: pH, protein concentration and genetic variant. Townend et al. (1960) studied the reversible dissociation below pH 3.5, using ultracentrifugal and light-scattering techniques. They reported the subunits to be identical and corresponded to the two polypeptide chains of the protein dimer. Townend et al. (1961) using radioactively labeled β-Lg A and B showed that no hybrid β-Lg dimers are formed when a mixture of the two genetic variants are acidified and reneutralized. This would indicate a

specific structural difference in the area of subunit contact of the genetic variants. However, Basch and Timascheff (1967) found no difference in the titration curves or maximum hydrogen ion binding capacity (40 H^+ per molecule) of β-Lg A, B, and C.

Above pH 4.5 the titration curves of β-Lg are dependent upon the genetic variant and can be accounted for in terms of normal ionization of all groups, with the exception of two histidine residues in the C variant and the two hidden carboxyl groups in all three variants. These data in conjunction with other physical properties of β-Lg suggest that sufficient attractive forces exist between ionizable groups to cause the genetic variants of β-Lg to behave differently between pH 3.5 and 5.2.

Townend et al. (1960) using ultracentrifugation and electrophoresis, Kumosinski and Timascheff (1966) using x-ray crystallography, and McKenzie and Sawyer (1967) using optical rotary dispersion, have shown β-Lg A, B and AB to tetramerize (a cubic array of eight dimer spheres (molecular weight 144,000) in the pH range of 3.5 to 5.2 with maximum association occurring at pH 4.6. McKenzie (1967) found that tetramerization involves hydrogen bonding of the carboxyl groups in the aspartic-glutamic rich areas of the protein dimers, which in the case of the A variant is enhanced by the additional aspartic residues. Townend (1965) reported that approximately 30 carboxyl groups of

the native protein are buried by the formation of the tetramer.

At present no associative interaction has been observed for β-Lg C in the pH range 3.5 to 5.2 (Bell and McKenzie, 1964 and 1966).

Above the Isoelectric Point of Beta-Lactoglobulin.--Wirtz et al. (1964) have carried out small-angle x-ray scattering investigations of β-Lg at pH 5.7 and found no evidence of molecular aggregation. The first evidence of physical change in β-Lg above its isoelectric point was reported in the original ultracentrifugation studies of Pedersen (1936). Tanford et al. (1959) observed a change in optical rotation while studying the titration curves of β-Lg. They observed a reversible change in configuration near pH 7.5 at 25 C which parallels the titration of imidazole groups and the two buried carboxyl groups from the interior of the molecule. Tanford et al. (1959) regarded this reversible change in optical rotation as a refolding of part of the polypeptide chain rather than an unfolding of the protein molecule. Basch and Timascheff (1967) support these data with their hydrogen ion equilibrium experiments on β-Lg A, B and C.

Georges et al. (1962) have shown that at alkaline pH (above 8) the β-Lg dimers are reversibly dissociated into their monomeric subunits.

Studies by McKenzie and Sawyer (1966 and 1967) involving zone electrophoresis and optical rotary dispersion have also shown a non-reversible, time dependent, conformational transition in β-Lg at pH 8.6 and above. The reaction involves polymerization to β-Lg polymers of 200,000 molecular weight. The association reaction was believed to involve oxidation of sulfhydryl groups and rupture of disulfides, since polymerization was nullified in the presence of N-ethylmaleimide. This would support the finding of Dunhill and Green (1965), who observed the N conformation of β-Lg at low pH shielded the sulfhydryl groups, while the transition to the R-state in the region of pH 7.4, caused by the release of the two hidden carboxyls, made the sulfhydryls more accessible by the refolding of the polypeptide chain.

Beta-Lactoglobulin: Binding Complexes

Various workers have reported the binding of non-protein anions to β-Lg and modified β-Lg. Ray and Chatterjee (1967) studied the binding of methyl orange and dodecyl-pyridine to β-Lg by equilibrium dialysis. Ray (1968) and Seibles (1969) have demonstrated the binding of dodecylsulfate to β-Lg, showing a complex of two moles of dodecylsulfate per mole of protein. All research concerning the binding of anions to β-Lg has shown the binding capacity of the protein to increase by a factor of 3-10 as the pH is raised from its isoionic

point to pH 7.5. However, no new binding sites were exposed by the conformation change occurring at pH 7.5 (Ray and Chatterjee, 1967). The exact nature of the binding sites on the protein is as yet undetermined, although Seibles (1969) suggested the influence of histidine residues as a possible factor.

β-Lg has also been shown to bind inorganic cations. Carr (1953) found β-Lg to exhibit a binding capacity for calcium ions, which increased with a rise in pH. These findings were supported by the work of Zittle et al. (1957). King et al. (1959) showed the copper and iron inherently associated with skimmilk protein was not affected by a decrease in pH from 6.7 to 3.0. However, the ability of the skimmilk and whey proteins to bind additional copper and iron decreased as the pH was lowered from 6.7 to 3.0. Aulakh (1967) reported a binding capacity of 2.5 moles of copper per mole of β-Lg at pH 6.5. Barker and Saroff (1965) have shown that β-Lg, one of the few milk proteins that binds sodium ions, exhibits no binding of the monovalent cations at its isoelectric point (IEP). However, the binding of sodium ions is known to increase as the pH is raised above the IEP of β-Lg and reaches a maximum at pH 9.48. Baker and Saroff (1965) have also indicated that the binding of sodium and the configuration changes associated with β-Lg appear to be controlled by the same ionization reaction.

Other β-Lg binding complexes have been reported. Kim et al. (1965) have reported β-Lg releases 203 micro-micrograms vitamin B_{12} per mg of protein, when autoclaved in the presence of acid and cyanide. In a later report Gizis et al. (1965) indicated that native milk proteins do not exhibit their maximum vitamin B_{12} binding capacity. They observed β-Lg to bind a total of 850 μμg/mg of protein of vitamin B_{12} in borate buffer, pH 9.0. Dorris (1968) reported that electrodialysis of β-Lg released a peptide mixture with an average vitamin B_{12} binding capacity of 5,410 μμg/mg of protein.

Recently Ford et al. (1969) have confirmed the presence of a reversible folate-β-Lg complex in milk, which exhibited a pH dependence. At pH values of 8.8, 7.1 and 6.0 the folate was wholly associated with the β-Lg. At pH 5.0 only 61 per cent was bound and at pH 3.6 all folate was dissociated from the protein.

EXPERIMENTAL PROCEDURE

Preparative Methods

Beta-Lactoglobulin: Isolation

The isolation of β-Lg was performed essentially as described by Aschaffenburg and Drewry (1957) with minor modifications. Details are presented in the appendix.

Beta-Lactoglobulin: Recrystallization

Details are presented in the appendix.

Chemical Methods

Nitrogen

Nitrogen determinations were performed using a micro-Kjeldahl technique. Details are presented in the appendix.

Tryptophan

Tryptophan, an acid labile amino acid, must be determined apart from the rest of the amino acids. Tryptophan was determined spectrophotometrically as described in Procedure W of Spies (1967). Details are presented in the appendix.

Sulfhydryl

The method used for the determination of sulfhydryl groups was that of Ellman (1959). Details are presented in the appendix.

Amino Acid Analyses

Amino acid analyses were carried out on 22 h hydrolysates of the β-Lg, (E)β-Lg, and isolated peptides, employing a Beckman Amino Acid Analyzer Model 120 C according to the method of Moore, Speckman and Stein (1958). Details are presented in the appendix.

N-Terminal Amino Acid Analyses

The n-terminal amino acids of β-Lg and (E)β-Lg were determined as dansyl chloride derivatives (Gray, 1967). Details are presented in the appendix.

Colorimetric Determination of Protein

Protein determinations were made using the Folin-Lowry procedure described by Layne (1955). Details are presented in the appendix.

Physical Methods

Method of Electrodialysis

The equipment employed for electrodialysis consisted of an ice bath to insure low temperature during electrodialysis, a power source reading voltage and

amperage, and the electrodialysis cell. The electrodialysis cell consisted of three parts: a glass cylinder (45 cm in length and 4.8 cm in diameter) to hold the dialysis water; a U-shaped glass rod to form an internal frame for the dialysis membrane; and two platinum foil electrodes.

Visking cellulose membrane was used as the semipermeable dialysis membrane and was treated with ethylenediaminetetraacetic acid (EDTA) and thoroughly washed with deionized water prior to use in the electrodialysis experiments. The membrane was knotted at one end and slipped over the U-shaped glass frame, which served as a place of stable attachment for the platinum foil electrode and ensured the membrane was held apart.

The β-Lg to be electrodialyzed was suspended in deionized water and poured into the dialysis sac. The membrane being held by the frame was positioned in the glass cylinder, which was filled with deionized water. The second platinum electrode, also supported by a glass frame, was positioned in the dialysis water outside the membrane and the entire electrodialysis cell was placed in an ice bath. To ensure even protein suspension during electrodialysis a glass stirring rod was placed inside the membrane and attached to a variable-speed Lightning Mixer. The power source was connected to the platinum electrodes of the dialysis system with the electrode in

the dialysate serving as the cathode and the electrode inside the membrane serving as the anode.

Two hundred volts of direct current were applied to the system. Initially, there was zero amperage. However, as charged particles began diffusing through the membrane, the current began to increase and reached a maximum. At maximum amperage, the dialysate water was changed and the electrodialysis continued. The current maximum was attributed to a charge equilibrium across the membrane. Therefore, to force the dissociation reaction, it was necessary to change the water whenever the amperage reached a maximum. The dialysate water was changed until little or no amperage could be detected in the system. This implies that few if any charged particles were being transferred across the dialysis membrane to complete the electrical circuit.

The electrodialysate was concentrated by pervaporation in EDTA treated cellulose membranes to approximately 200 ml, shell-frozen and lyophilized.

Polyacrylamide Gel Electrophoresis

The procedure for preparing and performing discontinuous polyacrylamide gel electrophoresis was essentially similar to that described by Melachouris (1969). The discontinuous flat-bed gel system required two solutions: a running gel solution and a spacer gel

solution. The running gel solution (9% gel) was prepared by dissolving 45 g Cyanogum 41 in 0.380M tris-hydroxymethylaminomethane (TRIS)-HCl buffer, pH 8.9, and made to a volume of 500 ml. To this solution 0.5 ml of N, N, N', N'-tetramethylethylenediamine (TEMED) was added. The spacer gel solution (5% gel) was prepared by adding 25 g of Cyanogum 41 to 0.062M Tris-HCl buffer, pH 6.7, and made to a volume of 500 ml. To the latter solution, 0.5 ml TEMED was added. The running and spacer gel solutions were stored at 5 C and brought to room temperature before use.

The running and spacer gels were poured into a flat Plexiglas gel bed (26 x 12 x 0.4 cm) as described. A Plexiglas spacer was placed 15 cm from one end of the gel bed to form a front for the running gel. The large area of the gel bed was filled with 190 ml of running gel solution containing 2 ml of freshly prepared 10% (w/w) ammonium persulfate solution. The running gel was polymerized under a nitrogen atmosphere.

The divider was removed and excess moisture was blotted from the gel bed. The smaller vacated area of the gel bed was filled with 90 ml of spacer gel solution containing 1 ml of a 10% ammonium persulfate solution. A slot former was placed in the spacer gel solution, 0.5 cm from the interface with the running gel. The spacer gel was also polymerized under a nitrogen atmosphere.

Following polymerization of the polyacrylamide gel, the slot former was carefully removed and the gel covered with Saran Wrap to prevent drying of the gel bed.

The protein samples were dissolved in spacer-gel buffer at a concentration of 2%, and Bromophenol Blue added to each sample as a marker dye. Varying amounts of protein solution ranging from 15 to 35 µl, were applied to the gel slots which were covered with Plexiglas cover. Again the entire gel was covered with Saran Wrap to reduce evaporation. The gel was connected to the buffer tanks by gel-filled legs which rested in the electrode vessels, each of which was filled with approximately 1600 ml of 0.046M Tris-glycine buffer, pH 8.3, and fitted with platinum electrodes. Electrophoresis was carried out at approximately 15 C at a voltage of 180 to 200 volts (Heathkit Power Supply) until the buffer front had migrated 13 cm from the sample slots.

A modification of the Melachouris (1969) procedure was used for verticle polyacrylamide gel electrophoresis in an E-C Verticle Gel Electrophoric Chamber. The gel bed was formed with 7% Cyanogum 41 in 190 ml 0.380M Tris-HCl buffer pH 8.9 containing 2 ml of freshly prepared 10% (w/w) ammonium persulfate. The buffer tanks were filled with 0.046M Tris-glycine buffer at pH 8.3 and were fitted with platinum electrodes.

The protein samples were dissolved in running-gel buffer at approximately 2% protein concentration. Sucrose was added to the protein samples to insure a density differential so that they could be introduced into the sample slot without diffusing into the buffer. Bromophenol blue was added to the samples as a marker dye. Electrophoresis was started with 40 mA of current until samples entered the gel (15 minutes); then increased to 80 mA (190-200 volts) and electrophoresis continued until the buffer front had migrated approximately 13 cm from the sample slots.

The technique of Melachouris (1969) was also adapted to polyacrylamide disc gel electrophoresis. β-Lg and whey standards were dissolved in spacer gel buffer at approximately 2% protein concentration. Sucrose was added to the protein samples to insure a density differential.

The samples were electrophoresed at 2 mA per disc. Discs were removed at 10 min intervals during the 30 min electrophoretic run.

Discs removed at 10 to 20 min intervals were stained with ninhydrin reagent (see high-voltage electrophoresis, p. 24) to check for the presence of fast moving peptides.

Staining Polyacrylamide Gels

Upon completion of the electrophoretic runs, the polyacrylamide gels were removed from their Plexiglas frames or glass discs and stained for 10 min in a dye solution consisting of 250 ml of deionized water, 250 ml of methanol, 50 ml of glacial acetic acid and 5 g of Amido Black 10 B (napthol blue black). The excess dye in the gel was removed in an electrolytic destaining cell containing 7% acetic acid solution.

The electropherograms were photographed with a Polaroid MP-3 Camera.

High-Voltage Electrophoresis

Paper.--The high-voltage paper electrophoretic (HVPE) technique employed was a modification of the technique originally described by Smith (1960). HVPE is ideal for the separation of small molecular components, with rapid diffusion rates and a net charge. Peptides meet these requirements.

Peptides isolated by the electrodialysis of 4X recrystallized β-Lg were streaked on Whatman 3 MM filter paper (20 x 56.5 cm) in 30 µl aliquots. The electrophoretic apparatus consisted of a Plexiglas cell in which the filter paper could be draped over a horizontal supporting bar, dipping each end of the filter paper into separate buffer compartments. The buffer tanks were filled with 500 ml of a volatile buffer (pyridine-acetic

acid-water, P 1:AA 10:H_2O 189, pH 3.5). Varsol (mineral spirits) was added to the Plexiglas cell to overlay the buffer and completely cover the paper. The electrophoretic chamber was fitted with two glass cooling coils for circulating water, which cooled the Varsol and minimized heat build-up on the filter paper during electrophoresis.

A field strength of 100 volts/cm was applied to the system for 1 h. Upon completion of the electrophoretic run, the filter paper was removed from the electrophoretic chamber and dried at 90 C in a forced-air oven. The peptides were located by spraying the paper with ninhydrin reagent (1 g ninhydrin, 700 ml absolute ethanol, 29 ml of 2,4,6-trimethylpyridine and 210 ml glacial acetic acid) and drying at 90 C until the spots became visible. The HVPE chromatograms were also sprayed with anisaldehyde-sulfuric acid reagent according to the procedure of Stahl and Kaltenbach (1961) to determine the presence of carbohydrates.

Thin-layer.--High-voltage electrophoresis was used primarily as a preparative procedure for separating the isolated peptide mixture into its components. Subsequent separations were performed on thin-layer cellulose plates, rather than paper, to facilitate the recovery of peptides. Thin-layer plates were prepared by suspending 15 g of MN

300 cellulose in 90 ml of deionized water and homogenizing the suspension in a Waring Blender. The slurried cellulose was applied to 20 x 20 cm thin-layer plates at a thickness of 500 microns with a Desaga Brinkmann thin-layer spreader. The plates were air-dried for 12 h. No activation of the MN-300 cellulose plates was required before TLHVE if the ambient relative humidity was under 75%.

Approximately 600 µl of the peptide mixture (peptides dissolved in 0.1M ammonium acetate) were applied to the thin-layer plates with Cordis disposable microapplicators and thoroughly dried. The plates were placed in a Reco Model E-800-2 water-cooled electrophoretic migration chamber and wicked to the buffer tanks with filter paper. The plates were sprayed with the same buffer used in the carbon electrode buffer tanks, i.e., pyridine-acetic acid-water, pH 3.5 (see paper high-voltage electrophoresis). A field strength of 40 V/cm was applied to the thin-layer plates for 25 min. Upon completion of electrophoresis, the plates were dried at 90 C in a forced air over. Location of the peptides was accomplished by spraying narrow strips (1.5 cm wide) on the plate edges in the direction of migration with ninhydrin reagent (see paper high-voltage electrophoresis). The plates were dried at 90 C until the peptide spots were evident. A ruler was used to mark off the areas containing the individual peptide streaks and the cellulose layer was scraped from these areas.

The peptides can be eluted from the MN-300 cellulose with various solvent systems. However, 5% acetic acid solution was the most suitable for this purpose. The eluted peptides were shell-frozen, lyophilized and stored in a dessicator over $CaSO_4$ until needed.

Gel Filtration Chromatography

Gel filtration chromatography, using Sephadex G-25 Fine, exclusion limit 5,000 M.W., was used to separate unbound vitamin B_{12} from the β-Lg and (E)β-Lg vitamin B_{12} complexes.

The Sephadex G-25 was rehydrated in deionized water for 4 h prior to pouring the column. A Sephadex chromatographic column (2.5 x 45 cm) was filled with the G-25 slurry. Following the formation of a 1 inch layer of beads at the bottom, the column outlet was opened to allow the bed to complete packing. The final column height was 27 cm, having a void column of 62 ml. A flow rate of 120 ml per h was used.

Prior to its use, the column was equilibrated with the same buffer used in the cyanocobalamin equilibrium binding experiment. This was accomplished by eluting approximately 300 ml of buffer through the column.

The same procedure was used in preparing Bio-Rad P-2, P-4 and P-10 polyacrylamide bead resins, and Sephadex G-15.

Determination of Protein Binding Capacity

To ensure consistency throughout binding experiments, a standard procedure was established for determining protein-bound vitamin B_{12}. The protein whose binding capacity was to be determined was dissolved in the appropriate buffer, at a concentration of 1 to 4 mg per ml of buffer. One milliliter of the protein solution was chromatographed on a Sephadex G-25 column previously equilibrated with the same buffer. An Isco Model UA-2 Recording Ultraviolet Analyzer with an operating wavelength of 254 nm was used to monitor the column effluent. Thus, an absorption profile of the protein prior to the addition of vitamin B_{12} was obtained.

The binding capacity of the protein was determined by adding 1 ml of a standard B_{12} solution (either 51, 600 µµg $Co^{60}B_{12}$ per ml, or 12,000 µµg $Co^{57}B_{12}$ per ml) to 1 ml of protein solution. The mixture was allowed to equilibrate for 1 h at room temperature with intermittant mixing. The unbound B_{12} was separated from the protein using Sephadex G-25 gel filtration chromatography. The protein was excluded from the gel beads and was recovered in the void volume effluent. The unbound $Co^{60}B_{12}$ permeated the gel beads and exhibited a characteristic elution volume, which was determined by counting eluent fractions with a Tri-Carb Scintillation Spectrometer.

The effluent containing the protein was collected, shell-frozen and lyophilized.

The lyophilized sample was dissolved in either 2 ml of volatile sample buffer or 2 ml of deionized water. A 1 ml aliquot was then counted in a Tri-Carb Scintillation Spectrometer and the average counts per minute (all counting done for 10 min intervals) were converted to vitamin B_{12} content by reference to previously prepared standard curves for $Co^{60}B_{12}$ and $Co^{57}B_{12}$. An aliquot of this sample was also used for protein determination by the Folin-Lowry procedure.

All binding capacities were expressed as micromicrograms (μμg) vitamin B_{12} per mg protein.

Determination of Peptide Binding Capacity

Sample preparation and binding equilibrium procedures were the same as those described for proteins. However, peptides were separated from unbound vitamin B_{12} by thin-layer chromatography (TLC) or TLHVE. The TLHVE system used was the same system as previously described. The TLC separation of unbound vitamin B_{12} was carried out on prepoured Silica Gel-G plates (250 microns). Two mobile phases were employed to effect the separation of the unbound vitamin B_{12} from the peptides: 30 ml glacial acetic acid, 10 ml acetone, 50 ml methanol and 110 ml of benzene; and deionized water.

After the chromatograms were developed they were dried in a forced-air oven at 90 C, sprayed with ninhydrin reagent and redried at 90 C until color developed.

The peptide bands were then scraped from the thin-layer plates and the bound vitamin B_{12} determined by counting gamma emmissions with a Tri-Carb Scintillation Spectrometer as described for proteins.

The migration characteristics of unbound $Co^{60}B_{12}$ and $Co^{57}B_{12}$ on the TLC plates were determined by spotting a 0.05% standard solution of crystalline B_{12} on each plate. The vitamin B_{12} standard was readily apparent because of its red color. This was confirmed by placing the plates in a chlorine atmosphere for 5 to 10 min. Following chlorination of the cyanocobalamin, the excess chlorine vapor was removed from the plates by directing a current of air over the plates. The plates were then sprayed with o-tolidine-potassium iodide reagent (160 mg o-tolidine dissolved in 30 ml glacial acetic acid, made up to 500 ml with deionized water and 1 g KI added (Bollinger, 1965). Under these conditions, cyanocobalamin is visible as a violet spot which corresponded exactly with the visual identification of B_{12}. The corresponding area where peptide-$Co^{57}B_{12}$ had been spotted was then scraped from the plates and the solid support counted with a Tri-Carb Scintillation Spectrometer.

Enzymatic Hydrolysis of Proteins

Enzymatic hydrolysis experiments were performed on β-Lg and (E)β-Lg using trypsin and chymotrypsin. Hydrolyses were performed at 37 C. A Sargent Recording pH Stat was employed to measure peptide cleavage by automatic titration of protons with standardized 0.05N triethylamine. The procedure and conditions used are described by Fasold and Gundlach (1963).

Ultracentrifugation

The moisture content of the protein was determined by drying samples over phosphorus pentoxide at reduced pressure for 48 h. The protein samples were weighed on a Mettler Type H 16 balance which was sensitive to 0.01 mg. The samples were dissolved in the running buffer and dialyzed for 24 h against the solvent.

Solvent densities were determined by pycnometry at 20 C ± 1 C. The viscosities of the solvent after equilibrium dialysis were measured in a standard Ubbelohde Dilution Suspended Level Type ASTM D 445 viscometer. The relative viscosities were calculated according to the following equation:

$$\frac{\eta}{\eta_o} = \frac{t}{t_o}\,\frac{\rho}{\rho_o}$$

where η, t and ρ are the viscosity, efflux time and

density of the solution and η_o, t_o and ρ_o are the corresponding values for water at 20 C.

The partial specific volume of the protein solution was obtained by reference to literature values.

Sedimentation coefficient.--The sedimentation-velocity experiments were carried out at 59,780 rpm (259,700 x g) in a Beckman Model E Analytical Ultracentrifuge with two double sector synthetic boundary cells.

The sedimentation coefficient is defined as the velocity of the sedimentating molecules per unit field as shown by the following formula:

$$s = \frac{1}{\omega^2 \cdot 60} \cdot \frac{dx}{dt}$$

where x is the distance of boundary from the center of rotation, ω is the angular velocity in radians per sec, and t is the sedimenting time in seconds.

The actual equation used in calculating the sedimentation constant is:

$$s = \frac{2.303}{\omega^2 \cdot 60} \cdot \frac{\log x}{t},$$

By plotting the log of distance (x) against time (t), the sedimentation coefficient may be obtained from the slope by the following formula:

$$S = \frac{2.303}{\omega^2 \times 60} \cdot \text{slope} \cdot 1 \times 10^{13}$$

Sedimentation coefficients are usually reported as $S_{20,w}$ values, which is the sedimentation coefficient the protein would exhibit in a solvent with a density and viscosity equivalent to water at infinite protein dilution. The following is the formula containing the terms necessary for these corrections:

$$S_{20,w} = S_{obs} \quad (\frac{\eta_{w,t}}{\eta_{w,20}}) (\frac{\eta_{w,t}}{\eta_{w,t}}) (\frac{1 - \bar{v}\rho_{w,t}}{1 - \bar{v}\rho_{s,t}})$$

the first term $(\frac{\eta_{w,t}}{\eta_{w,20}})$ is the correction factor for the viscosity of water at experimental temperature to that at 20 C. At the experimental conditions, this term had a value of unity since all runs were performed at 20 C. The second term $(\frac{\eta_{s,t}}{\eta_{w,t}})$ corrects the relative viscosity of the solvent to that of water at the same temperature. In the last term, $\bar{v}$, the partial specific volume of the protein was obtained from literature values and was assumed to be the same in all solvent systems. The density factor $(\frac{\rho_{w,t}}{\rho_{s,t}})$ is the correction ratio for the density of the solvent at any temperature to that of water at 20 C.

RESULTS

Beta-Lactoglobulin

Nitrogen Content

The nitrogen content of the isolated β-Lg as determined by micro-Kjeldahl was 15.53% which compared to the literature value of 15.6% reported by Larson and Jenness (1955). The nitrogen content was converted to per cent protein using a conversion factor of 6.25.

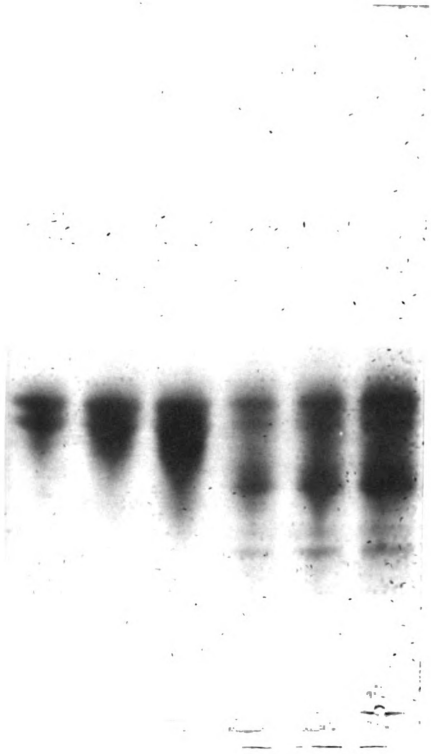
The concentration of all β-Lg solutions used during this study was based on this experimentally determined nitrogen content.

Sulfhydryl Content

The number of available sulfhydryl groups present in the isolated β-Lg was determined by Ellman's procedure. In the presence of urea, the protein exhibited two available sulfhydryl groups per mole of β-Lg (36,000 M.W.). No sulfhydryl groups were detected in the absence of urea.

Gel Electrophoresis of Beta-Lactoglobulin

Horizontal polyacrylamide gel electropherograms of the isolated β-Lg, whey standards and pure β-Lg A and B are shown in Figures 1 and 2. The electrophoretic



1 2 3 4 5 6

Figure 1.--Polyacrylamide gel electropherogram of recrystallized μ-Lg and whey standard at pH 8.3; left to right: slot 1 - 5 μl β-Lg; slot 2 - 10 μl β-Lg; slot 3 - 20 μl β-Lg; slot 4 - 20 μl whey; slot 5 - 25 μl whey; slot 6 - 35 μl whey.

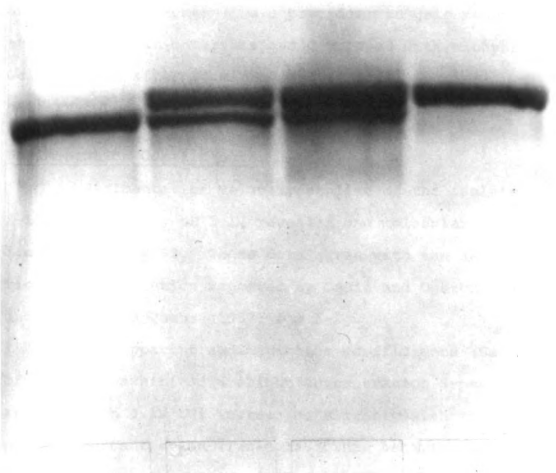


Figure 2.--Polyacrylamide gel electropherogram of recrystallized β-Lg and β-Lg A, B and AB standards at pH 8.3: left to right; slot 1 - 10 µl β-Lg B standard; slot 2 - 10 µl β-Lg AB standard; slot 3 - recrystallized β-Lg AB; slot 4 - 10 µl β-Lg A standard.

patterns confirm the presence of both genetic variants (A and B). No contaminating proteins were apparent. Polyacrylamide disc gel electrophoresis of β-Lg in a discontinuous buffer system did not show the presence of any fast moving zones (i.e., peptides) in gels removed at 10 and 20 min intervals and developed with ninhydrin. Gels removed after 30 min showed typical β-Lg patterns when stained with Amido Black 10B.

Sedimentation Coefficients

Sedimentation velocity studies of the isolated β-Lg in 0.1N KCl, pH 5.1, revealed one molecular species, i.e., $S_{20,w}$ = 2.92. These data agree with the sedimentation characteristics reported by Cecil and Ogston (1949) and Ogston and Tombs (1957) for β-Lg.

The apparent sedimentation coefficients (Sapp) of the β-Lg exhibited a slight concentration dependency at pH 5.1 in 0.1N KCl increasing slightly with increasing protein concentration (Table 1). This behavior is typical for a slow association-dissociation interaction (Gilbert, 1963).

The sedimentation coefficient of β-Lg was also determined in borate buffer, pH 9.0, I = 0.24. At this pH β-Lg should be dissociated to its monomeric state (i.e., 18,000 M.W.) and evidence of this was apparent by the decrease in the sedimentation coefficient to

TABLE 1.--The apparent sedimentation constants of β-Lg in 0.1M potassium chloride and borate buffer at 20 C

Protein	Protein Concentration (mg/ml)	Apparent Sedimentation Coefficients	
		KCl	Borate
Beta-Lactoglobulin	0.0[a]	2.93	2.1
	2.5	3.07	--
	5.0	3.32	2.6
	7.5	3.56	2.7
	10.0	--	3.3

[a]Values obtained by extrapolating plot of Sapp vs. protein concentration to infinite dilution.

$S_{20,w}$ = 2.16. This value compares with the value of $S_{20,w}$ = 2.4 reported by McKenzie and Sawyer (1967) for β-Lg at pH 8.6.

Electrodialyzed Beta-Lactoglobulin

Sulfhydryl Content

Sulfhydryl determinations on the (E)β-Lg, using Ellman's procedure, showed no loss or addition of -SH groups. Sulfhydryl determinations carried out on the (E)β-Lg in the absence of urea showed no available -SH groups.

Gel Electrophoresis

Discontinuous polyacrylamide gel electrophoresis of the (E)β-Lg together with a whey standard and β-Lg A and B standards, showed identical electrophoretic gel patterns. As shown in Figure 3, the (E)β-Lg exhibited electrophoretic mobilities identical to the β-Lg prior to electrodialysis.

Sedimentation Coefficient

Sedimentation velocity studies performed on the (E)β-Lg in 0.1 M ammonium acetate, pH 6.8, showed a single component with an $S_{20,w}$ = 3.0. This value was in agreement with the sedimentation coefficient determined for native β-Lg. However, apparent sedimentation coefficients calculated for (E)β-Lg did not exhibit the same concentration dependency as was shown for β-Lg (Table 2).

TABLE 2.--The apparent sedimentation constants of (E)β-Lg in 0.1N ammonium acetate and borate buffer at 20 C

Protein	Protein Concentration (mg/ml)	Apparent Sedimentation Coefficients	
		KCl	Borate
Electrodialyzed	0.0[a]	3.02	2.1
Beta-Lactoglobulin	5.0	3.06	2.69
	7.5	3.1	2.65
	10.0	3.08	3.3

[a]Values obtained by extrapolating plot of Sapp vs. protein concentration to infinite dilution.

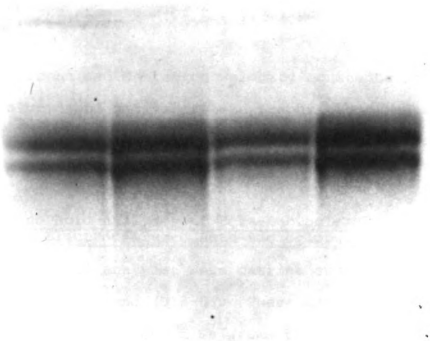


Figure 3.--Polyacrylamide gel electropherogram of β-Lg and (E)β-Lg at pH 8.3: slots 1 and 2 - (E)β-Lg at 1 and 2% protein concentration; slots 3 and 4 - β-Lg at 1 and 2% protein concentration.

The $S_{20,w}$ value of (E)β-Lg in borate buffer, pH 9.0 was 2.1. Apparent sedimentation coefficients for the (E)β-Lg (Table 2) corresponded exactly with those calculated for the native protein (Table 1) in the same buffer system with the exception of the 0.75% protein concentration.

Apparent sedimentation velocity constants for the electrodialyzed and native protein preparations indicate that both preparations exhibit a concentration dependent association-dissociation equilibrium.

Amino Acid Analyses of Beta-Lactoglobulin and Electrodialyzed Beta-Lactoglobulin

Amino acid analyses were carried out on the 22 h hydrolysate of β-Lg and (E)β-Lg. These data are presented in Table 3 and are expressed as grams of amino acid per 100 grams of protein.

N-Terminal Amino Acids of Beta-Lactoglobulin and Electrodialyzed Beta-Lactoglobulin

N-Terminal amino acid analyses, using the dansyl chloride procedure confirmed iso-leucine as the N-terminal amino acid for both β-Lg and (E)β-Lg.

TABLE 3.--Amino acid composition of beta-lactoglobulin and electrodialyzed beta-lactoglobulin

Amino Acid Residue	Beta-Lactoglobulin	Electrodialyzed Beta-Lactoglobulin
	g amino acid residues/100 g protein	
LYS	12.58	11.41
HIS	1.71	1.53
ARG	2.30	2.45
ASP	10.06	10.20
THR	4.39	4.41
SR	3.18	3.10
GLU	17.00	16.58
PRO	4.14	4.34
GLY	1.07	1.15
ALA	5.66	5.85
1/2 CYS	2.31	2.51
VAL	6.15	6.21
MTH	1.24	0.76
I-LEU	5.77	5.82
LEU	14.15	14.33
TYR	3.47	3.76
PHEN	2.65	3.43
TRYP	2.16	2.16
TOTAL*	99.99	100.00

*Total g amino acid residues/100 g protein

Rate of Enzymatic Hydrolysis of Beta-Lactoglobulin and Electrodialyzed Beta-Lactoglobulin

The relative enzymatic hydrolysis rates of β-Lg and (E)β-Lg preparations provided a means of assessing the possible effects of electrodialysis on β-Lg.

The terms used to compare proton release from the substrates examined were percentage hydrolysis versus hydrolysis time as shown in Figures 4 and 5. This method of expression eliminates inconsistencies that may have resulted from the proteolytic cleavage of a structured protein and the subsequent appearance of new cleavage sites heretofore unavailable due to conformational characteristics. As evidenced from Figures 4 and 5, no major differences can be seen in the tryptic or chymotryptic hydrolysis of β-Lg and (E)β-Lg. However, the calculated relative K_m values for tryptic and chymotryptic hydrolyses showed a lower K_m for both enzymatic hydrolyses when (E)β-Lg was the substrate (see Figures 6 and 7).

Paper High-Voltage Electrophoresis of the Tryptic Digest of Beta-Lactoglobulin and Electrodialyzed Beta-Lactoglobulin

Figure 8 shows the results of high-voltage paper electrophoresis of the tryptic hydrolysates of β-Lg and (E)β-Lg. Similar peptide patterns were obtained for the β-Lg and (E)β-Lg preparations.

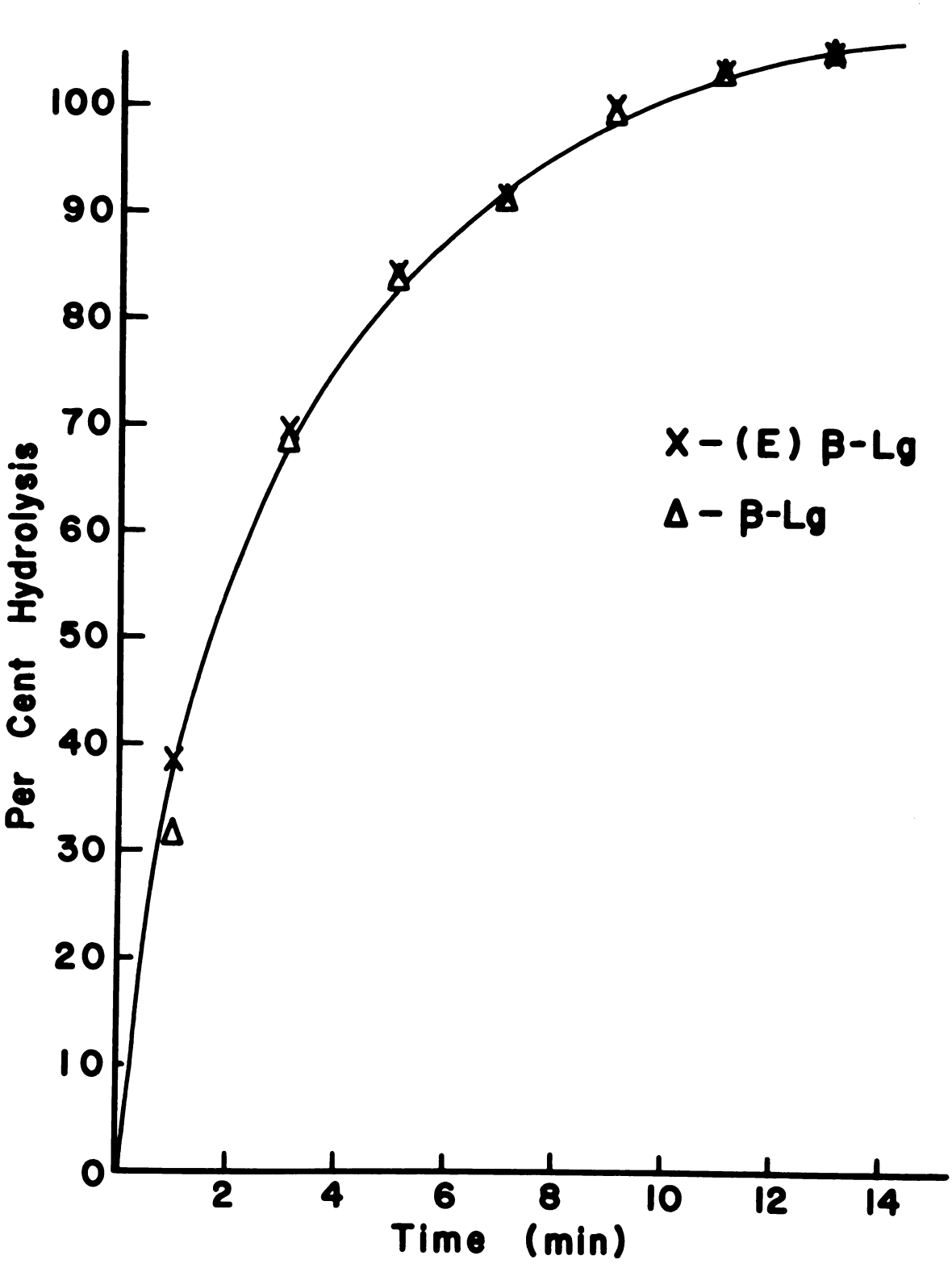


Figure 4.--Hydrolysis of β-Lg and (E)β-Lg with trypsin.

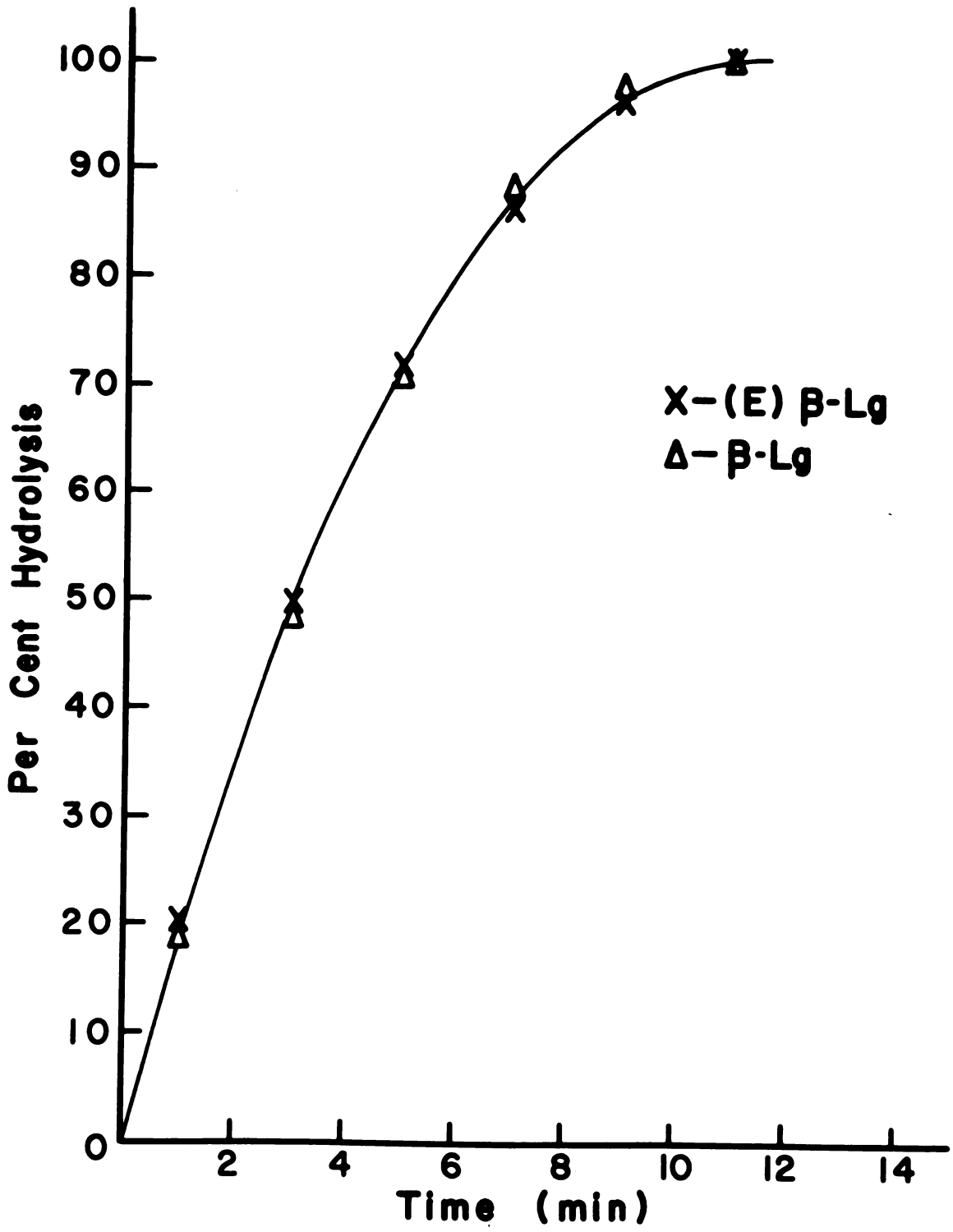


Figure 5.--Hydrolysis of β-Lg and (E)β-Lg with chymotrypsin.

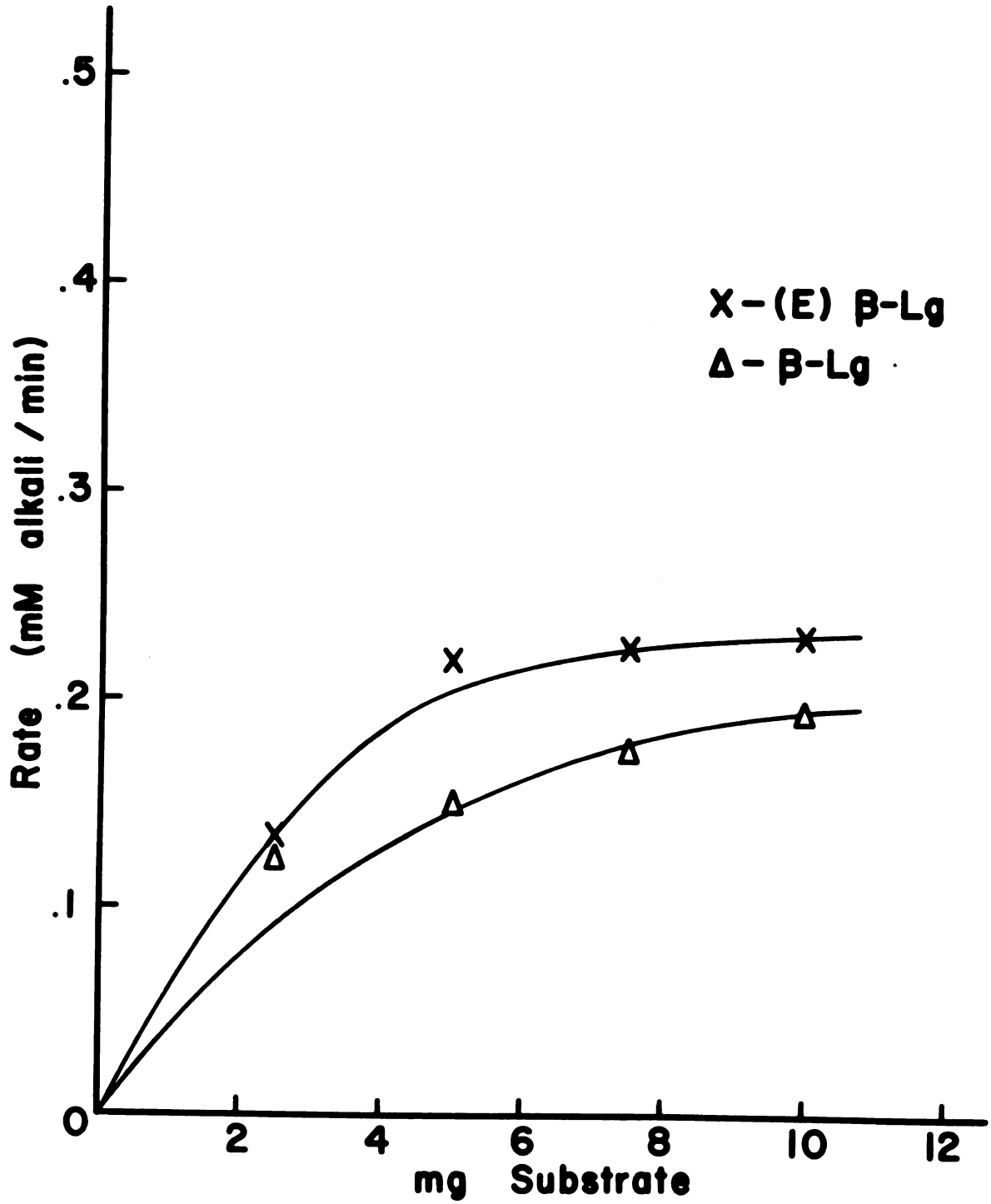


Figure 6.--Rate curves for tryptic hydrolysis of β-Lg and (E)β-Lg per 60 sec.

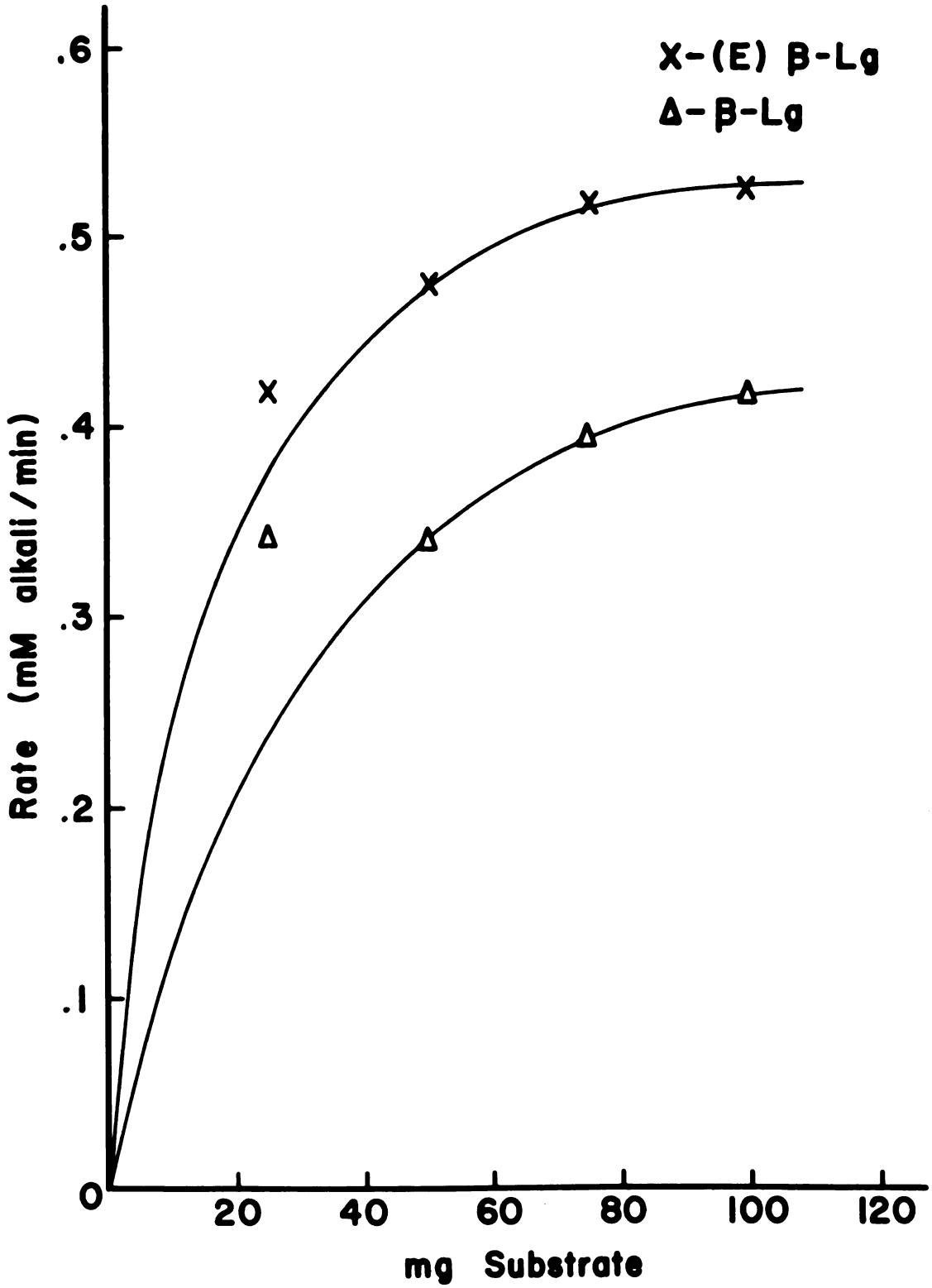


Figure 7.--Rate curves for chymotryptic hydrolysis of β-Lg and (E)β-Lg per 60 sec.

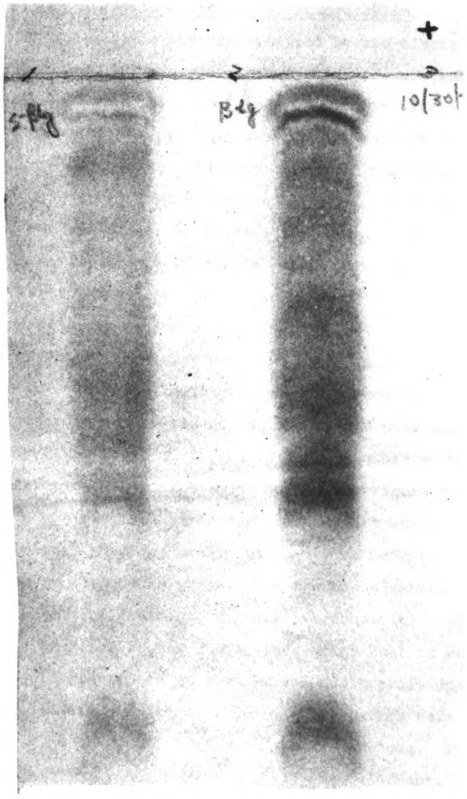


Figure 8.--High-voltage paper electropherogram of the tryptic hydrolysate of β-Lg and (E)β-Lg in pyridine-acetic acid-water buffer, pH 3.5: left to right; (E)β-Lg hydrolysate and β-Lg hydrolysate.

Peptides from Beta-Lactoglobulin

The peptide mixture obtained by the electrodialysis of β-Lg was light brown and had a sticky consistency following lyophilization of the electrodiffusate. Further dehydration of the peptides was accomplished by drying over phosphorous pentoxide under reduced pressure.

Initially, each electrodiffusate change during electrodialysis was pervaporated and lyophilized separately. The electrodiffusate fractions were rehydrated with deionized water and passed over a Bio-Rad P-2 column using deionized water as the eluate in an effort to remove salts or free amino acids that may have been electrodialyzed from the β-Lg. The electrodialyzed peptides were recovered in the void volume from the column. A second peak was eluted shortly after the peptides but did not exhibit a positive test for protein or free amino acids (see Figure 9).

The electrodiffusate fractions were analyzed by high voltage electrophoresis at pH 3.5 on both paper and thin layer supporting media. All fractions exhibited the same characteristic peptide pattern (Figure 10). One peptide migrated to the anode, indicating that it was negatively charged at pH 3.5 and was arbitrarily designated as the "negative peptide." Two other peptides were observed, both of which migrated to the cathode. These were arbitrarily designated as "+2" and "+3" peptides.

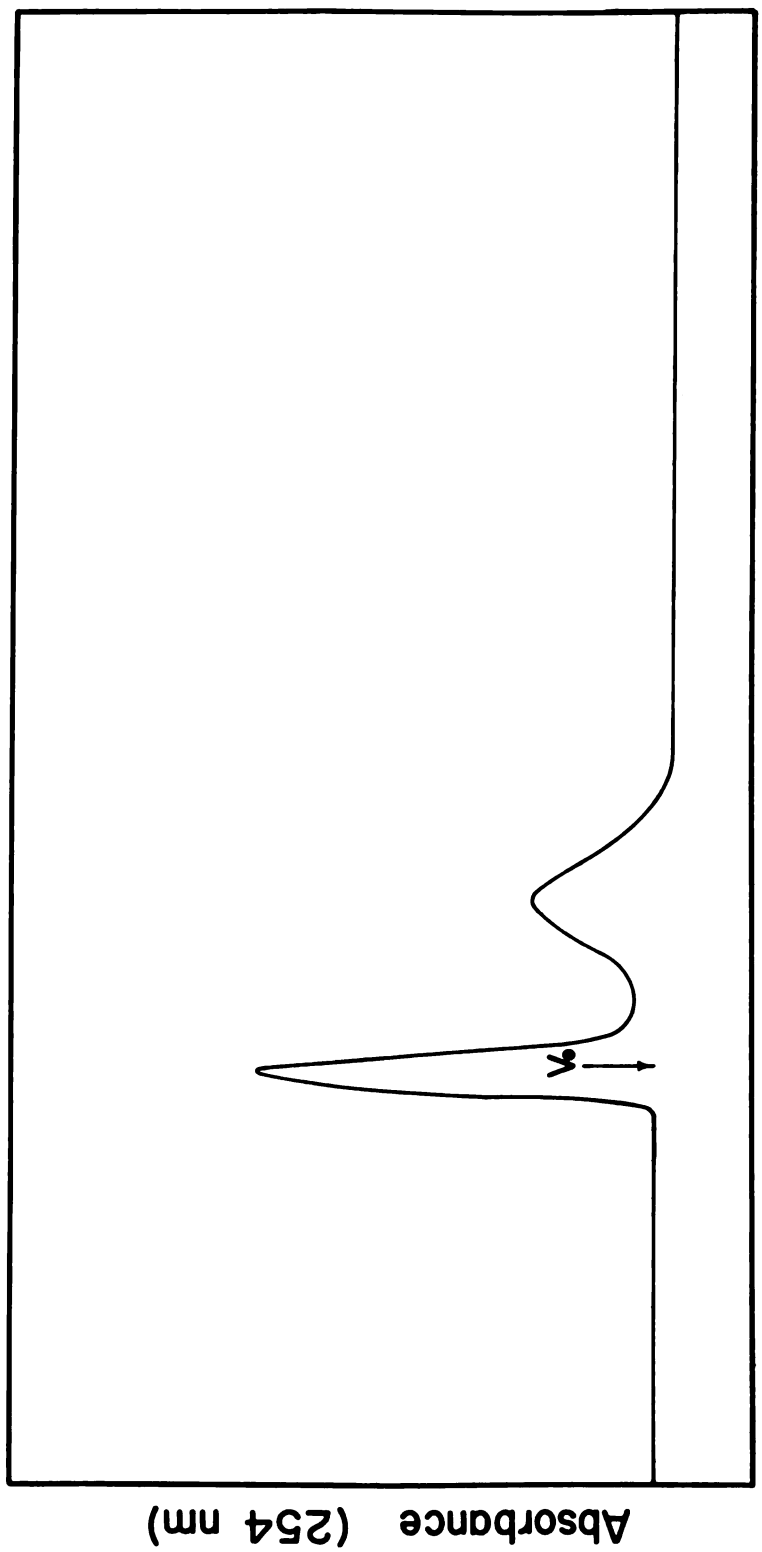


Figure 9.--Elution pattern of electrodialyzed peptides from Bio-Rad P-2 column with deionized water. Peak 1, peptide mixture; Peak 2, salts. Flow rate, 4 ml/min.

Figure 10.--Thin-layer high-voltage electropherogram of the peptide mixture electrodialyzed from β-Lg. Support; MN 300 cellulose:Buffer; pyridine-acetic acid-water, pH 3.5.

TLHVE confirmed that the peptide concentration was the only variable in the individual electrodiffusate fractions. The highest peptide concentrations were obtained between 2 and 4 h of electrodialysis. Subsequent to these findings, the total electrodiffusate was combined following pervaporation and lyophilized. The recovery rate of peptides from β-Lg was calculated as 7-8 mg of dried peptide mixture per g of β-Lg.

Separation and Recovery of the Peptide Mixture

The lyophilized peptides were separated using thin-layer high voltage electrophoresis as described in the experimental procedure. Excellent resolution of the peptides was obtained using this technique as shown in Figure 10.

Recovery of the isolated peptides from the cellulose (MN 300 cellulose) support was accomplished by elution with a solution of 5% acetic acid in deionized water, or a 1:1 (v/v) mixture of pyridine-deionized water. The latter system was discarded because of the difficulty of removing residual pyridine from the sample. Of the two systems, although both effected the extraction of some cellulose along with the peptides, the 5% acetic acid gave a more complete recovery of the peptides with fewer extractions. The per cent recovery, calculated on the basis of the

weight of peptides recovered from electrodialysis and the calculated weight of peptides present in the extracted material from the thin-layer support, was about 60-70%.

Physical and Chemical Properties of Isolated Peptides

The molecular weights of the isolated peptides appear to be greater than 1,500, but less than 3,600 on the basis of the gel filtration experiments performed. The peptide mixture was chromatographed over Bio-Rad P-2 polyacrylamide beads and was eluted from the column in the void volume at 62 ml (Figure 9). This indicated a molecular weight of the peptides above the exclusion limit of Bio-Rad P-2 (approximately 1500 M.W.). Gel filtration chromatography of the peptide mixture over Bio-Rad P-4 (Figure 11) showed an elution volume of 110 ml (Bio-Rad P-4 column V_o = 63 ml). This behavior indicated that the peptides may have a molecular weight of less than 3,600.

Gel filtration chromatography of the individual peptides on P-2 and P-4 gave elution patterns similar to those exhibited by the peptide mixture.

Minimum Molecular Weights of Isolated Peptides

Minimum molecular weights of the isolated peptides were calculated from the amino acid composition of individual peptides according to the following formula:

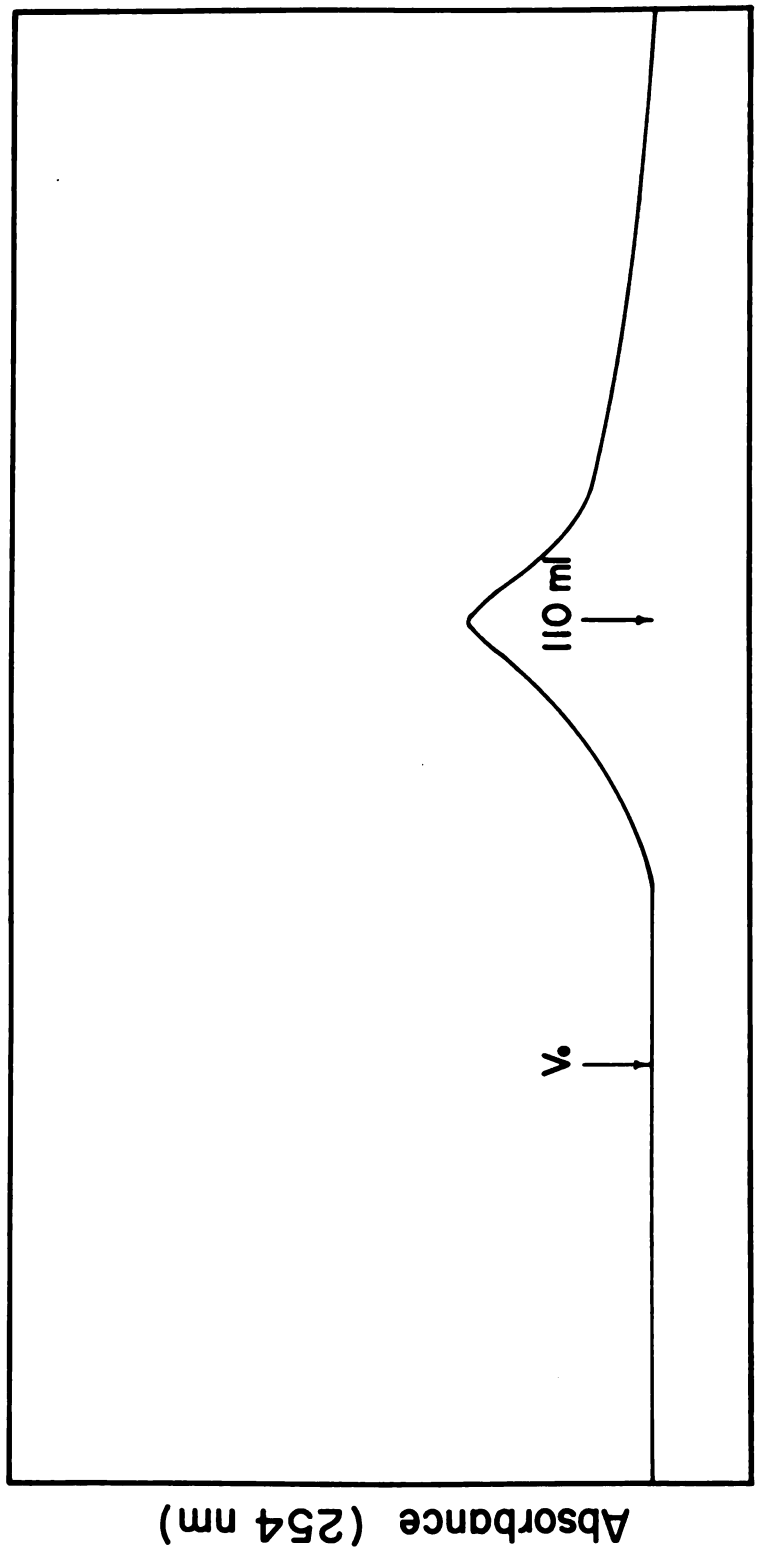


Figure 11.--Elution pattern of electrodialyzed peptide mixture from Bio-Rad P-4 column with 0.1M NH_4OAc. Flow rate was 3.0 ml/min.

$$\frac{M_w \text{ A.A.}}{M_w} = \frac{\text{Conc. A.A.}}{100}$$

where:

M_w A.A. = residue weight of amino acid residue

M_w = estimated minimum molecular weight of peptide

Conc. A.A. = concentration of amino acid residue in sample (g/100g protein).

Tryptophan, phenylalanine and tyrosine were not used in minimum molecular calculations of the peptide because the concentration of these amino acid residues was so low that they could not be determined accurately. All other amino acid residues present in the peptides were used in minimum molecular weight calculations and the least common denominator of these values was recorded as the estimated molecular weight of the peptide.

The estimated molecular weights of the peptides were calculated to be: negative peptide 4,000; +2 peptide 6,000; +3 peptide 6,000.

Amino Acid Analyses of Isolated Peptides

Amino acid analyses were performed on acid hydrolysates of the isolated peptides. These data are presented in Table 4 as grams of amino acid per 100 grams of protein.

TABLE 4.--Amino acid composition of isolated peptides from beta-lactoglobulin by electrodialysis

Amino Acid Residue	"Negative" Peptide	"+2" Peptide	"+3" Peptide
	g amino acid residues/100 g protein		
LYS	6.66	6.79	28.37
HIS	2.10	2.26	4.65
ARG	2.46	0.75	3.26
ASP	7.37	6.79	6.98
THR	2.81	5.28	3.26
SER	6.32	26.42	9.77
GLU	11.58	8.30	9.30
PRO	2.81	3.02	1.86
GLY	30.53	22.64	22.79
ALA	4.21	9.06	5.12
1/2 CYS	--	--	--
VAL	27.02	6.42	4.65
MTH	--	--	--
I-LEU	2.81	1.89	3.72
LEU	3.51	4.53	4.65
TYR	1.75	1.51	2.79
PHEN	1.40	1.51	2.79
TRYP	--	<0.38	<0.47
TOTAL*	113.34	107.55	114.43

*Total g amino acid residue/100 g protein

It should be noted that sulfur containing amino acids were not detected in any of the peptides. Tryptophan was not determined in the "negative" peptide because of insufficient sample.

Separation of Unbound $Co^{60}B_{12}$ from Protein and Peptide-Bound $Co^{60}B_{12}$ Using Gel Filtration

Bio-Rad P-2 polyacrylamide beads were unsuitable for separating $Co^{60}B_{12}$ from the protein or from peptide-bound $Co^{60}B_{12}$ because the exclusion limit of the P-2 was too close to the molecular weight of hydrated cyanocobalamin. Data contained in Table 5 illustrate the elution pattern of $Co^{60}B_{12}$ from a P-2 column as monitored with a Tri-Carb Scintillation Spectrometer. $Co^{60}B_{12}$ begins its elution from the P-2 column in the void volume (63 ml) and continues until approximately 130 ml of eluate is passed from the column. The summation of net counts per minute (cpm) for all fractions counted in Table 5 totaled 5293. One milliliter of the stock $Co^{60}B_{12}$ solution gave a total of 5333 cpm, indicating a recovery of 99.2%.

Similar elution volumes were exhibited by β-Lg and the peptides which have molecular weights above the exclusion limit of the polyacrylamide beads.

Experimental data have shown that Sephadex G-25 with an exclusion limit of 5,000 can be used to separate

TABLE 5.--Elution pattern of $Co^{60}B_{12}$[a] from Bio-Rad P-2 gel filtration column versus counts per minute of 5 ml elute fractions

Fraction	Gross Counts per minute[b] (10 min basis)	Fraction	Gross Counts per minute[b] (10 min basis)
1	52	26	71
2	53	27	59
3	41	28	80
4	50	29	55
5	51	30	59
6	51	31	52
7	53	32	61
8	50	33	45
9	47	34	56
10	44	35	54
11	84	36	60
12	96	37	41
13 (void volume)	657	38	48
14	653	39	37
15	776	40	42
16	773	41	42
17	499	42	49
18	503	43	31
19	326	44	47
20	324	45	37
21	208	46	48
22	191	47	43
23	173	48	31
24	132	49	31
25 (125ml V_e)	114		

[a] 1 ml of $Co^{60}B_{12}$ stock solution (51,600 μμg B_{12}/ml) diluted to 4ml with 0.1N ammonium acetate.

[b] Background gamma radiation count was 37 counts per minute (10 min basis).

free $Co^{60}B_{12}$ from the protein-$Co^{60}B_{12}$ complex by gel filtration chromatography. However, separation of unbound $Co^{60}B_{12}$ or $Co^{57}B_{12}$ from the peptide mixture or isolated peptides could not be accomplished by gel filtration chromatography using Bio-Rad P-2, P-4, P-6, P-10; Sephadex G-15 or G-25.

$Co^{60}B_{12}$ or $Co^{57}B_{12}$, at the concentrations being used, do not absorb at 254 or 280 nm. Consequently, the presence of cyanocobalamin that is not bound to proteins or peptides cannot be detected in the elution volume by monitoring with an Isco UA-2 ultraviolet analyzer. Rather, the elution pattern must be obtained by counting gamma emission in the eluate from the column.

Vitamin B_{12} Binding Capacity of Beta-Lactoglobulin and Electrodialyzed Beta-Lactoglobulin

The results of vitamin B_{12} binding studies with β-Lg and modified β-Lg are shown in Table 6. The binding capacity of β-Lg in 0.1M ammonium acetate, pH 6.8, and Jenness and Koops buffer, pH 6.6, where the pH and ionic strength is equivalent to that of normal milk, were 460 μμg vitamin B_{12}/mg of protein. (E)β-Lg and recombined (E)β-Lg and peptides showed no measurable vitamin B_{12} binding capacity in either the ammonium acetate or Henness and Koops buffer systems.

At pH 9.0 the binding capacities of all three forms of β-Lg for vitamin B_{12} varied with the buffer

system being used (see Table 6). In borate buffer the recombined (E)β-Lg and peptides showed a binding capacity of 200 μμg vitamin B_{12}/mg of protein, whereas β-Lg was shown to bind only 150 μμg vitamin B_{12}/mg of protein. In TRIS-HCl, pH 9.0, all three forms of β-Lg exhibited binding capacities that were not evidenced at any other pH or in any other buffer system. The binding capacities ex exhibited by β-Lg and (E)β-Lg in ammonium acetate-sodium hydroxide solvent, pH 9.0 were essentially equivalent (100 μμg vitamin B_{12}/mg of protein), whereas the re-equilibrated (E)β-Lg and peptides showed only a slightly lower vitamin B_{12} content.

In contrast to the results obtained at pH 9.0, β-Lg and the modified forms of β-Lg showed no measurable binding capacity at pH 2.0.

Confirmation that vitamin B_{12} was bound to the β-Lg was obtained by dissolving the protein-vitamin B_{12} complex (460 μμg $Co^{60}B_{12}$/mg of protein) in 0.1M ammonium acetate and dialyzing the solution against the solvent for 24 h. The vitamin B_{12} bound to the β-Lg in the dialysate was redetermined and showed no loss of vitamin B_{12}.

TABLE 6.--Vitamin B_{12} binding capacities of β-Lg, (E)β-Lg and recombined (E)β-Lg and peptides in various buffers and pH values

Buffer	pH	Average $Co^{60}B_{12}$ Bound (μμg $Co^{60}B_{12}$ per mg protein)[a]		
		β-Lg	(E)β-Lg	Recombined (E)β-Lg and Peptides
Ammonium-acetate	6.8	460	TLTD[c]	TLTD
Jenness and Koops	6.6	460	TLTD	TLTD
Borate	9.0	150	40	200
TRIS-HCl	9.0	580	550	380
Ammonium acetate sodium hydroxide	9.0	110	100	90
Sodium chloride-[b] hydrochloric acid	2.0	TLTD	TLTD	TLTD

[a]Sephadex G-25 column was used to separate unbound B_{12} from the protein bound vitamin B_{12}.

[b]Solvent used by Timascheff and Townend (1961) to ensure β-Lg was in its monomer form.

[c]Too low to determine.

Discontinuous Polyacrylamide Gel Electrophoresis of Beta-Lactoglobulin and Electrodialyzed Beta-Lactoglobulin Equilibrated with $Co^{57}B_{12}$

β-Lg and (E)β-Lg with 460 and 10 μμg/mg protein of bound $Co^{57}B_{12}$, respectively, were subjected to vertical gel electrophoresis at pH 8.3. The results of this experiment are shown in Figure 12. As shown in the electropherogram, both genetic variants (A and B) were present. The $Co^{57}B_{12}$ initially bound to either β-Lg or (E)β-Lg did not appear to affect the electrophoretic mobilities of the proteins. The zones containing β-Lg A and B were cut from the gel and counted in a Tri-Carb Scintillation Spectrometer. The $Co^{57}B_{12}$ was not found associated with either genetic variant, nor was there any evidence that the vitamin remained in the sample slots of the gel.

Vitamin B_{12} Binding Capacity of Peptides Isolated by Electrodialysis of Beta-Lactoglobulin

Following the equilibration of the peptide mixture with $Co^{60}B_{12}$, the peptide-$Co^{60}B_{12}$ mixture was chromatographed on a Bio-Rad P-4 gel filtration column. No absorption at 254 nm was monitored with elution of the column's void volume, indicating that the peptides had not formed a complex with vitamin B_{12} having a molecular weight in excess of 3,600.

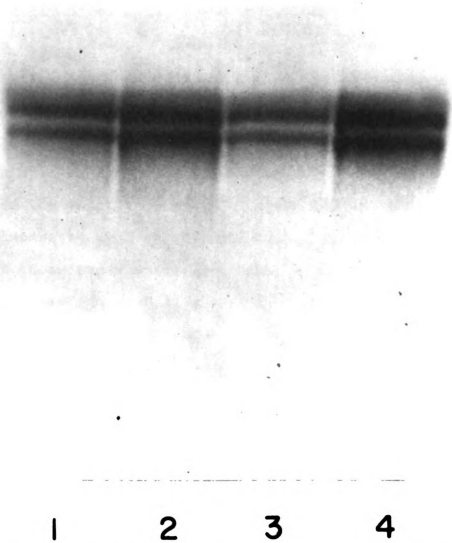


Figure 12.--Vertical polyacrylamide gel electropherogram of β-Lg and (E)β-Lg at pH 8.3. Column 1- (E)β-Lg-B_{12} complex; Column 2- (E)β-Lg; Column 3- β-Lg-B_{12} complex; Column 4- β-Lg.

Free $Co^{60}B_{12}$ was separated from the peptide B_{12} equilibration mixture by thin-layer high-voltage electrophoresis. The results of this experiment (Figure 13 and Table 7) showed only background radiation associated with the negative and the +3 peptides, while the +2 peptide exhibited a gamma radiation count equivalent to 200 μμg of vitamin B_{12}. However, close inspection of the crystalline B_{12} standard (column 1 of TLHV electropherogram) indicated that vitamin B_{12} migrated in the direction of the cathode at pH 3.5 in the field of 40 V/cm.

Thin-layer ascending chromatography was also employed in the separation of unbound $Co^{57}B_{12}$ from the peptide mixture as well as the separated peptides. The results of these experiments are shown in Figures 14, 15 and 16. The peptide remaining at the origin was identified as the +3 peptide, whereas the +2 peptide and negative peptide exhibited Rf values of 0.12 and 0.23, respectively. Determinations of the vitamin B_{12} binding capacities of the negative and +2 peptides showed only background radiations.

The crystalline B_{12} standard remained at the origin in this system. Gamma radiation accounting, performed on the $Co^{57}B_{12}$ reference spot, indicated that the $Co^{57}B_{12}$ applied to the thin-layer plate also remained at the origin. Thus, the binding capacity of the +3 peptide for $Co^{57}B_{12}$, if any, could not be determined by this TLC technique.

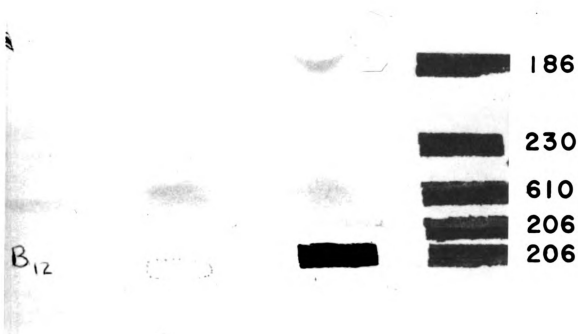


Figure 13.--Thin-layer high-voltage electropherogram of the peptide mixture equilibrated with $Co^{57}B_{12}$. Support - MN 300 cellulose; buffer - pyridine-acetic acid-water, pH 3.5. Left to right: column 1- cyrstalline B_{12}; column 2- electrodialyzed peptides; column 3- electrodialyzed peptides plus $Co^{57}B_{12}$; column 4- $Co^{57}B_{12}$ standard. (Numbers refer to radiation level in cpm--background radiation 183 cpm.)

TABLE 7.--Vitamin B_{12} binding capacities of the peptide after separation of unbound $Co^{57}B_{12}$ from the peptides by thin-layer high voltage electrophoresis

Sample	Net Counts/min[a]	Vitamin B_{12} Bound
Negative peptide	9	too low to determine
+2 peptide	426	200 μμg
+3 peptide	--	too low to determine
Origin	22	too low to determine

[a]Background radiation was measured at 184 counts/min. All counting was made on a 10 min basis.

Figure 13.--Thin-layer high-voltage electropherogram of the peptide mixture equilibrated with $Co^{57}B_{12}$. Support - MN 300 cellulose; buffer - pyridine-acetic acid-water, pH 3.5. Left to right: column 1- cyrstalline B_{12}; column 2- electrodialyzed peptides; column 3- electrodialyzed peptides plus $Co^{57}B_{12}$; column 4- $Co^{57}B_{12}$ standard. (Numbers refer to radiation level in cpm--background radiation 183 cpm.)

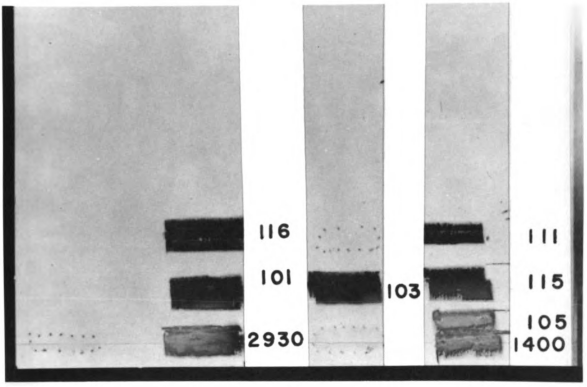


Figure 14.--Thin-layer chromatographic separation of unbound $Co^{57}B_{12}$ from the electrodialyzed peptide mixture. Support - Silica Gel G; mobile phase - 55% benzene, 25% methanol, 5% acetone and 15% glacial acetic acid. Left to right: column 1- crystalline B_{12} standard; column 2- electrodialyzed peptide plus $Co^{57}B_{12}$; column 3- electrodialyzed peptide; column 4- $Co^{57}B_{12}$. (Numbers refer to radiation level in cpm--background radiation 108 cpm.)

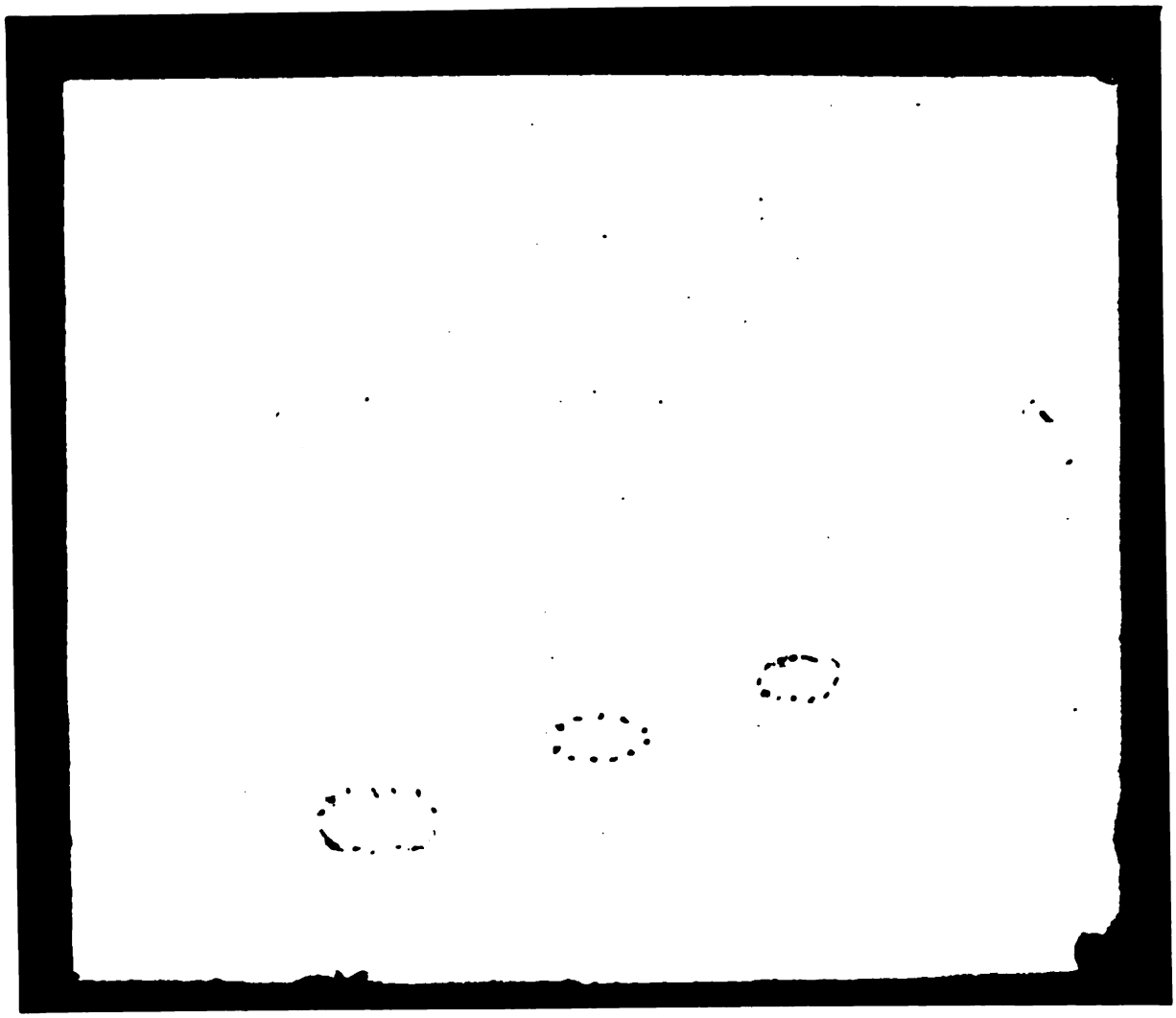


Figure 15.--Thin-layer chromatography separation of unbound $Co^{57}B_{12}$ from the negative, +2 and +3 peptides. Support - Silica Gel G; mobile phase - 55% benzene, 25% methanol, 5% acetone and 15% glacial acetic acid. Left to right: column 1- +3 peptide plus $Co^{57}B_{12}$ (cpm 2450); column 2- +2 peptide plus $Co^{57}B_{12}$ (193 cpm); column 3- negative peptide plus $Co^{57}B_{12}$ (188 cpm). Background radiation 186 cpm.

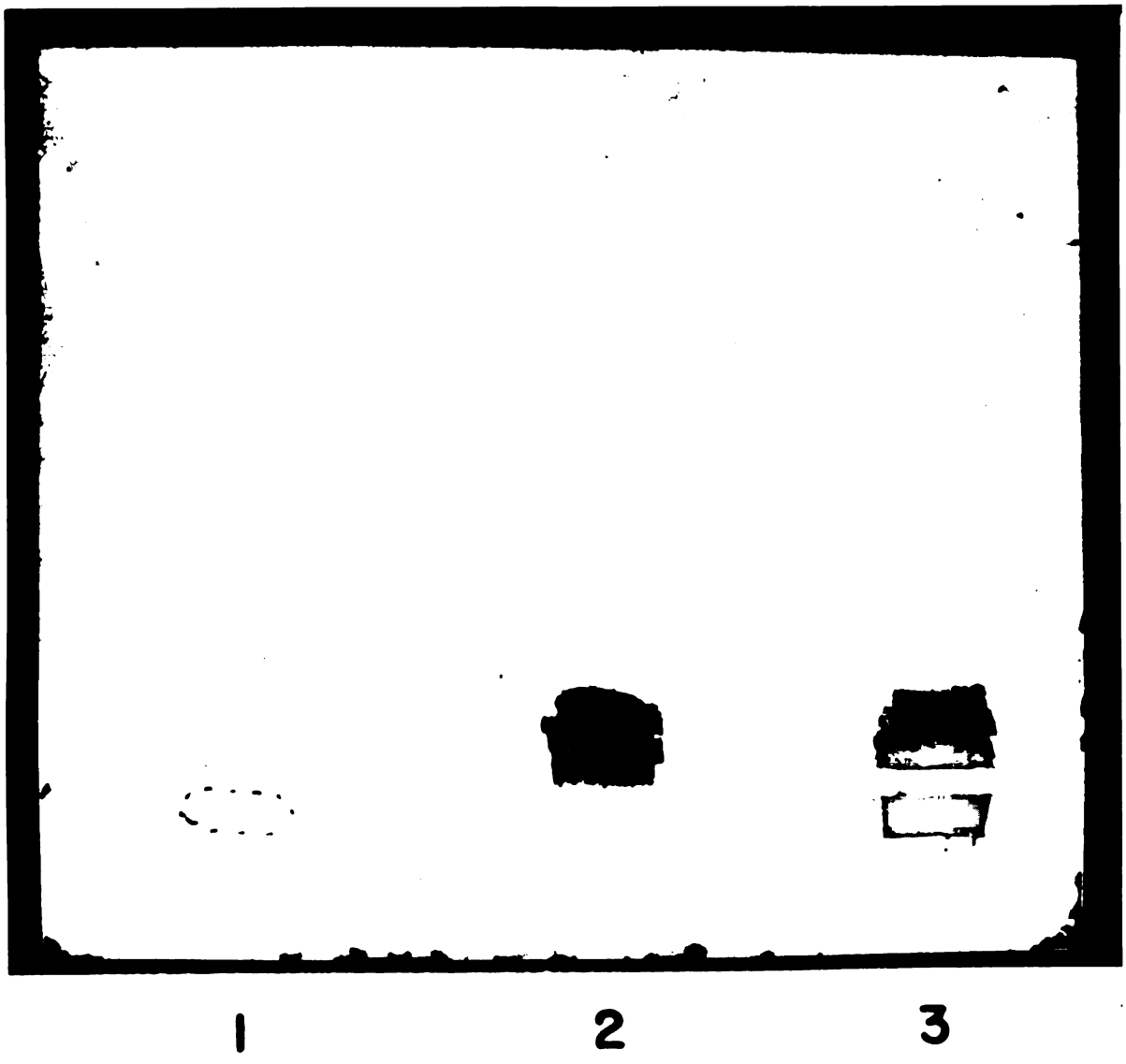


Figure 16.--Thin-layer chromatographic separation of unbound $Co^{57}B_{12}$ from the +2 peptide. Support - Silica Gel G; mobile phase - 55% benzene, 25% methanol, 5% acetone and 15% glacial acetic acid. Left to right: column 1- crystalline B_{12} standard; column 2- +2 peptide plus $Co^{57}B_{12}$ (192 cpm); column 3- $Co^{57}B_{12}$ standard. (Origin 2360 cpm; area corresponding to +2 peptide 185 cpm). Background radiation 187 cpm.

TLC technique using deionized water as the mobile phase was then employed to effect the resolution of the free $Co^{57}B_{12}$ from the peptide. The results of this experiment (Figure 17) showed that ideal resolution between the peptides and vitamin B_{12} was not obtained. However, the absence of gamma radiation at the trailing edge of the peptide band indicated that peptides did not bind vitamin B_{12}.

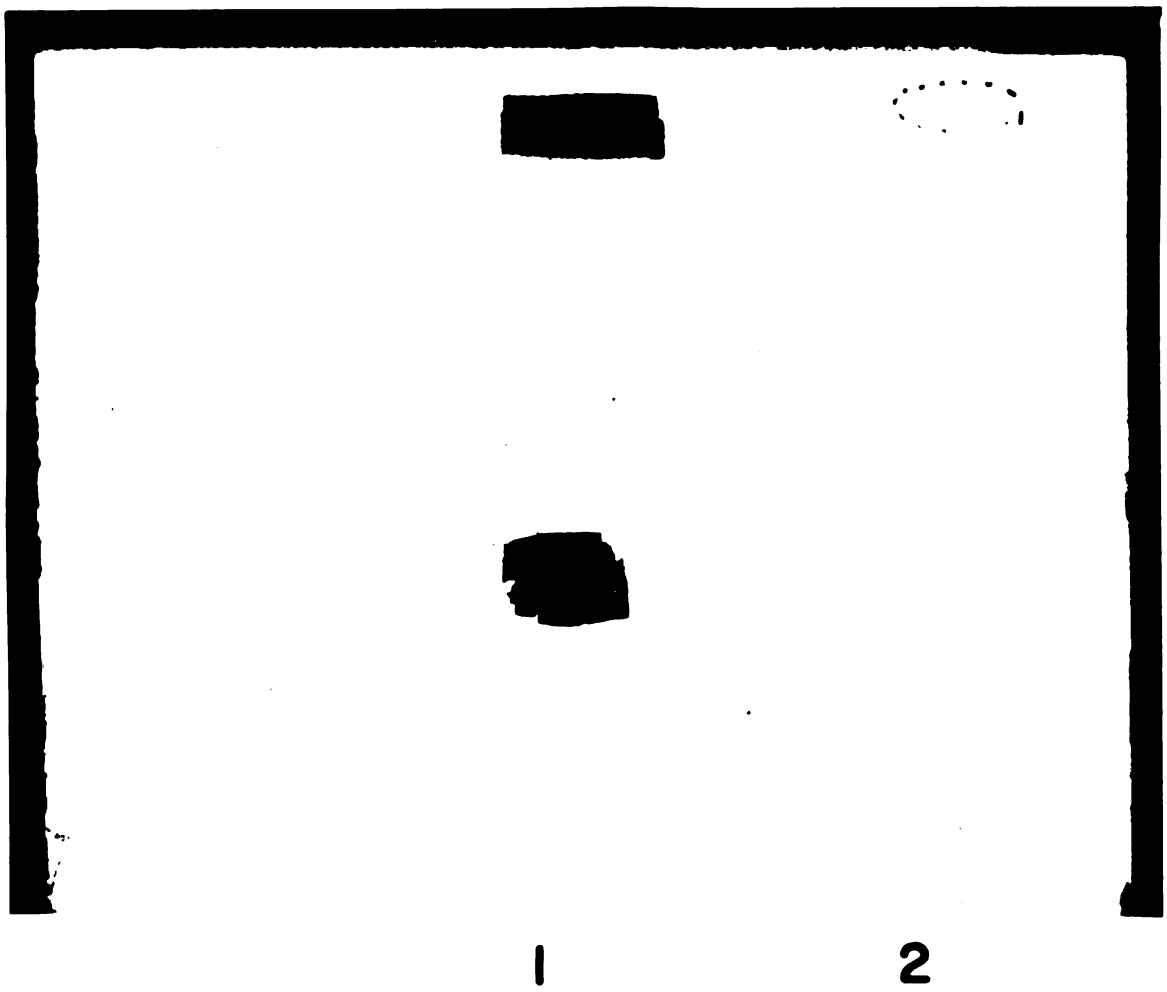


Figure 17.--Thin-layer chromatographic separation of unbound $Co^{57}B_{12}$ from the peptide mixture. Support - Silica Gel G; mobile phase - deionized water. Left to right: column 1- peptide mixture equilibrated with $Co^{57}B_{12}$ (radiation level at center of plate 194 cpm; trailing edge of peptide mixture 197 cpm; center of peptide band 2160 cpm); column 2- crystalline B_{12} standard. Background radiation 188 cpm.

DISCUSSION

In view of previous data concerning the binding of vitamin B_{12} to the peptides electrodialyzed from β-Lg reported by Gizis (1965) and Dorris (1968), the rationale developed for this thesis was to identify the binding site on the peptides for the *in vitro* binding of vitamin B_{12} in model systems. Because β-Lg was reported to have the highest concentration of associated peptides of all the milk proteins, it was chosen as the experimental system.

The β-Lg used in this research was isolated from mixed herd milk and was purified by repeated recrystallization. Reported chemical analyses, sedimentation velocities and polyacrylamide gel electropherograms (see pages 33-48 Results) are in agreement with comparable values published by Tilley (1960), Bell and McKenzie (1964) and Melachouris (1969).

Association of Vitamin B_{12} with Peptides

Following electrodialysis of the crystalline β-Lg as described under experimental methods, the desorbed peptides were recovered from the electrodiffusate. The vitamin B_{12} binding capacity of these peptides was determined by equilibration with $Co^{60}B_{12}$ for 1 h in

various buffer systems. The unbound $Co^{60}B_{12}$ was separated from the peptides by gel filtration chromatography using Bio-Rad P-2 as previously described by Dorris (1968). The results of this experiment indicated that the peptides exhibited an apparent binding in the range of 5,000 μμg $Co^{60}B_{12}$/mg of peptide. This value agreed with the binding value reported by Dorris (1968).

Because a maximum B_{12} binding value by the peptides was not previously reported, an experiment was designed to evaluate this parameter. The peptides were equilibrated with varying levels of $Co^{60}B_{12}$ and the free $Co^{60}B_{12}$ separated by gel filtration chromatography using Bio-Rad P-2. Increasing levels of a $Co^{60}B_{12}$ solution added to the peptide equilibration mixture provided data which indicated that the binding capacity of the peptides was unreasonably high. These experiments provided the first indication that this procedure for determining the B_{12} binding capacity of the peptides in model systems may be erroneous.

The unreasonably high B_{12} binding capacities measured for the peptides were only explainable by assuming that there was an incomplete separation of free $Co^{60}B_{12}$ from the peptide-$Co^{60}B_{12}$ equilibration mixture on the P-2 column. To confirm this postulate, a standard $Co^{60}B_{12}$ solution was chromatographed over the P-2 column and the eluate monitored with a scintillation

spectrometer to determine the elution volume of the $Co^{60}B_{12}$ standard. The results of this experiment showed that $Co^{60}B_{12}$ exhibited no UV absorbance at 254 nm as previously assumed by Dorris (1968), and that the $Co^{60}B_{12}$ was eluted from the P-2 column in the void volume along with the peptide mixture. Further study of the elution profile of the $Co^{60}B_{12}$ standard over the Bio-Rad P-2 column indicated that the adsorbance monitored during the chromatographic separation of the B_{12} standard was the result of the methyl and propyl esters of para-hydroxy benzoic acid added to the $Co^{60}B_{12}$ solution as an anti-microbial agent. The results obtained from these experiments indicated that the $Co^{60}B_{12}$ binding to the peptides as reported by Dorris (1968) was due to the elution of free $Co^{60}B_{12}$ with the peptides.

In subsequent experiments Bio-Rad P-4 and other gel filtration supports were employed in an effort to effect a separation of the unbound $Co^{57}B_{12}$ ($Co^{57}B_{12}$ was used in place of $Co^{60}B_{12}$) from the peptide-$Co^{57}B_{12}$ mixture. However, when column eluates from the peptide mixture and $Co^{57}B_{12}$ standard systems were monitored by both UV absorbance and scintillation spectrometry, the peptides and $Co^{57}B_{12}$ exhibited similar elution characteristics on all gel filtration supports employed. This observation indicated that the peptides and $Co^{57}B_{12}$ behaved like molecules having similar molecular weights.

Although separation of free $Co^{57}B_{12}$ from the peptide mixture with Bio-Rad P-4, a procedure reported by Gizis et al. (1965), proved unsuccessful, the fact that no UV absorbance at 254 nm was monitored at or near the elution of the void volume (peptide-$Co^{57}B_{12}$ complex would have a molecular weight near 3,600) gave the first indication that vitamin B_{12} was not bound to the peptides. It was not immediately ascertained that a peptide-$Co^{57}B_{12}$ complex, if present, could not elute with the free peptides and $Co^{57}B_{12}$.

In view of these experimental findings, it was apparent that a different method for separating free $Co^{57}B_{12}$ from the peptides was needed before the presence of a peptide-$Co^{57}B_{12}$ complex could be established.

Gizis and Meyer (1968) reported that peptides electrodialyzed from human blood bound vitamin B_{12} by non-electrostatic association. Therefore, TLHVE was employed in an attempt to separate the unbound $Co^{57}B_{12}$ from the peptides to determine if the peptides bound $Co^{57}B_{12}$ by a mechanism other than electrostatic attraction. The results of these experiments (Figure 13) showed no trace of the $Co^{57}B_{12}$ associated with either the +3 or negative peptides. The TLHVE data were inconclusive with regard to the binding capacity of the +2 peptide. The center of the ninhydrin stained +2 peptide zone, when removed and counted in the

scintillation spectrometer, showed a radiation level equivalent to 200 μμg of vitamin B_{12}. Although these data indicated that the +2 peptide had bound B_{12}, close inspection of the crystalline B_{12} standard, which had been spotted on the same plate, showed it migrating toward the cathode under the electrophoretic conditions employed. Thus, the vitamin B_{12} which was associated with the +2 peptide could have resulted from the concomitant migration of the free $Co^{57}B_{12}$ mixture. This postulate was further supported by the observation that the radioactivity determined at the leading front of the +2 peptide band showed only a background level of radiation.

These inconclusive results led to the development of an ascending TLC technique in an attempt to separate the unbound $Co^{57}B_{12}$ from the peptide. In this manner the migration of peptide and labeled B_{12} in an electrostatic field would be avoided. The mobile phase consisting of 55% benzene, 25% methanol, 15% glacial acetic acid and 5% acetone produced a migration of the negative and +2 peptides, whereas, vitamin B_{12} remained at the origin. As shown in Figures 14, 15, and 16, the migrating peptide zones had no $Co^{57}B_{12}$ associated with them. Hence, it was established that the +2 peptide did not bind B_{12}. A possible electrostatic binding of B_{12} by the +3 peptide could not be determined with this mobile

phase as a result of its inability to migrate. Therefore, a different mobile phase was employed to determine if the +3 peptide was forming a binding complex with the $Co^{57}B_{12}$.

In subsequent TLC separations, deionized water was used as the mobile phase. The resolution between the peptide mixture and the $Co^{57}B_{12}$ obtained with this system was not ideal (Figure 17). However, the $Co^{57}B_{12}$ standard indicated sufficient resolution between the peptides and free $Co^{57}B_{12}$ that the trailing edge of the peptide band could be analyzed for radioactivity. The cellulose was removed and monitored, showing no $Co^{57}B_{12}$ present. Subsequent TLHVE of the peptides isolated from the trailing edge of the peptide zone confirmed the presence of all three peptides in the cellulose. Thus, it was concluded that vitamin B_{12} is not bound by any of the identified peptides electrodialyzed from β-Lg.

These B_{12} binding characteristics of the isolated peptides do not agree with the findings of Gizis et al. (1965) and Dorris (1968), both of whom reported that B_{12} bound to the isolated peptides in model systems. However, their experiments were developed on the premise that Bio-Rad P-2 (Dorris, 1968) and Bio-Rad P-4 (Gizis et al., 1965) could separate unbound $Co^{57}B_{12}$ from the peptide-$Co^{57}B_{12}$ complex.

The experiments reported herein, indicated that what Gizis et al. (1965) and Dorris (1968) interpreted as a B_{12}-peptide complex was an unfortunate artifact resulting from the elution of free vitamin B_{12} with the peptides. Thus, it must be concluded that the peptides isolated from β-Lg by electrodialysis do not bind B_{12} when mixed in model systems. In view of these experimental findings, the original rationale of establishing the binding mechanism between B_{12} and the protein-associated peptides was abandoned.

Association of Vitamin B_{12} with Beta-Lactoglobulin

Vitamin B_{12} Binding at pH 6.6-6.8

Beta-lactoglobulin when dissolved in 0.1M NH_4OAc, pH 6.8, and in Jenness and Koops buffer, pH 6.6, exhibited an average binding capacity of approximately 460 μμg $Co^{60}B_{12}$/mg of protein. Using Avagadro's number, the molecular weights of vitamin B_{12} and β-Lg (36,000), and the amount of B_{12} bound per mg of protein, a molar binding ratio of 10 moles of vitamin B_{12} per mole of β-Lg was estimated. If the 460 μμg of vitamin B_{12} bound/mg protein as determined experimentally and the vitamin B_{12} content inherently associated with β-Lg (i.e., 870 μμg B_{12}/mg protein; Gizis et al., 1965) are combined, the amount of vitamin B_{12} bound to β-Lg would be in the range

of 1300 μμg B_{12}/mg protein. This B_{12} binding value at pH 6.6 and 6.8 represents approximately one-half that reported by Gizis et al. (1965) and twice that reported by Dorris (1968) for experiments performed in borate buffer at pH 9.0.

Vitamin B_{12} Binding at pH 9.0

At pH 9.0 the B_{12} binding capacity of β-Lg varied with the buffer system employed. In borate buffer at pH 9.0, β-Lg bound 150 μμg of vitamin B_{12}/mg of protein, less than half the binding capacity exhibited by β-Lg in the pH range of 6.6 to 6.8. The molar binding ratio of vitamin B_{12} to β-Lg in borate buffer decreased to approximately one-fifth the value observed at pH 6.6 to 6.8. This reduced binding capacity of β-Lg was also exhibited in the experiments performed with an ammonium acetate-sodium hydroxide solvent at pH 9.0 (Table 6). The calculated molar binding ratio indicated that 1 mole of vitamin B_{12} was bound per mole of β-Lg.

Some insight into these binding characteristics in both borate buffer and ammonium acetate-sodium hydroxide solvent at pH 9.0 can be obtained from the ultracentrifuge data reported in Tables 1 and 2. Sedimentation coefficients for the β-Lg in a borate buffer system was 2.1 $S_{20,w}$. When compared to a sedimentation coefficient of 2.9 $S_{20,w}$ at pH 5.1 (a dimeric unit of

36,000 MW) it is apparent that the β-Lg preparation exists in its monomeric state, i.e. 18,000, in borate buffer at pH 9.0. Thus, it is proposed that the loss of vitamin B_{12} binding capacity by β-Lg at pH 9.0 could be the result of a decrease in electrostatic binding sites concomitant with the increase in pH and/or a change in the conformation of the dissociated β-Lg.

The unusually high vitamin B_{12} binding capacity of β-Lg in TRIS-HCl buffer system at pH 9.0 (Table 6) is attributed to the direct association of $Co^{60}B_{12}$ with the TRIS ions. The basis for this postulate arises from the relatively more diffuse elution pattern of the $Co^{60}B_{12}$ in TRIS buffer when compared to the elution of $Co^{60}B_{12}$ in other buffer systems. Thus, the apparent binding was considered to be a peculiarity associated with the TRIS-HCl buffer system.

Vitamin B_{12} Binding at pH 2.0

The vitamin B_{12} binding capacity of β-Lg was measured at pH 2.0 where the protein also exists in its monomeric state. At this low pH, there was no apparent association of B_{12} to the protein; a characteristic attributed to the electrostatic repulsion of the protonated protein molecules. This binding characteristic has also been reported by Ford et al. (1969) concerning

the dissociation of the folate-β-Lg complex below pH 3.65.

These data concerning the binding of B_{12} to β-Lg at various values of pH supplement the results reported by Gizis et al. (1965) who reported that β-Lg in borate buffer, pH 9.0, bound B_{12} in greater quantities than inherently associated with the native protein. However, the data from these experiments indicate that the optimum pH range for the in vitro adsorption of B_{12} by β-Lg was 6.6-6.8, the normal pH range of fresh milk.

Effects of Electrodialysis on Beta-Lactoglobulin

The reduced vitamin B_{12} binding capacity exhibited by (E)β-Lg was believed by Dorris (1968) to be a result of the loss of the associated peptides which he reported as having a high B_{12} binding capacity. However, the present investigation has established that these peptides do not bind vitamin B_{12} in model systems. Consequently, alternate explanations for the reduction in B_{12} binding capacity as a result of electrodialysis were sought.

Chemical analyses, sedimentation velocities and polyacrylamide gel electropherograms reported for β-Lg and (E)β-Lg (see Results section) were in agreement with comparable values for β-Lg published by Tilley (1960), Bell and McKenzie (1964) and Melachouris (1969). The

interpretation of these data suggests that electrodialysis exhibits a subtle change in the protein.

In view of this observation an investigation was initiated to determine the relative rates of enzymatic hydrolysis of β-Lg and (E)β-Lg as an indication of molecular change. The rate of proteolysis of β-Lg and (E)β-Lg by trypsin and chymotrypsin (Figures 6 and 7), as estimated by pH Stat-monitored proton release at pH 8.0, illustrated that electrodialysis could have affected the conformation of the β-Lg molecules. Relative Km values calculated for the proteolytic hydrolysis of both β-Lg and (E)β-Lg (Figures 6 and 7) showed a lower value for (E)β-Lg with both trypsin and chymotrypsin, indicating a more rapid rate of hydrolysis.

Although Monnot et al. (1967) reported that hydrolysis of β-Lg with trypsin did not follow the Michaelis-Menten law because of allosteric kinetics, it is proposed that the observed reaction velocity data are significant if interpreted only as a difference between the relative reaction rates of β-Lg and (E)β-Lg and not as a means of determining specific reaction kinetics of the proteolytic reactions. Therefore, within the limits imposed upon these data, it was concluded that the increased rate of hydrolysis observed for (E)β-Lg is indicative of a change in its molecular conformation. Although the experimental data do not contain the

information required to discern the cause of this change, it is postulated that the removal of the protein bound salts and/or peptides from the β-Lg molecules resulted in an increase in intramolecular bonding. This hypothesis is supported by published findings of Tanford (1961) concerning the increased hydrophobicity of protein molecules in low ionic strength solutions.

Association of Vitamin B_{12} with Electrodialyzed Beta-Lactoglobulin and Electrodialyzed Beta-Lactoglobulin Peptide Mixture

Vitamin B_{12} Binding at pH 6.6-6.8

Previous studies have shown that following electrodialysis, β-Lg exhibited a large reduction in vitamin B_{12} binding capacity in borate buffer at pH 9.0. Dorris (1968) attributed this behavior of the protein to the loss of its associated peptides. He supported these findings by demonstrating that (E)β-Lg had a B_{12} binding capacity equal to that of β-Lg when the electrodialyzed protein was equilibrated with the peptide mixture prior to the addition of $Co^{60}B_{12}$. As previously stated the results from the current research indicated that the peptides recovered from (E)β-Lg did not bind to B_{12}. Therefore, the binding capacity of the (E)β-Lg was determined at various levels of pH and in various buffer systems to determine the effect of pH and specific buffer ions on the B_{12} binding capacity of (E)β-Lg.

(E)β-Lg and (E)β-Lg in combination with the peptides in 0.1M NH_4OAc, pH 6.6 showed binding capacities for $Co^{60}B_{12}$ that were less than 10 μμg B_{12}/mg protein. However, slight variations associated with the gamma emissions of $Co^{60}B_{12}$ required that binding values of 10 μμg/mg protein or less be reported as too low to determine.

The inability of the (E)β-Lg and the recombined (E)β-Lg-peptide mixture at pH 6.6 and 6.8 to bind B_{12} is attributed to a reduction in the number of primary binding sites previously proposed. In view of the binding of B_{12} by the electrodialyzed protein at pH 9.0 in both borate and ammonium acetate-sodium hydroxide solvents, the loss of protein bound salts and the subsequent loss of secondary binding sites for the B_{12} was not believed to be of principal importance in the loss of B_{12} binding by the (E)β-Lg.

Vitamin B_{12} Binding at pH 9.0

Whereas the vitamin B_{12} binding capacity of β-Lg in borate buffer at pH 9.0 decreased, the binding capacities of the (E)β-Lg and (E)β-Lg-peptide mixture increased to 40 and 200 μμg $Co^{60}B_{12}$/mg protein, respectively. These data represent a molar binding ratio of 1 mole of vitamin B_{12} for every 2 moles of (E)β-Lg and 3 moles of vitamin B_{12} for every 1 mole of recombined (E)β-Lg-peptide

mixture in borate buffer at pH 9.0. The binding capacities of the (E)β-Lg and recombined (E)β-Lg-peptide mixture reported here are lower than those reported by Dorris (1968) (i.e., 133 μμg $Co^{60}B_{12}$/mg of (E)β-Lg and 244 μμg $Co^{60}B_{12}$/mg of recombined mixture). The discrepancies arising between the vitamin B_{12} capacities for these two forms of modified β-Lg are attributed to differences in the electrodialysis technique.

The electrodialysis system employed in this study consisted of placing the anode inside the dialysis membrane with the cathode positioned in the diffusate. Placement of the electrodes in this manner together with a constant stirring of the protein suspension assured that the electrostatic potential, which is believed responsible for the dissociation of the salts and peptides from the β-Lg, was directed at the protein inside the membrane. The electrodialysis technique described by Dorris (1968) indicated that both electrodes were placed in the diffusate. Placement of the electrodes in this manner would result in dissociation of salts and/or peptides from the β-Lg when the electrostatic potential was initially applied. However, as the charged particles diffuse through the dialysis membrane, the maximum electrostatic potential of the system would no longer be directed toward the β-Lg inside the membrane.

A protein system electrodialyzed as described by Dorris (1968) would be subjected to a lower electrostatic potential, contributing to a lower recovery of peptides and salts from the protein. Consequently, the protein would be exposed to less stress and possibly undergo less change in conformation than if one electrode had been placed inside the dialysis sac. This hypothesis would be difficult to prove, but is offered as a possible explanation for the higher vitamin B_{12} binding activities of the (E)β-Lg and the (E)β-Lg-peptide mixture reported by Dorris (1968).

Although (E)β-Lg showed no binding at pH 6.6 and 6.8, a significant binding of B_{12} by the (E)β-Lg was observed at pH 9.0. Ultracentrifugation data (Tables 1 and 2) show that the (E)β-Lg in this buffer system dissociated to its monomeric state. Therefore, vitamin B_{12} binding by (E)β-Lg may be a result of secondary binding to the borate ions associated with the protein monomers. An alternate explanation for the B_{12} binding capacity of (E)β-Lg (1 mole B_{12} per 2 moles of (E)β-Lg) is that the change in protein conformation resulting from electrodialysis and subsequent monomerization of the (E)β-Lg yielded only one monomer from the original (E)β-Lg dimer (36,000 M.W.) with an available binding site for vitamin B_{12}.

The recombined system of (E)β-Lg and peptides in borate buffer at pH 9.0 showed a regain in vitamin B_{12} binding capacity similar to that reported by Dorris (1968). The binding capacity of 200 μμg vitamin B_{12}/mg of (E)β-Lg-peptide mixture resulted in a molar binding ratio of 3 moles of $Co^{60}B_{12}$ per mole of (E)β-Lg (18,000 M.W.). This regain in the binding capacity of the (E)β-Lg when equilibrated with the peptides in borate buffer at pH 9.0 may be indicative of an interaction between the borate buffer ions and peptides, negating the effects of the proposed conformational change in (E)β-Lg.

The extremely high binding capacities measured for all three forms of β-Lg in TRIS-HCl buffer as previously discussed are attributed to TRIS-associated $Co^{60}B_{12}$ and, as such, are considered artifactual.

The static binding capacities measured for the (E)β-Lg and (E)β-Lg-peptide mixture in NH_4OAc-NaOH solvent at pH 9.0 (Table 6) as well as β-Lg in the same solvent system are believed to reflect the actual electrostatic binding sites available on the β-Lg molecules at pH 9.0. The calculated molar binding ratio indicated that 1 mole of vitamin B_{12} is bound per mole of β-Lg. These data support the interpretation that the peptides are not directly responsible for the higher binding capacity of β-Lg and that the peptides alone are

responsible for the regain in the binding capacity of (E)β-Lg at pH 9.0.

Mode of Association Between B_{12} and Beta-Lactoglobulin

Although the binding of B_{12} by β-Lg has been reported previously, the type of association involved in the formation of the protein-B_{12} complex was not determined. In an effort to elucidate the type of binding suspected a β-Lg-$Co^{60}B_{12}$ complex (460 μμg $Co^{60}B_{12}$/mg protein) was dissolved in 0.1M NH_4OAc and dialyzed for 24 h against pure solvent. Scintillation spectrometry data showed that the B_{12} was not dialyzed from the protein, indicating that the B_{12} was associated with the protein. Aliquots of both β-Lg and (E)β-Lg-vitamin $B_{12}(Co^{60})$ complexes were then subjected to polyacrylamide gel electrophoresis at pH 8.3 (see Figure 12). At the conclusion of the electrophoretic run, the $Co^{60}B_{12}$ was not associated with the protein, nor was the B_{12} located anywhere in the running gel. These data indicate a dissociation of the $Co^{60}B_{12}$ from the protein prior to entering the gel, which would imply a loose association of the B_{12} with the protein, possibly through electrostatic or polar association.

Peptides Isolated from Beta-Lactoglobulin

HVPE and TLHVE showed three peptides present in the electrodiffusate from β-Lg (see Figure 10). It is

assumed that these peptides were not contaminants in the protein system, since non-associated peptides should have been removed from the system during the protein purification procedure. Furthermore, no evidence of protein decomposition was established from amino acid analyses, n-terminal amino acid analyses, -SH determinations, sedimentation velocities and gel electropherograms of the β-Lg and (E)β-Lg as reported in the results. These data in addition to the findings of Moretti et al. (1958) and Gordon (1960), who have shown that proteins recovered by electrodialysis (500 V potential) of starch gels following electrophoresis showed no indications of protein decomposition, support the hypothesis that the peptides electrodialyzed from the β-Lg were originally associated with the protein.

The recovery of peptides from β-Lg was in the range of 7 to 8 mg/g of β-Lg (0.75% of the protein). A more accurate determination of the peptide recovery from the β-Lg by electrodialysis was difficult to obtain because of the large volumes of electrodiffusate (8,000 to 9,000 ml) which had to be concentrated prior to lyophilization. The percentage recovery reported, herein, was higher than has been reported in previous studies (Dorris, 1968), and is attributed to a more complete electrodialysis of the β-Lg.

TLHVE data established the relative concentrations of the peptides in the electrodiffusate as: +2 peptide > +3 peptide > negative peptide. During preliminary studies this pattern of peptide concentration was reversed when the electrode inside the dialysis sac was connected to the cathode of the power supply unit. Placement of the electrodialysis electrodes in this manner significantly lowered the recovery of peptides because the positively charged peptides did not traverse the membrane as readily. In view of these results the cathode was positioned outside the dialysis sac as a standard procedure.

Estimated Molecular Weight of Peptides

Gel filtration chromatography of the peptide mixture over Bio-Rad P-2 (Figure 9) indicated that the peptides possessed molecular weights greater than 1,500. When the peptides were passed over the Bio-Rad P-4 column (V_o of 63 ml, Figure 11) their elution volume was 110 ml, indicating that the peptides appear to have similar molecular weights. The shoulders exhibited on the elution pattern (Figure 11) were not interpreted as resolution of the peptides, since each collected eluate fraction was shown by TLHVE to contain the peptide mixture.

Following the separation of the peptides by TLHVE, individual peptides were again chromatographed to

ascertain if molecular interactions between the peptides were responsible for the elution characteristics exhibited by the peptide mixture. The elution volumes of the individual peptides indicated that each peptide exhibited the elution patterns similar to that of the peptide mixture, i.e., characteristics of a globular protein with a molecular weight range of 1,500 to 3,600. However, little is known concerning these peptides and it is possible that they may have molecular weights larger than is indicated from gel filtration chromatography data because of unusual composition or structural characteristics.

The inadequacies of the gel filtration data inferred that the minimum molecular weights of the isolated peptides be estimated from their amino acid composition. These estimations also generated problems in that the limited amount of peptides available for amino acid analysis necessitated the calculation of the limiting amino acid residue by integration of its absorbance curve. Consequently, only amino acid residues which could be accurately measured were used to make these calculations. The estimated minimum molecular weights were: negative peptide, 4,000; +2 peptide, 6,000; +3 peptide, 6,000. Although these values did not agree with the values determined by gel filtration chromatography, they do compare favorably with values of 3,000 to 9,000

Daltons reported by Gizis et al. (1965) for two peptides obtained in a similar manner from electrodialyzed skim-milk. Dorris (1968) also reported an approximate molecular weight of 4,000 for a peptide mixture recovered from electrodialyzed β-Lg from gel filtration chromatography data. These data support the hypothesis that the peptides may have a secondary structure causing them to behave like much smaller molecules on gel filtration.

Mode of Binding Between Peptides and Beta-Lactoglobulin

The question now arises, "Does the β-Lg contain a specific binding site for the associated peptides?" The protein-peptide binding ratio as estimated from the average molecular weight of the peptides (2,500 Daltons) indicated approximately one mole of peptides per 10 moles of β-Lg. If the binding ratio is based on the average minimum molecular weight of the peptides as determined from amino acid composition (5,000 Daltons) the molar binding ratio is reduced to one mole of peptide per 20 moles of β-Lg. Regardless of which value is considered, it appears certain that the binding of peptides to β-Lg is non-specific and is a random electrostatic association between peptides and protein.

This small number of β-Lg molecules involved in the peptide-protein association, as well as the large molecular weight differential between the peptide and

β-Lg, also answers the question of why the sedimentation coefficients of β-Lg did not vary significantly from that of (E)β-Lg.

SUMMARY AND CONCLUSIONS

The adsorption of vitamin B_{12} by β-Lg, (E)β-Lg and the (E)β-Lg-peptide mixture was determined by equilibrating a known $Co^{60}B_{12}$ or $Co^{57}B_{12}$ standard solution with the proteins for 1 h. The unbound vitamin was separated from the protein by gel filtration chromatography. The radioactive B_{12} associated with the protein was measured by scintillation spectrometry. These experiments showed that adsorption of the vitamin by β-Lg, (E)β-Lg and the (E)β-Lg-peptide mixture was pH dependent.

β-Lg exhibited its greatest binding capacity at pH 6.6 and 6.8 (460 μμg B_{12}/mg protein). The reduction in binding capacity by (E)β-Lg and the (E)β-Lg-peptide mixture at this pH range was attributed to a reduction in the number of primary binding sites on the electrodialyzed protein molecules.

At pH 9.0 β-Lg exhibited a binding capacity of 150 μμg vitamin B_{12} per mg of protein in a borate buffer and 100 μμg per mg of protein in an ammonium acetate-sodium hydroxide solvent. The experimental results indicated that this reduction in the binding capacity of β-Lg could have resulted from a decrease in its electrostatic binding sites and/or a conformational change in

the dissociated dimers. Whereas, β-Lg showed a decreased adsorption of vitamin B_{12} at pH 9.0, (E)β-Lg and the (E)β-Lg-peptide mixture showed an increase in vitamin B_{12} adsorption. This increase was attributed to the secondary binding of the vitamin to the buffer ions associated with the (E)β-Lg and/or the dissociation of the (E)β-Lg molecules at pH 9.0 which may have negated the effects of electrodialysis on the β-Lg.

The static binding capacities of β-Lg, (E)β-Lg and the (E)β-Lg-peptide mixture for vitamin B_{12} in ammonium acetate-sodium by droxide solvent support the interpretation that the protein-associated peptides are not directly responsible for the higher binding capacities of β-Lg, or that the peptides alone are responsible for the regain in the binding capacity of (E)β-Lg at pH 9.0.

The inability of the vitamin to bind to either β-Lg or the (E)β-Lg model system at pH 2.0 was an indication of electrostatic repulsion by the protonated protein molecules.

High-voltage paper-electrophoresis and thin-layer high-voltage electrophoresis showed that three peptides were electrodialyzed from the purified β-Lg. Experimental findings revealed that these peptides were electrostatically associated to the protein. Calculations of the protein-peptide molar binding ratio showed that the binding of peptides is non-specific and probably

a random association between peptides and protein. The molecular weights of the isolated peptides were not accurately determined. However, gel filtration chromatographic parameters and minimum molecular weight calculations made from amino acid analyses indicated molecular weights in the range of 2,500 to 5,000, which may indicate the presence of an unusual secondary structure in the peptides.

Vitamin B_{12} binding experiments carried out with the peptide mixture and the individual peptides showed no binding of the vitamin by the peptides. Recombination experiments with (E)β-Lg and the peptide mixture at pH 6.6 (0.1M NH_4OAc), pH 6.8 (J and K buffer) and pH 9.0 (NH_4OAc-NaOH) showed no regain of binding capacity. From these data it was concluded that the decrease in vitamin B_{12} binding capacity by electrodialyzed β-Lg cannot be attributed solely to the loss of the protein-associated peptides.

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APPENDIX

APPENDIX

Isolation of Beta-Lactoglobulin

Ten liters of fresh, mixed-herd milk were collected from the Michigan State Dairy. Without allowing the milk to cool, it was heated to 40 C. Twenty grams of anhydrous sodium sulfate were slowly added to each 100 ml of milk, stirring continuously until all the sodium sulfate was dissolved.

After the addition of sodium sulfate, the temperature was lowered to 25 C, effecting the precipitation of globulins, proteose peptones, casein and fat. The precipitate was filtered out with E and D No. 515 fluted filter paper. The filtrate, containing β-Lg, α-lactalbumin and serum albumins, was collected for further fractionation.

Concentrated hydrochloric acid was added to the filtrate (1 ml conc. HCl per 100 ml filtrate) to adjust the pH to approximately 2. The resulting precipitate, containing all of the proteins except β-Lg, was removed by centrifugation in an International High-Speed Refrigerated Centrifuge, Model HR-1 with a No. 856 head, operated at 8,000 rpm for 30 min.

The supernatant, containing β-Lg, was filtered through E and D No. 515 fluted filter paper and refiltered through S & S No. 478-1/2 analytical grade fluted filter paper to insure the removal of precipitated proteins from the β-Lg.

The filtrate was adjusted to pH 6 with concentrated ammonium hydroxide (approximately 0.6 ml per 100 ml of filtrate was required) and 20 g of ammonium sulfate per 100 ml of filtrate was slowly added with continuous stirring. This resulted in the precipitation of the β-Lg. When the ammonium sulfate had completely dissolved, the solution was allowed to sit quiescently for one hour to ensure complete flocculation of the β-Lg. Beta-lactoglobulin was recovered by filtering the solution under vacuum through a thick layer of Johns-Mansvill Hyflo Super-Cel or Super Cel filter aid deposited on S & S No. 597 filter paper in a Buchner funnel. The filtrate was discarded. It was important to keep the filter aid covered with solution once filtration had begun or a gelatinized layer of β-Lg formed on the top of the filter aid, dramatically reducing the flow rate.

Following filtration, the filter cake was slurried with deionized water and placed in Visking cellulose dialysis tubing which had been boiled in dilute ethylenediaminetetraacetic acid (EDTA) solution and dialyzed against deionized water. After 12 h of dialysis

the contents of the dialysis tubing were filtered with suction through Whatman No. 1 filter paper in a Buchner funnel.

The filtrate containing the β-Lg was collected and the pH adjusted to pH 5.8 with 1N hydrochloric acid. A few drops of toluene were added as a preservative. The solution was placed in EDTA treated dialysis tubing and dialyzed for 48 h against deionized water (dialysate was changed every 12 h).

The β-Lg solution was then removed from the dialysis membrane and the pH adjusted to 5.2 with 1N HCl, which produced some cloudiness, and the dialysis continued. A few hours after the pH was lowered to 5.2, small β-Lg crystals began to form, having the appearance of small platelets.

Previous researchers have reported the presence of a heavy oily layer of β-Lg instead of crystals, a phenomenon also encountered in this study. However, by refiltering the supernatant after centrifugation to insure removal of precipitated α-lactalbumin and serum albunims, this problem was eliminated.

Recrystallization and Purification

The β-Lg crystals from five separate isolations were suspended in approximately 300 ml of deionized water and analytical grade sodium chloride was added until all the crystallized β-Lg was dissolved. The

solution was filtered through analytical grade filter paper and the pH adjusted to 5.17 with 1N HCl. The solution was placed in an EDTA treated cellulose membrane along with one or two ml of toluene and dialyzed against deionized water at 4 C until crystallization was complete.

Beta-lactoglobulin was recrystallized four times using this procedure.

Nitrogen

Nitrogen determinations were performed using a micro-Kjeldahl technique. The digestion mixture consisted of 5.0 g $CuSO_4 \cdot 5H_2O$ and 5.0 g SeO_2 in 500 ml of concentrated sulfuric acid. Approximately 6 to 8 mg of dried protein samples were prepared in triplicate for each analysis. The samples were digested with 4 ml of the digestion mixture over an open gas flame for 1 h. After the samples had cooled, 1 ml of 30% H_2O_2 was added to each digestion flask and the digestion continued for 1 h. When the contents of the digestion flasks had cooled, the sides were rinsed with approximately 10 ml of deionized water.

Twenty-five ml of 40% NaOH were added to each digestion mixture and the ammonia released was steam distilled into 15 ml of a 4% boric acid solution containing 5 drops of methyl red-bromcresol green indicator. The indicator consisted of 400 mg of bromcresol green

and 40 ml of methyl red in 100 mg of 95% ethanol. The distillation was continued until a total of 75 ml of distillate were collected in the receiving flask. The ammonium-borate complex was titrated with 0.0194N HCl. Both a blank and recovery standard were determined by the above procedure.

The average recovery for the ammonium sulfate standard was 98.5%.

Tryptophan

A 15 mg sample was weighed directly into a small vial. To each sample vial, 100 µl of freshly prepared pronase solution was added (containing 10 mg pronase per ml of 0.1M phosphate buffer, pH 7.5), and a drop of toluene. The vials were stoppered and incubated for 24 h at 40 C. After cooling, the sample vials were placed in 50 ml Erlenmeyer flasks containing 9.0 ml of 21.2N sulfuric acid (387 ml of H_2O to 500 ml concentrated H_2SO_4) and 30 mg of p-dimethylaminobenzaldehyde which had been prepared immediately prior to use. To each sample vial was added 0.9 ml of 0.1M phosphate buffer, pH 7.5. The vials were tipped over and the contents were quickly mixed by gentle swirling.

The Erlenmeyer flasks were then placed in the dark at 25 C for 6 h. After adding 0.1 ml of 0.045% sodium nitrite the reaction mixtures were shaken and

the color allowed to develop for 30 min in the dark at ambient temperature. Transmittance of the samples was read at 590 nm.

Duplicate blanks were treated as described above. One set of blanks contained everything but the protein being analyzed, and the other contained everything but the protein and pronase. Using this method, the tryptophan inherent in the pronase was not attributed to the protein being analyzed.

A standard curve for tryptophan, having a range of 0 to 120 mg of tryptophan, was prepared according to Procedure E of Spies and Chambers (1948).

Sulfhydryl

A 5-10 mg sample of protein was dispersed in 1 ml of deionized water. Nine ml of pH 7.0 phosphate buffer, which was 8M with respect to urea, was added to the sample solution and allowed to stand for 10 min at room temperature. To a 3 ml aliquot of this sample solution was added 0.02 ml of 5,5' dithiobis 2-nitrobenzoic acid (DTNB) reagent (39.6 mg of DTNB in 10 ml of pH 7.0 phosphate buffer). The tubes were shaken and the transmittance was read immediately.

Because it was very difficult to obtain a suitable standard curve, the sulfhydryl content was determined using the extinct coefficient as reported by Ellman (1959).

Amino Acid Analyses

Five mg dried protein and 20 mg dried peptide samples were weighed directly into 10 ml ampules. Five ml 6N constant boiling HCl were added to each ampule. The contents of the ampules were frozen in a dry ice-ethanol mixture and air was evacuated with a high-vacuum pump by allowing the samples to slowly thaw. Upon complete removal of gases, the samples were refrozen and sealed under vacuum.

The sealed ampules were placed in an oil bath at 110 C for a hydrolysis period of 22 h. Upon completion of hydrolysis, the samples were removed and cooled to room temperature.

The sealed ampules were opened and 1 ml of non-leucine standard was added to determine transfer losses. The contents of the ampules were transferred to a pear-shaped evaporation flask and evaporated to dryness with a rotary evaporator at 50 C. After evaporation to dryness, each sample was taken up in deionized water and re-evaporated. This procedure was continued until all the remaining hydrochloric acid residue was removed.

The dried hydrolysates were dissolved in 0.067M citrate-hydrochloric acid buffer, pH 2.2, and brought to a final volume of 5 ml in the case of β-Lg, and to 1 ml in the case of peptides. Varying aliquots of these

protein hydrolysates were required for amino acid analysis which depended upon the protein content of the samples.

Amino acid standards were chromatographed with the same buffers and ninhydrin solution used for the protein samples to ensure accurate quantitation of amino acids present.

The amino acid composition of the protein samples was expressed as gram residue of amino acids per 100 g protein.

N-Terminal Amino Acid Analysis

Approximately 10 mμ moles of protein were dissolved in 0.5 ml of 8M urea solution (ammonia and cyanate free), buffered with 0.5M sodium bicarbonate. To this was added 0.5 ml of a strong dansyl chloride solution (20 mg 1-dimethylaminonapthalene-5-sulfonyl chloride per ml of acetone). The mixture was allowed to react for 12 h at ambient temperature or several hours at 37 C.

Salts, urea and dansyl hydroxide were removed by passing the reaction mixture over a small Sephadex G-25 column. The dansyl-labeled, desalted protein was collected in a small test tube and dried by lyophilization. To the dried sample, 0.5 ml of 6.7N HCl (made from constant boiling HCl) was added and the tube sealed

with a propane torch. The sealed tube was placed in a 105 C oil bath and hydrolyzed for 18 h. Upon completion of hydrolysis the tubes were opened and the HCl removed by drying over sodium hydroxide pellets in a dessicator.

The dried samples were taken up in 10 µl of 50% (v/v) pyridine-water solvent. This solvent ensures transfer of any insoluble dansyl-amino acid derivatives. The samples were spotted on 20 x 20 cm thin-layer chromatography plates precoated with Silica gel G F (250 microns). The plates were placed in a Reco Model E-800-2, water-cooled electrophoretic migration chamber, containing pyridine/acetic acid/water (10/20/2500 v/v) buffer at pH 4.4 in the buffer tanks. The plates were sprayed with the same buffer and electrophoresed for 30 min at a field strength of 50 volts/cm. Upon the completion of electrophoresis, the plates were dried in a forced air oven at 90 C. The n-terminal dansyl amino acid derivatives were visualized under long-wave (365 nm) ultraviolet radiation.

Colorimetric Determination of Protein

The reagents required were: Reagent A, 2% sodium carbonate in 0.1N sodium hydroxide; Reagent B, 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium or potassium tartrate; Reagent C, alkaline copper solution prepared by mixing 50 ml of reagent A with 1 ml of reagent B (this should

be prepared fresh each day); Reagent D, Folin-Ciocalteu reagent diluted to 1N. Reagent D can be obtained commercially from Fisher Scientific Company.

One milliliter of protein solution was placed in a test tube and 5 ml of reagent C were added. The tube was shaken and allowed to stand for 10 min. To this solution 0.5 ml of reagent D was added and the contents of the tube were mixed and allowed to stand for 30 min. The transmittance was read at 500 nm with a Beckman Model DU-2 Spectrophotometer. A control blank was prepared containing 1 ml of water in place of the protein solution.

The protein content was determined by conversion of transmittance to μg of protein per ml, using a standard curve. The standard curve was prepared with bovine serum albumin having a range of 0 to 100 μg protein per ml.

Enzymatic Hydrolysis of Proteins

The pH stat was standardized at pH 8.0 and 37 C with phosphate buffer. β-Lg and (E)β-Lg samples were prepared in deionized water and the pH raised to 8.0 with 2N triethylamine before adjusting the protein concentration to 10 mg per ml.

Enzyme solutions were prepared in 0.02M $CaCl_2$ at a concentration of 2.5 mg of enzyme per ml. The

enzyme preparations were stored at 4 C after adjustment of pH to 8.0 with triethylamine. Immediately prior to each enzymatic hydrolysis a syringe containing 1 ml of the enzyme preparation was placed in a 37 C water bath to insure optimum temperature for enzymatic activity.

The substrate concentrations were varied by quantitative dilutions of the standard protein solution. The final volume of protein solution applied to the pH-stat was 10 ml. Each 10 ml substrate sample was permitted to equilibrate at 37 C and pH 8.0 in the pH-Stat prior to adding enzyme. The electrode control was then activated to the titration position and 1 ml of the enzyme solution at pH 8.0 and 37 C was added. During enzymatic hydrolysis in the pH-Stat reaction vessel, 0.05N triethylamine was automatically titrated into the reaction mixture to maintain a constant pH of 8.0. The volume of 0.05N triethylamine titrant was automatically plotted relative to time. A blank containing deionized water with pH adjusted to 8.0 with 0.05N triethylamine was employed to determine alkaline consumption due to enzyme degradation and carbon dioxide absorption.

Hydrolysis data were analyzed by plotting percentage total hydrolysis versus time. Thus, the hydrolysis curves for each concentration of native and electrodialyzed β-Lg could be compared and the relative Km values for β-Lg and (E)β-Lg for both trypsin and chymotrypsin calculated.