THE EFFECT OF ELECTRODIALYZABLE PEPTIDE FRACTIONS ON THE BINDING OF VITAMIN B_{12} BY β-LACTOGLOBULIN AND OTHER PROTEINS

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ABSTRACT

THE EFFECT OF ELECTRODIALYZABLE PEPTIDE FRACTIONS ON THE BINDING OF VITAMIN B_{12} BY β-LACTOGLOBULIN AND OTHER PROTEINS

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Vitamin B_{12} is an essential nutrient for man and for many animals. The presence of intrinsic factor, a glycoprotein, is required for the absorption of vitamin B_{12} in man. Intrinsic factor is known to bind vitamin B_{12}. Many proteins and other materials bind significant quantities of vitamin B_{12} and yet show no intrinsic factor activity in man.

The milk proteins have been shown to bind vitamin B_{12}, and among them, β-lactoglobulin has a higher binding capacity for the vitamin than the other proteins tested. Prior preliminary experiments indicated that β-lactoglobulin contains electrodialyzable peptides.

β-Lactoglobulin was chosen for the initial experimental work because of its relatively high vitamin B_{12} binding capacity. The protein used was crystalline and electrophoretically pure. Crystalline β-lactoglobulin was found to have a vitamin B_{12} binding capacity of approximately 260 micromicrograms per milligram. Upon electrodialysis β-lactoglobulin released peptides, accounting for about 0.2% of the protein, which had a vitamin B_{12} binding capacity of over 5,000 micromicrograms per milligram. After electrodialysis the β-lactoglobulin

remaining inside the dialysis sac had a binding capacity of only 130 micromicrograms of vitamin B_{12} per milligram of protein. When the peptide, removed by electrodialysis, was added back to electrodialyzed β-lactoglobulin the vitamin B_{12} binding capacity increased to 240 micromicrograms per milligram.

Amino acid analyses of β-lactoglobulin and of the peptides removed from β-lactoglobulin by electrodialysis showed wide variations between the amino acid compositions of the two. This indicates ordered cleavage rather than a random splitting of the β-lactoglobulin molecule. From the amino acid analysis of the peptide, a minimum molecular weight of 6,000 was calculated, based on the most limiting amino acid, histidine. Work with gel columns indicated a molecular weight of approximately 4,000. Because the indicated molecular weight of the peptide is near that of proteose peptone, and realizing the possibility that a small amount of proteose peptone might be bound by the β-lactoglobulin, the vitamin B_{12} binding capacity of proteose peptone was determined. An average binding capacity of 330 micromicrograms of vitamin B_{12} per milligram of proteose peptone compared to over 5,000 micromicrograms per milligram of peptide ruled out the possibility that the two are identical.

α-Lactalbumin was also found to release a peptide fraction on electrodialysis. Its vitamin B_{12} binding capacity was found to be approximately 3,600 micromicrograms per milligram. By the same

treatment, intrinsic factor preparation released a peptide fraction which had a vitamin B_{12} binding capacity of 11,665 micromicrograms per milligram.

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INTRODUCTION

Vitamin B_{12} (cyanocobalamin) is an essential nutrient for man and many animals. The vitamin usually occurs in a bound form in which it is attached to a protein. Intrinsic factor, a glycoprotein which binds large amounts of vitamin B_{12}, must be present in the intestine in order for absorption of the vitamin to take place. Many other proteins are capable of binding significant quantities of vitamin B_{12} and yet exhibit no intrinsic factor activity in man.

β-Lactoglobulin is one of the major milk proteins comprising from 7 to 12% of the total proteins in milk. It binds significant amounts of vitamin B_{12} and more than the other milk proteins. Because vitamin B_{12} is an essential nutrient and milk often serves as the sole source of food in the young mammal, one of the functions of β-lactoglobulin may be to serve as a carrier of vitamin B_{12}. The various techniques for protein separation and purification such as gel filtration, electrophoresis, centrifugation and etc. are applicable to studies with vitamin B_{12}-binding proteins.

In the present studies peptide fractions were obtained from different vitamin B_{12}-binding proteins, including β-lactoglobulin, α-lactalbumin and intrinsic factor, by electrodialysis. The peptides

from each protein had a much greater vitamin B_{12} binding capacity than the respective protein from which each was obtained while the proteins appeared to undergo a loss in vitamin B_{12} binding capacity when electrodialyzed.

The peptides appear to play a similar role in vitamin B_{12} binding by the different proteins. The data indicate that intrinsic factor activity may, in fact, depend on the presence of a particular peptide rather than the protein to which it and bound vitamin B_{12} may be attached.

REVIEW OF LITERATURE

History

The study of vitamin B_{12} began over a hundred years ago with the clinical recognition of pernicious anemia (1). In pernicious anemia the blood forming cells become enlarged while immature. Whipple (100) found that liver accelerated the regeneration of red blood cells in anemic dogs. Similar results were obtained when liver extracts were administered (98). Castle and Locke (15) postulated that atrophied stomach glands, accompanying achlorhydria in pernicious anemia patients, might be failing to secrete some essential substance in digestive juice which they called "intrinsic factor." This intrinsic factor, they concluded, could be necessary for the absorption of an extrinsic factor present in food (now known to be vitamin B_{12}), and could yield the liver factor as the product of the reaction (9).

It was suggested (89) that the animal protein growth factor required by chicks and other animals and the antipernicious anemia factor are identical.

Vitamin B_{12} was isolated almost simultaneously by two independent teams in 1948 (80, 87).

Source

Vitamin B_{12} is synthesized by various microorganisms (28, 75). Ruminants require no dietary source of vitamin B_{12}, as microorganisms in the rumen produce an excess of this nutrient, provided the diet contains sufficient cobalt. Animal tissue, especially organ meats, is a good source of vitamin B_{12}.

Organisms from the proactinomycetes genus Nocardia are used for the commercial production of vitamin B_{12} (28).

Structure

Cyanocobalamin is the largest known vitamin molecule. It has a molecular weight of 1,355 and is a complex coordination compound. The bulk of the molecule consists of a porphyrin-like corrin ring system surrounding trivalent cobalt. A cyanide group is joined to the cobalt which is coordinately bonded to 5,6-dimethylbenzimidazole. Other moities contained in the molecule are ribose-3-phosphate, aminoisopropanol and propionic acid. Hodgkin (58) used x-ray crystallography to definitely establish the structure of the molecule as we know it today. The cobamide coenzyme differs structurally from cyanocobalamin in that the cyanide group of cyanocobalamin is replaced by a molecule of deoxyadenosine.

Function

The cobamide coenyzme is active in many biologically important reactions (19, 101). Among those enzyme systems in which it is known to have a function, it acts as a hydrogen transfer agent during enzymatic ribonucleotide reduction (7). It also catalyzes the transfer of hydrogen in the conversion of 1,2-propanediol to propionaldehyde. It has a role in the interconversion of succinyl coenzyme A and methylmalonyl coenzyme A, the methylation of homocysteine to form methionine and the methylation of deoxyuridylic acid to form thymidylic acid.

Bound Form

Vitamin B_{12} is usually found in a conjugated form in which it is bound to protein (36). In cow's milk, less than 10% of the total vitamin B_{12} present has been reported to be unbound, while the remainder is bound to the various proteins present in the milk (66). The binding of vitamin B_{12} by intrinsic factor has been intensely investigated (91). Among the methods which have been used to study the binding of vitamin B_{12} by proteins are microbiological growth (12, 49, 60, 61, 86), microbiological absorption (22, 23), charcoal absorption (77), dialysis (9, 81), ultrafiltration (42), paper chromatography (55), zone electrophoresis (38, 73), column chromatography (27), gel filtration (20, 63), and competitive binding of vitamin B_{12} and cobalt-57 labeled vitamin

B_{12} (83). Many substances that have no intrinsic factor activity bind significant amounts of vitamin B_{12}.

Hedbom (52, 53, 54) isolated and characterized a cobalamin-polypeptide complex from liver. The storage of vitamin B_{12} in liver has been demonstrated (85). Other organ meats have also been shown to contain a high level of the vitamin (84). The vitamin B_{12} binding capacity of human serum has been reported (59, 82). Human serum is reported to contain at least two vitamin B_{12} binders (35). Gizis et al. (34) in preliminary studies, demonstrated the binding of vitamin B_{12} by electrodialyzable peptides in cow's milk.

Intrinsic Factor

Glycoproteins which are fully active in facilitating vitamin B_{12} absorption in doses as low as 50 micrograms have been isolated from hog pylorus (26). Intrinsic factor preparations with a number average molecular weight of 50,000 have been shown to slowly produce a 100,000 molecular weight form on incubation with vitamin B_{12} at 37^{o}(56).

Milk

Gregory (40) assayed the milk from cows and several other species and found that almost all the vitamin B_{12} present was in the cobalamin form.

The milk of some species contains peptides and proteins that bind cyanocobalamin and make it unavailable for the test organism (45, 47). Milk has been shown to have a considerable vitamin B_{12} binding capacity (41, 43, 45, 46); however, the binding materials in milk have no intrinsic factor activity (96). The findings of several workers indicate that there are no significant differences in the vitamin B_{12} content of milk from cows of various breeds (17, 18, 48, 51).

Pasteurization appears to destroy about 10% or less of the vitamin B_{12} in milk depending on the method employed (68, 69). More drastic heat treatment, however, may lead to considerable or nearly complete destruction of the vitamin (29, 88). Samples of milk subjected to ultraviolet irradiation showed no significant loss of vitamin B_{12} (30).

Milk Protein Separation

The major protein in milk is the phosphoprotein casein. It can be precipitated by acidification of milk to pH 4.6 or coagulated by rennin at neutral pH (16). Casein has been resolved into alpha-, beta- and gamma-casein by fractional precipitation with acid (99). α-Casein comprises about 75% of the total casein (76).

After casein is removed from milk, α-lactalbumin and β-lactoglobulin can be isolated from the acid whey by precipitation with ammonium sulfate (3). β-Lactoglobulin is insoluble at low ionic strength and can be obtained in crystalline form when the protein

precipitate is redissolved and dialyzed against dilute acid (79). The amount of β-lactoglobulin present in milk is about 0.3 gram per 100 milliliters (8).

Acetone precipitation (10) has been reported to be an aid in the preparation of α-lactalbumin. Fox et al. (31) have outlined a procedure for the separation of β-lactoglobulin from other milk serum proteins by precipitation with trichloroacetic acid.

Whey is reported to contain a red protein (50). Brunner and Thompson (11) have reviewed the characteristics of the minor milk proteins.

β-Lactoglobulin

Genetic variants. In 1955 Aschaffenburg and Drewry (4) discovered that β-lactoglobulin was actually a mixture of two genetically linked proteins. β-Lactoglobulin A was found to have two more free carboxyl groups per 35,500 molecular weight unit than β-lactoglobulin B (94). β-Lactoglobulin A has two more aspartic acid and two more valine residues than β-lactoglobulin B, whereas β-lactoglobulin B has two more glycine and two more alanine residues than β-lactoglobulin A (65). β-Lactoglobulin C has two more residues of histidine and two fewer residues of glutamine than β-lactoglobulin B per molecule of 36,000 molecular weight (64).

Conformational changes. The β-lactoglobulins have been found to undergo conformational changes between pH 4 and 6, and pH 6.5 and 9.5 respectively (90, 93). The tetramer which forms at about pH 4.65 (70) is composed of either an aggregate of homozygous β-lactoglobulin or heterozygous species, thus accounting for the variety of different species of aggregates found. The tetramer is reported to have a molecular weight of 144,000 (93), while β-lactoglobulin in milk has a molecular weight of about 36,000. Below pH 3.7 β-lactoglobulin dissociates into two identical half units of approximately 18,000 molecular weight (102).

β-Lactogloublin A and B have been shown to be immunologically identical (24). Several crystalline forms (2) of β-lactoglobulin have been demonstrated. A partial sequence of the peptide chain of β-lactoglobulin A has been published (33). β-Lactoglobulin consists of two peptide chains, the N-terminal groups being leucine and the C-terminal groups isoleucine. β-Lactoglobulin is the only milk protein which contains cysteine. It has free sulphhydryl groups and therefore is apparently the precursor for the cooked flavor of heated milk (62, 72, 97).

Complex formation. Several reports of complex formation between β-lactoglobulin and various non-protein materials have been made. Carr (13) found that the calcium binding activity of β-lactoglobulin was high and comparable with that of serum proteins. Zittle et al. (103) have

studied the effect of heat on the binding of calcium. Zittle (104) considered that pH, through its influence on the net charge of the protein, was the most important factor governing calcium binding. Complex formation with cadmium and with para-chloromercuribenzoate or para-chloromercuribenzene-sulphonate has been investigated (39). Carr (14) found that 3 atoms of sodium were bound per molecule of β-lactoglobulin. Klotz and Urquhart (67) compared the binding of methyl orange by β-lactoglobulin and serum albumin and showed that the uptake of the dye was related to the carbonyl, hydroxyl and amino group characteristics of the proteins. Fraenkel-Conrat (32) studied the combination of β-lactoglobulin with lactose. Uptake of lactose was associated with a decrease in free amino groups.

Davis and Dubos (21) found that 3 to 4 molecules of oleic acid could be bound per molecule of protein. Hill and Briggs (57) studied the interaction with n-octylbenzene-para-sulphonate and noted that as the concentration of the detergent increased, only two or three molecules were taken up initially per molecule of protein. Later the protein molecules appeared to open up and combine with up to 22 molecules of detergent.

It is apparent that β-lactoglobulin binds a variety of substances, depending upon experimental conditions, characteristics of the test substance, etc. While previous studies have shown that vitamin B_{12} is bound by β-lactoglobulin and other proteins no definite evidence is

available on the nature of the binding sites.

Since β-lactoglobulin has a greater binding capacity for vitamin B_{12} than other milk proteins (66) it was of interest to prepare crystalline β-lactoglobulin and study factors influencing the binding of vitamin B_{12} by this and other proteins. Other studies (34) have indicated that peptides are associated with the binding of vitamin B_{12} by milk proteins. Additional studies were carried out, therefore, to extend these observations, primarily with respect to the nature and influence of the peptides involved.

EXPERIMENTAL PROCEDURE

The Microbiological Assay

The microbiological assay for vitamin B_{12} used was a modification of Gregorys (40) method described in U.S.P. (92) and A.O.A.C. (5). The assay organism used was Lactobacillus leichmannii ATCC 7830, which requires vitamin B_{12} for growth. Vitamin B_{12} content of samples was determined by measuring the amount of acid produced by the organism during a 72 hour incubation period at 37° Centigrade. A standard curve was prepared from samples of known vitamin B_{12} content which were included in each assay. The acid produced was titrated with alkali solution. The vitamin B_{12} content of samples was read from the standard curve where milliliters of alkali required for titration of the acid produced were plotted on one axis and vitamin B_{12} content was plotted on the other axis.

Cobalt-60 Labeled Vitamin B_{12} Determination

The monitoring system used for cobalt-60 labeled vitamin B_{12} determinations consisted of a Packard Model 2001 Tri-Carb Scintillation Spectrometer equipped with a Model 5052 Well-Type Scintillation

Detector.

A standard curve (Figure 1) relating corrected counts per minute to cobalt-60 labeled vitamin B_{12} content, was prepared using dilutions of a known concentration of cobalt-60 labeled vitamin B_{12}. The cobalt-60 labeled cyanocobalamin, supplied by Merck and Company, had a label claim of 1.205 micrograms of cyanocobalamin per milliliter. Microbiological assay gave a figure of 1.20 micrograms per milliliter. The experimentally determined value was used in this work.

The sample size for counting was kept constant at one milliliter. All samples were counted for 100 minutes in order to minimize counting errors. A background count was determined either immediately preceeding or following sample counting. The background counts per minute value was subtracted from the sample counts to give corrected counts per minute.

The cobalt-60 labeled vitamin B_{12} content of samples was read directly from the standard curve (Figure 1). Since a standard one milliliter sample was used throughout, the value read from the curve was the cobalt-60 labeled vitamin B_{12} content per milliliter of sample. A schematic diagram outlining the procedure is given in Figure 2.

Nitrogen Determination

Micro-Kjeldahl nitroten analyses were performed in the following manner. A 5 to 10 milligram protein sample was digested over a gas

flame for one hour in 4 milliliters of digestion mixture consisting of 5.0 grams of SeO_2 and 5.0 grams of $CuSO_4.5H_2O$ in 500 milliliters of concentrated H_2SO_4. Following the initial digestion, the mixture was cooled, one milliliter of 30% H_2O_2 was added and digestion was again continued for one hour. After the second digestion the flasks were again cooled, 10 milliliters of water were added to each, the contents of each flask were neutralized with 25 milliliters of 40% sodium hydroxide and the released ammonia was steam distilled into 15 milliliters of 4% boric acid containing 3 drops of indicator (400 milligrams bromocresol green and 40 milligrams methyl red dissolved in 100 milliliters of 95% ethanol). Distillation was continued until the receiving flask contained a total volume of 50 milliliters. The ammonia was titrated with 0.02 N HCl utilizing a 10 milliliter microburet with 0.02 milliliter graduations. The hydrochloric acid used was standardized against trishydroxymethyl aminomethane (Sigma 121) as the primary standard by titrating weighed samples of Sigma 121 with the acid and then calculating its normality. An internal standard was used so that any losses occurring could be compensated for.

Amino Acid Determinations

Amino acid analyses of the protein fractions were performed on 20 and 70 hour acid hydrolysates (78). The analyzer was a Beckman Model 120C which utilizes high speed spherical resins. Approximately

4 milligrams of dry protein were weighed and transferred into 10 milliliter ampules. Six milliliters of 6 N HCl were added to the ampule. The contents of the ampule were frozen in a dry ice-ethanol bath, evacuated, allowed to melt slowly to remove gases, refrozen and sealed with a propane torch. The sealed ampules were placed in an oil bath which was placed inside an 110° C oven. The samples were hydrolyzed for either 20 or 70 hours.

After hydrolysis for the specified time intervals, the samples were individually evaporated to dryness in a small flask which was fitted onto a rotary evaporator and partially submerged in a 55° C. hot water bath. Each residue was redissolved in a few milliliters of deionized water and redried. This procedure was repeated until no HCl odor was discernable. Five milliliters of citrate-HCl buffer (pH 2.2) were added to the dried hydrolysate. Aliquots of 0.2 milliliter were removed from each sample for amino acid analysis.

The area under the curve, on the recorder chart paper, corresponding to each respective amino acid was determined by multiplying the calculated net height by the width (number of dots in the peak above the half-height). The area under the curve corresponding to each amino acid was divided by the experimentally determined standard for each respective amino acid in order to obtain the number of micromoles of each amino acid in the aliquot assayed. Tabulated data are expressed either as grams of amino acid per 100 grams of protein or as moles of

amino acid per 100 moles of amino acid.

Colorimetric Protein Estimation

Due to the limited quantities of certain fractions available a protein estimation method was needed which would be rapid and also sensitive to very low protein concentrations. A modification of the Folin-Lowry (74) Method was used.

The reagents required for this method are: Reagent A - 2% Na_2CO_3 in 0.1 N NaOH, Reagent B - 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate, Reagent C - a mixture of 50 milliliters of Reagent A and one milliliter of Reagent B and Reagent D - Phenol Reagent 2N Solution (Folin-Ciocalteau), diluted to 1N. Reagent D is commercially available from Fisher Scientific Company.

One milliliter of sample containing about 100 micrograms to 250 micrograms of protein was measured into a 16 by 145 millimeter test tube. Five milliliters of Reagent C were added, the tube was shaken to mix reagent and sample and allowed to stand for 10 minutes. Then 0.5 milliliter of Reagent D was added, the tube contents were mixed well by shaking and allowed to stand for 30 minutes. A blank was always included which contained the same reagents but one milliliter of water was used in place of the protein solution. The samples were placed in matched cuvettes and percent transmittance was read in the

Cenco-Sheard-Sanford Photelometer. A Corning filter with a transmission range of 600 microns or greater wavelength was used. The instrument was adjusted to read 100% transmittance with the blank. Sample values obtained were interpreted by reference to a standard curve (Figure 3) which had been prepared by running samples containing known amounts of β-lactoglobulin ranging from 54 to 400 micrograms per milliliter. A graphical plot was made of percent transmission versus protein concentration. Protein content of samples could be read directly from the standard curve.

Polyacrylamide Gel Electrophoresis

Electrophoresis in polyacrylamide gels was used to determine the purity of β-lactoglobulin during and after preparation. This technique was used for other proteins in this study. Different buffers including borate and veronal and concentrations of polyacrylamide varying from 5 to 8% in the gel were tried and yielded varying degrees of success. Gels consisting of 8% polyacrylamide in pH 8.7 borate buffer were most frequently used. A stock buffer solution was prepared by dissolving 190 grams of sodium hydroxide and 880 grams of boric acid in water sufficient to yield a final volume of 19 liters. The final buffer consisted of one part of stock buffer solution plus two and one half parts of water.

The gel solution was prepared by dissolving 80 grams of

Cyanogum-41 in sufficient buffer to yield one liter of solution. The gels were prepared by adding 0.4 milliliter of N,N,N^1,N^1-tetramethylenediamine (T.M.E.D.) to 250 milliliters of Cyanogum-41 solution and agitating until well mixed. Next, 0.4 gram of ammonium persulfate was added and stirring was continued until it was dissolved. The solution was poured immediately into a previously prepared gel bed, the sample slot former was introduced and the gel bed was placed under an atmosphere of nitrogen until the solution polymerized to a firm gel. The slot former was removed and the protein sample solution (0.2 milliliter of 2% protein solution) was introduced into the slots with a small bore glass capillary. The buffer tanks were filled with borate buffer to give a continuous buffer system, platinum electrodes were placed into the buffer tanks and 100 milliamperes (approximately 16 milliamperes per centimeter^2, cross section) of current were applied. After a 12 to 16 hour run the gel was removed from the bed and stained for five minutes in a solution consisting of 5 grams of the anionic dye, Amido Black 10B, dissolved in 250 milliliters of methanol plus 250 milliliters of water and 50 milliliters of acetic acid. The gel was removed from the staining solution, rinsed very briefly and the excess dye, not bound by protein was electrophoretically extracted in 7% acetic acid with a current of approximately 4 amperes. Protein bands in the destained gels appeared as stained zones and those considered of value were photographically recorded.

Preparation of β-Lactoglobulin

The method of preparation of β-lactoglobulin was essentially that of Aschaffenburg and Drewry(3), with minor modifications (Figure 4).

Fresh whole milk was collected at the Michigan State University Jersey Milking Barn. The milk was a mixed sample from several cows. The milk, without being allowed to cool following collection, was heated to 40° C. Twenty grams of anhydrous sodium sulfate were added, per hundred milliliters of milk, while stirring. Stirring was continued until all the sodium sulfate was dissolved.

After the temperature had dropped to 25° C., the batch was filtered through fluted Whatman #4 filter papers. The filtrate containing the "lactalbumins" (α-lactalbumin, β-lactoglobulin and serum albumin) was saved for further fractionation. The precipitate containing the globulins, the proteose peptone components and the casein and fat was discarded.

The volume of the filtrate was measured. One milliliter of concentrated hydrochloric acid was added per hundred milliliters of filtrate. This imparted a pH of approximately 2 and precipitated all the proteins present except β-lactoglobulin. The resulting precipitate was removed by centrifugation for 30 minutes at 8,000 rpm in the Sorvall Model RC 2-B. The supernatant was decanted from the centrifuge bottles and filtered through Whatman #4 filter paper to assure removal of the precipitated proteins.

The volume of the resultant filtrate was measured. Concentrated ammonia solution (NH_4OH) was added to bring the pH to 6. Approximately 0.6 milliliter per 100 milliliter of filtrate was required. Twenty grams of ammonium sulfate were added, per hundred milliliters of filtrate, to precipitate the β-lactoglobulin. The preparation was stirred until all the added ammonium sulfate was dissolved. The solution was permitted to stand undisturbed for one hour to allow the protein to flocculate, followed by filtration through a thin pad of Hyflo Super-Cel deposited on Whatman #1 filter paper. The filtrate was discarded.

The filter cake was triturated with water to a paste, placed in 3/4 inch diameter Visking cellulose dialysis tubing which had been boiled in the presence of ethylenediaminetetraacetic acid (E.D.T.A.) and dialyzed overnight. The contents of the dialysis tubing were filtered with suction through Whatman #1 filter paper. The Hyflo Super-Cel remaining on the filter paper was discarded.

The filtrate was adjusted to pH 5.8 with hydrochloric acid, a few drops of toluene were added and the material was dialyzed again for 48 hours against several changes of deionized water. The pH was reduced to 5.2 with dilute hydrochloric acid, which produced some cloudiness, and dialysis was continued.

A few hours after the pH was reduced to 5.2, a viscous oily layer appeared at the bottom of the dialysis sac. After one week no crystals were present. At this point two batches, each from twenty liters of

milk, were combined. The material was transferred to a tightly stoppered Erlenmeyer flask and stored in the refrigerator. After about five weeks all the protein had crystallized in the form of flat plates.

Recrystallization and Purification

Upon examination by polyacrylamide gel electrophoresis it was observed that the crystalline β-lactoglobulin was not homogeneous. In addition to β-lactoglobulin, it contained a slow moving component smearing forward from the sample slot (Figure 5). The supernatant yielded only one band (β-lactoglobulin).

The bulk of the crystalline material was dissolved in pH 9 borate buffer and then dialyzed to remove the buffer salts. Crystallization occurred in the dialysis sac. Again, the crystalline material contained the contaminating protein believed to be euglobulin, while the β-lactoglobulin in the supernatant was homogeneous.

The crystalline protein was redissolved in pH 9 borate buffer, placed in dialysis tubing and observed closely. When crystallization had begun, but before the bulk of the protein had crystallized, the material was removed from the dialysis sac and filtered. The filtrate was placed in a fresh dialysis sac and dialysis was continued; whereupon, further crystallization occurred. Three fractions were examined; consisting of, the first crystals filtered off, the final crystals and the final supernatant. The first crystals contained the contaminating

protein, while the protein in the final crystals and supernatant was homogeneous on polyacrylamide gel electrophoresis. The final ten grams of crystals of β-lactoglobulin were used for these studies. This represents a yield of only about 10% of the theoretical value.

Preparation of α-Lactalbumin

α-Lactalbumin was prepared by the method of Aschaffenburg and Drewry (3), Figure 6. Whole milk from the Michigan State University Holstein Herd was warmed to 40° C. Anhydrous sodium sulfate (20 grams per hundred milliliters) was added and dissolved by stirring. After the temperature had fallen below 25° C., the mixture was filtered through Whatman #4 fluted papers.

The precipitate containing the globulins, proteose-peptone components, casein and fat was discarded. The volume of the filtrate was measured and concentrated HCl (one milliliter per hundred milliliters of filtrate) was added with vigorous stirring. This lowered the pH to approximately 2, precipitating all the protein constituents except β-lactoglobulin. The precipitate was recovered by centrifugation in the Sorvall Model RC 2-B for 30 minutes at moderate speed (8,000 rpm). The precipitate was removed from the centrifuge bottles, combined and dissolved in water equal to one tenth the volume of the original filtrate with the addition of dilute ammonia solution. The pH was lowered to 3.5 with dilute HCl in order that any residual β-lactoglobulin

would be left in solution and not contaminate the α-lactalbumin which was precipitated. After the precipitated α-lactalbumin was recovered by centrifugation it was dissolved in one fourth the previous volume of dilute ammonia solution. Dilute HCl was again added until pH 4 was reached. The mixture was stored in the refrigerator overnight where the α-lactalbumin slowly precipitated. The mixture was brought to room temperature after which the α-lactalbumin precipitate was recovered by centrifugation. The α-lactalbumin was dried and stored for subsequent use.

Method of Electrodialysis

The equipment required for electrodialysis consisted of an ice bath to maintain a low temperature during operation, a power source reading voltage and amperage and the electrodialysis cell. The cell (Figure 11) was constructed of acrylic plastic. It consisted of an outer chamber for holding the deionized water, an inner chamber for supporting the dialysis sac containing the material to be electrodialyzed and the platinum electrodes. The outer chamber was constructed by cementing a 9 centimeter square base onto a 17 centimeter long, 4 centimeter diameter tube. The inside chamber was made of a 2.2 centimeter diameter plastic cylinder 18 centimeters long. A 3.7 centimeter diameter plastic disc was cemented to the bottom end to assure that the inside chamber would be properly centered in the outer chamber.

Slits 1.2 centimeters wide by 12 centimeters long were cut on opposite sides of the inner chamber to allow exposure of the dialysis sac and contents in line with the electrodes. A top or collar was constructed in such a way that it would hold both the inner chamber and the electrodes in place and help to cover the vessel.

The Visking cellulose dialysis tubing was always prepared prior to use by boiling in the presence of E.D.T.A. for a few minutes. Before electrodialysis, protein samples were dissolved in water or buffer solution, placed in prepared dialysis tubing (2.54 centimeters diameter, measured flat) and dialyzed for 24 hours against 150 volumes of deionized water which was changed 3 or 4 times during the 24 hours. Slow agitation with a magnetic stirrer was used to facilitate the dialysis.

Next, the dialysis sac with its contents was placed inside the inner chamber of the electrodialysis cell which was then lowered into the outer chamber. The outer chamber was filled with deionized water, the top was set in place, the electrodes were positioned opposite the slits in the inner chamber, the entire assembly was placed in the ice bath, the leads from the power source were attached to the electrodes and 200 volts were applied. Initially, there was no noticable amperage reading as there were not sufficient ions in the deionized water to carry the current; however, as material was pulled from the contents of the dialysis sac, the amperage reading increased. The deionized

water in the chamber was periodically replaced with fresh water until finally after 3 or 4 changes in as many hours, no detectable amperage reading occurred. All of the fluid from the chamber was saved, shell frozen and lyophilized. A very small amount of material comprising only about 0.2% of the original protein was obtained from the dialysate.

Vitamin B_{12} Binding Capacity

A standardized procedure was adopted for the determination of vitamin B_{12} binding capacity so that comparisons between different experiments would be as meaningful as possible. The protein or peptide, whose vitamin B_{12} binding capacity was to be determined, was dissolved in pH 9 borate buffer. An estimated excess of cobalt-60 labeled vitamin B_{12} was added. The mixture was allowed to stand at room temperature for one hour. The excess unbound cobalt-60 labeled vitamin B_{12} was then separated from the protein or peptide with its bound vitamin B_{12} by use of a Bio-Gel P-2 polyacrylamide bead desalting column. The protein or peptide with its vitamin B_{12} was excluded from the beads because of its large molecular size while the excess free vitamin B_{12} permeated the gel beads. This resulted in the early elution of the protein or peptide followed by the later elution of the free vitamin (Figures 7-10). An Isco Model UA Recording Ultraviolet Analyzer with an operating wavelength of 254 millimicrons was used to monitor the column effluent. The cobalt-60 labeled

vitamin B_{12} and the protein or peptide both absorbed at 254 millimicrons giving a peak on the recorder chart paper corresponding to their respective locations in the column effluent. The effluent fraction containing the protein or peptide was collected. A one milliliter sample was used for the cobalt-60 labeled vitamin B_{12} assay by determining the counts per minute in the Tri-Carb Scintillation Spectrometer and relating counts per minute to vitamin B_{12} content by reference to the previously prepared standard curve. A sample was also used for protein estimation by the Folin-Lowry method previously outlined so that binding capacity could be expressed as vitamin B_{12} bound per unit (milligram) of protein. In the case of the peptides, the amount available was so small that it was necessary to lyophilize the entire peptide effluent and use all the material available for protein estimation. Even then the amount of material available was sometimes so small as to limit the accuracy of the estimation.

Recombination Experiments

The determination of the vitamin B_{12} binding capacity of electrodialyzed β-lactoglobulin recombined with the peptides removed by electrodialysis was conducted in the following manner. An estimated excess of peptides, over the amount removed by electrodialysis, was added to a 5 to 10 milligram sample of electrodialyzed β-lactoglobulin in one milliliter of borate buffer (pH 9). After equilibrating for one hour

at room temperature, the mixture was passed through a P-6 polyacrylamide bead column. Unbound peptide and the protein with bound peptide were separated. The protein effluent was collected and lyophilized. Vitamin B_{12} binding capacity was determined in the manner previously described.

RESULTS AND DISCUSSION

The Microbiological Assay

The microbiological assay for vitamin B_{12} was used only two times in the course of this work. It was employed to establish the amount of unlabeled, naturally occurring vitamin B_{12} present in the crystalline β-lactoglobulin used throughout these studies. This amount was determined to be 133 micromicrograms of vitamin B_{12} per milligram of β-lactoglobulin.

The microbiological assay was used again to establish the vitamin B_{12} potency of the cobalt-60 labeled cyanocobalamin used in the binding studies. This assay gave a value of 1.20 micrograms per milliliter which compared favorably with the label claim of 1.205 micrograms per milliliter.

Nitrogen Content of β-Lactoglobulin

Duplicate nitrogen analyses of crystalline β-lactoglobulin by the micro-Kjeldahl technique gave an average value of 15.75%. The reported value is 15.6% (71). The experimentally determined value was corrected for any procedural losses encountered, by simultaneously

analyzing for percent recovery of a purified amino acid. The percentages of nitrogen found by assay (14.6% and 14.7%) were divided by the percent recovery (92.95%) in order to arrive at the values 15.7% and 15.8% respectively, which were averaged to give the value 15.75% reported above.

Amino Acid Analyses

Amino acid analyses were run on hydrolysates of β-lactoglobulin and electrodialyzed β-lactoglobulin. These data are presented in Table 1 where the values are expressed in grams of amino acid per 100 grams of protein. Also presented in the same table are amino acid analyses for the β-lactoglobulin phenotypes A, B and AB (37).

As expected there are no striking differences in the amino acid content of β-lactoglobulin and electrodialyzed β-lactoglobulin. The amount of peptide removed during electrodialysis accounted for only about 0.1 to 0.2% of the original β-lactoglobulin, as estimated by the Folin-Lowry colorimetric protein procedure previously outlined. Because the amount of the protein removed, as peptide, by electrodialysis is small, the amino acid composition of the protein remaining would not be markedly changed from the original value.

It may be reasoned that no great changes in the amino acid composition of the β-lactoglobulin remaining in the sac after electrodialysis would be expected due to removal of only a fraction of the

total protein. At the same time there could quite well be some remarkable differences in amino acid composition of the small amount of peptide material removed by electrodialysis from the β-lactoglobulin as compared to the original β-lactoglobulin. In Table 2 are presented, in moles per 100 moles, the amino acid distribution for β-lactoglobulin before and after electrodialysis and for the peptide material removed from β-lactoglobulin by electrodialysis. Here, we do notice some striking differences. For example, the peptide contained no cystine or cysteine and only an immeasurable, slight trace of tyrosine and methionine. It was also much lower in leucine and lysine content. Phenylalanine and isoleucine were also slightly lower in the peptide than in β-lactoglobulin, while proline, valine, threonine, aspartic acid and arginine were present in essentially equal amounts in peptide and protein. On the other hand, the peptide contained a slightly greater amount of alanine, considerably more glutamic acid, about twice as much serine and almost four times as much glycine as is present in β-lactoglobulin.

These differences in amino acid composition between β-lactoglobulin and the peptide material released on electrodialysis are certainly great enough to suggest a specific cleavage rather than a random cleavage of β-lactoglobulin.

From the amino acid analysis of the peptides from β-lactoglobulin a minimum molecular weight was calculated, based on the most

limiting amino acid, histidine. A value of 6,200 was obtained. This is considerably higher than the estimated molecular weight of about 4,000, based on the elution characteristics of the peptides from gel filtration columns in comparison to compounds of known molecular weight. On attempts to resolve the peptides on high voltage paper electrophoresis (6) two very faint bands were observed, indicating a two component mixture of peptides. The presence of two peptides rather than one could account for the discrepancy between the values obtained for the calculated minimum molecular weight based on amino acid composition and the estimated molecular weight based on elution characteristics from polyacrylamide bead columns.

Vitamin B_{12} Binding Characteristics of β-Lactoglobulin

β-Lactoglobulin was chosen for the major part of this work because of its relatively high vitamin B_{12} binding capacity (25, 66). The presence of vitamin B_{12} binding peptides in cow's milk has been reported (34). The removal of a peptide material by electrodialysis has also been briefly investigated (25).

Table 3 summarizes the vitamin B_{12} binding capacity data for β-lactoglobulin, electrodialyzed β-lactoglobulin, for the peptide removed from β-lactoglobulin by electrodialysis and for the recombined β-lactoglobulin and peptide.

The average vitamin B_{12} binding capacity for β-lactoglobulin was found to be 259 micromicrograms per milligram of protein. This value does not include the unlabeled vitamin B_{12} which was present in the β-lactoglobulin. If this value (259 micromicrograms) and the value found by microbiological assay (133 micromicrograms) are added, a value of 392 micromicrograms per milligram is obtained, which is much lower than the value of approximately 1,500 micromicrograms per milligram reported in earlier work (25) using a less purified β-lactoglobulin.

The values given for all the vitamin B_{12} binding studies include only the added cobalt-60 labeled vitamin B_{12}. After electrodialysis the binding capacity of the material remaining inside the sac was decreased to an average value of 133 micromicrograms per milligram which is equal to the original vitamin B_{12} content of the β-lactoglobulin found by microbiological assay. It would seem logical that the material removed from the sac by electrodialysis would have a higher vitamin B_{12} binding capacity than β-lactoglobulin itself. This logic would follow, if the total vitamin B_{12} binding capacity in the original β-lactoglobulin is to be retained by the two subsequent fractions which are the electrodialyzed material remaining inside the dialysis sac plus the peptide or low molecular weight material removed by electrodialysis. The average vitamin B_{12} binding capacity for the peptide was found to be 5,410 micromicrograms per milligram and this value for the vitamin B_{12} binding capacity of the peptide was comparatively high as was

expected. We may next logically ask whether the combined binding capacities of peptide plus electrodialyzed β-lactoglobulin are equivalent to the binding capacity of the original β-lactoglobulin. Since the peptide constitutes only about 0.2% of the original β-lactoglobulin, while the electrodialyzed material remaining inside the sac constitutes the other 99.8% we can multiply each components percentage contribution to the total by its respective vitamin B_{12} binding capacity and should arrive at the same figure found for the original β-lactoglobulin. It is seen that 0.998 x 133 plus 0.002 x 5,410 equals 144 while the value for the binding capacity of β-lactoglobulin was 259. It may be concluded, therefore, that some of the total binding capacity was lost during electrodialysis. It was visibly apparent that the protein agglomerated and precipitated inside the dialysis sac during electrodialysis. It is quite conceivable that agglomeration was brought about by intermolecular bonding which, in effect, "tied up" some of the vitamin B_{12} binding sites, thus lessening the number of such sites available in the electrodialyzed material to bind vitamin B_{12}. This could effectively decrease the combined vitamin B_{12} binding capacity of the two final components.

Upon electrodialysis peptides which had a high vitamin B_{12} binding capacity were obtained. The protein remaining inside the dialysis sac after electrodialysis had a considerably lowered vitamin B_{12} binding capacity. It was of interest therefore, to determine whether or not

electrodialyzed β-lactoglobulin and peptide removed by electrodialysis could be recombined, and in so doing, to determine if the vitamin B_{12} binding capacity could be restored to a value somewhat near the binding capacity of untreated-β-lactoglobulin. Because of the extremely small amount of peptide available and due to the unavoidable losses encountered in transferring such a small sample, an estimated two fold excess of peptide, over the amount originally removed by electrodialysis, was used in the recombination experiments. The recombined β-lactoglobulin and peptide has an average vitamin B_{12} binding capacity of 244 micromicrograms per milligram (Table 3) which compares quite closely with the value of 259 micromicrograms per milligram for untreated β-lactoglobulin. Both values are considerably greater than the 133 micromicrograms per milligram value obtained for the electrodialyzed protein.

Time of Electrodialysis; Effect on Binding Capacity

It was of importance to determine the optimum length of time of electrodialysis because, at best, the amount of peptide which could be obtained by electrodialysis of β-lactoglobulin was quite small. Initially, at the beginning of electrodialysis, at a constant voltage, the amperage reading on the meter of the power source was zero. However, after 15 to 30 minutes there was a deflection of the needle.

Completeness of electrodialysis could be effectively reached, based on removal of all the peptide possible, simply by repeatedly removing the fluid in the outer electrodialysis cell and replacing it with fresh deionized water until no current flow was detectable. The fluid fractions removed from the outer cell were combined and lyophilized in order to obtain the peptide. If the current flow which developed after electrodialysis was in progress was due, as believed, to the peptide material being pulled from the dialysis sac by the charge, the presence or absence of peptide could be detected, relative to the flow or lack of flow of current. From the elution chromatograms (Figure 8) of β-lactoglobulin after 1, 2, 4 and 8 hours of electrodialysis it can be seen that no detectable amount of peptide is present after 8 hours. These samples were taken from inside the dialysis sac. The peptide evidences its presence by the hump on the descending side of the protein peak. This same result was duplicated with a second set of samples.

Table 4 presents the vitamin B_{12} binding capacity data for β-lactoglobulin after 0, 1, 2, 4 and 8 hours. There is a very large decrease in binding capacity after the first hour. On continued electrodialysis there appeared to be a steady decrease in binding capacity, but the amount of peptide obtained by electrodialysis beyond 4 hours was insufficient to warrant further treatment.

Comparison of Proteins: B_{12} Binding Capacity

Proteose peptone. The peptides obtained by electrodialysis of β-lactoglobulin have an estimated molecular weight of 4,000. This estimation was arrived at by virtue of the elution characteristics on Bio-Gel P-2 and P-6 columns in comparison to the elution characteristics of proteins and peptides of known molecular weight. Bio-Gel P-2 has a range of separation of about 200 to 2,500 while P-6 has a range of about 1,000 to 4,000. The peptide was not separated from β-lactoglobulin which was eluted immediately following the void volume on P-2; however, the peptide did trail the larger protein, as was evidenced by the hump on the descending side of the protein peak on the recorder chart. On Bio-Gel P-6 the peptide was separated from the protein. This leads to the conclusion that the molecular weight is somewhere between 2,500 and 4,000, as this is the prescribed range of the column. When the elution characteristics of these peptides were compared, using the same column, to those of insulin, oxytocin and oxidized glutathione whose molecular weights are known, it appeared that the molecular weight of the peptides is near 4,000.

It is recognized that the peptide or part of it could be proteose peptone which was bound by the protein. A physical mixture of the separate proteose peptone components was obtained and the vitamin B_{12} binding capacity of this mixture was determined in the usual manner.

In Table 5, the data show that the proteose peptone mixture had an average vitamin B_{12} binding capacity of 330 micromicrograms per milligram, which is in the same range found for β-lactoglobulin, and nowhere near the values found for the peptide.

Intrinsic factor. It was of interest to determine the vitamin B_{12} binding capacity of intrinsic factor in the system used for the milk proteins. Samples of two different intrinsic factor preparations were obtained from Lederle Laboratories Division of American Cyanamid Company through the courtesy of Dr. Leon Ellenbogen. The vitamin B_{12} binding capacity of these materials was determined in the manner previously described. One of the intrinsic factor samples gave an average vitamin B_{12} binding capacity of 2,900 micromicrograms per milligram while the more highly purified sample had a binding capacity of 3,840 micromicrograms per milligram of protein.

Peptides from Various Proteins; Comparison of B_{12} Binding Capacity:

α-Lactalbumin. It was important to determine vitamin B_{12} binding with at least one other major milk protein besides β-lactoglobulin and see if it also would yield a peptide on electrodialysis. α-Lactalbumin was prepared as previously described. It was electrodialyzed by the same procedure used for β-lactoglobulin. The material obtained exhibited two peaks on its elution chromatogram (Figure 10). From the

elution volume of these two peptide components it appears that one is larger and one is smaller in molecular size than the peptides from β-lactoglobulin. The peptides obtained by electrodialyzing α-lactalbumin have an average vitamin B_{12} binding capacity of 3,595 micromicrograms (Table 6) per milligram which is in the same general range but considerably less than the average value of 5,410 micromicrograms per milligram found for the peptide from β-lactoglobulin.

Intrinsic factor. Because of its essential nature in the absorption of vitamin B_{12} by man it was worthwhile to determine whether or not intrinsic factor itself would release a vitamin B_{12} binding peptide on electrodialysis. The less purified sample of intrinsic factor preparation was electrodialyzed in the same manner used for β-lactoglobulin and α-lactalbumin. Material was obtained by electrodialysis which had an average vitamin B_{12} binding capacity of 11,665 micromicrograms per milligram (Table 6). The peptide material obtained by electrodialysis of intrinsic factor has a vitamin B_{12} binding capacity approximately twice that of the peptides obtained from β-lactoglobulin. From the elution characteristics of the peptide components it appears that one is smaller and one is about the same molecular size as the material obtained from β-lactoglobulin.

We cannot definitely conclude from presently available data whether the peptides we obtained by electrodialysis were a part of the

protein molecule or if they were simply bound to it. The amount of peptide present was so small that its presence, or absence, could easily go unnoticed in amino acid analyses or molecular weight determinations

The fact that peptides with high vitamin B_{12} binding capacity were obtained from intrinsic factor as well as from the two milk proteins, β-lactoglobulin and α-lactalbumin, makes speculation much more interesting. First, we may ask why these peptides are found on the milk proteins. It is possible that they enable these proteins to carry bound vitamin B_{12}. Likewise, the peptides removed from intrinsic factor by electrodialysis may have the sole function of binding vitamin B_{12} to aid in its absorption through the intestine. We may quite naturally wonder if the peptides removed from the milk proteins by electrodialysis are identical to, similar to or quite different from those removed from intrinsic factor by the same treatment. There was not sufficient material available so that adequate observations could be made to answer this question. The peptides from the different proteins have some distinct similarities and may indeed be identical in functional properties if not in amino acid structure. Since peptide was obtained from each protein by the same treatment and because the material removed from each protein bound large amounts of vitamin B_{12} under conditions as nearly similar as possible, we can at least say that the peptides have behavior in common.

The cyanocobalamin-peptide complex which Hedbom (52) isolated from bovine liver is reported to have a molecular weight just over 7,000. The peptide has 15 micrograms of cyanocobalamin per milligram bound to it, as isolated. This peptide has a higher molecular weight than those isolated from the milk proteins appear to have, and it obviously has a much higher vitamin B_{12} binding capacity.

It seems quite probable that some of the peptides could be identical. Let us consider a situation in which the milk proteins serve as a vitamin B_{12} source. Upon ingestion of the milk proteins with their peptide bound vitamin B_{12}, the vitamin B_{12} and peptide could quite conceivably be cleaved intact by acid hydrolysis or enzymatic action. Then the peptide-vitamin B_{12} complex could be bound to intrinsic factor because of a greater affinity. Similar peptides, free of vitamin B_{12}, could also be picked up and bound by intrinsic factor, thus accounting for its high vitamin B_{12} binding capacity.

Let us again refer to the vitamin B_{12} binding capacity of the peptides we have been studying. The peptides from α-lactalbumin have a lower binding capacity for vitamin B_{12} than those from β-lactoglobulin just as α-lactalbumin itself has a lower vitamin B_{12} binding capacity than β-lactoglobulin (66). We can see from the elution chromatograms that the peptides removed from α-lactalbumin by electrodialysis are not as homogeneous as those from β-lactoglobulin. The higher ratio of vitamin B_{12} binding peptide from β-lactoglobulin could suggest the possible role

of β-lactoglobulin as the major carrier for vitamin B_{12} in cow's milk. While not the sole carrier, β-lactoglobulin does contain and is capable of binding more vitamin B_{12} than the other proteins in cow's milk which have been investigated (25, 66). The peptides from intrinsic factor, we again note, have a much higher vitamin B_{12} binding capacity than those from the milk proteins. This is what we should expect when we consider the role of intrinsic factor. Perhaps intrinsic factor may accept vitamin B_{12} already bound to peptide leaving that complex intact, or possibly it receives vitamin B_{12} from a peptide or protein complex but transfers it to its own peptide or a combination of these two could be occurring simultaneously.

While this research problem has not answered all the questions it raised, it has provided some clues on the binding of vitamin B_{12} by proteins. It suggests a role for the milk protein β-lactoglobulin. It points out the probable role of peptides as a binding link between proteins and other materials. It suggests the possibility of altering the characteristics of proteins by adding peptides which would bind to both the protein and other materials one wished to bind by the protein.

Vitamin B_{12} is generally found to be bound to protein. This can aid in absorption of the vitamin. It can protect the vitamin from destruction by light, heat and chemical treatment. The fact that vitamin B_{12} is

usually found bound to protein, in conjunction with our finding of a peptide or peptides that appear to bind mutually both vitamin B_{12} and proteins helps to explain how this complex is so effectively brought about.

SUMMARY AND CONCLUSIONS

1. Crystalline β-lactoglobulin, on electrodialysis, releases peptides, and in so doing undergoes an alteration in its ability to bind vitamin B_{12}.

2. As peptides are removed, by electrodialysis, from β-lactoglobulin there is a successive decrease in the ability of the remaining protein to bind vitamin B_{12}.

3. The peptides removed by electrodialysis from β-lactoglobulin can recombine with the electrodialyzed protein. The recombined protein and peptide product has a vitamin B_{12} binding capacity almost equal to that of the untreated protein.

4. Amino acid analyses of the protein fractions suggest that specificity is involved in the removal of peptide by electrodialysis. If random cleavage were involved one would expect to find about the same amino acid distribution in peptide and protein. There were wide variations in the amino acid distribution. Some amino acids found in the protein were not found in the peptides. High voltage paper electrophoresis showed only two peptide spots. This also indicates that a random cleavage was not involved.

5. Proteose peptone, whose molecular size is similar to that of the peptides removed from β-lactoglobulin by electrodialysis, has been shown to have a vitamin B_{12} binding capacity in the range of the binding capacity of β-lactoglobulin and appreciably less than the values found for the peptides derived from β-lactoglobulin and α-lactalbumin. This leads to the conclusion that the peptides are not simply proteose peptone components which were bound to the β-lactoglobulin molecule.

6. α-Lactalbumin and intrinsic factor preparations have similarly been shown to release peptides upon electrodialysis. These peptides, just as the peptides from β-lactoglobulin, have a much higher vitamin B_{12} binding capacity than the respective protein from which each was obtained.

TABLE 1

Amino acid composition of β-lactoglobulins

Amino acid residue	β-Lactoglobulin[1]	Electrodialyzed β-Lactoglobulin[1]	A[2]	B	AB
	grams/100 grams of protein				
LYS	11.1 ± 0.3	11.6 ± 0.6	12.0	11.7	11.5
HIS	1.8 ± 0.1	1.7 ± 0.1	1.6	1.6	1.5
ARG	2.6 ± 0.4	2.9 ± 0.4	2.8	2.7	2.8
ASP	10.2 ± 0.3	10.9 ± 0.7	11.4	10.6	11.2
THR	4.2 ± 0.3	4.2 ± 0.4	4.9	4.8	4.8
SER	2.7 ± 0.5	2.7 ± 0.5	3.3	3.2	3.4
GLU	18.4 ± 0.2	17.9 ± 2.2	19.1	18.7	19.1
PRO	4.8 ± 0.2	4.5 ± 0.4	5.3	5.0	5.0
GLY	1.2 ± 0.1	1.3 ± 0.1	1.2	1.5	1.4
ALA	5.8 ± 0.1	5.8 ± 0.4	6.7	7.0	6.9
1/2 CYS	2.7 ± 0.1	2.8 ± 0.5	2.1	2.0	2.0
VAL	4.9 ± 0.1	3.9 ± 0.2	6.0	5.4	5.8
METH	2.5 ± 0.3	1.9 ± 0.5	3.2	3.1	3.2
I-LEU	6.0 ± 0.3	6.1 ± 0.3	6.1	6.1	6.2
LEU	14.0 ± 0.1	14.2 ± 0.6	15.1	14.9	15.1
TYR	3.7 ± 0.1	3.9 ± 0.4	3.8	3.8	3.9
PHEN	3.6 ± 0.1	3.8 ± 0.6	3.5	3.5	3.5

[1]The variance from the mean of duplicate 20 and 70 hour hydrolysates is given.

[2]The designations (A, B and AB) represent the corresponding phenotypes of β-lactoglobulin as reported by Gordon, et al. (37).

TABLE 2

Amino acid composition of β-lactoglobulin before and after electrodialysis and of the peptide removed[1]

Amino acid residue	β-Lactoglobulin	Electrodialyzed β-Lactoglobulin	Peptide from β-Lactoglobulin
	moles/100 moles		
LYS	9.6 ± 0.1	10.4 ± 0.2	6.8 ± 2.2
HIS	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
ARG	1.8 ± 0.2	2.1 ± 0.1	2.0 ± 0.1
ASP	10.0 ± 0.1	11.1 ± 0.1	10.4 ± 0.5
THR	5.0 ± 0.1	5.1 ± 0.1	5.5 ± 0.1
SER	4.2 ± 0.1	4.1 ± 0.1	8.5 ± 0.3
GLU	16.0 ± 0.2	14.9 ± 1.3	18.1 ± 2.4
PRO	5.4 ± 0.1	5.1 ± 0.3	5.5 ± 0.1
GLY	2.4 ± 0.1	2.5 ± 0.1	9.4 ± 0.0
ALA	9.1 ± 0.2	9.0 ± 0.4	10.0 ± 1.3
1/2 CYS	3.0 ± 0.1	3.3 ± 0.3	0.0 ± ---
VAL	5.5 ± 0.1	4.5 ± 0.2	5.3 ± 0.0
METH	2.0 ± 0.1	1.3 ± 0.1	Trace
I-LEU	5.8 ± 0.1	6.0 ± 0.2	5.0 ± 0.2
LEU	13.9 ± 0.2	14.3 ± 0.4	10.0 ± 0.2
TYR	2.6 ± 0.1	2.6 ± 0.1	Trace
PHEN	2.8 ± 0.1	2.8 ± 0.1	2.2 ± 0.1

[1]The variance from the mean of duplicate 20 hour hydrolysates is given.

TABLE 3

A comparison of the vitamin B_{12} binding capacity of β-lactoglobulin before and after electrodialysis, of the peptide removed by electrodialysis and of electrodialyzed β-lactoglobulin with the removed peptide added back

Material	Expt. 1	Expt. 2	Average
	micromicrograms/milligram		
β-Lactoglobulin	268	250	259
Electrodialyzed β-Lactoglobulin	153	113	133
Electrodialyzed β-Lactoglobulin plus peptide	267	222	244
Peptide	4,220	6,600	5,410

TABLE 4

Vitamin B_{12} binding capacity of β-lactoglobulin after various periods of electrodialysis

Time (hours)	TRIAL 1	TRIAL 2	AVERAGE
	micromicrograms/milligram		
0	268	250	259
1	174	153	163
2	92	143	117
4	153	113	133
8	60	108	84

TABLE 5

Comparison of the vitamin B_{12} binding capacity of various protein preparations

Material	TRIAL 1	TRIAL 2	AVERAGE
	micromicrograms/milligram		
β-Lactoglobulin	268	259	264
α-Lactalbumin	182	206	194
Crude Intrinsic Factor	2,375	3,417	2,896
Purified Intrinsic Factor	3,791	3,889	3,840
Proteose Peptone	280	380	330

TABLE 6

Vitamin B_{12} binding capacity of the peptides removed from various proteins by electrodialysis

Protein Source	TRIAL 1	TRIAL 2	AVERAGE
	micromicrograms/milligram		
β-Lactoglobulin	4,220	6,660	5,410
α-Lactalbumin	3,330	3,860	3,595
Crude Intrinsic Factor	11,670	11,660	11,665

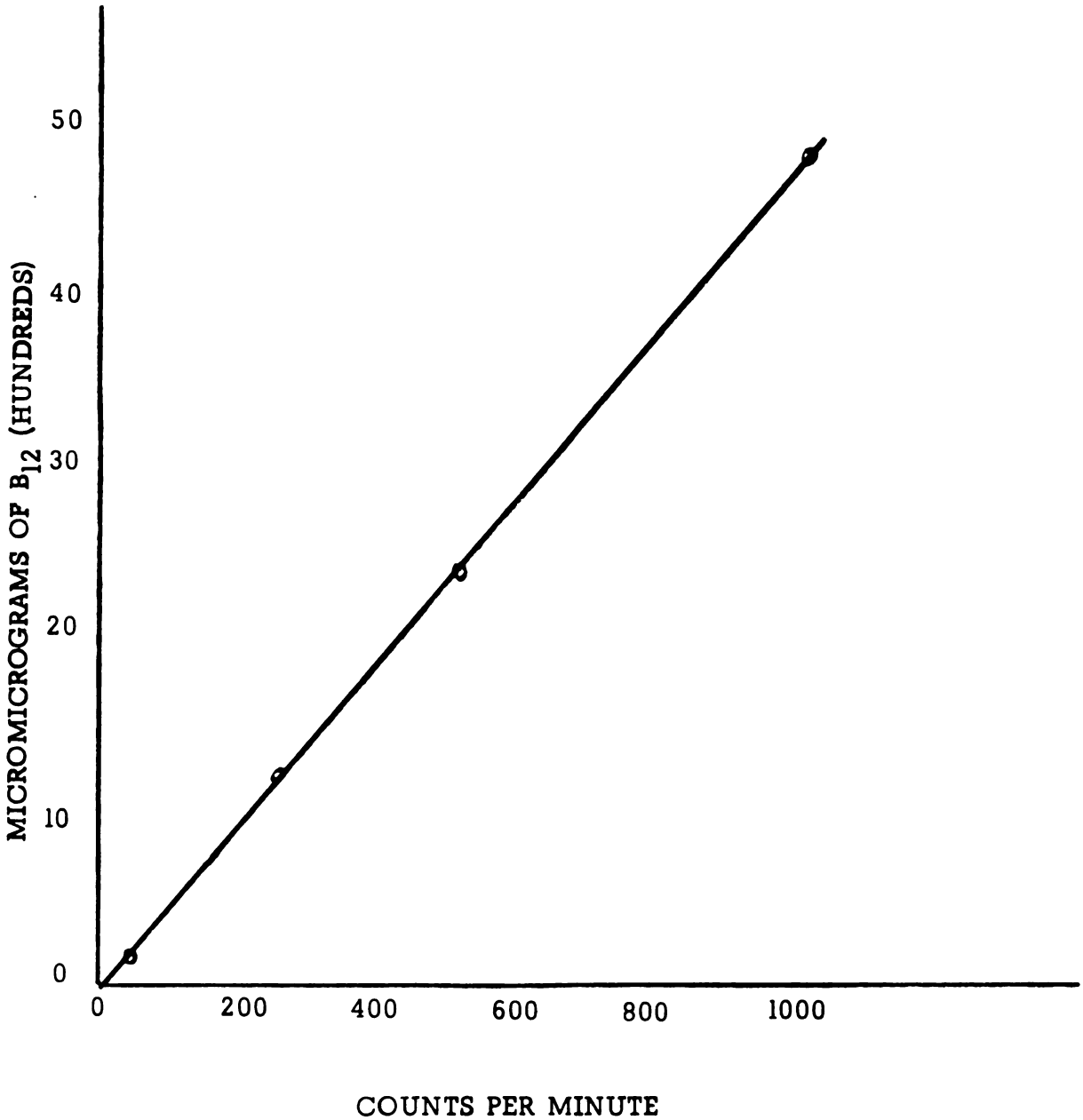


Figure 1. Standard curve for cobalt-60 labeled vitamin B_{12}.

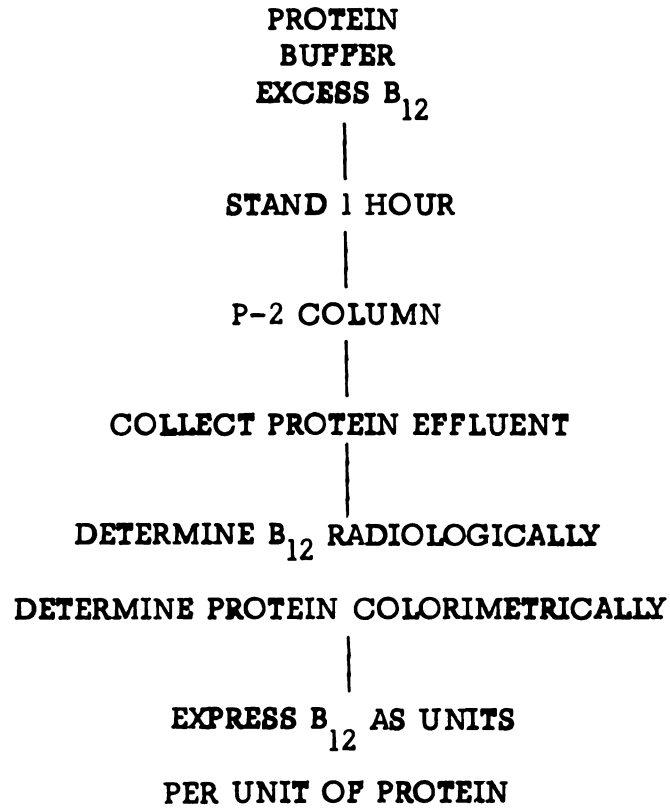


Figure 2. A schematic diagram outlining the procedure used to determine cobalt-60 labeled vitamin B_{12} binding capacity.

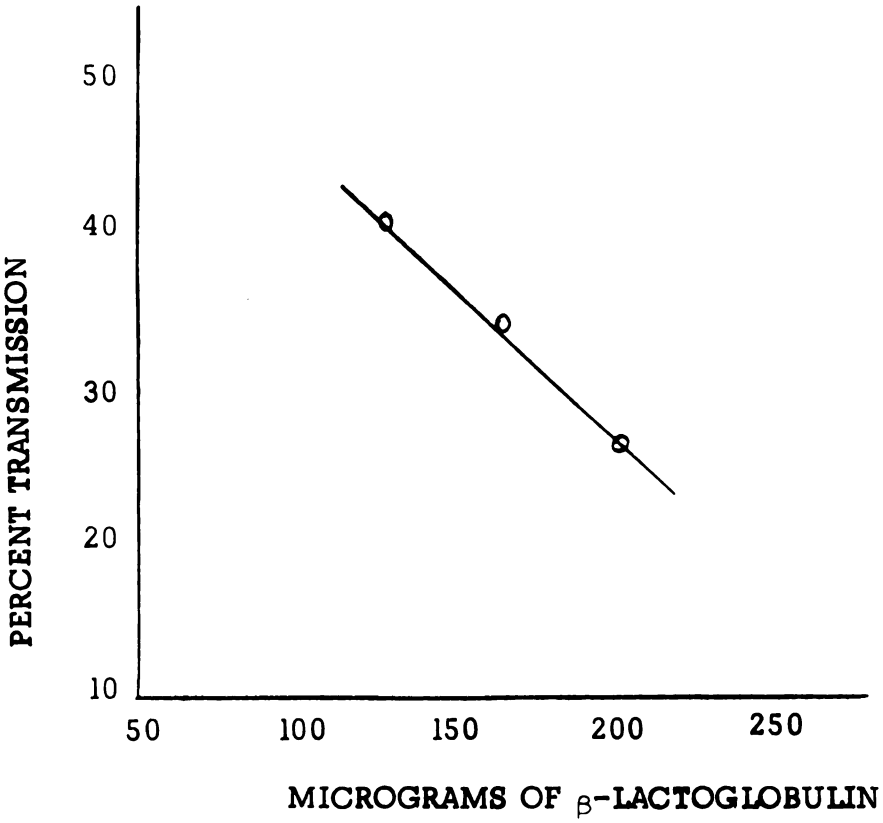


Figure 3. Standard curve for colorimetric protein estimation.

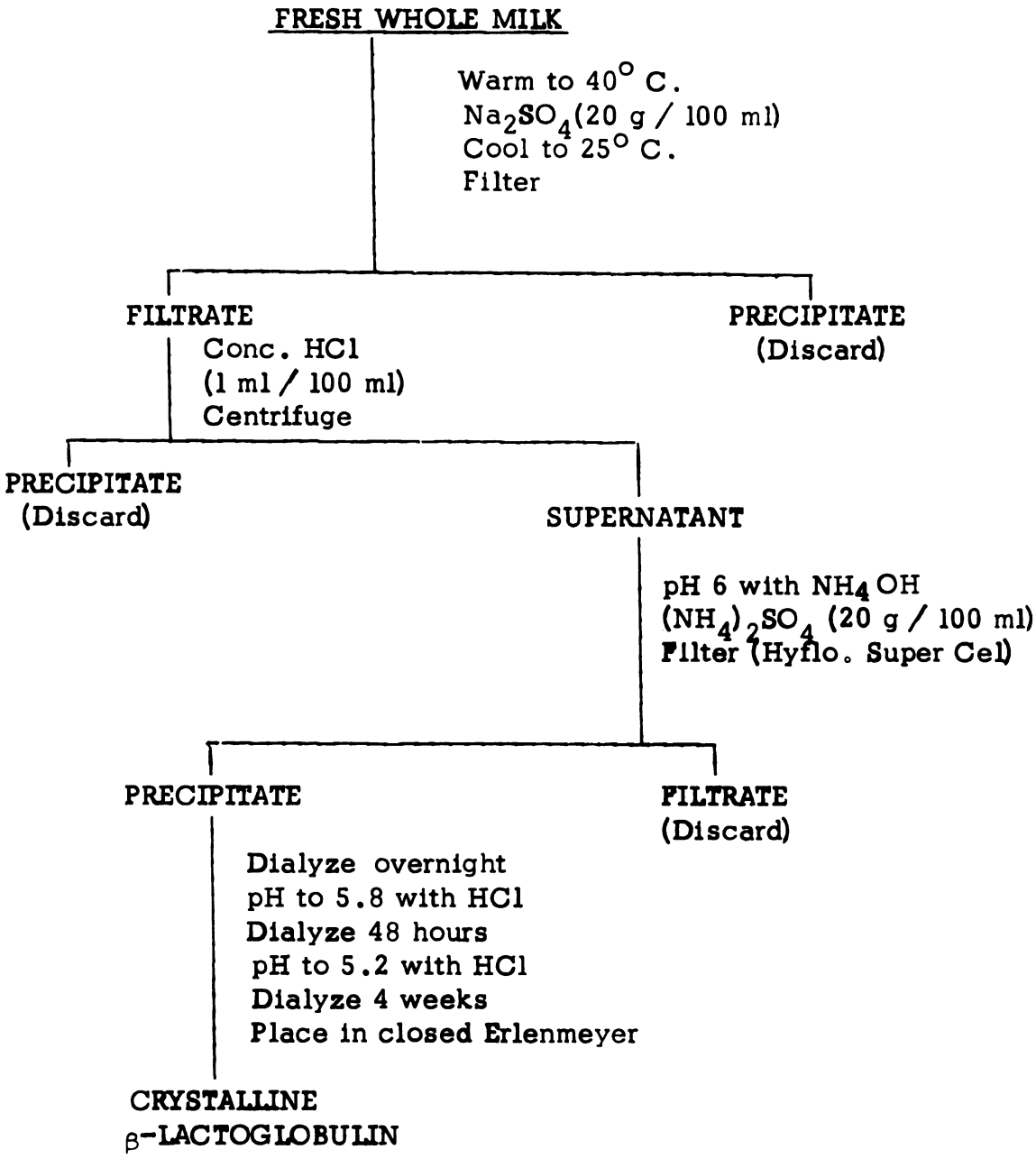
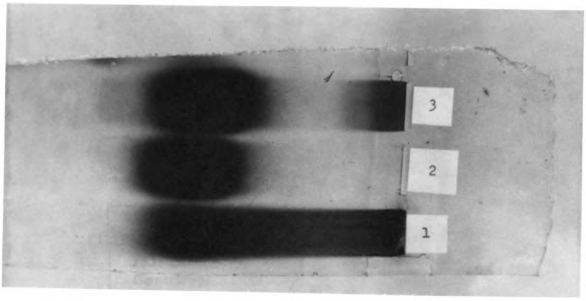


Figure 4. A schematic diagram showing the procedure used to obtain crystalline β-lactoglobulin.



1. Crude
2. Final crystals
3. First crystals

Figure 5. Polyacrylamide gel electrophoretogram of β-lactoglobulin at various stages of purification, as indicated.

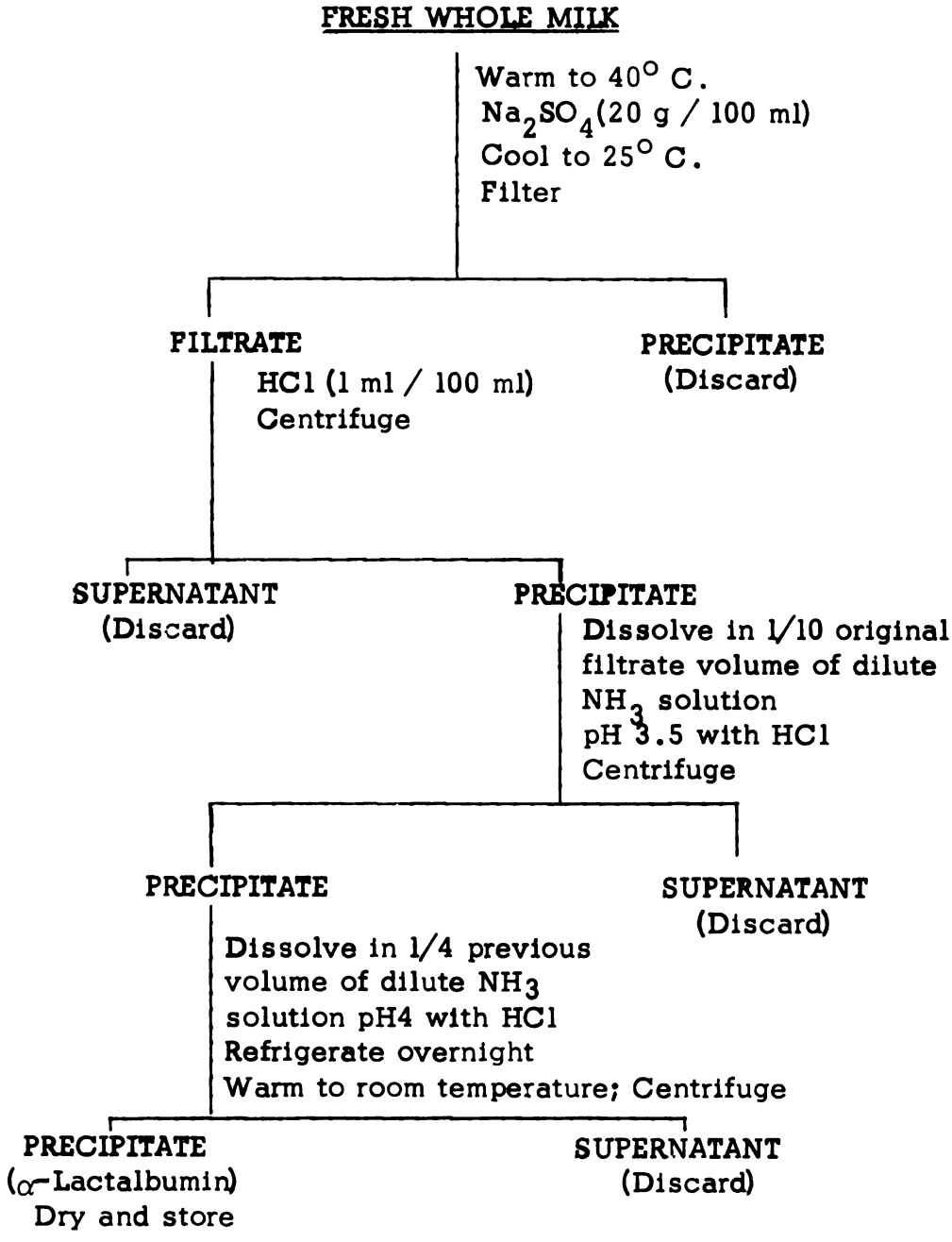
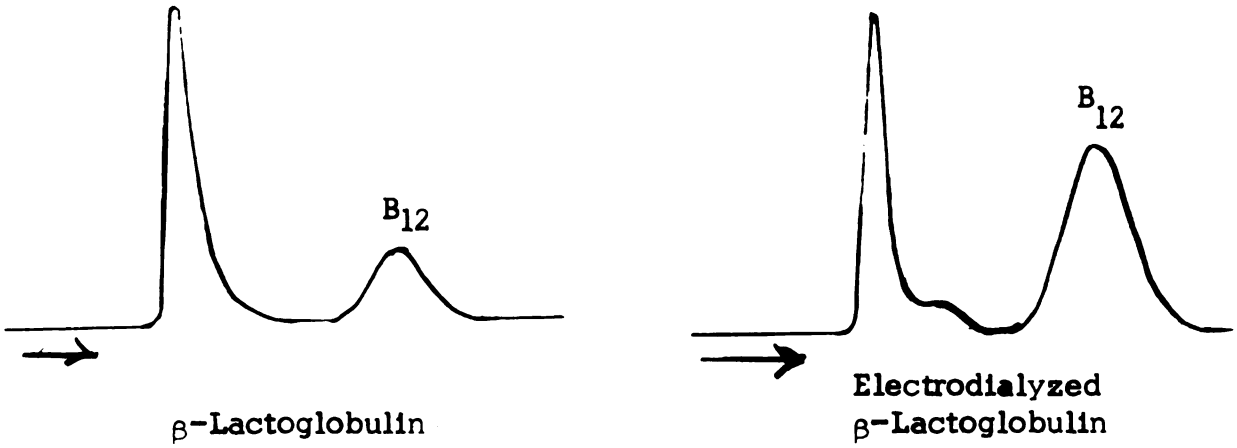


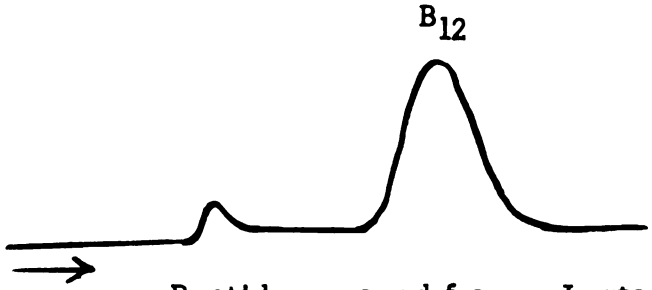
Figure 6. A schematic diagram showing the procedure used to prepare α-lactalbumin.



β-Lactoglobulin

Electrodialyzed
β-Lactoglobulin

BIO-GEL P-2



Peptide removed from β-Lactoglobulin
by electrodialysis.

Figure 7. Elution chromatograms of the component indicated plus an excess of cobalt-60 labeled vitamin B_{12}.

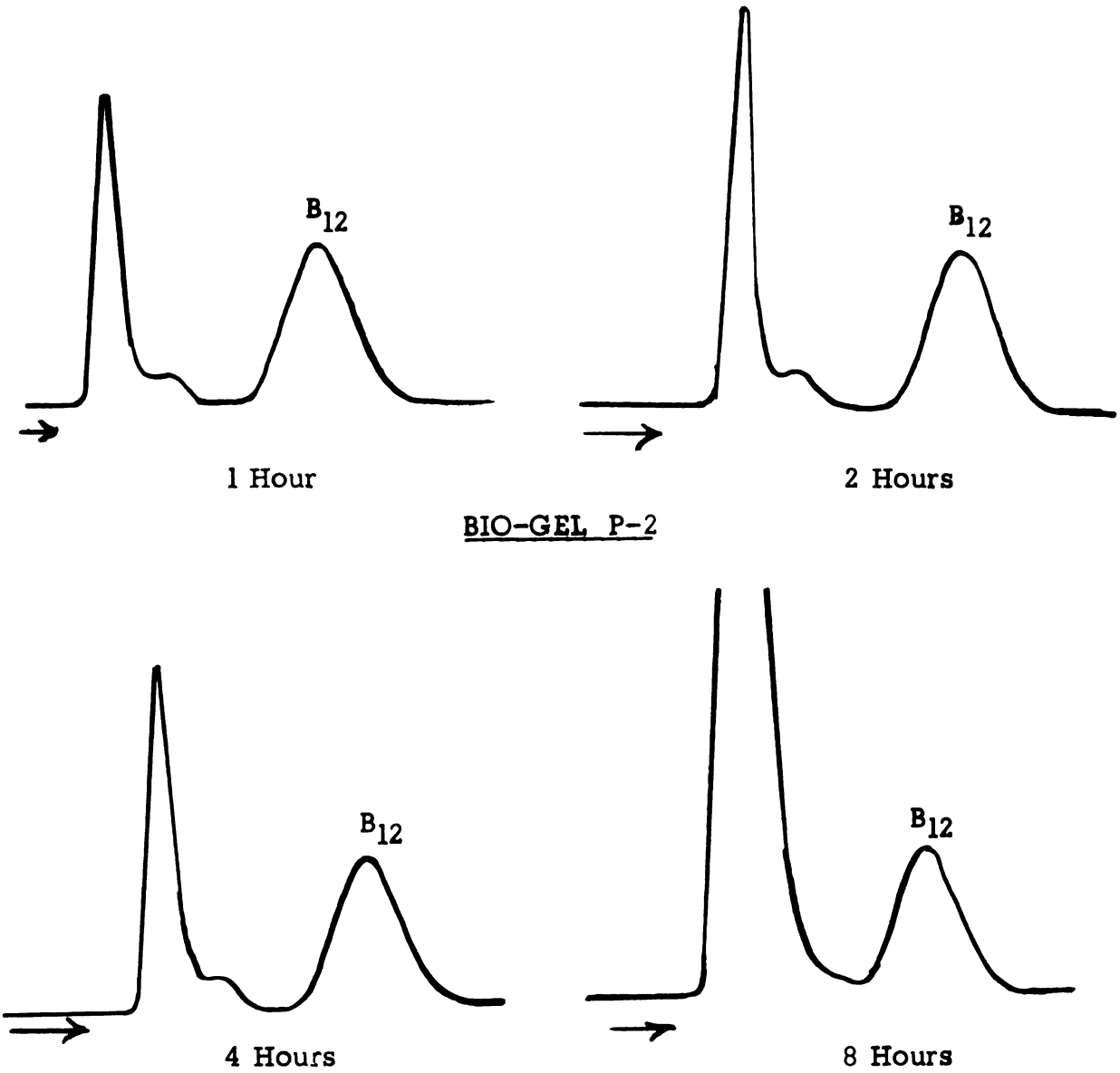
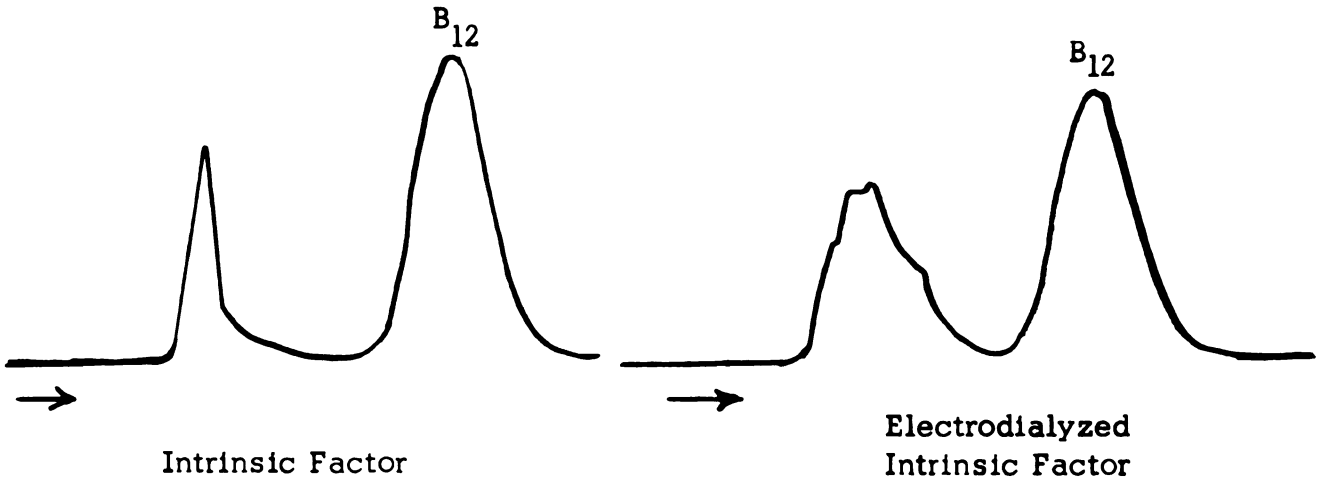
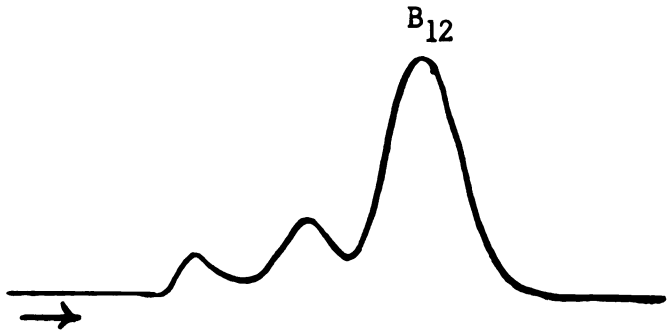


Figure 8. Elution chromatograms of β-lactoglobulin plus an excess of cobalt-60 labeled vitamin B_{12} after the β-lactoglobulin had been electrodialyzed for the indicated period of time.



BIO-GEL P-2



Peptides removed from Intrinsic Factor
by electrodialysis.

Figure 9. Elution chromatograms of the component indicated plus an excess of cobalt-60 labeled vitamin B_{12}.

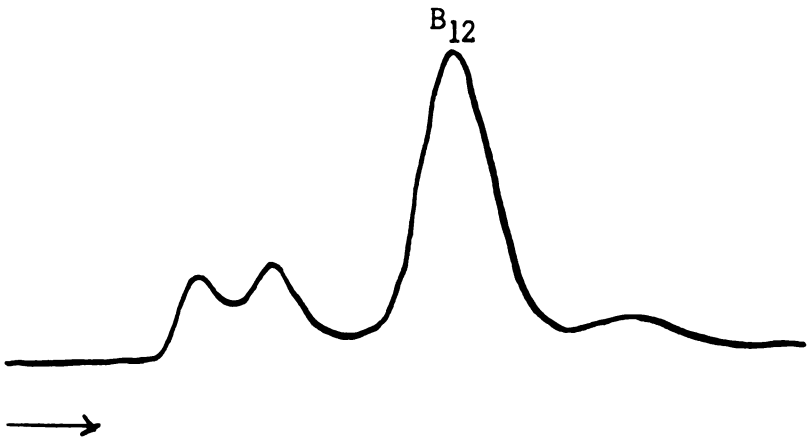


Figure 10. Elution chromatogram of the material removed by electrodialysis from α-lactalbumin, plus cobalt-60 labeled vitamin B_{12}.

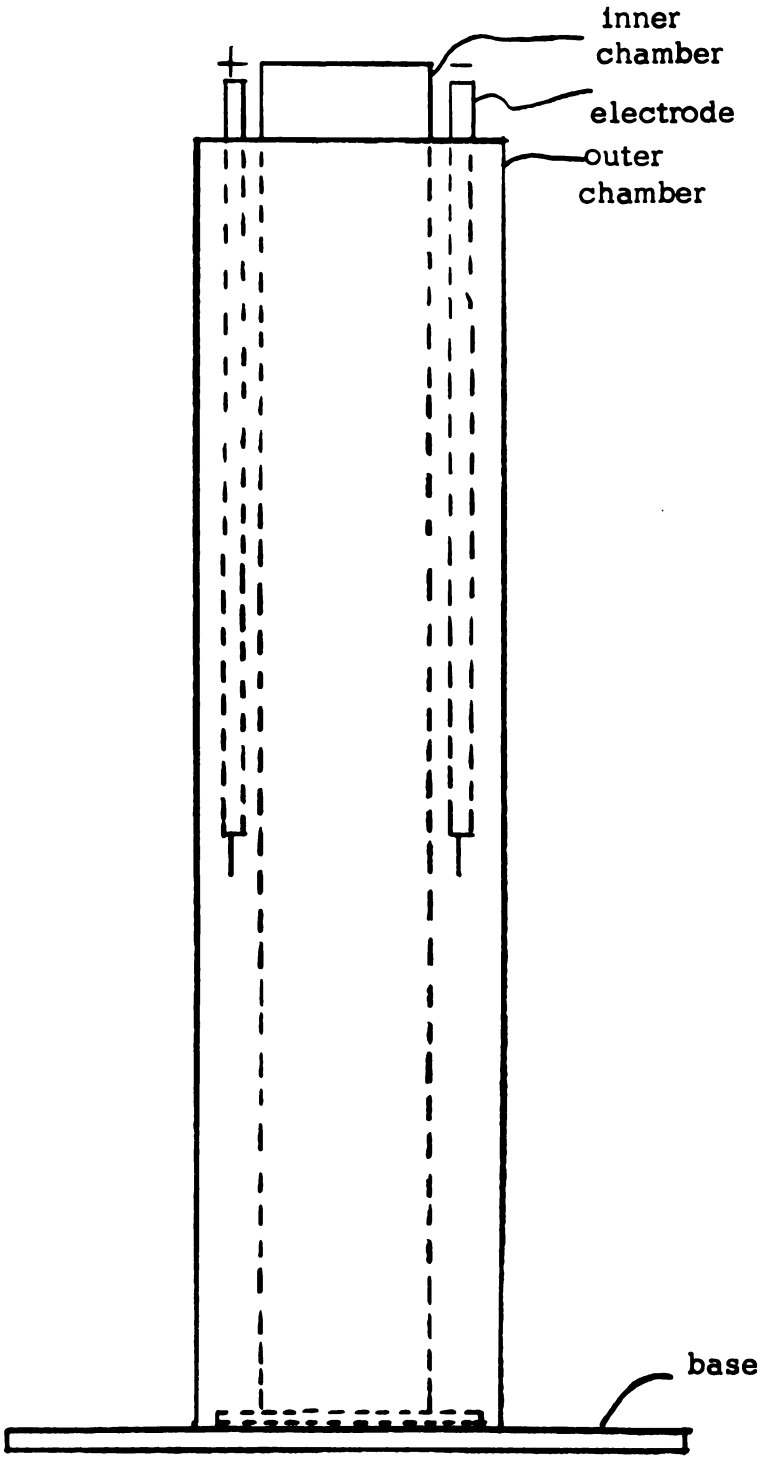


Figure 11. A sketch of the electrodialysis cell used.

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