THE BINDING OF VITAMIN B_{12} BY MILK PROTEINS

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

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1964

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By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science

1964

ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation to Dr. Bernard S. Schweigert, Professor and Chairman of the Department of Food Science, for his encouragement and motivating interest during the course of this study. The author also expresses her gratitude to Dr. J. Robert Brunner, Professor of Food Science, for his advice, efforts and counsel through this research.

Grateful acknowledgment is due to the Department of Food Science, Michigan State University and the National Institute of Health through Research grant EF-284 for the funds and facilities which made this research possible.

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INTRODUCTION

Vitamin B_{12} is required for the prevention of pernicious anemia in man. The relief of the symptoms of pernicious anemia, a disorder involving impaired intestinal absorption of vitamin B_{12}, by oral administration of vitamin B_{12} plus a source of intrinsic factor is evidence of its requirement in the human diet. It is a known dietary requirement for the growth of variety of animals.

For many years prior to the isolation of vitamin B_{12}, liver extracts were used for the treatment of pernicious anemia. Later came the important observations that the growth-factor potency of liver extracts for the microorganism Lactobacillus lactis Dorner, correlated well with the curative-potency when used in the treatment of pernicious anemia. Using this knowledge, rapid progress was made in the late 1940's in the isolation and identification of vitamin B_{12}.

Vitamin B_{12} is a cobalt-containing substance of high molecular weight and complex polycyclic structure with a basic porphyrin configuration resembling that of hemoglobin and chlorophyll.

Its metabolic function remains obscure, although various physiological roles at the cellular level, such as the synthesis of deoxyribosides, the metabolism of single carbon fragments, and the incorporation of amino acids into proteins, have been tentatively ascribed to vitamin B_{12}.

The occurrence of the vitamin is unique among vitamins in that it is absent in higher plants. Vitamin B_{12} is found exclusively in foods of animal origin (meats, fish, eggs, milk, etc.) and fermented products (cheese, silage, etc.) and most of the vitamin exists in the form of a protein complex. It originates either through synthesis by organisms within the animal's own digestive tract or from ingestion of foods derived

from animals. Extensive synthesis occurs within the forestomach of ruminants and these animals, therefore, do not show a dietary requirement of the vitamin. Man and certain animals are dependent upon dietary B_{12} because the vitamin is either not formed in sufficient quantities, or not released in adequate amounts from the cells of synthesizing organisms in the region of the intestinal tract from which absorption occurs. The vitamin is carried by the blood-stream to all parts of the muscles and organs, and mainly is stored in the liver.

Most of the vitamin is present as the bound form in the milk of different species of animals. Available evidence indicates that the vitamin is bound to proteins and unavailable to test organisms without special treatment of the sample. Free vitamin B_{12} is measured after Seitz filtration of samples. The value for bound vitamin B_{12} is obtained by subtracting the amount of free vitamin B_{12} from the amount of total vitamin B_{12}, which is measured after releasing and converting bound vitamin B_{12} to the more stable cyanocobalamin by autoclaving samples at pH 4.6 with a trace of cyanide added.

The purpose of this study was to isolate and characterize vitamin B_{12} rich protein fractions from cows' milk and carry out preliminary studies on the nature of the binding and equilibrium in model systems with isolated milk proteins and added vitamin B_{12}. As the first step of this study it was proposed to isolate and characterize vitamin B_{12} rich protein fractions from cows' milk. In cows' milk, approximately 95% of the total vitamin B_{12} was shown to be present as the bound form and the remainder of the vitamin was present as free vitamin B_{12}.

REVIEW OF LITERATURE

History

The study of Vitamin B_{12} actually began a century ago when pernicious anemia was recognized clinically (1). Pernicious anemia is a macrocytic anemia; the red blood cells are abnormally large, but relatively few in number, 1-3 million per cu. mm. instead of the normal 4.5-6 million. The bone marrow is megaloblastic; the blood-forming cells have become enlarged while still immature.

In 1920 Whipple (90) found that feeding liver accelerated the regeneration of red cells in dogs made anemic by bleeding. Minot and Murphy (61) in 1926 demonstrated that patients with pernicious anemia could be maintained in normal health by ingestion of liver. Subsequently it was discovered that the injection of liver extracts gave more reliable results with less inconvenience to the patients than oral feeding of liver preparations. Since that time, the use of liver extracts became routine practice prior to isolation and identification of Vitamin B_{12} in the treatment of not only of Addisonian anemia, but also of pernicious anemia due to tapeworm, pernicious anemia of pregnancy, nutritional megaloblastic anemia, megaloblastic anemia of infancy and childhood and megaloblastic anemia accompanying steatorrhea.

Cohn (17) started extraction of the "factor" from liver in 1928. Isolation of the pure crystalline factor was announced by two independent teams, Merck and Co., Inc. in America and Glaxo Laboratory in England, within a space of a few weeks (66, 78) in 1948 and the factor was called Vitamin B_{12}.

Pernicious anemia is also characterized by achlorhydria, a failure

to secrete hydrochloric acid and partial atrophy of the mucous membrane of the stomach. Castle (15), in 1928, thought that the atrophied stomach glands might be failing to secrete some essential substance present in normal gastric juice, which he later called "intrinsic factor". This factor was presumed to act upon something present in certain foods, such as liver, called "extrinsic factor" (8), which has now been identified as Vitamin B_{12}, and the substance was given successfully by mouth along with gastric juice to treat patients with pernicious anemia (90). It has been postulated that intrinsic factor acts with extrinsic factor to yield the "Liver factor", the product of this reaction.

Stokstad et al. (81) suggested that an animal protein growth factor required by chicks and other animals and the antipernicious anemia factor (vitamin B_{12}) are identical.

Vitamins of the B group are growth factors for most bacteria, as well as for higher animals. Some bacteria synthesize all they need, but others rely upon an external supply of one or more vitamins. Lactobacillus lactis Dorner would grow on a synthetic medium supplemented with tomato juice and liver extract (73). It was then found that the liver extract could be replaced by concentrates active against pernicious anemia and the "L.L.D. factor" required by the Lactobacillus has been shown to be identical with the antipernicious anemia factor (73).

Source

Vitamin B_{12} is unique among the vitamins in that it is synthesized by certain microorganisms but not by higher plants. Whenever it is found in nature, its origin can be traced back to bacteria or other microorganisms, growing in soil or water or in the rumen or intestine of some animals. The

activities of microorganisms in their natural habitats lead to the presence of Vitamin B_{12} at very low concentrations in soil, in pond-water and even in the sea. Some bacteria and actinomycetes however, produce much more than they need for their own growth, or at least they can be induced to do so under appropriate conditions of growth. Three out of twelve different proactinomycetes of the genus Nocardia produced the largest amounts of Vitamin B_{12}; N. erythropolis (12.2 mg/100ml of medium) (24). Organisms selected from these groups are employed for the commercial production of Vitamin B_{12}; among those recommended for this purpose are Streptomyces griseus, S. olivaceous, Bacillus megatherium and propionic bacterium and yields up to 3 μg of Vitamin B_{12} per ml of fermentation liquor have been claimed (53).

The presence of Vitamin B_{12} in higher plants is still controversial. The absence of Vitamin B_{12} in these plants is confirmed in part by the fact that deficiency symptoms have been induced in several species of animals and have also appeared in some human subjects on exclusively vegetable diets. The most recent claim, still unconfirmed, is that it is present in turnip greens (30).

Vitamin B_{12} is found in animal tissues and animal food products, milk and milk products and eggs. It originates either from organisms within the animal's own digestive tract or from ingestion of foods of animal origin. Strictly noncarnivorous non-ruminant animals must presumably acquire nearly all of their Vitamin B_{12} by absorption of what is synthesized within the gut. In man and some higher animals synthesis and absorption does not appear to occur to any significant degree, so that they are dependent upon dietary Vitamin B_{12}. Liver is the main storage depot in most animal species, although with some (e.g. the rat) kidney

levels tend to be higher.

Chemistry

Crystalline Vitamin B_{12} is a red, complex coordination compound containing a trivalent cobalt and a cyano group. Its empirical formula is $C_{63}H_{88}O_{14}N_{12}PCo$ and it has a molecular weight of 1355 which is considerably larger than that of any other known vitamin. Soon after Vitamin B_{12} was crystallized, considerable knowledge of its structure was obtained by degradation studies, but the molecular structure was definitely established after the brilliant work of Hodgkin and her colleagues (40) using x-ray crystallography as shown in figure 1 (20).

The cyanide group is attached to the cobalt atom which in turn is linked coordinately to a nitrogen of the 5,6-dimethylbenzimidazole group.

Vitamin B_{12} contains a nucleotide component. However, the base present is 5,6-dimethylbenzimidazole rather than the various purine and pyrimidine bases of the nucleic acids and the sugar ribose has an α-glycosidic linkage unlike the β-linkage in the nucleic acids. The D-1-amino-2-propanol moiety of the molecule is esterified to the nucleotide and joined in amide linkage to the porphyrin-like nucleus.

The term "cobalamin" has been suggested for the entire B_{12} molecule except for the cyanide group, Cyanocobalamin (the cyano derivative) is used synonymously with Vitamin B_{12}.

Naturally occurring Vitamin B_{12} derivatives in which the CN group is substituted by other groups are summarized in table 1 (2,77). All the Vitamin B_{12} derivatives listed are converted into Vitamin B_{12} itself by cyanide treatment. Therefore all the compounds listed show biological activity towards microorganisms, animals and human patients with pernicious

anemia.

Table 2 (77) shows naturally occurring Vitamin B_{12} analogues in which the 5,6-dimethylbenzimidazole group is replaced by a purine or pyrimidine base or related derivatives. All of these Vitamin B_{12} analogues are inactive or less active than Vitamin B_{12}, as shown in the table, for various organisms.

The role of Vitamin B_{12} has recently been brought into sharp focus on an enzyme level since the isolation of B_{12} derivatives acting as coenzymes by Barker and his colleagues (6,7,49). Although the precise mechanism of this action is not known, Vitamin B_{12} participates in several types of reactions. These reactions are: (1) the isomerization of carboxylic acids, (2) metabolism of one carbon compound, (3) conversion of ribonucleic acid to deoxyribonucleic acid (82).

Vitamin B_{12} is a neutral molecule, odorless and tasteless, and is soluble in water to the extent of 1.2% at 25° C. Vitamin B_{12} crystallizes as dark red needles or prisms. The crystals darken at 210-220°C but do not melt below 300°C. The crystalline cyanocobalamin is stable in the solid state, even for several hours at 100°C. Aqueous solutions are most stable between pH 4 and 6; within this range solutions can be sterilized by autoclaving at 121°C with the loss of only a few per cent of activity. Aquocobalamin is less stable, especially in alkaline solution, but both are about 90% inactivated by one hour at 100°C at pH 8 (27). On exposure to light, cyanide is slowly split off, leading to hydroxocobalamin, but this change is reversed on keeping the solution in the dark (87). Prolonged exposure to sunlight, however, causes irreversible destruction. The absorption spectrum of Vitamin B_{12} shows three characteristic maxima at 278, 361 and 550 mμ which are relatively independent of pH. The

extinction co-efficients ($E^{1\%}_{cm}$) at the above wavelengths are 115, 207 and 64 respectively (4).

Assay

Numerous procedures for the assay of Vitamin B_{12} have been developed. Very commonly the vitamin occurs in such minute concentration that at present the microbiological assays are the best methods. A description of the assay methods follows:

1. Chemical assays.

a. Spectrophotometric. As little as 25 μg of B_{12} per ml can be determined. This method is rapid and accurate but many of the Vitamin B_{12} analogues also have absorption maxima at 361 mμ, and therefore the usefulness of the assay is limited for the most part to pure samples of Vitamin B_{12}.

b. Colorimetric. Cyanide is liberated by reduction or by photolysis and is measured by a sensitive colorimetric procedure. An alternate method has been proposed which is based on the difference in the spectrum of cyanocobalamin and its purple dicyanide complex (71). Other colorimetric methods, less widely used, are based on the presence of 5,6-dimethylbenzimidazole (12) and on the hydrolysis products resulting from treatment of Vitamin B_{12} with strong hydrochloric acid (22).

c. Isotope dilution. This technique involves the addition of a known amount of radioactive Co-labeled Vitamin B_{12} to a crude sample. This method is highly specific but sufficient amounts of the sample must be taken to permit isolating a measurable quantity of the pure vitamin.

Chemical methods although very accurate are less sensitive than microbiological methods and may be time consuming and tedious, and therefore

are not amenable to routine assays of many food samples.

2. Microbiological assays. Microbiological assays are based on the fact that certain organisms grow slowly or not at all in the absence of Vitamin B_{12}. A few kinds of microorganisms are more commonly used as test organisms. Limitations on the use of these methods are similar to those with other microbiological assays for B vitamins and include lack of specificity of the method and problems in releasing bound forms of the vitamin prior to assay. Vitamin B_{12} has been shown to be bound to other constituents in different degrees (70, 30). The bound form cannot be assayed unless the vitamin is released in the free form. The bound form can be released by simple boiling of the sample in some cases such as for blood serum and liver, however, muscle tissues, human's and sows' milk have been reported to require treatment with trypsin and papain (72).

a. Lactobacilli. Vitamin B_{12} was first measured microbiologically by Shorb (74), with a strain of Lactobacillus lactis. In subsequent work reasonable satisfactory tube and cup-plate methods were developed for this organism, but it has since been almost entirely displaced by more reliable lactobacilli. Skegg et al. (75) have recommended L. leichmannii ATCC 4797, Hoffman et al. (42) independently pioneered the use of the strain ATCC 313, which has also been adopted by other workers (57). The method official in the U.S. Pharmacopoeia uses L. leichmannii ATCC 7830 in the titrimetric assays (84). The L. leichmannii tube assays are sensitive enough for the assay of B_{12} levels in blood serum (28). A complication with all the lactobacilli is that they respond not only to Vitamin B_{12} but to deoxyribonucleosides. The ratio of interfering substances, permissible compared to Vitamin B_{12} to avoid interference is 10^6 (DNA): 1 (Vitamin B_{12}), and permissible amount of desoxyribotides compared to

Vitamin B_{12} is 2×10^4:1 (39). In general, except when the test extracts are rich in nuclear material such as liver, interference in the assays is largely eliminated simply by dilution. L. leichmannii strains also respond in varying degrees to the range of Vitamin B_{12} analogues containing different nucleotides in place of the benzimidazole moiety (23) and this can be a serious complication when assaying samples of microbiological origin (Table 3). These analogues do not have significant Vitamin B_{12} activity for man and other animals and are synthesized by certain microorganisms.

b. E. coli mutant. One of the E. coli mutants, strain 113-3 has come into popular use as a test organism (55). It is somewhat less sensitive than the lactobacilli. This organism does not respond to deoxyribonucleosides, but it can use methionine as a substitute for Vitamin B_{12}. The levels of methionine required for interfering with the Vitamin B_{12} assay is 5×10^4 parts of methionine to 1 part of Vitamin B_{12}. This mutant responds to Vitamin B_{12} analogues, such as factor B and for this reason it has been widely used in the assay of Vitamin B_{12} analogues.

c. Euglena gracilis. This photosynthetic algae (44) was introduced for assay of the vitamin. Its advantages over other microorganisms are extreme sensitivity and the fact that it does not respond to deoxyribosides or other compounds unrelated to Vitamin B_{12}. Its disadvantages are slow growth, and the fact that fairly intense illumination must be provided during the growth period.

d. Other organisms. Ochromonas malhamensis (30) and Poteriochromonas stipitata have higher specificity towards true Vitamin B_{12} than any other organisms previously used. O. malhamensis (26) has an animal-like specificity for the vitamin and can be used to measure the vitamin

even when related compounds are present, and has been recommended for the assay of the vitamin in animal feeds.

3. Biological assays. Chicks (16) are most widely used, because it is relatively easy to rear depleted birds since little Vitamin B_{12} is synthesized in the gut. Rats are also used. There are, however, some difficulties; first young animals bred from normal parents may be endowed by the mother with an adequate reserve of the vitamin. Secondly, since the vitamin is a product of fermentation, it frequently results from bacterial activity in the gut and may be absorbed by the animal either directly through the gut wall or by intake of its own feces. Assays made in higher animals are somewhat more difficult and time consuming than microbiological assays. Large amounts of samples and approximately 2-4 weeks are needed for results.

4. Clinical assay. Before Vitamin B_{12} was isolated a great many assays of liver extracts were carried out with only semiquantitative precision in human subjects with pernicious anemia. Potency was determined by the magnitude of the increase in red blood cell counts, hemoglobin, and the rise in reticulocyte percentage.

We considered that among all these methods, the microbiological method using L. leichmannii was the most adequate one for this study because of its simplicity, the very small amount of the vitamin required, negligible interference from other substances in milk, and the relatively short time of the assay.

Stabilization of Vitamin B_{12}.

Many workers (79, 72, 65) have tested stabilizers of Vitamin B_{12} during autoclaving of the samples. Reducing agents show somewhat unpredict-

able effects. Thiol compounds at low concentrations are alleged to protect the vitamin from destruction but in larger amounts they can cause destruction (50). Sulphite has also been recommended especially for the stabilization of hydroxocobalamine (Vitamin B_{12b}). The addition of ascorbic acid destroys Vitamin B_{12b} quite rapidly, but shows no effect on cyanocobalamin itself (27). A combination of thiamine and nicotinamide, or nicotinic acid alone has been shown to result in slow destruction of Vitamin B_{12} in solution although either alone shows no effect (11). Iron has been reported to protect the vitamin from destruction by this combination (27). Lees et al. (56) reported that the growth response of L. leichmannii to Vitamin B_{12} is related to the oxygen content of many media. A larger surface area and smaller depth of medium increased the rate of diffusion of oxygen into the medium and caused a decrease in growth of the organism. Ford (25) studied the factors influencing the destruction of the vitamin by heat in milk. He concluded that destruction of the vitamin was caused mainly by oxygen dissolved in milk and this destruction could be reduced by deaeration of the milk before heating.

Vitamin B_{12} binding factors.

Several of the B vitamins occur naturally bound to peptides or proteins. As these conjugates differ in physical and biological properties from the free vitamin they may be more or less effective for any specific biological system. As early as 1928, Castle showed that pernicious anemia could be treated with intrinsic factor in the normal human gastric juice or from dried hog stomach, along with the so-called "extrinsic factor" present in meat and other foods. Crude intrinsic

factor preparations were shown to combine with Vitamin B_{12} *in vitro* (83). The resulting "bound Vitamin B_{12}" could be distinguished from the free vitamin in various ways. It was non-dialysable; it was not available to the microorganism used for Vitamin B_{12} assay (10); it was not appreciably taken up from dilute aqueous solutions by bacterial cells, e.g. the wild strain of *E. coli* (41). On the other hand it was much more effective than the free vitamin in the oral treatment of pernicious anemia. Vitamin B_{12} binding has been measured by microbial growth inhibition (83), electrophoresis (46, 54), absorption on charcoal (59), ultrafiltration (35), and dialysis binding (67). These are methods of separation of the free and bound form of the vitamin.

With the use of radioactive Vitamin B_{12}, work on the isolation of intrinsic factor proceeded in America, Britain, Holland and Scandinavia. Latner, Merrills and Raine (52) extracted intrinsic factor from hog mucosa by fractionation at pH 4.5 with ammonium sulfate and removal of salts by ultrafiltration. The molecular weight was between 15,000 and 20,000 with 6.5% hexosamine, 2.2% fucose, 10% N, 13% reducing sugar expressed as glucose, 10.4% tryptophan and 19.6% tyrosine and it was clinically active at 1 to 4 mg per day. Williams et al. (91) also applied ammonium sulfate precipitation to mucosal extract, but they introduced a new step of digestion with trypsin. The product was clinically active at 1-2 mg daily; however, it had a molecular weight of only about 5,000, contained 5.2% glucosamine and 11.8% N, and showed negligible binding capacity for Vitamin B_{12}. Latner suggested that the trypsin had degraded the mucroprotein molecule to a still active mucopeptide. Holdsworth (43) purified hog stomach extract by repeated treatment with DEAE-cellulose. The ultracentrifugally homogeneous product bound 15 μg of Vitamin B_{12} per mg and

the molecular weight was higher than Latner's preparation showed. Grüsbeck (29) obtained intrinsic factor which had a molecular weight of 70,000 by using electrophoresis on starch. O'Brien et al. (62) claimed to obtain a homogeneous product with a molecular weight of 40,000 from human stomach. Smith (77) suggested that the "native" substance as secreted has a high molecular weight, but it is degraded by relatively mild treatment to sub-units of several sizes, all retaining clinical activity. Grüsbeck (29) concluded from his experiment that binding of B_{12} was a prerequisite for intrinsic factor activity and that, in addition, another part of the intrinsic factor molecule was necessary for physiological activity. In 1961, Bromer and Davisson (13) presented a preliminary report on the preparation of a Vitamin B_{12}-intrinsic factor complex active at less than 50 μg per day. The complex contained 25 μg of Vitamin B_{12} per mg and had a molecular weight of 53,000.

Intrinsic factor is by no means the only natural substance that binds Vitamin B_{12}. Many substances without intrinsic factor also bind Vitamin B_{12}: bile constituents, hemoglobin, heparin, milk, saliva, and tear fluid (29). Rosenthal described multiple cyanocobalmin binding sites in serum from different species of animals (68). Normal serum contains about 300 μμg of Vitamin B_{12} per ml (63, 76) attached to proteins that are either identical with, or electrophoretically indistinguished from the α_1-α_2- and β-globulin (63). In chicken blood most of the vitamin is bound to α_1-globulin and α_2-globulin (68). In human and rabbit blood most of the Vitamin B_{12} is bound to α_2-globulin and β-globulin (68). Gregory (37) also reported that cyanocobalamin in sows' milk is bound only with one major protein constituent, lactalbumin.

Most sera have the capacity to bind additional Vitamin B_{12} in vitro,

thus rendering the vitamin non-dialysable and unavailable to assay organisms (63, 9). Pitney (64) showed that the Vitamin B_{12} binding of the liver was less firm than by serum, inasmuch as heat was not needed to release the vitamin (as with serum) before assaying with Euglena gracilis.

Gregory and Holdsworth have isolated binding factors in a relatively pure state from chick serum, rat stomach and sows' milk (36, 33). Kato (47) also reported that the Vitamin B_{12}-binding protein in the α-globulin fraction of human serum was assumed to be a kind of glycoprotein. These glycoproteins represent small fractions of the total proteins of the source material. Though similar in chemical and physical properties they are all different immunologically, even when derived from different organs of the same species (e.g. hog pylorus and sows' milk: chick serum and chick proventriculi). A few attempts have been made to ascertain the point of attachment of the peptide chain on the vitamin molecule. Hydroxocobalamin can easily combine to yield compounds of the "cobalichrome" type (47) when a suitable terminal amino acid, such as histidine, is present that can link directly to the cobalt atom. The binding of cyano- and hydorxocobalamin by normal serum proteins was studied by Meyer (60). Blocking of cyanocobalamin binding by analogues with substituted amide groups in the pyrrol ring side chains was also investigated. The results obtained suggested that some of these amide groups was involved in binding. More hydroxo- than cyanocobalamin is bound, suggesting that the cobalt atom is also involved in binding.

Milk

At the present time the most accurate and rapid method of measuring Vitamin B_{12} in milk is by microbiological assays. Lactobacillus strains

have been mostly widely used.

Certain proteins and peptides have the properties of combining with cyanocobalamin and inactivating it for microorganisms (36, 34). Gregory found that treatment of samples with cyanide increased the microbiological value for Vitamin B_{12} in milk sample extracts. This increase is probably due to action of cyanide in releasing the vitamin from bound forms, and to converting cobalamin derivatives to the more stable cyanocobalamin. The presence of cyanide in the extracts is therefore a necessary precaution, and in some instances treatment with proteolytic enzymes is also essential for releasing bound Vitamin B_{12}. Preliminary treatment of milk for releasing bound Vitamin B_{12} is necessary before the vitamin could be measured quantitatively by the test organism. Table 3 (31) gives some published values of Vitamin B_{12} in the milk of different species of animals (32). The term Vitamin B_{12} refers to the total Vitamin B_{12} activity measured by using Lactobacillus leichmannii.

A difference in the Vitamin B_{12} content of milk between morning and afternoon milking is controversial. Some reports (86) claimed the afternoon milk contain more Vitamin B_{12} than morning milk, but some other reports (3) were the other way. Other workers (48) studied the Vitamin B_{12} content of cows' milk for different seasons, and found the values to be the highest in the autmn and lowest in spring. On the contrary, Collins et al. (19) observed that the cobalamin content of cows' milk did not vary significantly with season or between the Jersey, Guernsey, Ayrshire, Friesian and Brown Swiss breeds.

Collins et al. (18) described that when cows were fed a trace-mineralized salt containing 0.02% cobalt, their colostrum contained significantly higher cobalamin than when iodized salt was given. Contrarily

other workers (38) observed that the milk of cows fed a cobalt supplement did not contain significantly more Vitamin B_{12} as measured by rat growth assay.

Koetsveld (86) found that Vitamin B_{12} activity of milk increased when the cows were put on to pasture after being indoors.

Gregory (32) and Lichtenstein (57) measured the comparative Vitamin B_{12} content in foods and milk using different microorganisms. The latter showed that higher Vitamin B_{12} contents were obtained by using Ochromonas malhamensis than using Lactobacillus leichmannii but the former group of workers showed approximately the same value of B_{12} content using each micro-organism.

The results of microbiological assays showed that 79-86% of added Vitamin B_{12} in milk was destroyed by heat treatment at 115° C for two hours, but heating at 116° C for 15 minutes showed no marked effect on the Vitamin B_{12} content (85). Almost all the Vitamin B_{12} was lost during the sterilization of evaporated milk or in-bottle sterilized milk. This loss was reduced by deaeration of the milk before heating (80). When raw milk was subjected to 0.125-1.0 Mrad γ-radiation either when no-gassed, air-gassed or N_2-gassed, no losses were observed for Vitamin B_{12}.

By the use of an ultrafiltration technique, Gregory (32) has shown that Vitamin B_{12} occurs in a bound form in the milk of the cow, goat, pig, rat, sheep and human. The bound Vitamin B_{12} in cows', goats', rats', and ewes' milk was available to the test organisms after treatment with cyanide but that the bound Vitamin B_{12} in sows' and human's milk was more firmly held and not released by autoclaving with cyanide, although digestion with a papain rendered it available. Gregory and Holdsworth (36) studied further

the occurrence of Vitamin B_{12}-binding proteins in milk. They reported that pseudo-Vitamin B_{12}, factor A and factor B were bound by intrinsic factor and by sows' whey concentrates to the same extent as cyanocobalamin. The binding capacity of both concentrates was much greater before than after heating. In 1954 (33), the cobalamin-binding protein of sows' milk was isolated, by continuous electrophoresis on paper, in the form of its complex with cyanocobalamin. The complex contained 16.1% of N and 23.6 μg of cyanocobalamin in 1 mg of the complex. They assumed that with equimolecular combination the molecular weight of the protein would be 55,000. It contained 17.6% of tyrosine and 7% of hexosamine. The cyanocobalamin-protein bond did not involve the -CN of the vitamin or the -SH of the protein.

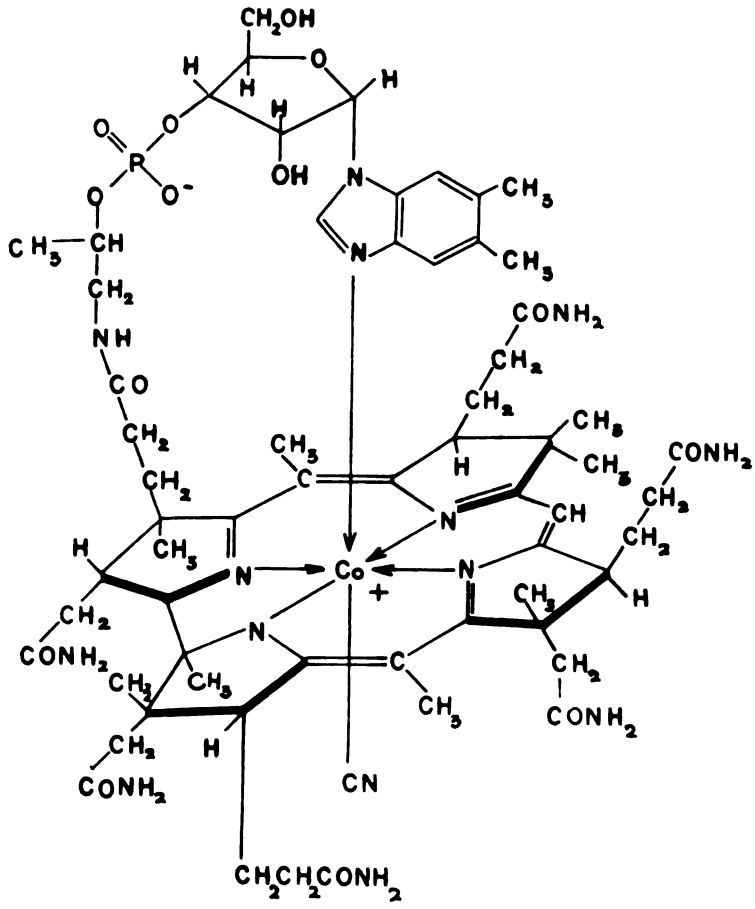


Fig. 1. Vitamin B_{12} (cyanocobalamin)

Table 1

Vitamin B_{12} Derivatives (77)

Original name	Ion or molecule co-ordinated	Semi-systematic name	Systematic name
Vitamin B_{12}	CN-	Cyanocobalamin	α-(5:6-dimethylbenziminazolyl) cobamide cyanide or α-(5:6-dimethylbenziminazolyl) cyanocobamide
Vitamin B_{12_a}	OH-(alkaline solution)	Hydroxocobalamin	α-(5:6-dimethylbenziminazolyl) hydroxocobamide
Vitamin B_{12_b}	H_2O (acid solution)	Aquocobalamin	α-(5:6-dimethylbenziminazolyl) aquocobamide chloride (or sulphate, etc.)
Vitamin B_{12_c}	ONO-	Nitritocobalamin or Nitrocobalamin	α-(5:6-dimethylbenziminazolyl) cobamide nitrite or α-(5:6-dimethylbenziminazolyl) nitritocobamide
	SCN-	Thiocyanatocobalamin	α-(5:6-dimethylbenziminazolyl) cobamide thiocyanate or α-(5:6-dimethylbenziminazolyl) thiocyanatocobamide
Ammonia Cobalichrome	NH_3		α-(5:6-dimethylbenziminazolyl) amminecobamide chloride (etc.)
Histidine Cobalichrome	Histidine		α-(5:6-dimethylbenziminazolyl)-(histidine)-cobamide
Chlorocobalamin	Cl		α-(5:6-dimethylbenzimidazoyl) cobamide chloride

Table 2. Naturally-occurring Vitamin B_{12} Analogues (77). (Activities expressed in relation to Vitamin B_{12} at 100).

Name	Base of Nucleotide	E. coli plate test	E. coli tube test	L. leishmanii tube test	Euglena tube test	Ochromonas tube test	Chick assay by mouth	Chick assay by injection	Clinical assay
Vitamin B_{12}	5:6-Dimethylbenziminazole	100	100	100	100	100	100	100	100
Ψ-Vitamin B_{12}	Adenine	100	10	50	100	0	Ant.	0	0
Factor A	2-Methyladenine	100	50	40	60	0	< 1	< 1	S. Act.
Factor B	No nucleotide	100-250	20	0	0	0	Ant.	0	0
Factor C	?(Guanine?)	100?	20?	10?		0			
Factor D	?	0	0	0	0	0	Ant.		
Factor E	?	Act.	0	0		0			
Factor F	? (2-Methylmercaptoadenine?)	100-130	50			2			
Factor G	Hypoxanthine	160		20-100		0			
Factor H	2-Methylhypoxanthine	280	40	15-40		0			
Factor I (B_{12}-factor III)	5-Hydroxybenziminazole	100-150	50	35		50	4:10-30	5	Act.
Factors J,K,L,M	?	0							
—	Unknown tertiary basic purine?	Act.							
—(Factor F?)	2-Methylmercaptoadenine	(100)	(50)	(40)	(60)	(0)			
—(Factor C?)	Guanine	Act.							
Factor 'A' Ribose Phos	No base	Act.							

Ant. - Antagonism Act. - Active S. Act. - Slightly active

Table 3. Vitamin B_{12} in the milk of different species (31).

	Average Vitamin B_{12} content	Range	Assay organism
	mμg/ml	mμg/ml	
COWS' MILK	--	1.0-6.0	L. leichmannii
	--	2.0-24.0	L. leichmannii
	6.6	3.2-12.4	L. leichmannii
	--	2.7-9.0	L. lactis
	3.0		L. leichmannii
	--	1.3-11.5	L. leichmannii
	3.9	3.2-4.8	L. leichmannii
COWS' COLOSTRUM	--	3.0-78.0	L. leichmannii
	--	5.8-38.0	L. leichmannii
	--	4.0-30.0	L. leichmannii
EWES' MILK	14.0	8.0-20.0	L. lactis
	1.4	1.0-2.0	L. leichmannii
	--	1.0-6.0	L. leichmannii
	7.0	--	L. leichmannii
GOATS' MILK	0.9	0.3-1.4	L. lactis
	0.1	0.1-0.2	L. leichmannii
	0.7	--	L. leichmannii
GOATS' COLOSTRUM	4.2	1.3-8.5	L. leichmannii
	5.0	--	L. leichmannii
HUMAN MILK	0.4	0.1-1.5	L. leichmannii
	--	0.06-0.16	L. lactis
	0.3	--	L. leichmannii
3rd-8th day of lactation	0.9	0.3-2.4	L. leichmannii
1st-8th month of lactation	0.3	0-0.8	L. leichmannii
HUMAN COLOSTRUM	0.2		
RATS' MILK	--	11.0-95.0	L. leichmannii
	--	4.5-139.0	L. leichmannii
	12.0	--	L. leichmannii
SOWS' MILK	1.1	0.03-2.7	L. leichmannii
	2.0	--	L. leichmannii
	--	0.5-7.6	L. leichmannii

Table 3. Vitamin B_{12} in the milk of different species (31).

	Average Vitamin B_{12} content	Range	Assay organism
	mμg/ml	mμg/ml	
COWS' MILK	--	1.0-6.0	L. leichmannii
	--	2.0-24.0	L. leichmannii
	6.6	3.2-12.4	L. leichmannii
	--	2.7-9.0	L. lactis
	3.0		L. leichmannii
	--	1.3-11.5	L. leichmannii
	3.9	3.2-4.8	L. leichmannii
COWS' COLOSTRUM	--	3.0-78.0	L. leichmannii
	--	5.8-38.0	L. leichmannii
	--	4.0-30.0	L. leichmannii
EWES' MILK	14.0	8.0-20.0	L. lactis
	1.4	1.0-2.0	L. leichmannii
	--	1.0-6.0	L. leichmannii
	7.0	--	L. leichmannii
GOATS' MILK	0.9	0.3-1.4	L. lactis
	0.1	0.1-0.2	L. leichmannii
	0.7	--	L. leichmannii
GOATS' COLOSTRUM	4.2	1.3-8.5	L. leichmannii
	5.0	--	L. leichmannii
HUMAN MILK	0.4	0.1-1.5	L. leichmannii
	--	0.06-0.16	L. lactis
	0.3	--	L. leichmannii
3rd-8th day of lactation	0.9	0.3-2.4	L. leichmannii
1st-8th month of lactation	0.3	0-0.8	L. leichmannii
HUMAN COLOSTRUM	0.2		
RATS' MILK	--	11.0-95.0	L. leichmannii
	--	4.5-139.0	L. leichmannii
	12.0	--	L. leichmannii
SOWS' MILK	1.1	0.03-2.7	L. leichmannii
	2.0	--	L. leichmannii
	--	0.5-7.6	L. leichmannii

Table 3. Vitamin B_{12} in the milk of different species (31).

	Average Vitamin B_{12} content	Range	Assay organism
	mμg/ml	mμg/ml	
COWS' MILK	--	1.0-6.0	L. leichmannii
	--	2.0-24.0	L. leichmannii
	6.6	3.2-12.4	L. leichmannii
	--	2.7-9.0	L. lactis
	3.0		L. leichmannii
	--	1.3-11.5	L. leichmannii
	3.9	3.2-4.8	L. leichmannii
COWS' COLOSTRUM	--	3.0-78.0	L. leichmannii
	--	5.8-38.0	L. leichmannii
	--	4.0-30.0	L. leichmannii
EWES' MILK	14.0	8.0-20.0	L. lactis
	1.4	1.0-2.0	L. leichmannii
	--	1.0-6.0	L. leichmannii
	7.0	--	L. leichmannii
GOATS' MILK	0.9	0.3-1.4	L. lactis
	0.1	0.1-0.2	L. leichmannii
	0.7	--	L. leichmannii
GOATS' COLOSTRUM	4.2	1.3-8.5	L. leichmannii
	5.0	--	L. leichmannii
HUMAN MILK	0.4	0.1-1.5	L. leichmannii
	--	0.06-0.16	L. lactis
	0.3	--	L. leichmannii
3rd-8th day of lactation	0.9	0.3-2.4	L. leichmannii
1st-8th month of lactation	0.3	0-0.8	L. leichmannii
HUMAN COLOSTRUM	0.2		
RATS' MILK	--	11.0-95.0	L. leichmannii
	--	4.5-139.0	L. leichmannii
	12.0	--	L. leichmannii
SOWS' MILK	1.1	0.03-2.7	L. leichmannii
	2.0	--	L. leichmannii
	--	0.5-7.6	L. leichmannii

EXPERIMENTAL PROCEDURE

The experiments in this study were undertaken primarily to determine which protein fractions of cows' milk contain the largest amount of bound Vitamin B_{12}. It is hoped that this study would provide some information on the relationship between the Vitamin B_{12} content of blood proteins as compared to milk proteins.

Throughout this study, a modified Gregory's method (32) was applied along with the method described in U.S.P. XVI (84) and A.O.A.C. 9th edition (5) for the determination of Vitamin B_{12}.

The Microbiological Assay

Assay organism

Lactobacillus leichmannii ATCC 7830 was used.

Media

Standard official media (Bacto-B_{12} culture agar, Bacto-B_{12} inoculum broth, and Bacto-B_{12} assay medium U.S.P. were used.

Inoculum

One loop of cells from the stock culture in inoculum broth was transferred to a tube of the inoculum broth. The culture was incubated for 16-24 hours at 37° C $\pm$ 0.5° C. This transferring had been done daily for three days before performing the Vitamin B_{12} assay. The culture was washed three times by means of centrifuging and decantation and was then suspended in 50 ml of saline solution. One drop of suspended cells was inoculated into each tube for assay of the vitamin.

Method of determination of Vitamin B_{12}

All procedures were carried out under minimum exposure of the samples

to light, and amber colored volumetric flasks were used.

Standard solution

One mg of Vitamin B_{12} (cyanocobalamin) from an ampule standard solution was diluted to contain 50 μμg per ml of working standard solution.

Sample solution

Samples were treated as outlined below and diluted to contain about 20-50 μμg of Vitamin B_{12} per ml of assay solution and kept in a refrigerator after addition of a small amount of toluene.

Procedure

Standard curve. Predetermined quantities of standard solution were added to the standard size test tubes, in triplicate, at 0.5 ml interval and made to 10 ml volume with 5 ml medium and sufficient water. These tubes were covered and autoclaved at 121° C for five minutes. After cooling to room temperature one drop of cell suspension was added to each tube. Following incubation at 37° C for 72 hours, the lactic acid produced was titrated with the use of an automatic titrimeter or by using bromthymol-blue solution as an indicator. A standard curve was prepared by plotting titration values, expressed in ml of alkali solution for each level of standard Vitamin B_{12} solution used, against the quantity of reference standard.

Determination of total Vitamin B_{12}.

To the same size of test tubes, in duplicate, 1.0 ml to 5.0 ml of sample solution was added at 1 ml interval. A procedure was followed

similar to that used for preparing the standard curve. The quantity of Vitamin B_{12} for each level of assay solution was determined by interpolation of the standard curve. The amount of Vitamin B_{12} in each original sample was obtained by multiplying by the dilution factor.

Determination of free Vitamin B_{12}

Each suitably diluted sample was passed through a Sietz filter or ultrafiltered through a regenerated cellulose tubing under vacuum, aseptically, and 0.0 ml to 5.0 ml of Sietz filtered or ultrafiltered solution was added aseptically to the tubes for assay. The concentration of free Vitamin B_{12} was obtained by interpolation of the standard curve.

Preparation of Samples and Analysis

A series of experiments were carried out in seven steps to isolate and characterize Vitamin B_{12} rich fractions from cows' milk.

1) Experiment 1 -- Determination of the total Vitamin B_{12} in pasteurized milk.
2) Experiment 2 -- Determination of total and free Vitamin B_{12} in pasteurized milk.
3) Experiment 3 -- Determination of total and free Vitamin B_{12} in raw skimmilk, raw whole milk, pasteurized skimmilk and pasteurized whole milk.
4) Experiment 4 -- Determination of total and free Vitamin B_{12} in casein samples and whey samples which were prepared from pasteurized skimmilk by three different methods (ultracentrifugally sedimented, rennin coagulation and isoelectric precipitation).
5) Experiment 5 -- (a) Preparation of crude lactalbumin and lactoglobulin from raw whole milk. (b) Determination of total and free

Vitamin B_{12} in the fraction of proteins on a dry basis. (c) Electrophoretic studies of the fractions.

6) Experiment 6 -- (a) Preparation of skimmilk, whey, casein, crude lactalbumin and lactoglobulin from raw whole milk. (b) Determination of total and free Vitamin B_{12} in the fractions of proteins on a dry basis. (c) Electrophoretic studies of the whey, crude lactalbumin and lactoglobulin.

7) Experiment 7 -- (a) Separation of α-lactalbumin and immune globulin from crude lactoglobulin. (b) Determination of total and free Vitamin B_{12} in the fractions of proteins on a dry basis. (c) Electrophoretic studies of the fractions.

The procedures used in each experiment are described in detail as follows:

Experiment 1. Determination of total Vitamin B_{12} in pasteurized milk

Six ml of pasteurized milk obtained from the university's dairy plant was diluted to 200 ml with water and used as a working sample solution.

Experiment 2. Determination of total and free Vitamin B_{12} in pasteurized milk

The purpose of this experiment was to determine the best method for determining total and free Vitamin B_{12}. Gregory's procedure (32) was applied with a slight modification; namely, final pH of the treated sample solution and medium was adjusted to pH 6.8 instead of pH 5.5.

Pasteurized whole milk was obtained from the university's dairy plant.

Determination of free Vitamin B_{12}

Method 1. Seitz filtration

One ml of the milk was diluted to 50 ml with water and Seitz filtered.

Method 2. Ultrafiltration

Regenerated cellulose tubing was suspended from the stem of a glass funnel held in the neck of a filtration tube by means of a rubber bung. The bag was made by knotting one end of the tubing tightly and tying the other with cotton over a piece of Polythene tubing fitted over the stem of the funnel. The milk was diluted 50 times with water and poured into the cellulose bag and the outer tube evacuated and sealed with a stopper. After approximately two hours, 2-3 ml of ultrafiltrate could be collected.

Determination of total Vitamin B_{12}

Method 3. Extraction with cyanide

Twenty ml of milk sample were diluted with an equal volume of water and 0.1 N - HCl added until the pH was reduced to 4.6. Twenty drops of 1% (w/v) NaCN solution were added, and the mixture was autoclaved for ten minutes at 10 p.s.i. pressure. After cooling, the extract was diluted to 100 ml with water and filtered. Five ml of the filtrate were adjusted to pH 6.8 with 0.1 N - NaOH and diluted to 200 ml with water.

Method 4. Extraction with cyanide

This procedure consisted of adding 20 ml of 0.1 M sodium acetate buffer at pH 4.6 and 20 drops of 1% (w/v) NaCN solution to a 20 ml sample of the milk under test. The mixture was heated in steam for 30 minutes, cooled, diluted to 100 ml, made to pH 4.6, and filtered. Five ml of the solution were adjusted to pH 6.8 and diluted to 200 ml with water for use as a working solution.

Method 5. Digestion with papain

For digestion with papain, 20 ml milk was mixed with 20 ml of 0.1 M

sodium acetate buffer (pH 4.6), warmed to 60° C in a water-bath, and 1 gram of papain in 10 ml water was added. The papain was activated by adding 10 drops of 1% (w/v) NaCN solution. The mixture was incubated for one hour at 60° C, steamed for ten minutes to inactivate the enzyme, made to pH 4.6, and filtered, after making to 100 ml, with water. Five ml of this solution was adjusted to pH 6.8 and diluted to 200 ml with water.

Samples prepared by methods 3, 4 and 5 were diluted further to provide samples of approximately 30 μμg of Vitamin B_{12} per ml for assay.

Determination of bound Vitamin B_{12}

The values for bound Vitamin B_{12} were obtained by subtraction of the free Vitamin B_{12} content from the total Vitamin B_{12} content.

Method 1 for free Vitamin B_{12} and method 3 for total Vitamin B_{12} were used for further study since these methods were simple and gave satisfactory results.

Experiment 3. Determination of total and free Vitamin B_{12} in raw skimmilk, raw whole milk, pasteurized skimmilk, and pasteurized whole milk

This experiment was undertaken to determine the effect of fat separation and pasteurization upon the Vitamin B_{12} content of milk.

Preparation of raw skimmilk

Raw whole milk, obtained from the university's dairy plant, was centrifuged at 2,000 r.p.m. for 30 minutes. The top cream layer was discarded. The supernatant skimmilk layer was used in this experiment.

Preparation of pasteurized whole and skimmilk

Portions of the whole milk and skimmilk were placed into an erlenmeyer

flask and pasteurized at 143-145° F for 30 minutes in a water bath.

Experiment 4. Determination of total and free Vitamin B_{12} in casein and whey samples prepared from pasteurized skimmilk by ultracentrifugation, rennin coagulation, and isoelectric precipitation methods

Preparation of casein and whey samples

a. Ultracentrifugal method

Ten ml of 2 M $CaCl_2$ were added to 200 ml of milk and ultracentrifuged at 21,000 r.p.m. for 90 minutes. Whey was separated from casein by decantation. To purify the casein: (1) The casein was resuspended in 250 ml of 0.085 M NaCl solution. (2) One hundred ml of 1.5 M Ca-oxalate along with sufficient oxalic acid to maintain constant pH were added. (3) Ca-oxalate precipitate was removed by centrifuging at 2,000 r.p.m.

b. Rennin coagulation method

One to two ml of rennin (0.45 mg/ml) were added to 200 ml of milk, and the mixture was allowed to stand in a water bath at 36-37° C until coagulation was complete. Coagulated milk was centrifuged at 2,000 r.p.m. for 30 minutes. Whey was separated from the casein by decantation. The casein was washed with water and centrifuged at 2,000 r.p.m. for 30 minutes.

c. Isoelectric precipitation method

Fifty ml of water were added to 200 ml of milk and the pH of the mixture was adjusted to 4.6 with 1 N HCl solution. The mixture was stirred with a magnetic stirrer and centrifuged at 2,000 r.p.m. for 30 minutes. Whey was removed from the casein by decantation; then the casein was washed with water by centrifugation at 2,000 r.p.m. for 30 minutes.

From these methods, about 20-30 grams of moist-crude casein and 170-180 ml of whey were obtained from 200 ml skimmilk. The original whey and washings from the casein were combined and considered as the whey fraction in this experiment.

Determination of total and free Vitamin B_{12} in casein samples

Total Vitamin B_{12}

Whole casein from each sample preparation was placed in a small size mortar and ground with a pestle with the addition of a small amount of water until the casein was in the form of fine particles. The ground casein was transferred to a 250 ml volumetric flask and made to 250 ml with water. Five ml of the diluted sample solution were taken, and Vitamin B_{12} was extracted under the same conditions as used in method 3. After cooling, the volume was made to 200 ml with water, and the solution was filtered. The pH of the solution was adjusted to 6.8, and the solution was diluted to 250 ml with water.

Free Vitamin B_{12}

Five ml of casein suspension were taken from the diluent, adjusted to pH 6.8, diluted to 200 ml with water, and Seitz filtered.

Determination of total and free Vitamin B_{12} in whey samples

The same procedure used for the milk and the casein samples was followed. The scheme used for sample preparation is outlined in Figure 2. Calculation of Vitamin B_{12} in μμg/mg of protein from skimmilk casein, and whey was based on the protein content of milk (45). The protein content of skimmilk used was estimated at 3.8%; of whey, 1.1%; and the protein contributed by casein, 2.7%. The specific gravity of skimmilk

(1.343) and of whey (1.0153) was obtained from the literature (45).

Experiment 5

Preparation of crude lactalbumin and lactoglobulin from raw whole milk

The scheme for the isolation of the crude lactalbumin and lactoglobulin is shown in Figure 3. The fresh raw whole milk was separated three times at 40° C in laboratory-size cream separator. The pH of the skimmilk was adjusted to 4.6 with 0.1 N HCl and the casein removed by filtration. The acid whey was adjusted to pH 6.5 with 0.1 N NaOH. Ammonium sulfate was added to 0.5 saturation in order to precipitate the crude globulin (Fraction A). Fraction A was dissolved in water at a concentration of approximately three per cent, the pH was adjusted to 4.6, and ammonium sulfate added to 0.25 saturation. The ensuing precipitate (Fraction C) was removed by centrifuging in a Servall centrifuge operated for 15 minutes at 25,000 x G. The resulting supernatant was filtered through a thick layer of glass wool. The lactoglobulin (Fraction D) was salted-out of the supernatant at 0.4 saturation with ammonium sulfate at pH 6.0. This fraction was redissolved in distilled water to about three per cent protein concentration, the pH adjusted to 4.5, and the insoluble residue filtered off. Fraction B was dialyzed and pervaporated. The solution was adjusted to pH 4.6, and the precipitate was filtered off. Fraction G was obtained by adjusting Fraction F to pH 6.5, adding ammonium sulfate to 0.5 saturation, and centrifuging at 2,000 x G for 15 minutes. Fraction E (crude lactoglobulin) and Fraction G (crude lactalbumin) were dialyzed against water and lyophilized. The lyophilized proteins were kept in the cold room at -10° C.

Determination of total and free Vitamin B_{12} in crude lactalbumin and lactoglobulin

The total and free form of Vitamin B_{12} in 5-10 mg and 20 mg, respectively, of these protein fractions was determined as previously described. The moisture and protein contents of the crude protein fractions, needed for the determination of the vitamin content on dry basis, were obtained as follows:

Moisture determination

Moisture was determined gravimetrically by means of Mojonnier milk tester.

Nitrogen and protein determination

The protein content of the crude proteins was estimated by multiplying their nitrogen content, obtained by micro-Kjeldahl determinations, by the factor 6.38 (5).

Electrophoretic studies of crude lactalbumin and lactoglobulin

Free boundary electrophoresis

Free boundary electrophoretic data were obtained with a Perkin-Elmer Model 38-A Electrophoresis apparatus. One per cent of protein solution was made up in veronal buffer, pH 8.6, $\mu = 0.1$, and dialyzed against the same buffer solution. The system was stirred overnight. The specific conductivity was calculated from the following equation:

$$K = \frac{CC}{R}$$

K - specific conductivity

CC - cell constant

R - resistance of solution in Ohms observed at 1° C

The electrophoretic mobilities (μ) were calculated by means of the following equation: $\mu\ (cm^2.v.^{-1}\ sec.^{-1}) = \frac{daK}{itRm}$

d - distance migrated (cm)

a - area of cross section of the cell (cm^2)

K - specific conductivity cell constant

i - current (amps)

t - time (sec.)

R - resistance of buffer (Ohms)

m - magnification factor (1.1)

Zone electrophoresis

Zone electrophoretic data were obtained by urea-starch gel electrophoresis (89).

Experiment 6

Preparation of skimmilk, whey, casein, crude lactalbumin and lactoglobulin from fresh raw whole milk

The scheme for the separation of each fraction is shown in Figure 4. Fresh raw whole milk obtained from the university's dairy barn was separated three times at 40° C in a laboratory size cream separator. The skimmilk was acidified with 0.1 N HCl to pH 4.6 and the casein removed by filtration. The acid whey was adjusted to pH 6.5 with 0.1 N NaOH, and ammonium sulfate was added to 0.5 saturation. A precipitate (Fraction A) and supernatant (Fraction B) were obtained. Fraction A was redissolved to about three per cent protein concentration, and the pH was adjusted to 4.6 and filtered. The supernatant and Fraction B were dialyzed against water and lyophilized. The volumes of skimmilk and whey and the weights of casein, crude lactalbumin and lactoglobulin were recorded.

Analytical methods

Concentration of total and free Vitamin B_{12} in the fractions was expressed in terms of the anhydrous protein.

Experiment 7

Separation of α-lactalbumin and the immune globulin fractions from crude lactoglobulin

Crude lactoglobulin prepared by ammonium sulfate precipitation was further fractionated by free boundary electrophoresis in a Perkin-Elmer Model 38-A Electrophoresis apparatus using the 6 ml cell. A 1.5% solution of the crude protein in veronal buffer, pH 8.6, and 0.1 ionic strength was dialyzed against the same buffer with stirring overnight. After electrophoresis was completed, the distance from the origin to the peaks, corresponding to α-lactalbumin and immune globulin, was measured. The solution within the distance of the first peak, corresponding to α-lactalbumin, in the ascending limb of the cell was collected with a syringe-type microsampler. The rest of the solution contained the immune globulin fraction and lesser quantities of α-lactalbumin. This electrophoretic purification step was repeated twice.

Analytical methods

Determination of total and free Vitamin B_{12} in the fractions and electrophoretic study on the fractions was the same as in Experiment 5.

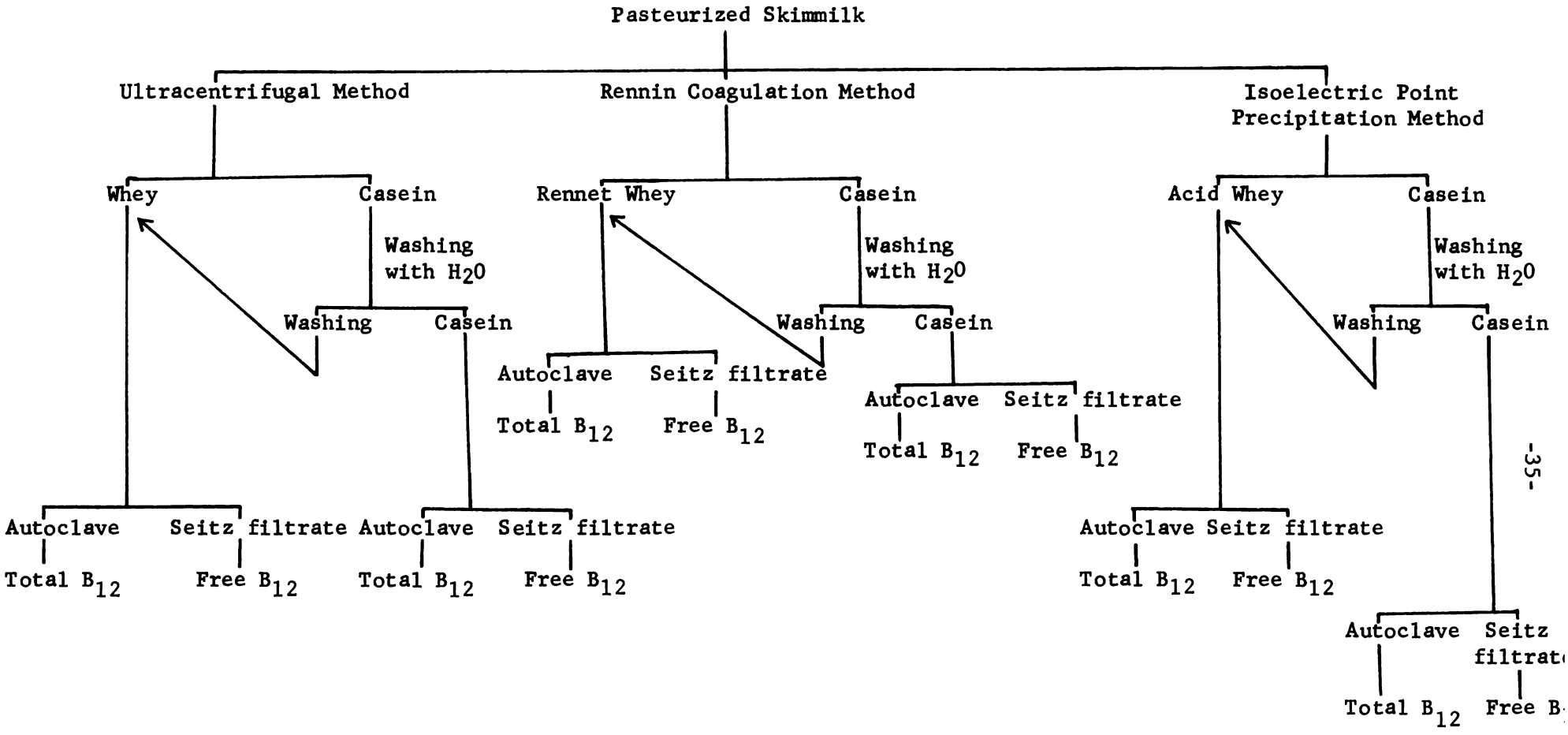


Figure 2. A schematic diagram showing the sample preparation for determination of total and free Vitamin B_{12}.

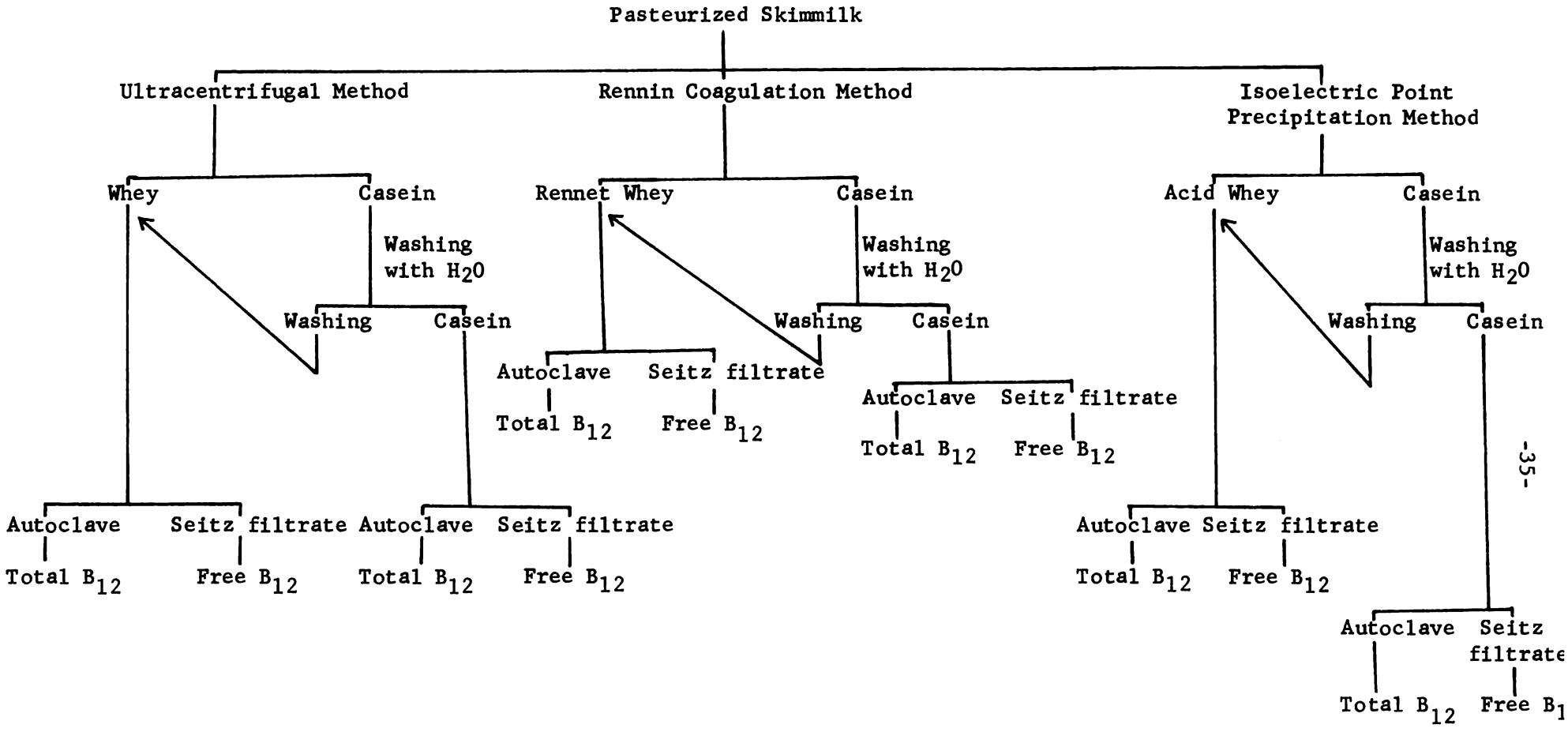


Figure 2. A schematic diagram showing the sample preparation for determination of total and free Vitamin B_{12}.

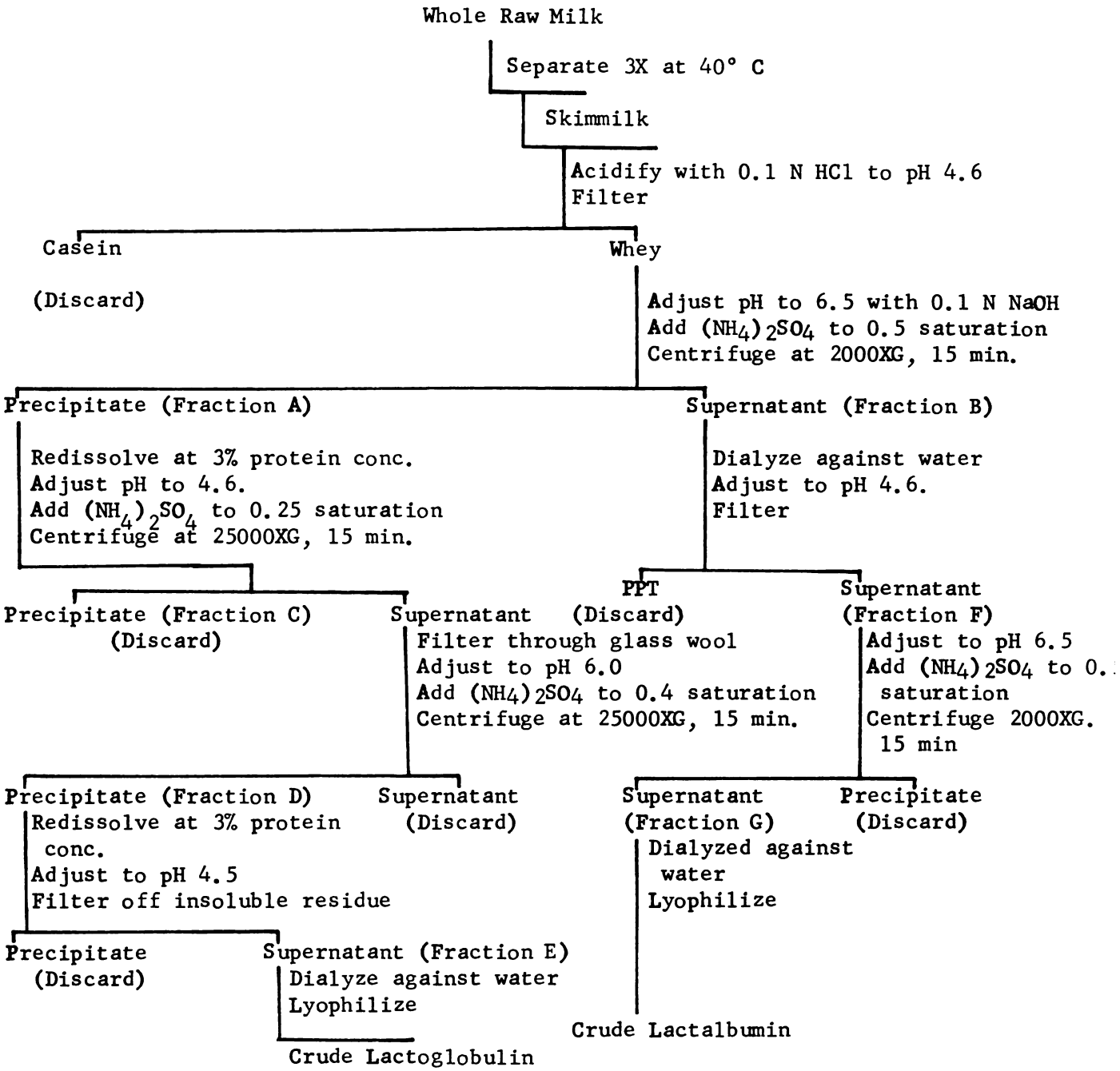


Figure 3. A schematic diagram showing the fractionation of cows' milk with ammonium sulfate (88).

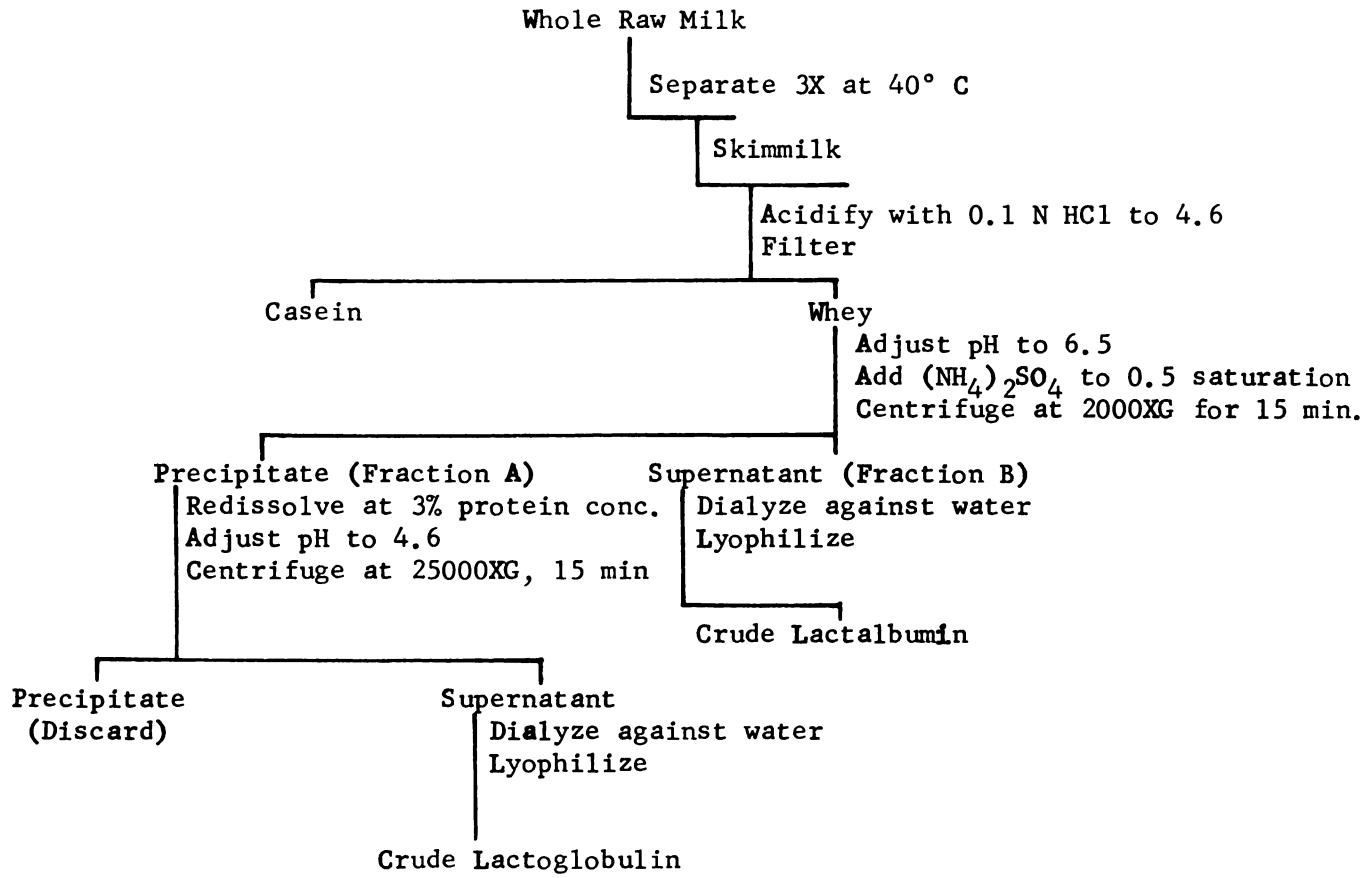


Figure 4. A schematic diagram showing the fractionation of cows' milk with ammonium sulfate (88).

EXPERIMENTAL RESULTS

Experiment 1

Four different samples of pasteurized whole milk were found to contain 212, 234, 338 and 531 mμg of total Vitamin B_{12} per 100 ml of milk. The average value obtained was 329 mμg per 100 ml of pasteurized whole milk.

Experiment 2

The content of total and free Vitamin B_{12} in pasteurized cows' whole milk is shown in Table 4. Values for the total Viatmin B_{12} content were found to be similar for the three methods used for the release of the vitamin (extraction with cyanide and HCl, extraction with cyanide and NaAc, and digestion with papain). The extraction method with cyanide and hydrochloric acid was used in all subsequent experiments. Seitz filtration was considered to be the method of choice for preparation of samples for Vitamin B_{12}, because of the small variation in the values obtained by this method and its simplicity as compared to the ultrafiltration method in maintaining aseptic condition.

Experiment 3

The amounts of total and free Vitamin B_{12} in raw skimmilk, raw whole milk, pasteurized skimmilk and pasteurized whole milk are shown in Table 5. The different samples gave similar results. However, to assure the greatest similarity in the history of milk samples, raw

milk obtained immediately after milking was used in all future experiments. The content of free Vitamin B_{12} was approximately five per cent of the total Vitamin B_{12}.

Experiment 4

The results for the total and free Vitamin B_{12} content of casein and whey samples which were prepared from pasteurized skimmilk by three different methods--ultracentrifugal method, rennin coagulation method, and isoelectric precipitation method-- are shown in Tables 6 and 7. The ultracentrifugal method gave the lowest content of Vitamin B_{12} in casein. The Vitamin B_{12} content of casein and whey prepared by the isoelectric precipitation of casein was in good agreement with the Vitamin B_{12} content of the skimmilk from which they were prepared. Casein contains slightly larger amounts of Vitamin B_{12} than the whey remaining after the precipitation of the casein. For further study the isoelectric precipitation method was used for preparing casein samples, since this was the simplest method. Table 7 shows that whey protein contains more Vitamin B_{12} than casein protein when expressed in $\mu\mu$g/mg of protein. Whey samples were used in future experiments, since the B_{12} content in whey proteins was higher than that in casein for all samples tested on the basis of B_{12} concentration per mg of protein.

Experiment 5

In the first preparation of crude lactalbumin and lactoglobulin from raw whole milk, 3.8 grams of crude lactalbumin and 0.5 grams of lactoglobulin were obtained from 3,400 ml of whey. In the second preparation, 4.0 grams of crude lactalbumin and 0.7 grams of crude lactoglobulin were obtained from 3,400 ml of whey.

The moisture contents were 8.3% and 15% in crude lactalbumin and lactoglobulin, respectively, for the first preparation, and 11.1% and 11.1% in crude lactalbumin and lactoglobulin, respectively, for the second preparation. Protein contents, obtained by multiplying the nitrogen content by 6.38 of the crude proteins were 99.5% and 90.0% in crude lactalbumin and lactoglobulin, respectively, for the first preparation, and 94.4% and 85.5% in crude lactalbumin and lactoglobulin, respectively, for the second preparation. The free boundary electrophoretic mobilities of these proteins are given in Table 8, and mobility patterns are shown in Figure 5. The proteins present in each of the fractions were identified from the descending electrophoretic patterns (14). Four bands were obtained from crude lactalbumin in the first and second preparations by zone electrophoretic study. They were tentatively identified as proteose-peptone, blood serum albumin, mixture of β-lactoglobulin A and B and α-lactalbumin. When the crude lactoglobulin fractions from the first and the second preparation were examined, the bands were spread out and no clear zones were obtained. The total and free Vitamin B_{12} contents of each fraction are shown in Table 9. It will be noted that crude lactalbumin was found to contain more Vitamin B_{12} per mg than crude lactoglobulin in this experiment.

Experiment 6

By ammonium sulfate fractionation, 2,990 ml of whey, 352.4 grams of moist-crude casein, 19.0 grams of crude lactalbumin, and 4.7 grams of crude lactoglobulin were obtained from 3,500 ml of skimmilk. The protein content for each fraction is shown in Table 10. Free boundary electrophoresis showed proteose-peptone and α-lactalbumin in the fraction of crude lactal-

bumin. Immune globulin or proteose-peptone or a mixture of these and α-lactalbumin were identified in the lactoglobulin fraction. Absolute identification is difficult since the descending mobilities of these proteins are very similar. The mobilities are shown in Table 11, and the mobility patterns are shown in Figure 6. By zone electrophoresis, five bands were observed in both fractions. The content of total and free Vitamin B_{12} in the skimmilk, casein, whey, crude lactalbumin, and lactoglobulin is tabulated in Tables 12 and 13. The total Vitamin B_{12} in casein and whey accounted for 95% of the total Vitamin B_{12} present in skimmilk. Total Vitamin B_{12} in the crude lactalbumin plus that in the crude lactoglobulin fraction accounted for 90% of the total Vitamin B_{12} found in the whey. Whey proteins again were shown to contain more Vitamin B_{12} per unit of protein than casein. Total Vitamin B_{12}, uncorrected to unit protein concentrations, in the crude lactalbumin fraction is twice as high as that found in crude lactoglobulin. A very high concentration of free Vitamin B_{12} was shown in casein. The values for free Vitamin B_{12} are very low in most cases, and precise values are difficult to obtain (Table 13). Data for total and free Vitamin B_{12} are shown in Table 14 and data for total B_{12} were also calculated in μμg B_{12} per mg on a dry protein basis in each fraction (Table 16). As is shown in the table, whey protein contains twice as much total Vitamin B_{12} per mg as casein; crude lactoglobulin contains more total Vitamin B_{12} per mg than crude lactalbumin.

The highest total Vitamin B_{12} content per mg was found in crude lactoglobulin; next highest, in crude lactalbumin; next, in whey; then skimmilk; and the smallest total Vitamin B_{12} content was observed in

the casein (all values were compared on the basis of Vitamin B_{12} content per mg of protein). Both total and free Vitamin B_{12} contents are high in the whey and crude lactoglobulin fraction.

Experiment 7

From the ammonium sulfate fractionation procedure less than 5 ml of immune globulin and α-lactalbumin solution in veronal buffer were obtained. The protein content in the immune globulin and α-lactalbumin solutions was 0.8 mg/ml and 1.26 mg/ml, respectively. The mobility patterns of crude lactoglobulin, immune globulins, and α-lactalbumin are shown in Figure 7. The total Vitamin B_{12} content in each fraction is shown in Table 17. As indicated in the table the immune globulin fraction contains approximately five times more Vitamin B_{12} than α-lactalbumin.

Table 4. Total and free Vitamin B_{12} in pasteurized whole milk (All values expressed as mμg per 100 ml of pasteurized whole milk).

	Total			Free	
	Treated with cyanide and HCl soln.	Treated with cyanide and NaAc soln.	Treated with papain	Ultrafiltered	Seitz filtered
Average	273	255	267	16	14
Range	210-360 (5)	180-294 (3)	255-303 (3)	8-28 (3)	4-17 (3)

Numbers in the parentheses are number of individual samples used in this study.

Table 5. Total and free Vitamin B_{12} content of raw skimmilk, raw whole milk, pasteurized skimmilk, and pasteurized whole milk.

B_{12} mμg/100 ml	Raw		Pasteurized	
	Skimmilk	whole milk	Skimmilk	whole milk
Total	514	504	550	515
Free	35	21	23	22

Table 6. Mμg of total and free Vitamin B_{12} in skimmilk, casein and whey contained in 100 ml skimmilk and percentage of total Vitamin B_{12} accounted for in fractions.

	Skimmilk	Ultracentrifuged		Rennin Coagulated		I.E.P. Precipitated	
Number of experiment		Casein	Whey	Casein	Whey	Casein	Whey
			Total				
1	332	128	120	156	139	147	103
2	350	156	-	156	102	217	131
3	332	125	-	147	171	176	127
4	350	140	139	146	140	187	147
Mean and	341	137	130	151	138	182	127
Std. dev.	± 9	±12	±10	±5	±25	±13	±16
% of total vitamin B_{12} accounted for in fractions	100	40	38	44	41	53	37
			Free				
1	21	2	10	12	11	50	14
2	26	6	13	5	14	-	18
Mean	24	4	12	9	13	50	16

Table 7. Total Vitamin B_{12} calculated in μμg per mg of casein, skimmilk protein and whey protein.

	Skimmilk	Ultracentrifuged		Rennin Coagulated		I.E.P. Precipitated	
		Casein	Whey	Casein	Whey	Casein	Whey
B_{12} μμg/mg	87	47	139	51	147	61	135

Table 8. Electrophoretic mobilities of crude lactalbumin and lactoglobulin fractions of cows' milk in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$, at 1° C.

Fraction	Electrophoretic Mobility Ascending ($x10^{-5}$)	Descending ($x10^{-5}$)	Protein Present (calculated from descending mobility)	Literature Value μ(14)	
1st Preparation					
lactalbumin	-4.12	-3.90	α-lactalbumin	α-lactalbumin	- 4.2
	-5.58	-5.49	β-lactoglobulin A or B, or mixture of A and B	β-lactoglobulin A	- 5.3
				β-lactoglobulin B (mixed A and B)	- 5.2
lactoglobulin	-2.21	-2.0	pseudoglobulin or mixture of pseudo-euglobulin	pseudoglobulin	- 2.0
				euglobulin	- 1.8
			α-lactalbumin	blood serum albumin	- 6.7
2nd Preparation					
lactalbumin	-3.25	-4.0	α-lactalbumin		
	-4.17	-5.4	β-lactoglobulin A or B, or mixture of A and B		
	-5.46	-7.2	Blood serum albumin		
lactoglobulin	-2.30	-2.13	pseudoglobulin or mixture of pseudo-euglobulin		
	-2.36	-4.23	α-lactalbumin		

Table 9. Total and free Vitamin B_{12} in μμg per mg of protein for whey, crude lactalbumin and lactoglobulin.

Prep. No.	Total			Free	
	whey	lactalbumin	lactoglobulin	lactalbumin	lactoglobulin
1	272	233	157	9	20
2	272	263	205	10	18

Table 10. Percent protein in various milk fractions on dry basis.

Skimmilk Solids	Casein	Whey Solids	Lactalbumin	Lactoglobulin
35.9	90.0	11.9	77.9	84.5

Table 11. Electrophoretic mobilities of crude lactalbumin and lactoglobulin of cows' milk in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$, at 1° C

Fraction	Electrophoretic mobility Descending ($\times 10^{-5}$)	Protein present determined from descending mobility
lactalbumin	-3.0	proteose-peptone (-3.0)
	-4.1	α-lactalbumin (-4.2)
lactoglobulin	-2.6	pseudoglobulin (-2.0)
		euglobulin (-1.8)
		proteose-peptone (-3.0)
	-4.5	α-lactalbumin (-4.2)

Data in parenthesis are from literature (14).

Table 12. Total and free Vitamin B_{12} in protein fractions obtained from 100 ml skimmilk and percentage accounted for in various fractions

Fractions	Skimmilk	Casein	Whey	Lactalbumin	Lactoglobulin
			Total		
B_{12} mμg/100 ml	545	243	265	155	84
Total in fractions		518		239	
% to total skimmilk	100	46.3	48.6	28.4	15.3
% in fractions		95.0		43.7	
% to total whey			100	58.4	31.5
% in fractions				90.0	
			Free		
B_{12} mμg/100 ml	10	81	19	1	3

Table 13. Total and free Vitamin B_{12} in μμg per mg of protein from skimmilk and protein fractions

Assayed	Skimmilk	Casein	Whey	Lactalbumin	Lactoglobulin
			Total		
B_{12} μμg/mg	160	105	404	461	550
			Free		
B_{12} μμg/mg	3	3	35	3	26

Table 14. Total Vitamin B_{12} in protein fractions obtained from 100 ml skimmilk and percentage accounted for in various fractions

Fractions	Skimmilk	Casein	Whey	Lactalbumin	Lactoglobulin
B_{12} mμg/100 ml	543	198	287	190	58
% to total skimmilk	100	36.5	52.9	35.0	10.7
% in fractions		89.4		45.7	
% to total whey			100	66.2	20.2
% in fractions				86.4	

Table 15. Percent protein in various milk fractions on dry basis

Skimmilk solids	Casein	Whey Solids	Lactalbumin	Lactoglobulin
38.0	101.5	12.3	74.2	85.2

Table 16. Total Vitamin B_{12} in μμg per mg of protein from skimmilk and protein fractions

Assayed	Skimmilk	Casein	Whey	Lactalbumin	Lactoglobulin
B_{12} μμg/mg	143	65	333	370	430

Table 17. Total Vitamin B_{12} in μμg per mg of protein from skimmilk and protein fractions

Assayed	Skimmilk	Casein	Whey	Crude lactalbumin	Crude lactoglobulin	Immune globulin	α-lactalbumin
B_{12} μμg/mg	160	105	404	461	550	392	80

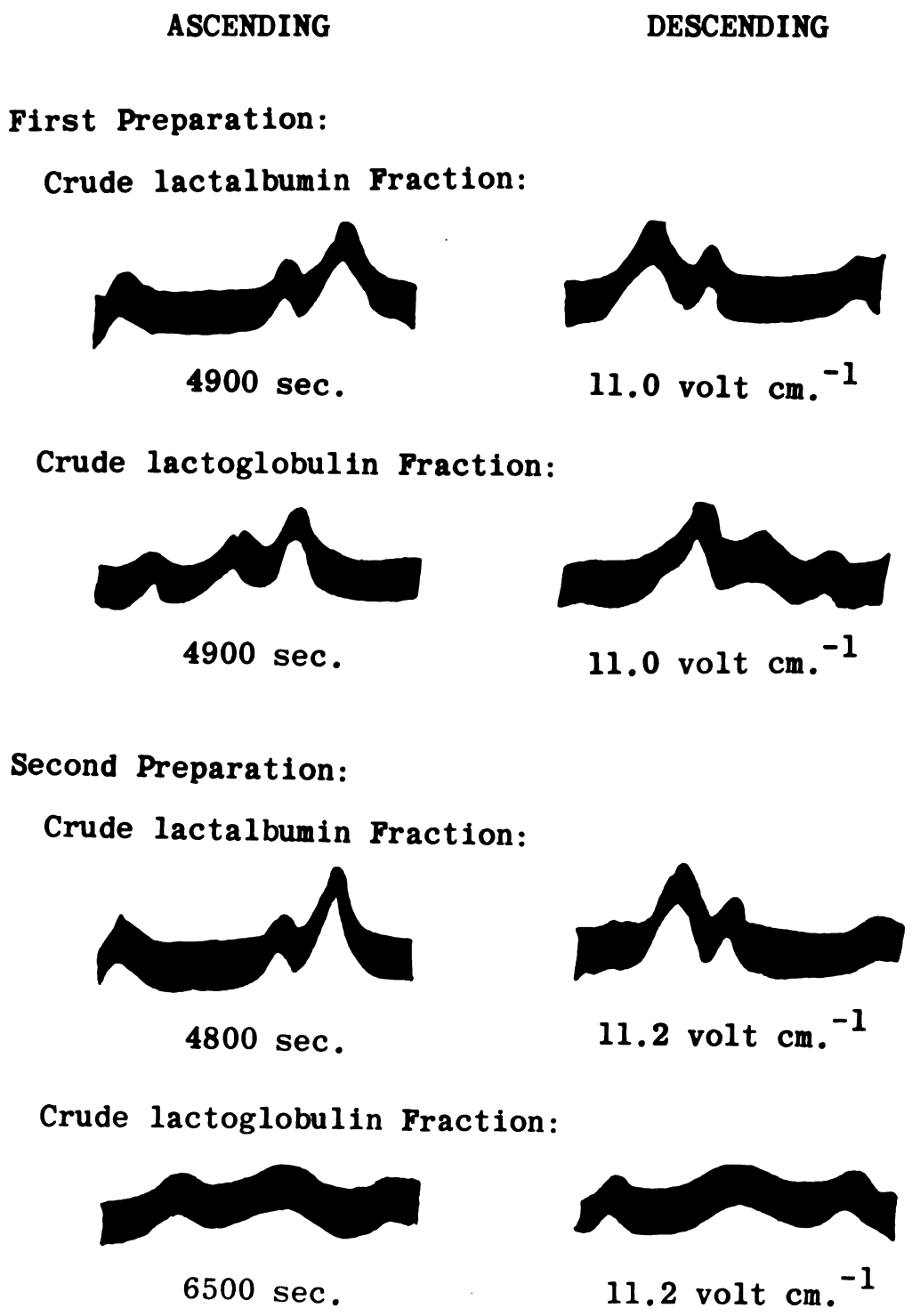


Figure 5. Electrophoretic patterns of crude lactalbumin and lactoglobulin in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$

ASCENDING DESCENDING

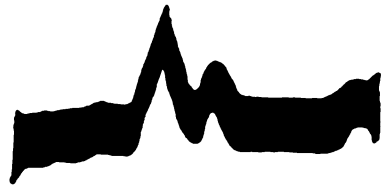
Crude lactalbumin Fraction:

5500 sec.

11.0 volt $cm.^{-1}$

Crude lactoglobulin Fraction:

4400 sec.



11.0 volt $cm.^{-1}$

Figure 6. Electrophoretic patterns of crude lactalbumin and lactoglobulin in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$

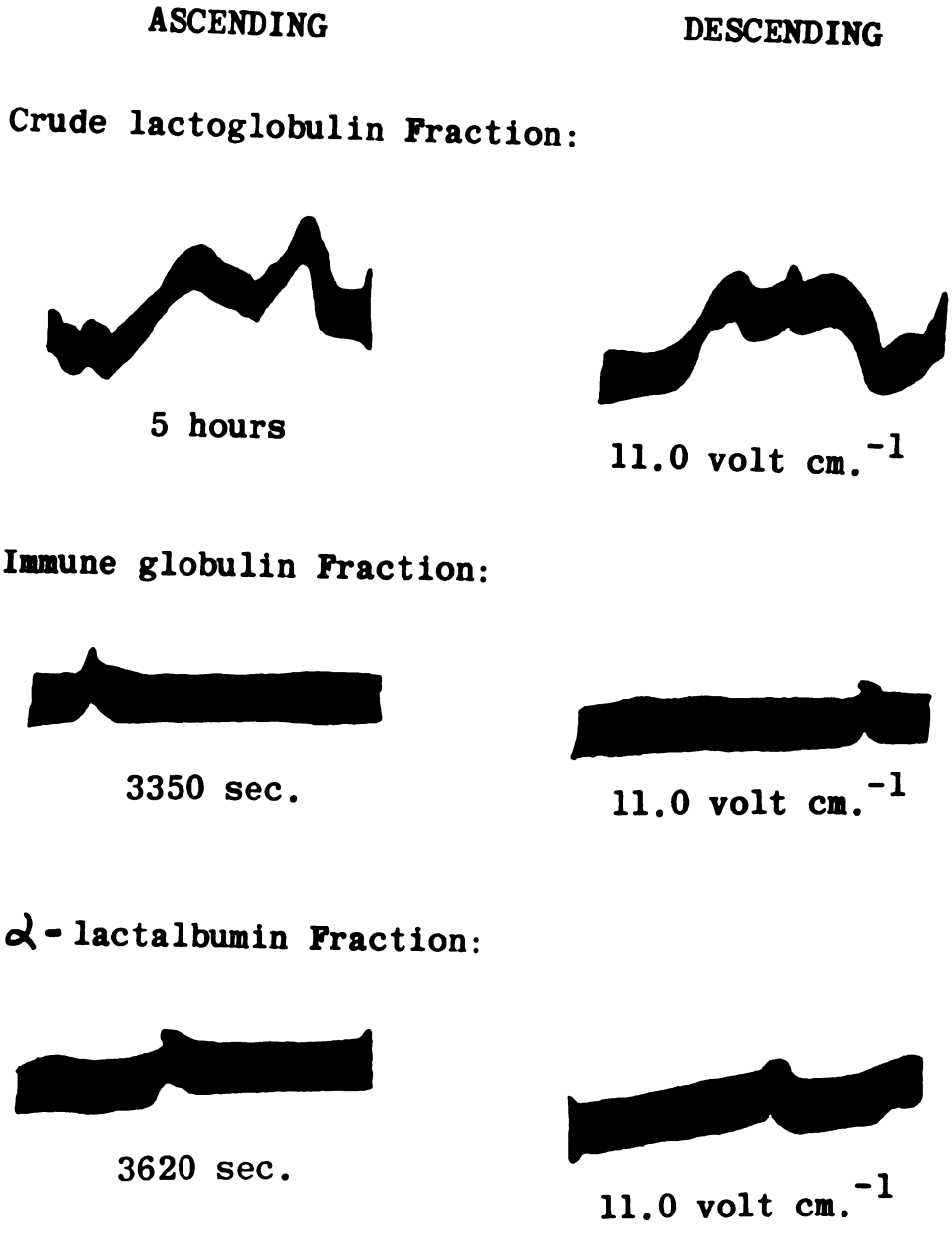


Figure 7. Electrophoretic patterns of crude lactoglobulin, immune globulin and α-lactalbumin in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$

DISCUSSION

The Vitamin B_{12} content of milk varies from species to species and from animal to animal. The vitamin content of milk from the same animal apparently varies with the season, the lactation period, the time of milking, and feeding regimen. The Vitamin B_{12} content in milk was reported to be significantly increased by adding cobalt in the feed. In spite of these factors, the values for total Vitamin B_{12}, 255-550 mμg per 100 ml of whole milk or skimmilk, from our experiments (Tables 4, 5, 6, 12, 14) were in good agreement with other worker's results (Table 3).

Samples of milk proteins were autoclaved after the addition of cyanide and HCl, in order to release the bound Vitamin B_{12} and convert any hydroxocobalamin into the more stable cyanocobalamin. Extraction by autoclaving with cyanide and HCl, or with cyanide and NaAc, and digestion with activated papain (Table 4) were satisfactory methods for this purpose. Recoveries of cyanocobalamin added before extraction by autoclaving with cyanide and HCl, the method selected for our experiment, were between 95-105% of the original amount added.

The free Vitamin B_{12} content in Experiment 2 was approximately 5% of the total Vitamin B_{12} content (Tables 5 and 6). Both methods, Seitz filtration and ultrafiltration, gave 60-80% recoveries of standard cyanocobalamin added to milk extracted by the cyanide and HCl autoclaving method. Gregory (32) described that when cows' milk was extracted by cyanide or papain before ultrafiltration, 50 to 75% of the total Vitamin B_{12} present in the milk could be ultrafiltered. An explanation for this

low recovery could be that some substances present in regenerated cellulose tubing combine with free Vitamin B_{12}. The same phenomenon could have occurred in the pad of the Seitz filtrater when free Vitamin B_{12} is passing through the pad of the Seitz filter.

Table 5 shows slightly different total Vitamin B_{12} contents between raw and pasteurized whole milk and between raw and pasteurized skimmilk. The Vitamin B_{12} content in raw and pasteurized skimmilk is slightly higher than in raw and pasteurized whole milk. This difference could be accounted for on the basis of dilution by the fat phase.

The data in Table 6 show that three different methods of whey preparation gave slight but probably nonsignificant differences in Vitamin B_{12} content, ranging from 102 to 171 mμg of total Vitamin B_{12} in the whey prepared from 100 ml of skimmilk. Casein contained more Vitamin B_{12} than whey when prepared by rennin coagulation or isoelectric preparation. The data in the table show that casein and whey contain approximately the same amount of Vitamin B_{12} when these fractions were prepared by centrifugation. The free Vitamin B_{12} in isoelectrically prepared casein showed an extremely high value. No rationale is apparent to explain this observation. Whey samples showed higher free Vitamin B_{12} contents than the casein preparations except for the casein prepared by isoelectric precipitation.

As shown in Table 7, whey protein contains two to three times more Vitamin B_{12} than casein from all preparations when the amount of the Vitamin per mg of protein is considered.

From the data in Tables 12 and 14 it could be concluded that Vitamin B_{12} is distributed among all the milk proteins, or that the specific protein-B_{12} adsorption complex was a contaminant in all preparations, since the Vitamin B_{12} content in skimmilk is randomly distributed approximately half and half between casein and whey. When the Vitamin B_{12} content was calculated per mg of milk protein, it is two to three times higher in whey protein than in casein as shown in Tables 7, 13, 14 and 17, and lactalbumin contains two to three times more Vitamin B_{12} than lactoglobulin in skimmilk. Lactalbumin contains more Vitamin B_{12} than lactoglobulin on a dry basis (Table 9), but, contrarily, a higher Vitamin B_{12} content was observed on the second experiment for lactoglobulin than lactalbumin on a dry basis (Tables 13, 16 and 17).

It is accepted that Vitamin B_{12} is synthesized in the digestive tract by rumen microorganisms, and it is carried to the mammary gland through the blood stream. The proteins (58) present in milk have been considered not only to arise from the synthetic activities of the mammary gland but also to include some preformed which enter the gland from the blood. The isolation and recognition in recent years of 97% of the protein of cows' skimmilk as specific chemical and biological entities have made it possible now to determine which of these proteins are synthesized in the mammary gland. Larson et al. (51) described from their radioisotope C^{14} experiment that the levels of C^{14} incorporated by α-casein, β-casein, α-lactalbumin and β-lactoglobulin suggest that these milk proteins are synthesized in the mammary gland from a common amino

acid pool. The levels of C^{14} incorporated by γ-casein, the immune globulins, and milk serum albumin, and similarity with the levels present in the blood proteins, suggest these proteins enter the milk preformed from the blood stream.

Vitamin B_{12} is possibly carried by the blood proteins and interchanged between the blood proteins and milk proteins synthesized by the mammary gland.

In one experiment lactalbumin was found to contain more Vitamin B_{12} than lactoglobulin (Table 9), while in other experiments lactoglobulin was found to contain more Vitamin B_{12} than lactalbumin (Tables 13 and 16). Whey proteins contained two to three times more Vitamin B_{12} than casein (Tables 7 and 13) and five times more Vitamin B_{12} in another experiment (Table 16). Probably no equilibrium exists between the Vitamin B_{12} bound with different milk proteins. Vitamin B_{12} is being randomly distributed among the binding sites, or, also, possibly the binding state of Vitamin B_{12} and different milk proteins is changed continuously with some biochemical mechanism.

Distribution of Vitamin B_{12} between different proteins of the same biological systems has been found in serum. Cresseri (21) described that six different fractions with microbiologically available Vitamin B_{12} exist in the serum of the normal rabbit, and Rosenthal (69) reported that more than one major binding site exists in the serum of man, dog, rabbit, frog and chick.

These reports are consistent with the concept that the cyanocobalamin-binding phenomena in sera is associated with multiple binding sites that may be different for various animal species and that special orientation

between the protein and cyanocobalamin moieties is necessary for binding to occur.

It appears from the results of this study that Vitamin B_{12} is distributed among more than one milk protein and that equilibrium does not exist between Vitamin B_{12} bound with different milk proteins, since there is no regularity in the distribution of the vitamin between different milk proteins.

The evidence suggests that Vitamin B_{12} is carried into milk via blood proteins (immune globulin, serum albumin, and possibly a blood protein in the casein complex) in the mammary gland.

For further study preparation of more highly purified milk protein is very necessary. Purified protein will give more information of the occurrence of B_{12} in milk protein and relationships between milk and blood proteins. Sampling of milk at certain intervals obtained from the same cow may also be of value to determine changes in Vitamin B_{12} binding between different milk proteins. If blood and milk are sampled at the same time from the same cow, important relationships in Vitamin B_{12} binding by blood and milk proteins may be obtained.

CONCLUSIONS

1) Extraction by autoclaving with cyanide and HCl was a satisfactory method for the release and conversion of the bound form of Vitamin B_{12} to cyanocobalamin in cows' milk prior to microbiological assay.

2) The Vitamin B_{12} content of whole milk or skimmilk was found to range from 255 to 550 mμg per 100 ml. The free vitamin accounted for 5% of the total Vitamin B_{12} content in whole milk or skimmilk.

3) Slightly higher Vitamin B_{12} contents in raw skimmilk and pasteurized skimmilk than in raw whole milk and pasteurized whole milk were found.

4) Higher Vitamin B_{12} contents in casein than in whey were obtained from a given amount of skimmilk by rennin coagulation or isoelectric precipitation than by ultracentrifugation. Vitamin B_{12} content in whey is two to three times higher than in casein when the results are expressed per unit weight of protein.

5) The Vitamin B_{12} content in crude lactalbumin is higher than in lactoglobulin from a given amount of whey.

6) Immune globulin contains more Vitamin B_{12} than α-lactalbumin.

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