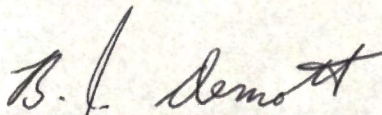


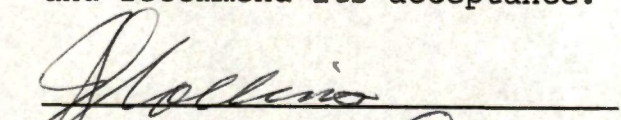
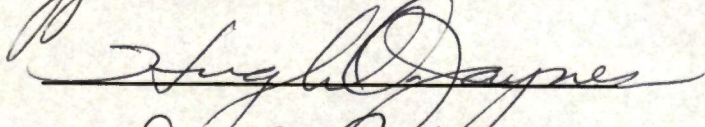
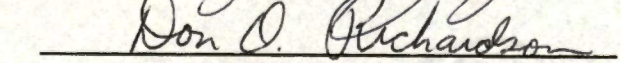
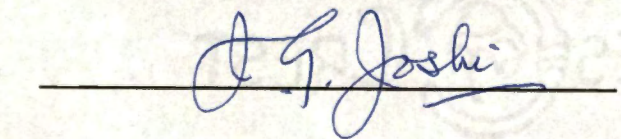
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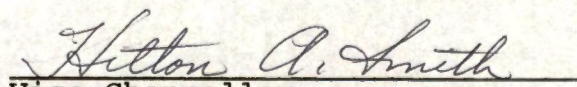


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Major Professor

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CHROMATOGRAPHIC IDENTIFICATION OF ADDED
IRON IN CASEIN AND CASEIN COMPONENTS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee

Burhan Dincer

December 1975

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ABSTRACT

The objective of this study was to determine the distribution of added radioiron in milk protein and casein components of cow's milk fractionated by the combination of Sephadex G-150 gel filtration and DEAE-cellulose chromatography.

Labelled $^{59}\text{FeCl}_3$ was added directly to raw milk at the concentration of 0.02 and 10 ppm isotope and carrier, respectively, and held overnight at 4°C. Five ml of the skim milk was first chromatographed on Sephadex G-150 column and fractionated into casein, whey protein, and nonprotein materials. The casein fraction was chromatographed on a DEAE-cellulose column and separated into its components, α_s , β -, and κ -caseins.

Radioactivity was determined first in skim milk and later in casein, whey protein, and casein components.

The data indicated that most of the added iron was associated with skim milk. Sephadex G-150 gel permeation of skim milk showed that the casein was associated with more than 85 percent of the activity in the skim milk. DEAE-cellulose chromatography of casein showed that the added iron was bound mostly to α_s -casein and β -casein. Surprisingly, very little radioactivity, about 5 percent, was bound to κ -casein.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
II. REVIEW OF LITERATURE	3
Distribution and Chemical Association of	
Natural and Added Iron in Milk	3
Components of Casein Protein Complex	5
Casein Micelles	10
Use of Gel Permeation and DEAE-Cellulose	
Chromatography for Separating Milk	
Proteins	12
Ion-Protein Binding Studies by Gel Filtration	
and Ion-Exchange Chromatography	16
III. MATERIALS AND METHODS	19
Preparation of ⁵⁹ Fe Labelled Milk	19
Fractionation of Milk	19
Gel filtration of skim milk on	
Sephadex G-150	21
Column chromatography on diethylaminoethyl	
(DEAE)-cellulose	24
Acrylamide Gel Urea Electrophoresis	27
IV. EXPERIMENTAL RESULTS	29
Separation of Casein From Skim Milk by	
Sephadex G-150 Gel Filtration	29

CHAPTER	PAGE
Separation of Casein into Its Components by DEAE-Cellulose Column Chromatography . .	31
Distribution of Added $^{59}\text{FeCl}_3$ in Skim Milk and Casein Components	33
V. DISCUSSION	40
VI. SUMMARY	47
LIST OF REFERENCES	49
VITA	58

LIST OF TABLES

TABLE	PAGE
1. Relative Amounts of the Three Skim Milk Fractions with the Distribution of ^{59}Fe Among Them Eluted From Sephadex G-150 Column	32
2. Relative Amounts of the Four Casein Fractions with the Distribution of ^{59}Fe Among Them Eluted From DEAE-Cellulose Column	34
3. Amount of Radioactivity Removed From Casein Containing ^{59}Fe by Dialysis and the DEAE-Cellulose Chromatography	39



LIST OF FIGURES

FIGURE	PAGE
1. Summarized scheme for the separation of casein and its components from milk	20
2. Chromatogram of 5 ml of skim milk eluted from a Sephadex G-150 column (2.6 x 30 cm) with deionized water at 4°C.	23
3. Chromatogram of Sephadex casein (fraction A from Figure 2) eluted from DEAE-cellulose column (1.6 x 28 cm) by a gradient of NaCl in 0.01 M imidazole-HCl buffer containing 3.3 M urea and 0.01 M 2-mercaptoethanol at 4°C.	26
4. Acrylamide gel urea electrophoretic pattern of whole casein, Sephadex casein fraction, and casein fractions obtained by DEAE-cellulose chromatography: W - whole acid casein; A - Sephadex casein; III - β -casein; IV - α_s -casein II - κ -casein	30
5. Chromatogram of 5 ml skim milk containing ^{59}Fe eluted from Sephadex G-150 column (2.6 x 30 cm) with deionized water at 4°C.	35
6. Chromatogram of casein containing ^{59}Fe obtained from Sephadex G-150, eluted from DEAE-cellulose	

FIGURE

PAGE

column (1.6 x 28 cm) with NaCl gradient in
0.01 M imidazole-HCl buffer (pH 7.0) containing
3.3 M urea and 0.01 M 2-mercaptoethanol. . . . 38

CHAPTER I

INTRODUCTION

Milk, which is one of the best sources of many nutrients, is poor in iron, ranging from 100 to 900 μg iron per liter (2, 5, 34). For this reason, fortification of milk and milk products has been proposed as a means of correcting iron deficiency anemia (13, 15, 18, 72).

The distribution and chemical association of iron added to milk and milk fractions has been studied by a number of workers. The early concept was that both added and natural iron were combined with the fat globule membrane and they were nonionic (1, 36). Later, the added iron was found to be mostly associated with the milk proteins (72). More recently, Demott and Park (14) and Basch (5) reported that added iron was primarily associated with the milk proteins and that casein bound about 70 percent of the iron.

Casein, the major protein in milk, has been accepted as a good source of essential amino acids and widely used as a protein supplement in many food products (22, 23). It occurs in milk as a colloidal calcium phosphate complex, the casein micelle (7, 40). Because of its great nutritional importance and its complexity, the separation and purification of the casein micelle into its components has become an

important subject in the study of proteins. Even now, scientists are not certain that all the components of the casein micelle have been separated and identified. However, in recent years, gel permeation and ion exchange chromatography have been used as simple and economical means of separating and identifying casein components (57, 82, 83, 84).

The purpose of this study was the identification of added radioiron in casein and casein components of cow's milk fractionated by Sephadex gel permeation and DEAE-cellulose chromatography.

CHAPTER II

REVIEW OF LITERATURE

I. DISTRIBUTION AND CHEMICAL ASSOCIATION OF NATURAL AND ADDED IRON IN MILK

Iron was the first of the trace elements to be recognized as essential to life functions (34). It was found in milk by Bunge in 1889 (2, 9, 34). Cow's milk is a poor source of iron, containing only 100 to 900 μg per liter (5, 9, 34, 47). The iron in milk exists in the form of complexes with proteins (33), such as lactoferrin (red protein) and serum transferrin. It was also found in the enzymes of milk such as xanthine oxidase, peroxidase, and catalase (5, 25, 27). Natural iron was not dialyzable at the normal pH of milk (36).

Allan (1) studied the kinetics of ascorbic acid oxidation in milk and butter serum. He found that both added and natural iron were associated with the fat globule membrane and were nonionic. Van der Waarden (71), investigating the distribution of natural and added iron and copper in butter, found that the proteins contained all of the iron. In a similar study, Davies (12) reported that added copper and iron were uniformly distributed in gravity-separated milk in direct proportion to the curd nitrogen. Herald et al. (28) found that the trace elements, copper, iron, and zinc, were

concentrated on the fat globule membrane possibly as constituent elements of various metabolic complexes.

King et al. (36) studied the distribution of iron in cow's milk after addition of ^{59}Fe as ferric chloride and found that most of the added iron was associated with the skim milk; casein accounted for most of the radioactivity in the original milk and iron added to milk was slightly dialyzable.

Ferrous iron in a prepared infant formula was reported by Vaughan and Knauff (72) to be associated almost entirely with the milk proteins. Before protein hydrolysis, only 3 percent of the added iron was dialyzable against either water or citrate solution, but after hydrolysis in vitro, 20 percent of the iron was dialyzable against water and 79 percent against citrate solution. They concluded that the iron was available nutritionally after the protein portion of the complex was digested.

More recently, Demott and Park (14) investigated the influence of processing upon the association of added iron with various milk protein fractions and found that skim milk contained about 87 percent of the added iron. Casein bound most of the iron. Isoelectric casein bound about 67 percent of the iron, whereas, rennin coagulated casein contained about 70 percent of the iron.

Basch et al. (5) studied the distribution of iron and polyphosphate phosphorus added to cow's milk and found that casein had a greater affinity for iron than for phosphorus: 85 to 90 percent of the added iron was bound to acid precipitated casein while 60 to 70 percent of the iron was bound to centrifugal casein.

II. COMPONENTS OF CASEIN PROTEIN COMPLEX

Milk and milk products provide proteins of excellent quality for nutrition of man. Casein, the principle protein of milk, makes up 80 percent of total protein content of milk which in turn contains 3.5 percent protein (7). It has been known as a good source of essential amino acids and has been used widely in food products as a protein supplement. Such food includes processed meat, breakfast cereals and coffee whiteners. The annual output of casein on the world market in 1970 was about 272 million tons. The United States consumes 40 percent of this production and one-third of this 40 percent is used in the food industry (22, 23).

Casein occurs in milk as a colloidal calcium phosphate complex which contains about 7 percent inorganic materials of which the most important are calcium, phosphate, and magnesium (7, 42). It has been defined as a heterogeneous

phosphoprotein complex (casein micelle) precipitated from skim milk at pH 4.6 at 20°C (26, 40).

Casein was long considered to be a pure protein (31), but Mellander (43) demonstrated the heterogeneity of bovine casein by using a Tiselius free-boundary electrophoresis cell and separated three fractions, α -, β -, and γ -caseins in order of decreasing mobilities. Following this impetus, Warner (73) succeeded in obtaining the α - and β -casein fractions by controlling the solubility through careful pH adjustment, and Hipp et al. (31) reported separation of three fractions of casein using differential solubility properties in alcohol and urea solutions. Waugh and Von Hippel (76) found that α -casein was a mixture of α_s which is now called α_{s_1} -casein (26) and κ -casein. From all the work on the characterization of casein, three major components of the casein protein complex have been described, namely α_{s_1} -, β -, and κ -casein.

The major protein of cow's milk, α_{s_1} -casein is insoluble in the presence of calcium ion concentration occurring in milk (16). α_{s_1} -casein was found as a single chain polypeptide with 199 amino acid residues and a molecular weight of 23,000 daltons (7, 16, 24, 44). The molecule of α_{s_1} -casein contained eight phosphate residues, seven of which occurred in a cluster between residues 43 and 80. This highly acidic segment also contained 12 carboxyl residues and accounted for almost all of the molecule's net negative charge (24, 44). The

hydrophobicity value of α_{s1} -casein was found very high by the method of Bigelow (6). The high degree of hydrophobicity exhibited by the C-terminal half of the molecule was probably responsible for the pronounced tendency to self-association of the α_{s1} -casein monomer in aqueous solution (16, 64). Schmidt (64) concluded that some ionic binding must occur in the reaction in addition to hydrophobic interaction. At calcium ion concentrations of 5 to 10 mM, α_{s1} -casein formed an insoluble precipitate (69) and calcium solubility of α_{s1} -casein was found temperature independent (64, 69).

A number of methods have been used for the preparation of the α_{s1} -casein. Waugh and his co-workers (77) described a unique preparation of α_{s1} -casein from the casein by using CaCl_2 . Zittle and Custer (85) developed a method in which urea, NaCl, and ammonium acetate were used and reasonably pure α_{s1} -casein was obtained in a relatively short time. α_{s1} -casein was also prepared using chromatographic methods. Thompson (68) purified α_{s1} -casein by direct chromatography of whole casein on a diethylaminoethyl (DEAE)-cellulose column. Fox et al. (19) fractionated α_{s1} -casein by combining Zittle and Custer's method (85) with DEAE-cellulose chromatography and concluded that isoelectric casein could be separated into its major components, such as α_{s1} -, β -, and κ -caseins. Rose et al. (62) separated the acid casein into three main

fractions α_{s1} -, β -, and κ -casein, and concluded that α_{s1} -casein comprised 50 percent of the total casein.

The second most abundant milk protein, β -casein, has been distinguished from the other caseins by the temperature dependence of its solubility in the presence of calcium ion. In an early study, Peterson et al. (56) isolated a phosphopeptide resulting from tryptic hydrolysis of β -casein. Later, Manson and Annan (39) isolated the same phosphopeptide from β -casein. More recently, Ribadeau-Dumas et al. (60) isolated this same phosphopeptide and determined the primary structure which yielded compositional values identical to those given previously by Peterson et al. (56). Ribadeau-Dumas et al. (60) showed that β -casein was a single chain polypeptide with a molecular weight of about 24,000 daltons containing five phosphoserine residues. β -casein contained 209 amino acids. The N-terminal portion of β -casein molecule contained essentially all of the protein's net charge while the C-terminal half of the molecule contained many apolar residues as demonstrated by its hydrophobicity (16, 17). The N-terminal concentration of charge and the highly hydrophobic C-terminal might account for the temperature dependence of the self-association of β -casein since hydrophobic interactions were temperature sensitive (16, 17, 54). β -casein was found to be insoluble at room temperature in the presence of calcium ion concentration below the

concentration in milk estimated that 28 to 30 mM. However, the calcium β -caseinate complex was found soluble at 1°C. This temperature dependence was probably due to the charge distribution of the β -casein monomer (69).

The first successful fractionation of β -casein was achieved by Warner (73). He added sodium hydroxide in a dilute solution of acid casein at pH 3.5 and 2°C. Under these conditions, β -casein was obtained from the supernatant. Warner's preparations (73) were not electrophoretically pure. Hipp, et al. (31) developed two methods. In the first method, separation was by using ethyl alcohol in the presence of ammonium acetate and β -casein was obtained from acid casein. The second method was based on urea fractionation. Whole casein was dissolved in 6.6 M urea and fractionation was achieved by diluting with water. Upon dilution to 3.3 M urea, β -casein was obtained again. Aschaffenburg (3) simplified the urea method of Hipp et al. (31) and the simplified method was found acceptable for fractionation of β -casein.

κ -casein, the third major component of the milk proteins, was found to be more complex than those of α_{s1} - and β -casein (74). κ -casein was discovered in 1956 when Waugh and Von Hippel (76) added 0.25 M calcium to casein which caused κ -casein to separate from micellar casein. Their discovery of κ -casein made possible a separation of whole casein into

fractions which were soluble and insoluble in the presence of different calcium ion concentrations (38; 76). The fraction soluble in the 0.25 M calcium chloride at 37°C was κ -casein and it was found to be the micelle stabilizer and substrate for rennin (76). κ -casein was reported to contain cystine (78). Kalan and Woychic (35) reported that the reduced κ -casein molecule contained 2-SH groups which could form disulfide bridges between molecules (66). It was also found as the major component of casein-containing carbohydrates (53). All the carbohydrate was associated with the macropeptide which is the soluble portion of κ -casein formed by rennin hydrolysis (30, 78).

After the discovery of κ -casein (76), several methods were developed for the isolation of the protein (78, 85). Zittle and Custer (85) dissolved whole casein in 6.6 M urea and when they added sulfuric acid until pH 1.5 was reached, the supernatant contained mostly κ -casein.

III. CASEIN MICELLES

The casein micelles of milk have been defined as the colloidal milk protein complex ranging in size from 1×10^7 to 3×10^9 daltons (26, 55, 74). There has been much speculation about the formation and structure of casein micelles since the discovery of κ -casein and its ability to stabilize and form casein micelles (4, 75, 76).

The formation of casein micelles is the result of the complex interaction between casein monomers, α_{s1} -, β -, and κ -caseins, and inorganic salts (7). Several methods have been developed to describe the micellar structure (74, 75). The core-coat model was proposed by Waugh et al. (75). They described that, in the absence of κ -casein, α_{s1} - and β -casein cores precipitated from casein solution. When κ -casein was present, colloidal stabilization occurred through the formation of a surface coat of κ -casein.

Rose (61) proposed a slightly different model which was based on polymer chains of β -casein with α_{s1} -caseins interacting with κ -casein. The internal structure was proposed as interwoven with calcium apatite chains of proteins.

A porous framework model was proposed by Garnier and Ribadeau-Dumas (21). In their models, κ -casein was the keystone of micelle structure, thus, trimers of casein were bound to three chains of α_{s1} - and β -caseins to form a large three dimensional network. Recently, Ashoor et al. (4) added a papain super polymer to hydrolyze micelles. They found that up to 70 percent of the total micelle has a constant average composition of α_s -, β -, and κ -caseins. From their results they concluded that κ -casein did not have a specific location in the casein micelle and the three major caseins, α_{s1} -, β -, and κ -casein, were distributed randomly throughout the micelles.

The casein micelles have been known to be very stable either at low or high temperature (20). This is thought to be due to the fact that the colloidal milk protein complex is composed of a mixture of calcium insoluble proteins, α_{s1} - and β -caseins, stabilized by a calcium soluble protein, κ -casein. The latter protein was readily split into two parts; para κ -casein and macropeptide, by the enzyme rennin, thus destabilizing and allowing coagulation to occur (17, 20). However, α_{s1} - and β -caseins were insoluble at calcium ion concentrations of 5 to 10 mM, whereas normal milk has a calcium ion concentration of about 30 mM. On the other hand, κ -casein was found completely soluble under similar conditions. At various κ/α_{s1} casein ratios, the κ -casein stabilized the α_{s1} -casein and formed a colloidal complex (16, 17).

IV. USE OF GEL PERMEATION AND DEAE-CELLULOSE CHROMATOGRAPHY FOR SEPARATING MILK PROTEINS

A number of classical methods have been applied for the fractionation of different individual milk proteins (31, 63, 73, 85). Although many milk proteins have now been isolated in a pure state, some are still only fractions of doubtful purity (32). However, in recent years gel filtration and ion exchange chromatography which have greater resolving capabilities on the protein mixtures, have been used

extensively for the isolation and purification of the individual milk proteins (82).

Gel filtration, which has also been called gel permeation chromatography, exclusion chromatography, and gel chromatography, is a technique in which separation of the protein depends primarily on molecular size. Large molecules cannot penetrate the gel particles and, therefore, they are eluted first. Smaller molecules, however, penetrate the gel particles to a varying extent, depending on their size and shape and, therefore, are eluted from gel particles in order of decreasing molecular size (58, 82).

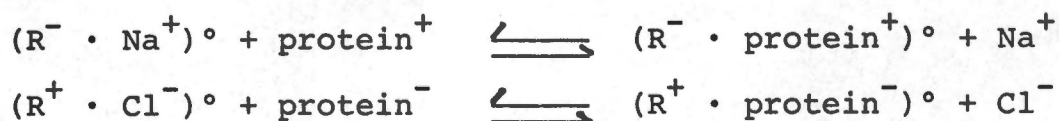
Gel filtration of skim milk and casein has been attempted by the use of different types of Sephadex, which is a trade name of dextran gel (45, 58). Hill and Hansen (29) devised a method for separating proteins of skim milk by use of Sephadex G-100 gel filtration. Four fractions were obtained; the first three corresponding to the casein micelle, lactoglobulin and lactalbumin, while the last contained no proteins. Sephadex G-75 and G-100 was used by Morr et al. (46) to determine the protein particle size distribution in skim milk. Sephadex G-75 failed to fractionate the skim milk satisfactorily. Sephadex G-100 fractionated skim milk into four heterogeneous components, but there was no clear information about the nature of the protein in each fraction. Yaguchi and Tarassuk (83) separated skim milk into six

fractions on Sephadex G-200; the first three of which contained casein. The last three fractions were β -lactoglobulin, α -lactalbumin, and the dialyzable nonprotein components, respectively. The behavior of skim milk passed through Sephadex G-100 and 200 was distinctly different, which might be due to types of Sephadex gels or temperature. Recently, Boulet et al. (8) eluted skim milk on Sephadex G-150. Three fractions were obtained. The first fraction was the casein complex; the second was whey proteins and the last peak was dialyzable materials in milk.

Gel filtration has been used to separate casein micelles by Nakai et al. (51); Nakahori and Nakai (49); Cheeseman (10); and Yaguchi et al. (81). These authors have proposed the necessity of the dissociation of the casein micelle into the monomeric caseins in the presence of dissociating agents such as urea and ethylenediaminetetraacetate (EDTA) to achieve their separation on gel filtration. Otherwise, separation was not possible on Sephadex gels. Nakahori and Nakai (49) separated the electrophoretically pure α_{s1} -casein from acid casein on Sephadex G-100 by eluting with 0.5 mM sodium dodecylsulphate (SDS). Nakai et al. (51) used phosphate buffer and isolated reasonably pure α_{s1} -, β -, and κ -casein from skim milk, however, additional procedures were used to purify the casein fractions after gel filtration.

Separation of proteins by ion-exchange chromatography involves the establishment of multiple electrostatic bonds

between ionized groups on the surface of the ion exchanger and opposite charges on the proteins which have different electrical properties and are released from the ion-exchangers by changes in salt concentration or pH of the eluant (57, 82). Schematically, this is shown as:



Where R^- and R^+ indicate a cation and anion exchanger, respectively (79).

Ion exchange chromatography has been used particularly for fractionation of the mixture of closely associated proteins, such as blood serum proteins and milk proteins (67, 84).

Yaguchi et al. (84) separated the casein fraction from the whey proteins without overlapping peaks and identified major components of casein on the elution pattern from DEAE-cellulose. However, their results indicated that the fractionation of milk proteins was not complete on the casein micelle, because of the strong tendency for casein monomers to aggregate. For this reason ion-exchange chromatography has also been carried out on the casein micelle in the presence of dissociation agents such as urea, sodium dodecylsulphate (SDS) with and without reducing agent, 2-mercaptoethanol (10, 84).

Ribadeau-Dumas et al. (59) reported the fractionation of whole casein by DEAE-cellulose chromatography in the presence of urea and a sodium chloride elution gradient. α_s - and β -casein were fractionated in a moderately pure form; κ -casein was not. It was found in the other fractions. Thompson and Kiddy (70) and Thompson (68) described the purification of α_s -casein and β -casein variants by DEAE-cellulose-urea chromatography with and without 2-mercaptoethanol. They concluded that DEAE-cellulose-urea chromatography containing 2-mercaptoethanol might be suitable for fractionation of whole casein into its major components. Rose et al. (62) eluted the alkylated and reduced whole casein from a DEAE-cellulose column with a sodium chloride gradient in buffer containing 6.6 M urea and obtained four fractions. The first was a slow moving component; the second contained κ -casein and probably some γ -casein; the third contained relatively pure β -casein and the last contained α_s -casein.

V. ION-PROTEIN BINDING STUDIES BY GEL FILTRATION AND ION-EXCHANGE CHROMATOGRAPHY

Proteins have the ability to bind a variety of ions to form complexes of varying stability (80). In regard to the binding of metal ions to proteins, two types of binding might be considered. In the first, the ions are bound to protein in a nonspecific manner, the binding site depending on the pH

of the solution. The extent of binding increases with increasing free metal ion concentration without approaching a well-defined value. In the second, the limiting stoichiometry is approached at comparatively low metal ion concentration; for example, the binding of two iron ions per mole of lactoferrin (41).

The studies of metal ion protein binding has involved physical separation of unbound metal ions and bound ions to the proteins by some equilibrium dialysis, ultracentrifugation, and ultrafiltration (8, 41, 80). Equilibrium dialysis had the disadvantage of prolonged equilibrium times, while ultracentrifugation required sophisticated equipment and was not suitable (8, 11, 41). A recent development has, to a large extent, overcome some of these disadvantages (80).

Gel filtration and ion-exchange chromatography have been found to be suitable methods for separating free metal ions (unbound ions) by overcoming difficulties in the classical methods (11, 80). Gel filtration has been considered as a rapid and effective dialyser and has the important feature that fractions are not exposed to chemicals and heat which might denature the proteins (8, 45).

Ion-exchange chromatography was also found useful for desalting and fractionation of milk proteins (37, 45, 52).

Boulet et al. (8) examined the mineral composition of the casein micelle by passing the skim milk through a Sephadex

G-150 column. He separated colloidal calcium from free calcium ions and removed 22 percent of calcium by gel filtration.

Lehmann and Kaplan (37) described a procedure for determining serum-iron binding capacity by using anion exchange resin and ultrafiltration procedures for the removal of excess iron from serum protein. They found very good correlation between these two methods. Shaikh and Lucis (65) isolated a cadmium-binding protein by using a Sephadex G-75 column and a DEAE-Sephadex column. Cadmium binding proteins obtained by Sephadex G-75 treatment applied to DEAE-Sephadex column separated into two fractions as cadmium binding protein I and cadmium binding protein II. They concluded that gel filtration and ion-exchange chromatography were useful for separating free metal ions from the protein bound ions.

CHAPTER III

MATERIALS AND METHODS

I. PREPARATION OF ^{59}Fe LABELLED MILK

Five hundred μCi of $^{59}\text{FeCl}_3$ obtained from New England Nuclear, Boston, Massachusetts, were diluted to 5 ml with deionized water and 0.02 ml of this solution was mixed with 0.1 ml of 0.19 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. One hundred twenty μl of this latter solution were added directly to 100 ml of raw milk giving a concentration of 0.02 ppm and 10 ppm isotope and carrier, respectively. The ^{59}Fe labelled milk was held overnight at 4°C to allow interaction of iron with the other components of milk.

II. FRACTIONATION OF MILK

The scheme for the separation of casein and its components from milk is shown in Figure 1. Raw milk was warmed to 37°C and centrifuged in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Connecticut) at $7710 \times g$ for 15 minutes at 37°C . The temperature control was then set at 4°C and centrifuging continued for a further 15 minutes. The solidified cream plug was carefully removed. The skim milk was then stirred for 30 minutes at 37°C to redisperse the milk proteins.

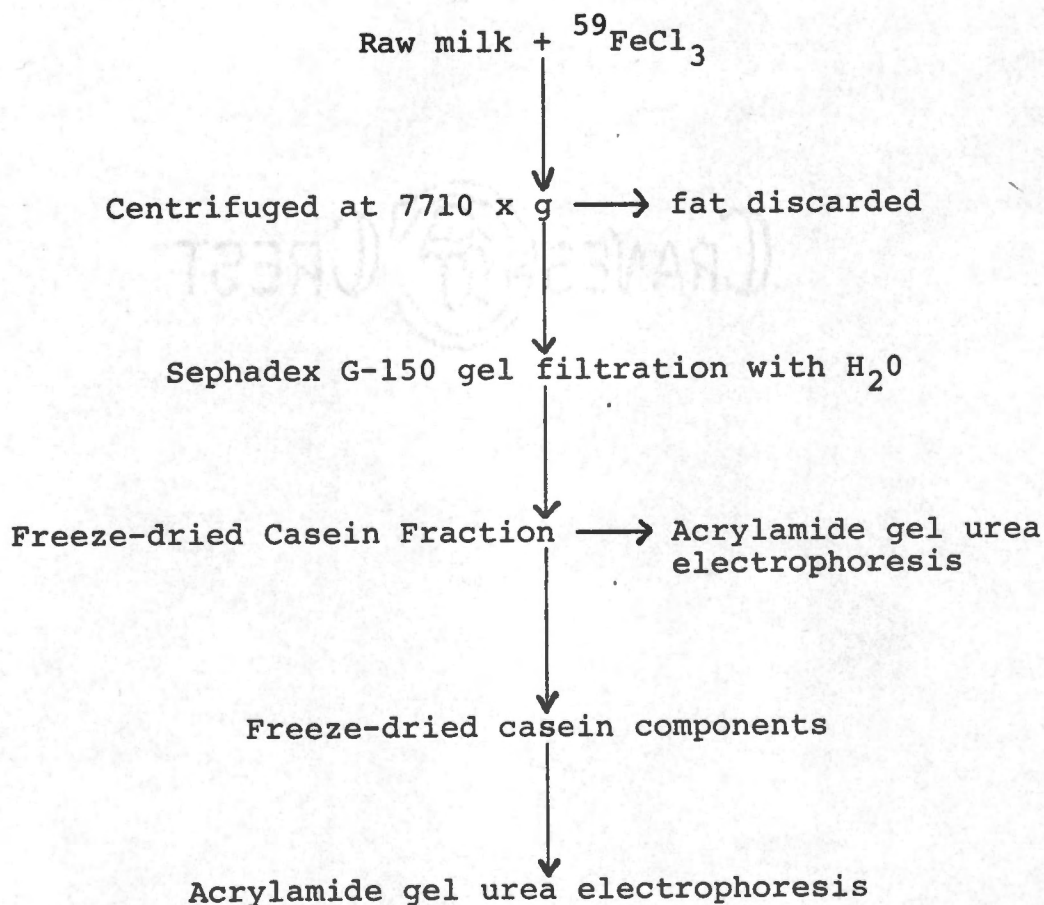


Figure 1. Summarized scheme for the separation of casein and its components from milk.

Gel Filtration of Skim Milk
on Sephadex G-150

About 25 g of Sephadex G-150 (Pharmacia, Fine Chemicals, Uppsala, Sweden) were suspended in deionized water and allowed to swell for 3 days. The fines were then removed by decantation. A thin slurry of Sephadex G-150 gel particles was then packed into a jacketed column (K26/40; Pharmacia, Fine Chemicals, Uppsala, Sweden) to give a gel bed measuring $2.6 \times 30 \pm 1$ cm. A flow rate of about 20 ml per hour was maintained by positioning the end of the air inlet tube in the reservoir about 10 cm above the end of outlet tubing. The distance between the inlet tubing and outlet was called the operating pressure (57). The Sephadex column was flushed with deionized water for 24 hours before applying the sample.

The skim milk, in 5 ml portions, was applied to the column by using a sample applicator (Pharmacia, Fine Chemicals, Uppsala, Sweden). Elution was accomplished with deionized water at 4°C and the effluent collected in 2.5 ml fractions by using a M75-15" Universal Fraction Collector (Medical Research Apparatus, Boston, Massachusetts).

The protein concentration in each 2.5 ml fraction was relatively determined as follows: a one ml sample from each fraction was diluted with 3 ml of a mixture of 0.3 percent each of disodium ethylenediaminetetraacetate (EDTA) and

sodium lauryl sulphate (SLS); one drop of 30 percent NaOH solution was added; the absorbance reading was then determined at a wavelength of 280 nm in a Hatachi, Coleman Model-124 Spectrophotometer, (Norwalk, Connecticut). The spectrophotometer was set at zero with a water blank prepared in the same manner (50).

The area under the pattern of absorbance values at 280 nm in Figure 2 was measured by a Compensating Polar Planimeter (Keuffel and Esser Co., New York). Each procedure was replicated 3 times, and 3 measurements were made on each replicate.

For radioactivity counting, each 2.5 ml fraction from the fraction collector tubes was transferred into the counting tubes. Radioactivity was then determined directly by an Automatic Gamma Well Counter System (Nuclear Chicago Corporation, Desplaines, Illinois) at a background of 114 counts per minute. Radioactivity was expressed as counts per minute (cpm) and corrected for decay. From each 2.5 ml fraction duplicate counts were taken and replicated 5 times.

The protein suspension of 12 tubes in fraction A shown in Figure 2 was freeze-dried in a Virtis Freeze-Dryer (The Virtis Company, Gardiner, New York) and stored in a desiccator at room temperature until used.

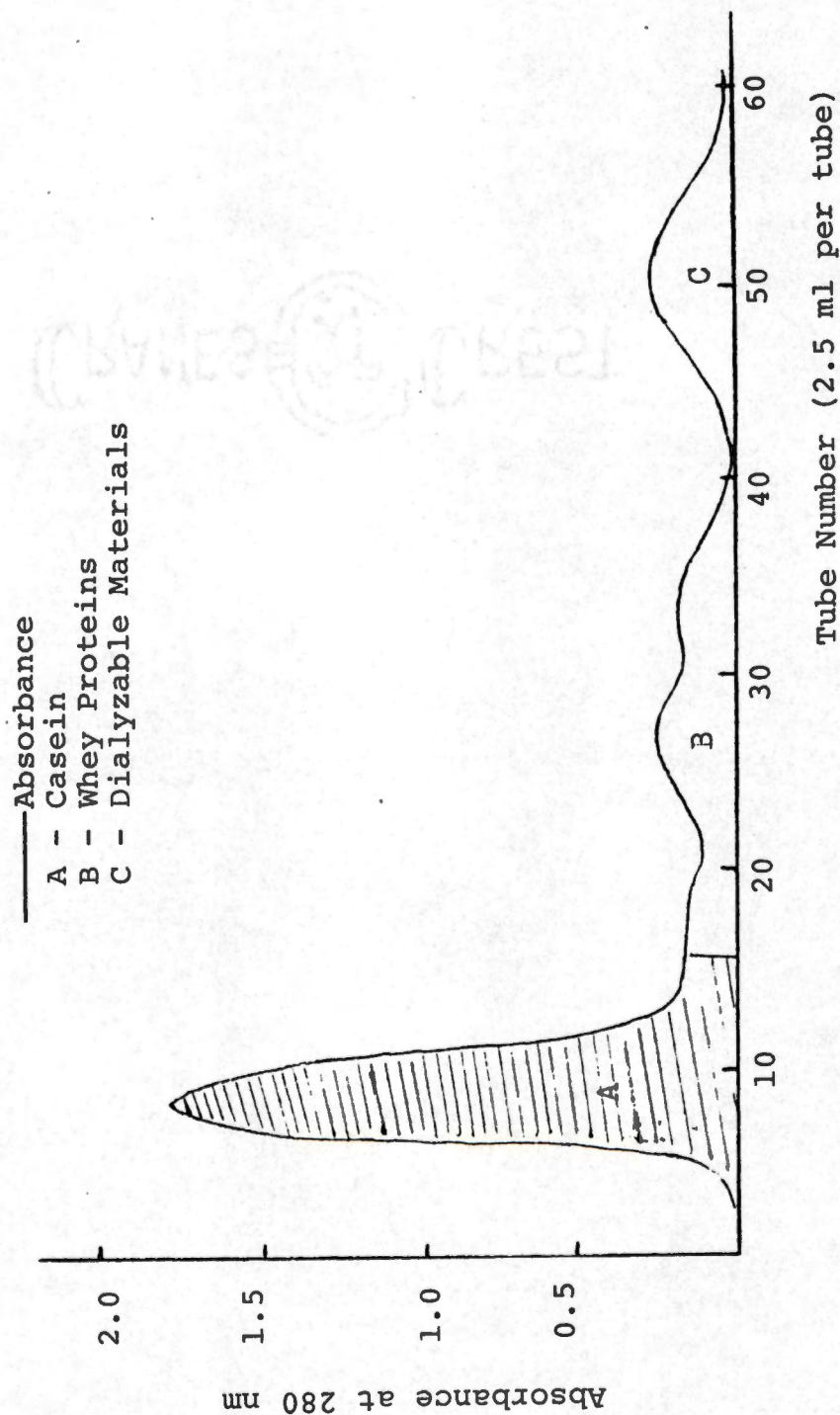


Figure 2. Chromatogram of 5 ml of skim milk eluted from a Sephadex G-150 column (2.6 x 30 cm) with deionized water at 4°C. Shaded area indicates the pooled sample which was used for DEAE-Cellulose chromatography and electrophoresis.

Column Chromatography on Diethylaminoethyl
(DEAE)-cellulose

DEAE-cellulose (Bio-Rad Laboratories, Richmond, California) was prepared by the procedure of Whitaker (79). The DEAE-cellulose was suspended in one liter of 0.25 M HCl. After about 90 percent of DEAE-cellulose had settled, the supernatant containing the fine particles was siphoned off. Sizing was continued in one liter volumes of water until the supernatant was clear. The DEAE-cellulose was then washed with 0.25 M NaCl-0.25 M NaOH solution and filtered using a Buchner funnel. The precipitate was then washed two times with water, again with 0.25 M HCl, and five times with water and again filtered. The filtered cake was then equilibrated in a buffer of 0.01 M imidazole-HCl (pH 7.0) containing 3.3 M urea and 0.01 M 2-mercaptoethanol by repeated suspension until no changes in buffer pH occurred. The slurry of DEAE-cellulose was then packed into a jacketed column (K16/40; Pharmacia, Fine Chemicals, Uppsala, Sweden) to form a column of DEAE-cellulose $1.6 \times 28 \pm 1$ cm. The DEAE-cellulose column was equilibrated with the same buffer for 6 hours before the sample application under the operating pressure of about 90 cm and flow rate of about 70-75 ml per hour.

The freeze-dried casein, fraction A obtained by Sephadex G-150 gel filtration, was dissolved in about 5 ml buffer solution and dialyzed against the same buffer solution

overnight. It was then applied to the DEAE-cellulose column. Elution was performed with the gradient mixer (GM-1; Pharmacia, Fine Chemicals, Uppsala, Sweden) which consists of two identical reservoirs; the mixing reservoir and buffer reservoir. It maintains automatically linear ionic strength gradients in the column. Three hundred ml of 0.01 M imidazole-HCl buffer (pH 7.0) containing 3.3 M urea and 0.01 M 2-mercaptoethanol were poured in the mixing chamber. Three hundred ml of the same buffer containing 0.4 M sodium chloride were put in the reservoir of the gradient mixer.

The effluent was collected in 5 ml fractions and the elution pattern was formed by the measurement of the absorbance value of each fraction at 280 nm with the spectrophotometer. The spectrophotometer was set at zero with a blank which was the effluent of the tubes 120-124.

The area under the elution pattern of absorbance at 280 nm in Figure 3 was determined with the polar planimeters. In each procedure, 3 readings were obtained on each of 3 replications.

The radioactivity counts of 5 ml fractions were determined as described previously.

The protein solutions in each peak were pooled and dialyzed at 4°C against deionized water to remove urea and then the proteins were precipitated with 0.7 saturated

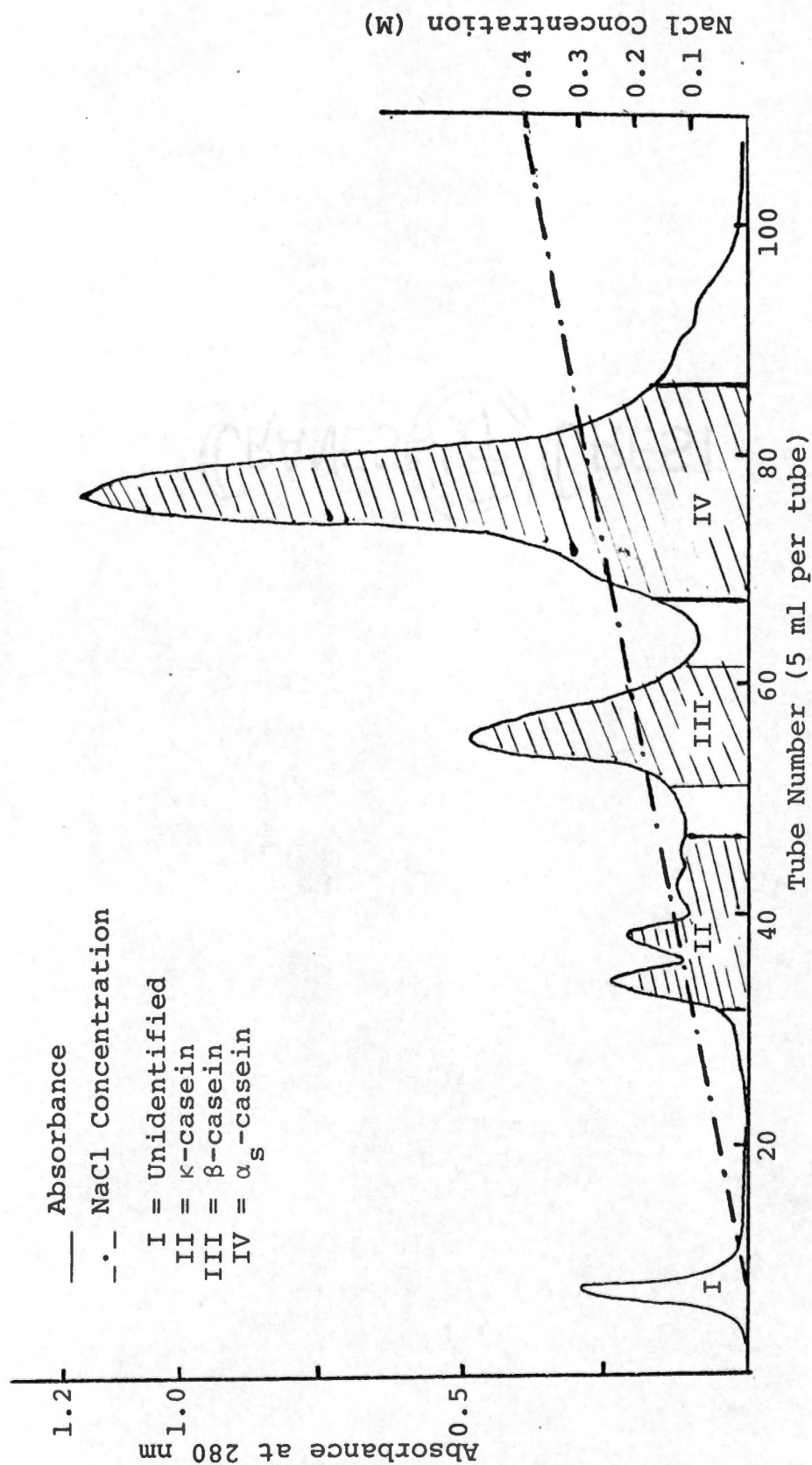


Figure 3. Chromatogram of Sephadex casein (fraction A from Figure 2) eluted from DEAE-cellulose column (1.6 x 28 cm) by a gradient of NaCl in 0.01 M imidazole-HCl buffer containing 3.3 M urea and 0.01 M 2-mercaptoethanol at 4°C. Shaded area indicates the pooled samples which were used for electrophoresis.

ammonium sulphate. The precipitated proteins were suspended in about 15 ml of water and dialyzed again against deionized water for 2 days at 4°C and freeze-dried. The freeze-dried proteins were stored in a desiccator at room temperature until used for electrophoresis.

III. ACRYLAMIDE GEL UREA ELECTROPHORESIS

Acrylamide gel urea electrophoresis was performed in a vertical gel electrophoresis E-C Apparatus (E-C Apparatus Corporation, St. Petersburg, Florida). Buffer and gel were prepared according to the method of Nagasawa et al. (48).

A tris-EDTA-boric acid buffer solution was made by mixing 0.045 M tris-(hydroxymethyl)aminomethane, 0.0013 M $\text{Na}_2\text{-EDTA}$ (disodium-ethylenediaminetetraacetic acid), and 0.025 M boric acid (pH 8.5).

The stock gel solution was made by dissolving 80 g Cyanogum 41 (Bio-Rad Laboratories, Richmond, California) in about 500 ml of buffer solution and adding 270 g urea and diluting to one liter with buffer. The resulting solution, which was 8 percent Cyanogum 41 and 4.5 M urea, was stored for further use.

For a single run the gel solution was prepared by adding 150 mg ammonium persulphate and 0.2 ml of N, N, N', N'-tetramethylethylenediamine (TEMED) to 150 ml of stock gel solution previously deaerated. This solution was poured

immediately into the cell and the appropriate slot former also was inserted. The gel was allowed to polymerize about 45 minutes. Tris-EDTA-boric acid buffer in the absence of urea was used in the electrode chamber.

Five mg protein samples were dissolved in 0.5 ml of tris-EDTA-boric acid buffer solution containing 7 M urea, and 0.02 ml of bromphenol blue solution added. After the slot former was removed, 30 μ l of protein solution were layered in the gel slots. The apparatus was set for a constant voltage of about 300 volts. The electrical current was discontinued when the dye bands reached a position about 14 cm from starting slot, usually in 5 hours.

After electrophoresis, the gel was removed from the cell and immersed for 3 minutes in one percent Amido Black 10 B solution (water-methanol-acetic acid, 50 : 50 : 10). The gel was then destained with 7 percent acetic acid solution until protein bands were visible clearly in about 2 days. The gel was then photographed.

CHAPTER IV

EXPERIMENTAL RESULTS

I. SEPARATION OF CASEIN FROM SKIM MILK BY SEPHADEX G-150 GEL FILTRATION

The elution pattern obtained by Sephadex G-150 gel filtration of skim milk with deionized water at 4°C is shown in Figure 2, page 23.

Skim milk was separated into 3 major fractions which were designated alphabetically, A, B, and C. Fraction A had the same white appearance as milk, because the milky appearance of milk is indicative of the casein micelle (32). This fraction was subjected to acrylamide gel urea electrophoresis for identification. Figure 4 shows the band patterns obtained by acrylamide gel urea electrophoresis of whole casein, W, and fraction A. They were found similar in their electrophoretic composition and both contained 3 major bands of casein components which were in order of increasing mobility, κ -, β -, and α_s -caseins. This result indicated that separation of casein from skim milk was complete and this casein was named "Sephadex Casein" and so designated in these experiments. Fraction B contained whey proteins, proteoses and peptones, fraction C

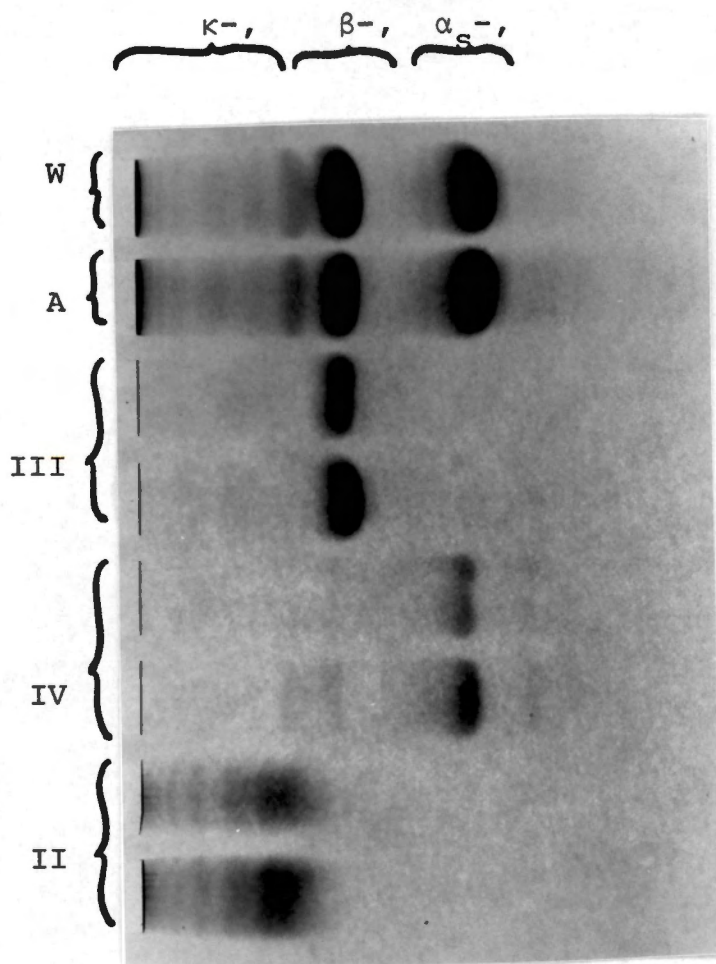


Figure 4. Acrylamide gel urea electrophoretic pattern of whole casein, Sephadex casein fraction, and casein fractions obtained by DEAE-cellulose chromatography: W - whole acid casein; A - Sephadex casein; III - β -casein; IV - α_s -casein; II - κ -casein. Electrophoresis conditions 5 hours, 300 V., in Tris-EDTA-boric acid buffer, 4.5 M urea, pH = 8.5, 25°C.

corresponded to dialyzable materials such as calcium, phosphate, lactose as described previously in detail (8, 29, 83).

Data in Table 1 show the percentage of the total areas of each fraction. Sephadex casein comprised 60.9 percent; fraction B, about 25 percent, and fraction C, 13.8 percent of the total pattern area. Sephadex casein made up 71 percent of the total protein area while fraction B made up 29 percent.

II. SEPARATION OF CASEIN INTO ITS COMPONENTS BY DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Freeze-dried Sephadex casein from Sephadex G-150 gel filtration of skim milk was subjected to DEAE-cellulose column chromatography. A typical chromatogram obtained with 120 mg of a sample of Sephadex casein is shown in Figure 3, page 26. Shaded areas of the elution pattern indicate the selectively pooled fractions named as fractions I, II, III, and IV.

To identify casein components in these fractions, the dialyzed and freeze-dried fractions were subjected to electrophoresis. The band patterns of fractions II, III, and IV, whole casein acid and Sephadex casein are shown in Figure 4, page 30.

TABLE 1

RELATIVE AMOUNTS OF THE THREE SKIM MILK FRACTIONS WITH THE DISTRIBUTION
OF ^{59}Fe AMONG THEM ELUTED FROM SEPHADEX G-150 COLUMN

Fractions	Peak Areas 280 nm (%) ^a	^{59}Fe Activity ^b (cpm)	Recovered Activity From Column (%)
A	60.90±4.66	51649	85.13±2.55
B	25.30±3.77	5804	9.57±0.71
C	13.80±1.09	3219	5.30±1.86
Total	100.00	60672	100.00
Total Activity in Skim Milk Before Elution		65987	91.95

^aMeans of nine observations with one standard deviation.

^bMeans of ten observations with one standard deviation.

The comparison of the band patterns of these fractions with those of whole acid casein and Sephadex casein indicates that DEAE-cellulose column chromatography fractionated the Sephadex casein into its electrophoretically distinct components. The quantity of fraction I was not enough to analyze electrophoretically. Fraction II shows a major band representing κ -casein and some slow moving components as minor bands which are probably γ -caseins (62). Fraction III shows a major band which represents relatively pure β -casein and fraction IV shows a major band representing α_s -casein and several fast moving minor bands.

The area of each fraction in Figure 3, page 26, is shown in Table 2. Fraction I comprised about 3 percent; fraction II 15 percent; fraction III, 19 percent, and fraction IV, 61 percent of the total pattern area.

III. DISTRIBUTION OF ADDED $^{59}\text{FeCl}_3$ IN SKIM MILK AND CASEIN COMPONENTS

The distribution of added radioactive iron in skim milk fractions is shown in Figure 5.

Skim milk containing radioactive iron was separated into 3 ultraviolet-absorbing fractions at about the same positions as those obtained from skim milk containing no $^{59}\text{FeCl}_3$. The absorbance values obtained at 280 nm were a little higher than those obtained from skim milk without $^{59}\text{FeCl}_3$. In the

TABLE 2

RELATIVE AMOUNTS OF THE FOUR CASEIN FRACTIONS WITH THE DISTRIBUTION OF ^{59}Fe
AMONG THEM ELUTED FROM DEAE-CELLULOSE COLUMN

Fractions	Peak Areas 280 nm (%)	^{59}Fe Activity ^b (cpm)	Recovered Activity from Column (%)
I	3.08±0.51	170	0.72±0.02
II	15.89±3.11	960	4.06±1.15
III	19.46±0.89	5148	21.77±2.41
IV	61.56±2.06	17186	72.67±2.21
Fraction between I and II		186	0.78 0.61
Total	100.00	23650	100.00
Total Activity in Casein Before Elution		51649	45.79

^aMeans of nine observations with one standard deviation.

^bMeans of ten observations with one standard deviation.

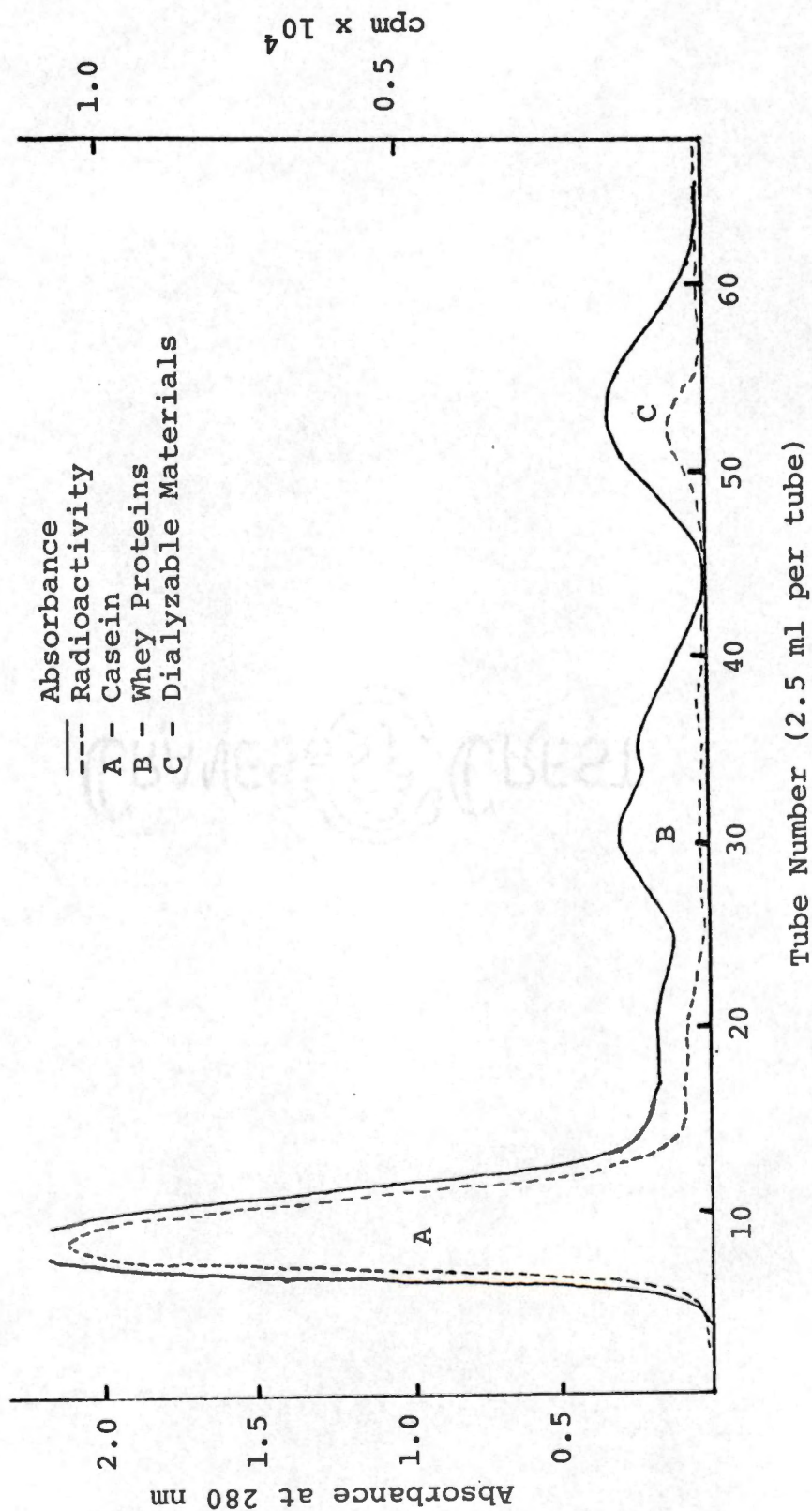


Figure 5. Chromatogram of 5 ml skim milk containing ^{59}Fe eluted from Sephadex G-150 column (2.6 x 30 cm) with deionized water at 4°C.

Sephadex casein fraction, tubes 3 and 4 gave absorbance readings higher than the spectrophotometric range.

The radioiron distribution from each tube gave a peak which closely paralleled the Sephadex casein fraction. Surprisingly, no clear radioactive iron peak was observed in the whey protein, fraction B, and only a small radioactive peak was obtained in the dialyzable material, fraction C.

Data in Table 1, page 32, indicate that about 85 percent of added iron was bound to the Sephadex casein fraction, 9 percent to the whey protein, fraction B, and about 5 percent was found in the dialyzable material, fraction C. Total recovery of radioactivity from Sephadex G-150 column was about 92 percent.

Although protein concentration was not determined by the nitrogen concentration in these experiments, the absorbance values showed that the distribution of added iron appeared to be about proportional to the concentration of casein and not to the whey proteins.

By DEAE-cellulose column chromatography, about 120 mg of Sephadex casin containing $^{59}\text{FeCl}_3$ was separated into 4 major fractions at about the same position as those obtained from Sephadex casein without radioactive iron. The proteins previously identified in these fractions are κ -casein, β -casein, and α_s -casein, in order of increasing mobility as shown in Figure 4, page 30.

The distribution of added radioactive iron in these fractions is shown in Figure 6 and Table 2, page 34. The two major radioactive peaks were found in the β -casein fraction (III) and α_s -casein fraction (IV). No radioactive peak was seen in fraction I or κ -casein, fraction II. α_s -casein bound most of the radioactive iron, about 72 percent, and β -casein bound 21 percent. Very little iron was bound by κ -casein, about 4 percent.

The total recovery of radioactivity from the DEAE-cellulose column was about 45 percent. To find the remainder of the radioactivity removed from Sephadex casein either by dialysis or during the passage through the DEAE-cellulose column, radioactivity of 5 ml of Sephadex casein solution were counted before and after dialysis. Only 1.7 percent of radioactivity was released during the dialysis as shown in Table 3. Later, this 5 ml casein solution was passed through the DEAE-cellulose column and 51 percent of radioactivity was recovered in the protein elution. Later washing of the column with 0.01 N NaOH showed that 34.9 percent of radioactivity was then removed from the column. The remainder of the radioactivity, 12.4 percent, was not recovered.

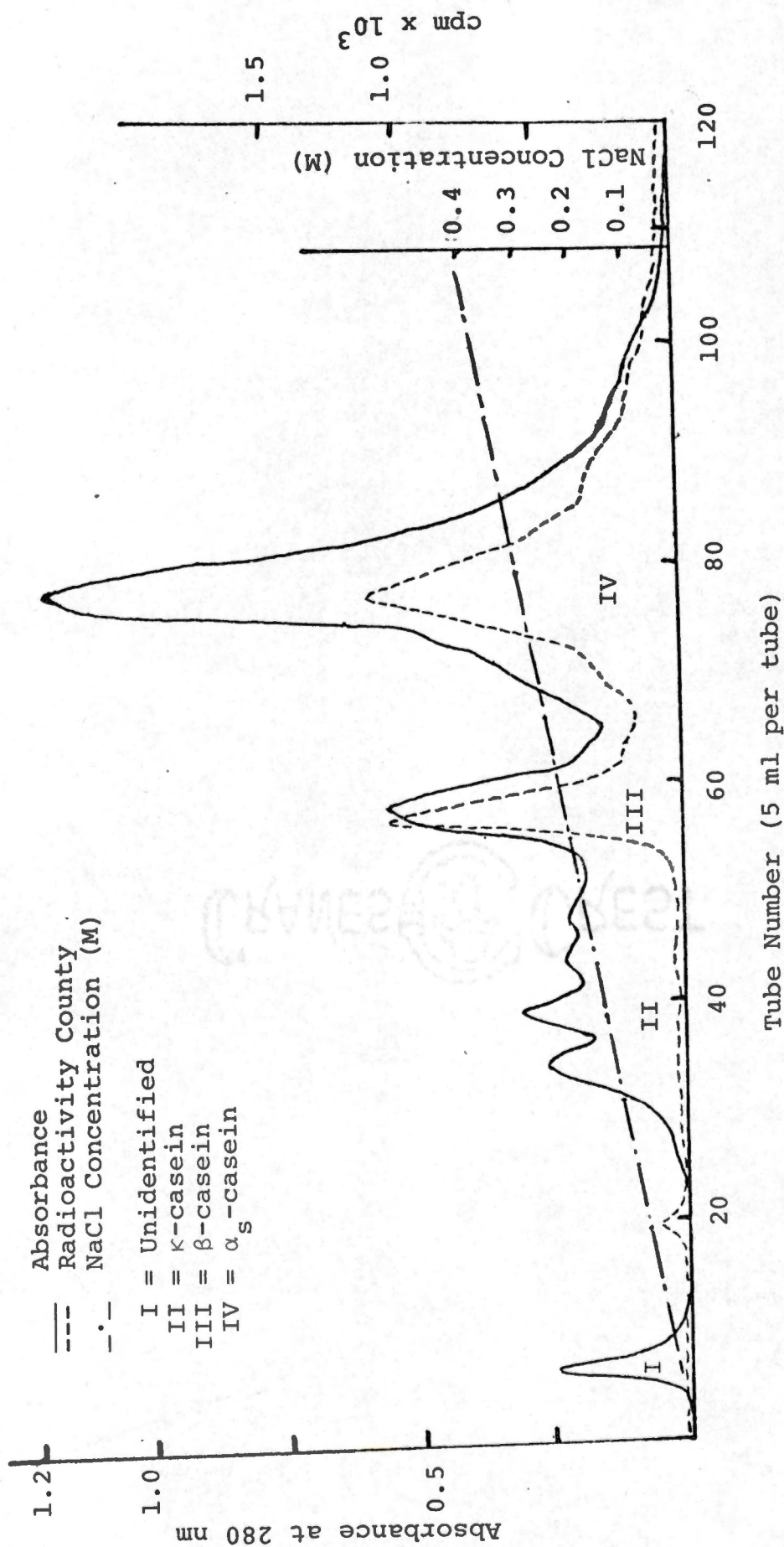


Figure 6. Chromatogram of casein containing ^{59}Fe obtained from Sephadex G-150, eluted from DEAE-cellulose column (1.6 x 28 cm) with NaCl gradient in 0.01 M imidazole-HCl buffer (pH 7.0) containing 3.3 M urea and 0.01 M 2-mercaptoethanol.

TABLE 3

AMOUNT OF RADIOACTIVITY REMOVED FROM CASEIN
CONTAINING ^{59}Fe BY DIALYSIS AND THE
DEAE-CELLULOSE CHROMATOGRAPHY

Components	Radioactivity ^a	
	(cpm)	(%)
Casein Before Dialysis	39481	100.0
Lost in Dialysis	-	1.7
Casein After Dialysis	38816	98.3
Recovered in Protein Elution	19708	51.0
Retained in Column	13547	34.9
Unrecovered		12.4

^aMeans of 3 observations

CHAPTER V

DISCUSSION

The objective of this investigation was to determine the distribution of added radioiron in milk protein and casein components fractionated by a combination of Sephadex G-150 gel filtration and DEAE-cellulose chromatography.

The elution pattern obtained from Sephadex G-150 gel filtration of skim milk using water as the eluant at 4°C showed 3 main peaks (Figure 2, page 23). Identification of the first peak by acrylamide gel urea electrophoresis indicated the separation of casein complex directly from skim milk.

Other workers (29, 46) chromatographed skim milk from Sephadex G-100 by eluting with sodium acetate and phosphate buffer at room temperature and obtained 4 peaks corresponding to the casein micelle, lactoglobulin, lactalbumin, and non-protein components. But they found some contamination between casein and whey protein fractions. Yaguchi and Tarassuk (83) chromatographed skim milk on Sephadex G-200 by eluting with phosphate buffer at 4°C and separated skim milk into 6 fractions. Boulet et al. (8) chromatographed skim milk on Sephadex G-150 and separated it into 3 fractions.

Generally, the results of this study are in agreement with those obtained in earlier experiments. The reasons for obtaining slightly different elution patterns might be due to conditions used for separations, such as type of the Sephadex, temperature, and eluant buffer.

Sephadex G-100 and G-150 have the molecular weight of exclusion levels of 4,000 to 400,000 (58). Obviously, Sephadex G-100 and G-150 are useful to separate the casein micelle from skim milk. Thus, casein micelles which are complex, globular aggregates, having molecular weights of 1×10^6 to 3×10^9 daltons (55, 74), cannot penetrate the pores of Sephadex G-100 and G-150 and are eluted in the void volume of the gels. On the other hand, the molecular weight of the whey proteins are relatively small; about 16,000 and 30,000 daltons (29). They can penetrate the gel pores of both Sephadex G-100 and G-150 and are eluted from the gel column after the casein micelle. Sephadex G-200, which contains a maximum molecular weight of exclusion value of about 800,000, may not separate the casein micelle from skim milk. Any buffer solution has some dissociating capacity on the casein micelle into its components having molecular weights between 20,000 to 30,000 daltons. As a result, the casein components can penetrate into the gel pores of Sephadex G-200. The explanation as to why 6 peaks were obtained by the methods of Yaguchi and Tarassuk (83) might be due to using Sephadex G-200 with phosphate buffer.

Temperature might be a factor also in the separation of casein from skim milk. At low temperature, casein cannot dissociate very easily.

Thus, gel permeation chromatography can be useful for direct fractionation of the casein micelle from skim milk, because of mild conditions and simplicity of procedure and the feasibility of automatic processing. A very critical step is the packing of the column very uniformly in order to get good results.

The anion exchanger DEAE-cellulose, was found to be a very useful tool for further separation of Sephadex casein obtained by Sephadex G-150 gel filtration. DEAE-cellulose chromatography of Sephadex casein was performed by a gradient of sodium chloride in imidazole-HCl buffer containing urea and 2-mercaptoethanol.

The results shown in Figure 3, page 26, and Table 2, page 34, demonstrate that a series of chromatographic separations of Sephadex casein on the DEAE-cellulose column was reproducible and satisfactory. Acrylamide gel urea electrophoresis of the fractions revealed relatively pure casein components like κ -, β -, and α_{s1} -caseins.

Earlier workers (67, 84) separated the whole casein on DEAE-cellulose column by eluting with a phosphate buffer and obtained several fractions. Electrophoretic patterns of these fractions indicated that none of the casein fractions

was homogeneous. Ribadeau-Dumas et al. (59) fractionated the whole casein on a DEAE-cellulose column by eluting with urea, including a gradient of sodium chloride. They obtained α_s - and β -casein in highly pure form but not κ -casein. Thompson (68) fractionated casein by DEAE-cellulose urea chromatography in the presence of 2-mercaptoethanol into 6 fractions.

Electrophoretic identification of these fractions showed α_s - and β -casein in pure form while κ -casein was contaminated with the other fractions. Rose et al. (62) chromatographed the reduced and alkylated freeze-dried casein on a DEAE-cellulose column by eluting with a tris-citrate urea buffer and obtained 3 main peaks. Electrophoretic patterns of these fractions indicated that κ -casein in its reduced form separated from α_s - and β -caseins.

The elution pattern obtained by Rose et al. (62) was found to be very close to the patterns obtained in these experiments, and the electrophoretic patterns of fractions were also in agreement in both methods.

From the foregoing discussion about the fractionation of the casein micelle by DEAE-cellulose column chromatography, some conclusion might be drawn regarding the similarities of buffer systems used as eluants. DEAE-cellulose column chromatography, some conclusion might be drawn regarding the similarities of buffer systems used as eluants. DEAE-cellulose chromatography is more effective for separation

and purification of casein micelles in the presence of dissociating agent in the fractionation of casein micelles (62, 69, 73). Urea and some other dissociating agents such as sodium dodecyl sulphate improve the separation of casein and might be due to reaction with the hydrophobic region of casein components and thus weakening the formation of the casein micelles (10). On the other hand, the reason for using the reducing agents such as 2-mercaptoethanol (ME) and dithiothreitol (DTT) on DEAE-cellulose is to reduce the disulfide linkage in κ -casein. Thus, in the presence of both reducing and dissociating agents, all three major casein components are dissociated extensively and highly purified κ -casein can be separated from α_s - and β -casein as shown in these experiments and previous experiments (32, 62). However, the separation of the casein micelle is dependent on proper packing of the column and setting the system of chromatography.

After successful fractionation of Sephadex casein and its components were achieved by using the above chromatographic methods, radioactivity was determined in casein and its components. Results obtained from Sephadex G-150 gel filtration, indicated that about 85 percent of the added radioiron was associated with casein. About 8 percent of radioiron was found in the whey protein fraction and 5 percent was in the nonprotein components.

The Sephadex G-150 gel filtration was found to be a rapid and sensitive technique yielding reproducible results. The gel filtration process was considered here as an effective water dialysis treatment, because of the molecular sieving effect of Sephadex G-150. As a result of this effect of Sephadex gel filtration, 5 percent of the iron was recovered in the dialyzable material fraction. Ninety-five percent of radioiron was found as bound iron to casein and whey proteins.

The distribution of added radioiron in casein components obtained after DEAE-cellulose column chromatography as shown in Figure 6, page 38, and Table 2, page 34, indicated that about 72 percent of added iron was bound to α_s -casein, and 21 percent was attached to β -casein. Only about 5 percent of the radioactivity was recovered in the κ -casein and slow moving components. From the DEAE-cellulose column, 45 percent of the total radioactivity was recovered in the protein elution volume of about 600 ml. Over 34 percent of the radioactivity was retained on the column while 12 percent of the total radioactivity was not recovered. The reason for this might be due to: sieving effect of DEAE-cellulose column, removing capacity of anion exchanger of loosely bound iron from protein bound iron, or during the dissociation of the casein micelle in the column, some of the bound iron in the casein micelle might be released from the casein micelle.

Previous workers (5, 14, 36) found that most of the added radioiron was bound to the milk proteins and that casein bound most of the added iron, about 67 to 90 percent. In this study, casein bound about 78 percent of added radioiron in skim milk. Thus much variation among the results obtained by the different workers might be due to using different methods, type of milk and type of iron added to milk. Other workers did not report on the amount of iron in casein fractions.

CHAPTER VI

SUMMARY

This study was undertaken to identify the distribution of added radioiron in caseins of cow's milk fractionated by Sephadex G-150 gel filtration and DEAE-cellulose column chromatography.

Based on the results of this study, the following conclusions were made:

1. Sephadex G-150 gel filtration was found to be a useful technique to separate skim milk into its components, casein, whey proteins, and dialyzable materials. Complete separation of casein from skim milk was confirmed by acrylamide gel urea electrophoresis.

2. The distribution of added radioiron in skim milk fractions showed that casein bound more than 85 percent of the added radioiron and whey protein fraction bound less than 10 percent. The binding of added iron to casein was found to be proportional to the concentration of casein but not so with the whey proteins.

3. DEAE-cellulose column chromatography with the buffer containing dissociating and reducing agents was found suitable for further separation of the Sephadex casein into its components: α_s -, β -, and κ -caseins.

4. Among the casein components, α_s -casein bound the highest amount of radioiron, about 72 percent. β -casein bound about 21 percent and κ -casein bound about 4 percent.

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