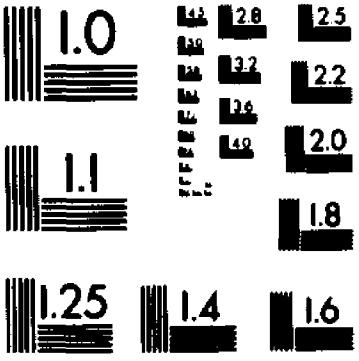

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BOVINE BETA-CASEIN: MODEL PROTEIN TO STUDY CYSTEINE MISINCORPORATION AND PHOSPHORYLATION

City University of New York

PH.D. 1986

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BOVINE β- CASEIN: MODEL PROTEIN TO STUDY CYSTEINE MISINCORPORATION AND PHOSPHORYLATION

By Anne T. Flateau

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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January 10, 1986
Date

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Abstract

Many theories have been proposed to explain aging. One theory in particular, Orgel's Error Hypothesis, has been tested here. Orgel's theory states that increasing levels of missynthesized proteins will accumulate with age and that this accumulation of protein errors is the cause of senescence and death. Bovine β-casein was chosen because it did not contain the amino acid cysteine and because it had multiple phosphorylation sites. The misincorporation rate of cysteine (error rate) into "normal" β-casein could be studied as well as any changes in phosphorylation with aging. Although no "error" containing β-casein was isolated, an upper limit of the error rate could be estimated, based on the limits of the detection methods used. This estimate of amino acid misincorporation is too low to account for aging and thus argues against Orgel's Error Hypothesis. The degree of phosphorylation of β-casein did not change with aging. However, techniques were developed which could have general applications both in aging and general research.

Acknowledgement

I thank Professor Aaron Lukton
for his faith in my abilities.

Dedication

To my husband, David C. Mochu

and my son, Paul Chikwe.

Table of Contents

List of Tables

List of Figures

I.

INTRODUCTION

The problems associated with the growing population of aged individuals in our society has become overwhelming. There are social, economic and political ramifications, as well as biological and medical problems which need solving because of the increase in life-expectancy. As scientists, we are primarily concerned with the biological and medical aspects of a population that continues to live longer and longer. "Aging is, in general, associated with a gradual decline in the performance of most organ systems with a loss in reserve capacities."[1] But, is the quality of this increased lifespan better than 30 years ago? Can't it be improved? I feel these questions are at the heart of biochemical research into the causes and effects of aging. By seeking to clarify and define the causes of aging we aim, ultimately, to stop or reverse such changes. This is, of course, a very "lofty" goal and it will take much "mundane" research and many years to achieve.

One approach to research in aging has been to derive theories based on empirical observations and then to devise experiments to test these theories. Nathan Shock[1] classifies such theories as either genetic, non-genetic or physiological. Genetic theories assume that the lifespan of an individual is "programmed" in the genes and that aging results from damage to DNA or errors in transmission of information vital to the formation of products. Non-genetic

theories emphasize changes that take place in proteins over time after they are formed. Physiological theories stress the effects of aging on regulatory mechanisms.

Non-genetic theories include: "Wear and Tear Theories," "Deprivation Theories," "Accumulation Theories" and "Free Radical Theories." Included under this heading would be evidence of post-translational modifications associated with aging (such as: cross linking, heat lability, deamidation, changes in conformation, glycosylation, etc.). Each theory and the experimental evidence in support of, or refuting it will be discussed.

"Wear and Tear" theories are based on the assumption that living organisms behave like machines and parts wear out or become defective and fail. These theories also assume a "rate of living," which means that increased temperature caused increased biochemical reactions and shortened lifespan by increasing "wear and tear." Evidence for these theories is mainly from experiments with Poikilothermic, non-mammalian species. Osanai and Yonezawa[2] showed that rearing Bombyx mori (adult silkmoth) at 35°C rather than the normal 25°C reduced their lifespan by one half. These results are complicated by the fact that the adult Bombyx mori is a closed system and cannot take in nutrients after emergence because its mouth degenerates.

This is certainly not the case in higher organisms. Osanai and Yonezawa also found increased levels of urea and arginase activity in the moths reared at 35°C suggesting an increase in protein catabolism (via the urea cycle). These authors conclude that an extrinsic factor, temperature, influences the lifespan of Bombyx mori by altering intrinsic aging processes via accumulation of a deleterious substance, urea. In other words, increased temperature and increased "rate of living" didn't cause aging, but caused harmful effects inside the cells which led to shortened lifespan and death.

"Deprivation Theories" argue that inadequate delivery of nutrients or O_2 cause impaired function and cellular death.[1] Evidence supporting this theory are effects of decreased cardio-vascular function on cells or tissue function. In this case, whole areas of cells or organs die, if oxygen is not delivered, e.g., the necrosis of heart muscle during and after a myocardial infarction. However, this situation doesn't really mirror aging where cells randomly disappear and are not replaced.

"Accumulation Theories" are based on the increased deposition of insoluble lipofuscin in neurons, muscle fiber, etc. with increasing age. Lipofuscin appears to be the result of autophagocytosis involving lysosomes.[3] This

accumulation is most notable in long-lived post mitotic cells such as neurons and cardiac myocytes. Lipid peroxidation reactions with release of harmful free radicals are believed to be involved in lipofuscin formation,[3] but this is still speculation. Also, it hasn't been shown whether the lipofuscin itself interferes with cellular function.

"Free Radical Theories" of aging emphasize the deleterious effects of the $\cdot OH$, $\cdot HO_2$ and $\cdot O_2^-$ (superoxide) radicals on cells. These radicals result from normal metabolism as well as spontaneous random reactions. Unsaturated fatty acids are particularly susceptible to free radical oxidation (removal of hydrogen). It is also thought that the main damage from ionizing radiation is from formation of free radicals from water. Harmon,[4] et al. added several anti-oxidants, such as 2-mercaptoethanol and Vitamin E to the diet of mice and found some mice had increased average lifespan while others did not. However, there was no record of food intake, and, if the anti-oxidant was distasteful, the mice might have simply not eaten the food. Food restriction has been shown to increase lifespan in mice.[5] More experiments need to be done before anything conclusive can be said about this theory.

Evidence of post-translational changes in protein correlation with aging is growing steadily and appears to be a universal phenomenon. Evidence that post-translational modification of proteins increases with age has been obtained chiefly from work done on collagen, eye lens proteins and a 148kDa protein (non-collagenous) from cartilage.

It has been reported by many investigators[6,7] that collagen demonstrates increased crystalline organization, decreased water content and increased tensile strength with increasing age. These changes are attributed to cross-linking of the collagen chains. Lysyl and hydroxylysyl residues are oxidized by lysyl oxidase to form aldehydes, allysine and hydroxyallysine. These aldehydes can undergo aldol condensation. This aldol cross-link can react with a histidine side chain and form an aldol-histidine cross link. The aldehyde group in the aldol-histidine cross-link can form a Schiff base with another side chain such as hydroxylysine and thus 4 side chains can be covalently linked. These links can be intramolecular (same chain) or intermolecular (between chains).[8] See Figure 1.1.

Recently, Paulssen, et al.[9] have isolated a non-collagenous protein from steer cartilage, a 148 kDa glycoprotein. The amount of this 148kDa protein isolated

from individual steers increased with age and the protein also became more insoluble in granidium chloride with age and the protein also became more insoluble in guanidium chloride with age. The mechanisms by which the protein bcomes insoluble is unknown but it is suggested by the authors that this may be a situation analagous to collagen, i.e., covalent cross-links are formed.

The eye lens structural proteins or crystallins are particularly suited to study post-translational modifications of proteins and aging. Since the lens never sheds its cells, all changes observed in the inner layers (nucleus) can be attributed to aging.[10] The physical changes observed are decreased elasticity of the lens and increased opacity of the lens. The biochemical changes observed with aging are formation of disulfide bridges,[11,12] other cross linking,[13] deamidation of asparagine and glutamine,[14,15] partial degradation of polypeptides at specified sites, racemization of aspartic acid residues[16] and non-enzymatic glycosylation.[17] Ozaki et al.,[18] using Raman Spectroscopy, have shown that tryptophan residues in eye lens proteins undergo micro-environmental changes with aging. These micro-environmental changes correlate with formation of S-S bonds, removal of water from the lens, and subsequent protein aggregation.

Cross linking of protein chains by glucose has also been implicated as a cause of aging. Arnetz et al.[19] studied the glycosylated derivative of hemoglobin A (HbA), HbA_{1c}. HbA_{1c} is an indicator of average blood glucose levels 8-12 weeks before sampling time. They studied the HbA_{1c} and glucose levels of non-diabetics between 50-89 years of age and found although fasting glucose levels were virtually the same, there was an increase in the amount of HbA_{1c} as the age of the subject increased. Pongor, et al.[20] have isolated a fluorescent chromophore from proteins exposed to glucose over long periods of time. The structure of FFI <2-(2-furoyl)-4 (5)-(2-furanyl)-1H-imidazole' is derived from 2 peptide amine nitrogens from lysine and 2 glucose residues. See Figure 1.2. This suggests that peptide-bound FFI precursors are involved in cross linking of proteins by glucose in vivo. Shub et al.[21] working on mucus glycoproteins from rat small intestine have found that the glycoproteins from old rats had more carbohydrate than that from young rats.

Other investigators have concluded that conformational changes in proteins are the primary cause of aging. Gershon[22] states that age dependent inactivation of enzymes result from very subtle changes since, Km, electrophoretic mobility and antigenic identity are changed very little, if at all, in older enzyme molecules. Goren et al.[23] using

very sensitive isoelectric focusing methods concluded that subtle changes due to changes in charge of peptides such as acetylation, phosphorylation, deamidation, glycosylation, amino acid substitution, etc. were not the cause of differences between young and old nematode aldolase or young and old rat liver superoxide dismutase. Rather the differences in specific activity and temperature sensitivity may be due to conformational changes. Singh and Rao,[24] working on acid deoxyribonuclease (acid DNase) from chick brain, used circular dichroism to study the enzyme in young and old chicks and found the old acid DNase molecules were more rigid and had more α-helical structure than the young molecules. Therefore, they also conclude conformational changes are the cause of differences between young and old enzymes. The above evidence correlating post-translational changes in proteins with aging can all be taken in support of "non-genetic" theories of aging.

Physiological Theories of aging emphasize either the failure of a single organ or central mechanism. Almost every organ has been implicated as the key to aging, at one time or another. Because so many elderly die from cardiovascular disease, cardiovascular system failure was once considered the primary cause of aging. But, because other lower animals, as well as plants, age and die, this theory doesn't have general significance. The thyroid and

gonads have also been emphasized, at one time or another. One physiological theory, that does seem to have validity is the Autoimmune Theory of aging because it has been shown that there is increased production of autoantibodies and autoimmune disease with aging.[25,26,27] This theory proposes that aging results from the production of antibodies to one's own proteins. These autoantibodies attack and destroy tissue and cause aging and death. More experimental evidence is needed to prove whether this is a universal phenomenon or not.

Genetic Theories of aging emphasize preprogrammed genetically determined aging. There is quite a bit of evidence to support this point of view. The average lifespan of species varies from one day for a fly to 70 years[28] for man. It would seem that there is some genetic program or biological clock which sets the maximum lifespan for each species. Within species, there are genetically determined differences, e.g., human offspring of long-lived parents and grandparents live an average 6 years longer than offspring whose parents and grandparents died before age 50.[29] It was demonstrated by Hayflick and Moorehead[30] that diploid human fibroblasts had a limited lifespan in culture (40-50 divisions). The number of cell doublings is also dependent on the age of the donor.[31] Cells from younger donors double more times than cells from old donors and

there seems to be finite total number of doublings possible. The evidence does seem to point to: 1) a genetic program that sets upper limits on lifespan within a species, 2) inherited characteristics that influence differences in lifespan, and 3) alteration of the genetic program by environmental factors.

Several genetic theories have been advanced including the Codon Restriction Theory, the Somatic Mutation Theory and Error Theories.

The Codon Restriction Theory, advanced by Strehler,[32] states that as a result of differentiation, cells lose their ability to translate genetic information. This is an extension of the theory of gene activation and repression. The observation that there are changes in types of tRNA synthetases in the cell with aging is important to this theory.[33] With aging, messages previously read and used by the cell are no longer accessible because of changes in the structure of DNA and histones. These changes could be post-translational modifications (methylation, phosphorylation, etc.) or conformational changes. This is a very difficult theory to test experimentally because it means characterizing tRNA's at different points in the life cycle. Strehler and his colleagues have produced evidence to support the Codon Restriction Theory.[33,34,35]

Somatic Mutation Theories emphasize DNA damage and subsequent alteration in the "message". This would lead to altered proteins with impaired function or to mutation of the cell line. If this theory were correct, mutagenic agents such as X-rays would cause accelerated aging. While it is true that exposure to nonlethal radiation shortens lifespan,[36] subsequent research has shown the primary lesions of aging and radiation are not the same.[37] Curtis[38] believed spontaneous mutations in somatic cells caused aging. His experiments with rats and mice showed the incidence of abnormal chromosomes is increased with aging. However, there are weaknesses with Somatic Mutation Theories: 1) calculations of the rate of somatic mutations are too low to account for overall changes in the animal,[39] 2) most cells contain DNA repair mechanisms and there is no evidence of a decline of this repair apparatus with aging,[40] 3) there is also a vast body of evidence that should defective proteins be made, they are quickly degraded.[41,42,43]

Species differences between animals in the ability to withstand DNA damage could also determine lifespan. The presence of redundant or satellite DNA (multiple copies of the same gene) could thus offer protection from DNA damage. Cutler[44] found a positive correlation between redundant DNA and lifespan in mammals. Medvehev[45] has postulated that the

evolutionary stability of rRNA versus tRNA is due to the fact that the principal rRNA gene is repeated 10-20 times more often than those of the different tRNA's. Thus it appears that the more redundant DNA a species has, the longer will be its lifespan.

Error Theories imply that aging and death are a result of errors which may occur in the sequence of information transferred from DNA to protein. These errors result in faulty proteins which can't function properly. Medvehev first recognized that such errors could cause aging and death, but it was Orgel[46,47,48] who formalized and amplified the theory. Orgel's error hypothesis stated that aging is a result of errors in key proteins, e.g., enzymes of transcription and translation, which can become amplified over time, in the absence of cell division, and cause an "error catastrophe". This theory is very attractive from a biochemist's point of view since all one need do is to detect these error containing proteins and determine if there is a correlation with aging. This, in fact, is a difficult task technically. Most evidence for and against this theory has been indirect. Holliday and Tarrant[49] showed that heat labile G6PD, and 6-phosphogluconate dehydrogenase accumulate in late passage human fibroblasts. Also that the RNA base analog 5-FU (5-fluorouracil) induces premature aging in human fibroblasts and induces the

appearance of heat labile enzymes. Lewis and Tarrant[50] showed that human fibroblasts accumulate more and more altered LDH (lactic dehydrogenase) of lower specific activity with age. Ryan et al.[51] used amino acid analogs, p-fluorophenylalanine and ethionine to study error accumulation in a human diploid cell line. When cells were grown in non-toxic levels of the analogs, they achieved the same number of population doublings as untreated cells. When cells were grown on inhibitory levels of analogs for one week and then transferred to fresh medium, they recovered and achieved the same number of population doublings. Ogrodnik et al.[52] found a decrease in the discrimination ratio of methionine versus ethionine in aged mouse liver, which supports the notion of decreased fidelity of the protein synthesis apparatus. Baird et al.,[53] in a review article, pointed out that there are many enzymes which do not accumulate altered forms with aging, e.g., rat liver catalase. He concludes that altered forms found in aging may be due to a loss of total enzyme or changes in the microenvironment of the enzyme.

Gallant and Palmer[54] measured mis-translation of a specific UAA codon in E. Coli and found that error frequency could be increased by a magnitude (using streptomycin, which induces misreading of certain codons) without generating an "error catastrophe". The error frequency increased

gradually over a few generations, reached a new higher plateau and remained there without apparent harm to the cells.

Misincorporationn rates have been measured by other investigators. Loftfield and Panderjaet[85] analyzed peptides of human and rabbit hemoglobin α chains for the misincorporation of Val for Ile. They found an error rate of 2-6 parts per 10,000 in an in vitro system. Harley et al.[86] used cultured human fibroblasts from fetal, young and old donors as well as cells from subjects with Hutchinson-Gilford and Werner Syndromes, to study missynthesis of proteins with and without histidine starvation. They examined two-dimensional gels of the fibroblast proteins and looked for satellite spots trailing the native protein spots. These satellite spots represented proteins with amino acid substitutions caused by histidine starvation. Harley et al. found error rates of about 1 per 10,000 for all cells. There was no correlation with age of donor or maximal lifespan in vitro.

Popp et al.[87] measured the error rates for misincorporation of isoleucine into human hemoglobin A_1, which does not normally contain isoleucine. They used conventional purification techniques such as molecular selving and ion

exchange to purify the hemoglobin A_1. They did not, however, use a two dimensional gel system to verify the purity of their samples. They estimated an error rate of $3x10^{-5}$ for isoleucine misincorporation for adults.

Hirsch et al.[88] used the amino acid analog AIBA (α-aminoisobutyric acid) to measure the misincorporation rate of this analog for leucine in mice. They found no statistical differences in young and old mice for misincorporation of AIBA. Their overall error rate was $3x10^{-5}$.

As I pointed out earlier, most of the evidence for and against Orgel's Error Hypothesis is indirect. The aim of my thesis was: 1) to directly test the theory on a molecular level, i.e., to try to isolate "error" containing molecules, 2) develop new techniques to study aging proteins. We wanted to work with a protein which lacked one of the amino acids and then to see if the missing amino acid was misincorporated as the source of the protein (an organism) aged.

The protein which was chosen to work with was β-casein from bovine milk. β-casein was selected for several reasons: 1) β-casein is available in large quantities, 2) β-casein doesn't contain cysteine, 3) purification schemes for β-casein had been worked out,[55] although not

for the degree of purity we required, 4) amino acid composition,[56] sequence data[57] and phosphorylation sites[57] are known. The experimental design was: 1) to isolate β-casein from different aged cows, 2) determine if any cysteine had been misincorporated, and 3) if there was cystein misincorporation, to determine if it was correlated with aging. If Orgel's Theory was right, we would expect to isolate more and more cysteine-containing β-casein as the age of the donor (cow) increases. This would test the Error Theory directly. During the isolation of cysteine containing β-casein, new techniques would be developed for isolating and characterizing "error" containing molecules. We also wanted to determine if total phosphorylation, as an example of post-translational modifications, changed with aging. That is, if there was an increase or decrease in the number of phosphoseryl residues with age.

β-casein accounts for about one-third of the total casein in bovine milk.[58] It is a phosphoprotein made up of a single polypeptide chain of 209 amino acids, among which there are five phosphoseryl residues.[57] It is an extremely hydrophobic protein of molecular weight 23,983.[57] The C-terminal region is much more hydrophobic than the N-terminal region. The five phosphoseryl residues are located close to the end of the N-terminal region.

We reasoned that a single amino acid substitution of cysteine for another amino acid would have little effect on the total charge or mobility of the molecule and that such mis-synthesized molecules would be similar enough to "normal" β-casein to co-purify with it. Cysteine containing β-casein could be purified by affinity chromatography using mercuro-agarose[59] which covalently binds molecules containing cysteine. β-caseins from different aged cows would be analyzed for cysteine.

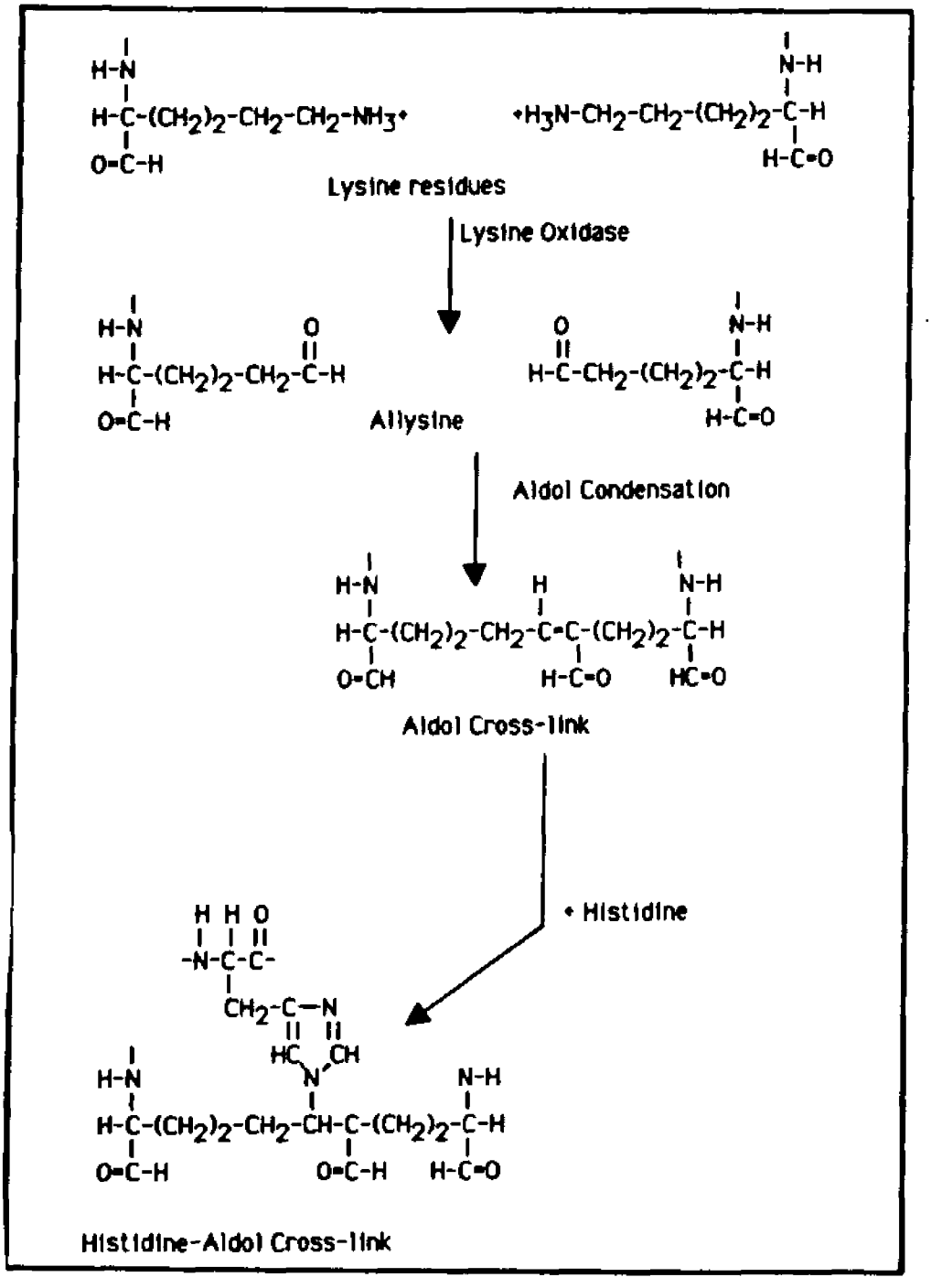
Error rates could then be calculated as number of cysteine per molecule (we expected only one) and assuming the cysteine substitution was random, we could calculate a total amino acid error rate (cysteine error rate X 20). We would thus be able to determine, on a molecular level, if error rates do occur and to measure them directly.

The degree of phosphorylation of β-casein would be measured enzymatically and by wet digestion. By analyzing β-casein from different aged cows, we could determine if phosphorylation changes with aging.

β-casein was an ideal model to prove or disprove the "error hypothesis" because we could directly measure misincorporation of an amino acid (cysteine) and directly determine the degree of phosphorylation. We also hoped to develop new and useful techniques for aging and more general research.

Fig. 1.1 Crosslinking of chains in collagen.[8]

Fig. 1.2 Tautometers of FFI implicated in crosslinking of protein chains by glucose.[20]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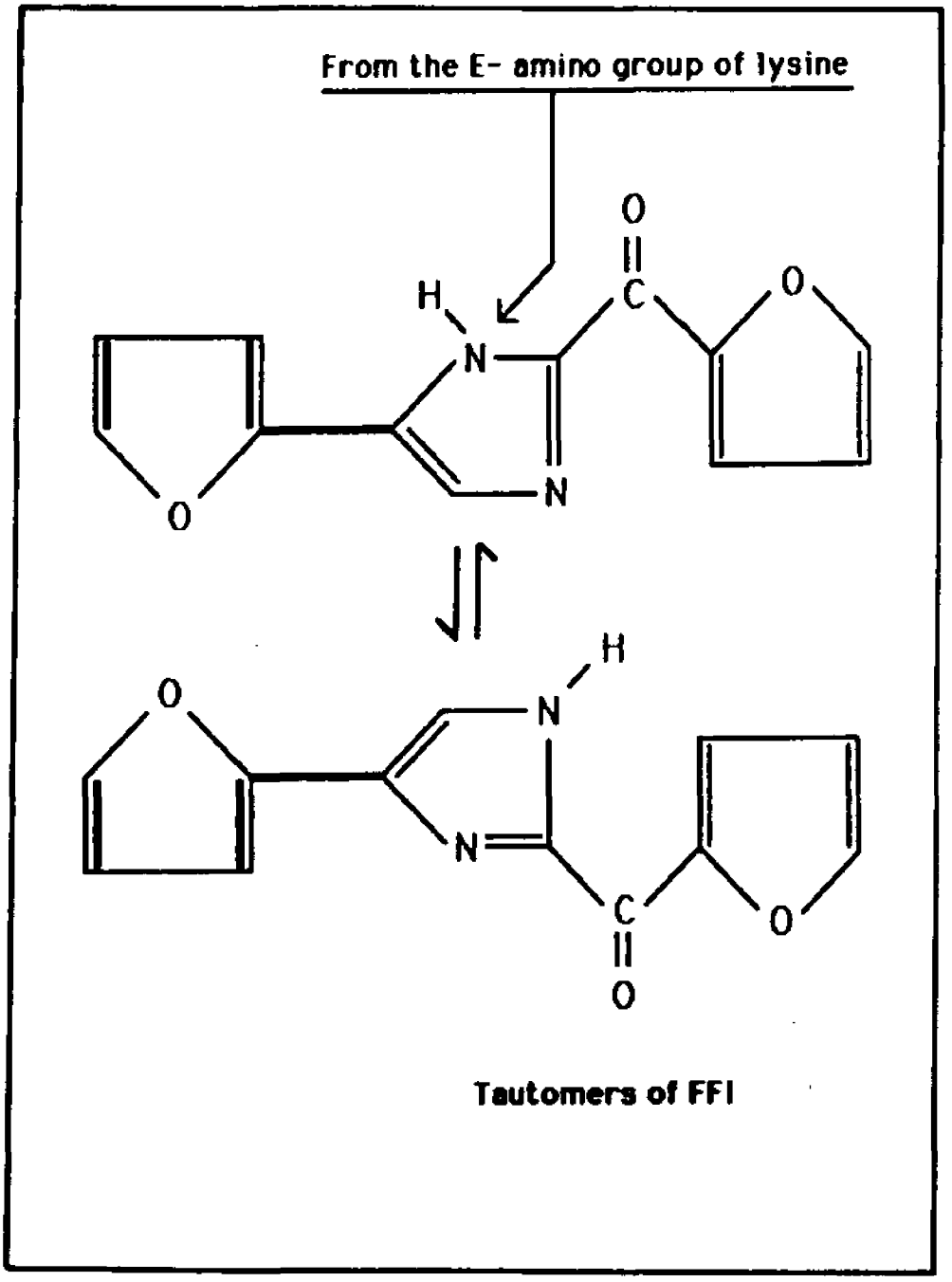
Lysine Oxidase
Allysine
Aldol Condensation
Aldol Cross-link
+ Histidine
Histidine-Aldol Cross-link

Fig. 1.1



Tautomers of FFI

Fig. 1.2

II.

MATERIALS AND METHODS

DEA (diethylaminoethylcellulose), mercuro-agarose, SDS (sodium dodecylsulfate), Coomassie R-250, Bradford Reagent (dye concentrate), acrylamide, bis-acrylamide, TEMED (N, N, N', N'-tetramethylethylenediamine), electrophoresis and isoelectric focusing apparatus were obtained from BioRad.

The electrophoresis power supply, fraction collector, and recorder were obtained from LKB.

PMSF (∝-toluenesulfonyl fluoride) and basic fuchsin (Rosanaline chloride) were obtained from Eastman Kodak.

Sepharose 4B, Sephades G-25 and chromatography columns were obtained from Pharmacia.

Acetic acid, mono and dibasic sodium phosphates, urea, 2-mercaptoethanol, sodium hydroxide, hydrochloric acid, TCA (trichloroacetic acid), acetone, di-sodium EDTA (ethylene diamine tetraacetic acid), sodium chloride, cyanogen bromide, 1,6-hexane diamine, iodoacetic acid, TRIS (tris hydroxymethyl aminomethane), periodic acid, bromophenol blue, citric acid, formaldehyde, silver nitrate, ammonia, boric acid, ethylene diamine, sulfuric acid, formic acid, glycine, glycerol, sucrose, ammonium bicarbonate, and dialysis tubing were obtained from Fisher. All chemicals were reagant grade.

Calf intestine alkaline phosphatase Type VII was obtained from Sigma.

Preparation of Milk Samples

Milk samples from the dairy farm of James Patsos of Waterloo, New York were collected in gallon plastic containers and transported on ice to Brooklyn College. About two liters of milk was collected from each of eight Ayrshire cows from the same herd. The cows, ranging in age from two to fourteen years were:

Rosebud	-	two years old
Jeannie	-	three years old
Japonica	-	five years old
Sugar	-	eight years old
Lucy	-	twelve years old
Valma	-	thirteen years old
Ruby	-	thirteen years old
Japetta	-	fourteen years old

Survival curves do not exist for dairy cows since, in general, they do not die a natural death, but are destroyed when their milk production falls too low. However, the following information was obtained from the Ayrshire Breeders Association: 1) Ayrshires begin milking at two years of age, 2) two to four years is considered a young cow, 3) five to ten years is considered a middle-aged cow, 4) milk production peaks at about four years of age and declines gradually after this age.[60]

The milk was allowed to sit in the refrigerator overnight so that most of the cream (lipid) would rise to the top. After standing overnight, a hole was punched in

the bottom of the container and the milk was collected in a large beaker. The cream, which was on top of the milk, was discarded. The milk, devoid of most lipids, was warmed at 30°C and 10% (v/v) acetic acid was added[61] until the milk curdled. The curd of whole casein was strained through several layers of cheesecloth and washed with warm distilled water (30°C).

The whole casein was then blended at low speed with 5mM phosphate, pH 7.0 containing 3.3M urea. This buffer is the same as Thompson's[62] except phosphate replaces the imidazole, and the pH was adjusted to 7.0 with 1M NaOH. Mercaptoethanol was added to 1% (v/v) and the whole casein was treated with PMSF solution[63] containing 6mg PMSF/mL ethanol. PMSF treatment was necessary to prevent auto-proteolysis by plasmin which is found in trace amounts in milk.[64] One volume of the PMSF solution was mixed with twenty volumes of whole casein solution. The whole casein mixture was stirred overnight in the cold, the pH adjusted to 7.0 with 1M NaOH again and then frozen for further fractionation later.

Fractionation of Whole Casein

First attempts to fractionate whole casein were based on DEAE column chromatography.[62] However, huge columns would have been required and the yield of β-casein was low. Instead, a modification of the Hipp Urea Fractionation Method[65] was used with minor changes, to isolate a crude β-casein fraction. Whole casein at 25°C in 5mM phosphate pH 7.0 with 3.3M urea and 1% mercaptoethanol was titrated to pH 4.6 with 1.0M HCl. A precipitate formed which is primarily α and χ casein. The mixture was centrifuged at 9,000 xg for 10 minutes and both precipitate and supernatent fraction were saved. The pH of the supernatent fraction containing mostly β-casein, was raised to 4.9 with 1.0M NaOH, diluted 1:3.3 (to make it about 1.0M in urea), and warmed to 30°C. A white flocculate of β-casein appeared and it was allowed to settle, undisturbed, for several hours or overnight. The flocculate was then collected either by filtration through Whatman #1 filter paper or by centrifugation. Both α plus χ casein and β-casein precipitates were redissolved in 5mM phosphate pH 7.0, with 3.3M urea and 0.1% mercaptoethanol and frozen. The α plus χ casein was usually recycled through the urea fractionation since it often contained large quantities of β-casein. The purity of the α plus χ and β-casein fractions was determined by alkaline gel electrophoresis.[66]

Mercuro-Agarose Chromatography

Protein X (protein containing cysteine and presumed to be β-casein) was purified from the bulk of the non-cysteine β-casein by mercuro-agarose chromatography. The method of Ruiz-Carrillo[59] was used with the addition of 3.3M urea to all buffers and with the following modifications. The crude β-casein fraction in 5mM phosphate pH 7.0, with 3.3M urea and 0.1% mercaptoethanol was thoroughly reduced by adding mercaptoethanol to approximately 1% (v/v) and warming to 37°C, with gentle shaking, for two hours. A solution 50% (w/v) TCA was then added to the β-casein solution to a final concentration of 20% (w/v). The precipitate of β-casein which formed was allowed to settle for 30 minutes then centrifuged at 9,000 xg for 10 minutes. The reduced β-casein was washed once with 0.1% HCl in cold acetone and three times more with cold acetone to remove the excess mercaptoethanol. The reduced β-casein was then dried under vacuum to prevent reoxidation.

The reduced β-casein was dissolved in 10mM phosphate pH 6.0 4mM EDTA and 3.3M urea. A solution of 1.0M NaOH was used to adjust the pH to 6.0. The reduced β-casein was then loaded onto a mercuro-agarose column (0.8cm wide, 15cm long), equilibrated in the buffer used to dissolve the reduced β-casein, at a flow rate of 6.5mL/hr. The column

was washed with buffer and buffer containing 0.2M NaCl overnight, to eliminate non-specific binding. The cysteine-containing proteins which remained on the column were eluted with 0.1% mercaptoethanol in the same buffer at a flow rate of 6.5mL/hr. Fractions were collected. The protein peak was verified by reading the fractions at 280 nm. The protein fractions were combined, dialyzed versus the same buffer and concentrated with Millipore filters in preparation for ω-NH_2-hexyl agarose chromatography.

ω-NH_2-Hexyl Agarose Chromatography

ω-NH_2-hexyl agarose was determined to be a good separation medium for β-casein by using the Shaltiel Test Columns.[67] The Shaltiel columns are a series of agarose columns, with various lengths of carbon-hydrogen spacers (2, 4, 6, 8, 10 and 12 carbons long) and an amino group attached at the end of the spacer arm. They act as hydrophobic columns and as ion exchangers. β-casein was found to bind well to the 6-12 carbon derivatives. It could be removed with a salt gradient (0-0.3M NaCl) from the hexyl derivative, but was virtually impossible to remove from the 8, 10 and 12 carbon derivatives. The ω-NH_2-hexyl agarose was prepared essentially as described by Cuatrecasas.[68] To 300mL of Sepharose 4B suspended in 300mL of water 75g of cyanogen bromide was added with stirring. A solution of 8M

NaOH was added immediately to raise and keep the pH=11. Pieces of ice were added as needed to keep the temperature about 20°C. After about ten minutes, the pH stopped dropping and indicated the reaction was complete. Crushed ice was added and the suspension transferred to a Buchner funnel and washed extensively with cold distilled water. Meanwhile a solution of 2m moles of 1,6-hexane diamine/mL was prepared in 300mL water and the pH adjusted to 10.0 with 6M HCl. The diamine solution was added to the activated sepharose and gently shaken in the cold for 48 hours. The derivatized gel was then washed with a large excess of cold distilled water and stored at 4°C. Three parts of the ω-NH_2-hexyl agarose were mixed with one part of Sephadex G-25 to give a constant bed volume and an adequate flowrate. The mixture was loaded into a small column (0.8cm wide, 8.0cm long) and equilibrated using the same mercuro-agarose buffer (see above) with the addition of 0.1% mercaptoethanol.

After loading the concentrated cysteine-containing proteins onto the column at a flowrate of 6.5mL/hr. and washing overnight with buffer, a gradient of 0.0-0.3M NaCl in the same buffer was applieβ-casein on ω-NH_2-hexyl luted. The elution profile of normal β-casein on ω-NH_2-hexyl agarose was used as a reference for determining the position of cysteine-containing β-casein.

Preparation of S-Carboxymethylated Proteins

It became apparent after several attempts at detecting cysteine, as cysteic acid, that the S-carboxymethylated derivative of cysteine would be easier to detect for amino acid analysis. The ω-NH_2-hexyl agarose peak containing protein X was combined. concentrated, dialyzed and a portion used for carboxymethylation according to Crestfield[69] with the exception that the samples (in buffer) were dialyzed versus a buffer containing TRIS, EDTA, urea and mercaptoethanol rather than adding these reagents as solids. The degree of derivatization was evaluated by isoelectric focusing.[70,71]

Purification of Protein X

Both alkaline and acid gel electrophoresis, as well as isoelectric focusing indicated that the ω-NH_2-hexyl agarose peak contained many other proteins besides protein X. Two dimensional electrophoresis, a modification of O'Farrell's Method,[70] was used to purify the protein X. The first dimension was isoelectric focusing in urea gels[71,72] (8cm long, 5mm wide tubes) using a pH range of 4-6. Gels were focused for 6 hours. The second dimension (a slab 16cm long, 14cm wide and 3mm thick) was a 15% acrylamide running gel, pH 8.8, with a 5% stacker pH 6.8.[73] Gels were run

overnight (18-20 hours) at 15mA/slab with bromophenol blue as tracing dye. Gels were stained with 0.1% Coomassie R-250 in 50% TCA for one hour, then destained for several days in many changes of 7.5% acetic acid.

The spot corresponding to "normal" β-casein was cut out of the gel and the protein (and dye) were electroeluted in a TRIS-borate-SDS buffer system. The same buffer used for alkaline electrophoresis[62] was used to electroelute the protein and dye from the gel. Small gel tubes (8cm long, 5mm wide) were constricted at the end by heat and filled to 1cm height with 1% agarose and 0.5% SDS in the TRIS-borate buffer. After the agarose hardened, the gel spot was cut into pieces with a razor. More molten agarose-buffer was added to the gel tube to about halfway and the gel pieces were quickly added to the tube, avoiding trapping any air bubbles. A little more molten agarose-buffer was added to completely seal in the gel pieces. All these operations were performed with surgical gloves on both hands. Then 8cm long pieces of specially prepared dialysis tubing which fit snugly on the constricted ends of the gel tubes were attached.[74] A very small amount (less than 0.5mL) of TRIS-borate buffer was included in the dialysis sack and the sack was clamped off to close it. The top electrode (cathode) buffer was TRIS-borate-0.5% SDS. The bottom (anode) buffer was plain TRIS-borate. A current of 6mA/gel was applied

for 5 hours during which time the dye and the protein concentrated in the bottom of the dialysis sack. The dialysis tubing containing the dye and protein was carefully removed after electroelution and dialyzed extensively against 0.2M ammonium bicarbonate in preparation for amino acid analysis. A blank piece of gel was worked up identically to the protein spots.

Amino Acid Analysis

Amino acid analysis was performed on electroeluted samples of carboxymethyl derivatives or samples cut directly out of gels. The analysis was done in the laboratories of Dr. S. Stein and Dr. Y. Pan, at Hoffman-LaRoche, Nutley, N.J., using fluorescamine as detector.[74,75]

Electrophoresis

Both alkaline [62] and acid[75] polyacrylamide gel electrophoresis was performed on samples during all stages of the purification procedures to determine the numbers and types of proteins present. Isoelectric focusing in urea gels was done essentially as described as Josephson.[70,71]

Protein Assays

Protein assays were performed according to Bradford[78] using ω-NH_2-hexyl agarose purified β-casein as the standard. β-casein was standardized by recording the absorbance of a solution at 280 nm and using the extinction coefficient for β-casein,[79] (ϵ = 4.6 for a 10mg/mL solution).

PAS (Periodic Acid - Schiff) Staining

PAS staining[80] was done on acid gel electrophoresis samples of the ω-NH_2-hexyl agarose peak containing protein X to determine if any of the contaminating proteins were glyco-proteins. Bovine Serum Albumin (BSA) was used as a negative control and ovalbumin was used as a positive control.

Phosphorylation of β-Casein

In an attempt to learn if the degree of phosphorylation of β-casein changed with age, the amount of phosphorylation was evaluated both enzymatically (with alkaline phosphatase) and chemically (wet digestion).

Phosphate content of β-casein was determined enzymatically using initial rate techniques and a colorimetric method to detect inorganic phosphate liberated by the action of alkaline phosphatase on β-casein.[79] The assay mixture contained about 1.5mg/mL β-casein, 0.20M TRIS, pH 8.0, 8.0 X 10^{-4}M $MgCl_2$, and aproximately 1 unit alkaline phosphatase (calf intestine) Type VII. The protein, buffer and $MgCl_2$ were mixed and warmed to 37°C and the reaction was started by adding the enzyme. Several assay tubes were run simultaneously and stopped at various time intervals by adding 0.2mL of 50% TCA. The protein precipitate was filtered out and 1mL of the filtrate was added to 1mL of the phosphate reagent. The Lin-Morales method[81] was used to determine phosphate content. This method makes use of a "one step" reagent containing ammonium molybdate and ammonium metavanadate as phosphate complexing agents. A series of standards was prepared at the same time. The absorbances were read at 350 nm and a standard curve was made. Phosphate released from β-casein was plotted versus time and a maximum value was obtained. From this maximum value and the known concentration of β-casein, the number of phosphate groups/molecule of β-casein could be determined.

Phosphate content of β-casein was also determined using wet digestion[82] and the colorimetric method of Kirsten and Carlsson.[83] The wet digestion procedure used to determine phosphate content was that of McKenzie and Murphy[82]. Nitric and sulfuric acids were added to the β-casein samples and the samples were heated in a digestion rack until no more brown nitrogen peroxide or sulfuric acid fumes evolved. Hydrogen peroxide was then added and the mixture heated until sulfuric acid fumes evolved. This treatment completely oxidizes the protein present and cleaves the phosphate from its seryl residues. The free inorganic phosphate was then assayed by the method of Kirsten and Carlsson.[83] This colorimetric method used molybdate as the phosphate complexing agent. However the molybdate-phosphate complex was extracted with amyl acetate from the aqueous phase. Then stannous chloride was used to reduce the phosphomolybdic acid to molybdenum blue and the absorbencies were read at 720nm. Standards were run along with the unknowns.

Calculation of Error Rates

$$\frac{\text{\# of cysteine residues in cys-}\beta\text{-casein}}{209 \text{ amino acids}} \times 100 = \%\ \text{cysteine error in the error containing molecules}$$

$$\frac{\text{grams cys-containing }\beta\text{-casein}}{\text{total grams }\beta\text{-casein}} \times 100 = \%\ \beta\text{-casein with cysteine error}$$

$$\%\ \beta\text{-casein with cys error} \times 20 = \%\ \beta\text{-casein with any amino acid error}$$

III.

RESULTS AND DISCUSSION

FRACTIONATION OF WHOLE CASEIN

An overall view of the purification schemes used to purify cysteine-containing β-casein is shown in Fig. 3.1. Out of a typical 10g of crude β-casein only approximately 10-100 μg was the putative cysteine β-casein isolated on two dimensional gels.

A typical urea fractionation[65] of the milk sample from Lucy, aged 12, yielded:

whole casein	34.3g
α+ χ casein's (Fractionations I-II)	15.9g
crude β-casein (Fractionations I-II)	18.1g

$$\% \text{ crude } \beta\text{-casein} = \frac{15.9}{34.3} \times 100 = 46.3\%$$

See Figure 3.2, alkaline gels of the various steps in the urea fractionation of the milk, sample from Ruby aged 13. $\alpha + \chi$ caseins from the first and second fractionations were combined after purity was assayed using alkaline electrophoresis. The same was done for the two crude β-casein fractions. Protein content of each fraction was determined by the Bradford Assay.[78]

To determine the % β-casein in crude β-casein, alkaline gels of the crude β-casein fractions were scanned at 580_{nm} with a Gilford gel scanner attached to a Gilford spectrophotometer. A tracing was made (See Figure 3.3), the

peaks were cut out and weighed, and the % β -casein was calculated as:

$$\frac{\text{weight of } \beta\text{-casein peak}}{\text{total weight of all peaks}} = \%\ \beta\text{-casein}$$

A typical calculation for Ruby, aged 13, was:

$$\frac{\text{weight of } \beta\text{-casein peak}}{\text{total weight of all peaks}} = \frac{0.063g}{0.088g} \times 100 = 72\%$$

Fig. 3.1 Flow Diagram of purification schemes

Flow Diagram

Purification of cysteine-containing β-casein

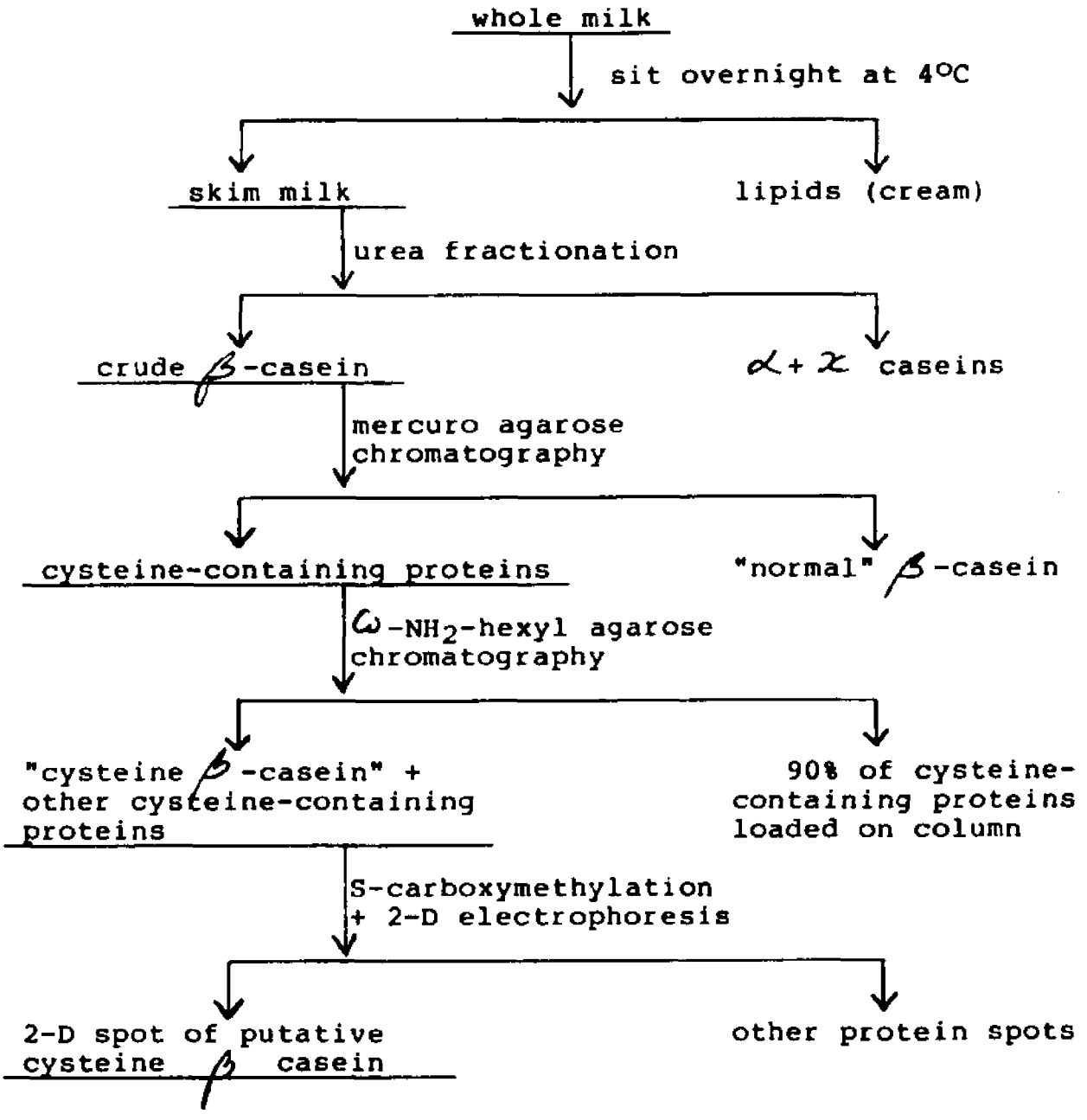


Fig. 3.1

Fig. 3.2 Alkaline gels of urea fractionation of whole casein from Ruby-13:
a) whole casein,
b) α+ κ caseins (1st fractionation),
c) crude β -casein (1st fractionation),
d) supernatant from crude β-casein (1st fractionation),
e) α+ κ casein (2nd fractionation),
f) crude β -casein (2nd fractionation),
g) supernatant from crude β-casein (2nd fractionation).

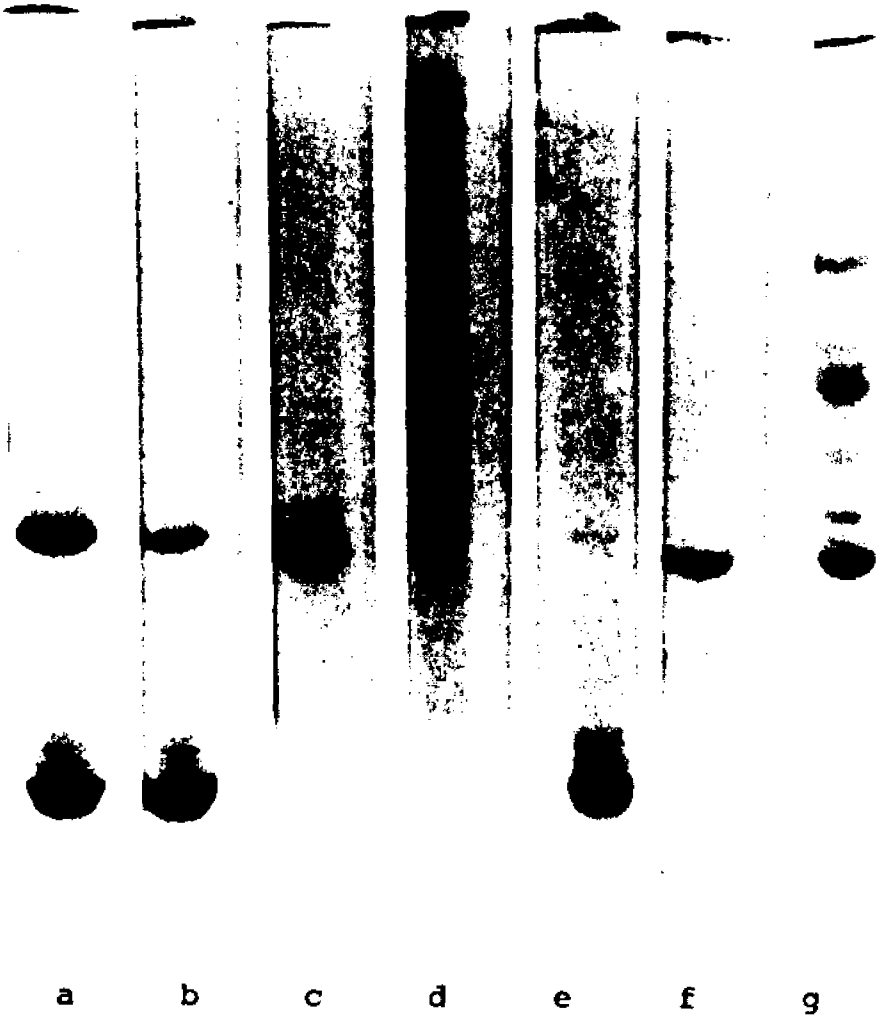


Fig. 3.2

Fig. 3.3a Gel scanning at 580mm of Lucy-12 crude β-casein.

Fig. 3.3b Alkaline gel of Lucy-12 crude β-casein.

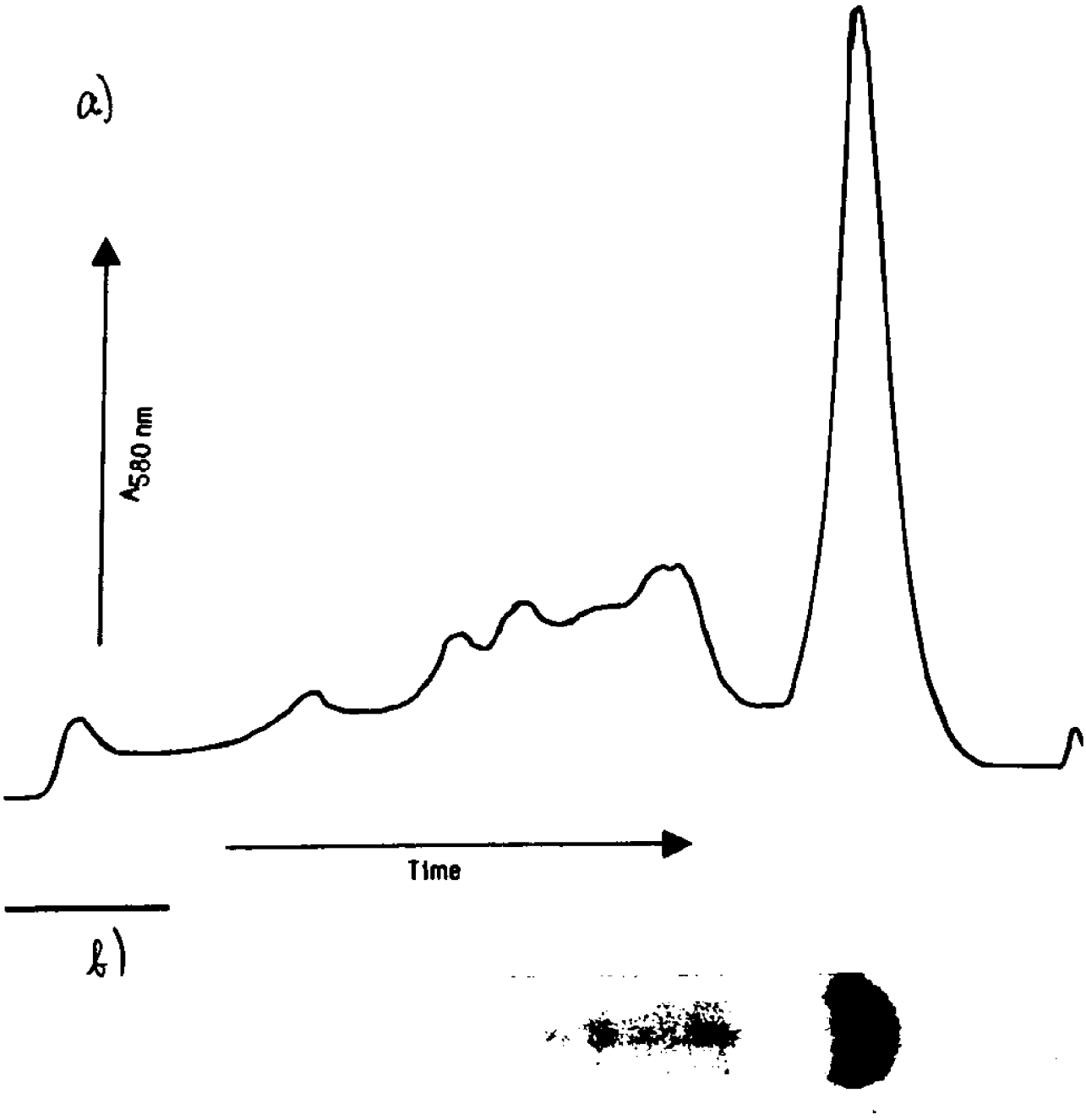


Fig. 3.3

IV.

RESULTS AND DISCUSSION

MERCURO AGAROSE CHROMOTOGRAPHY OF CRUDE β-CASEIN

A mercuro-agarose chromotograph is shown in Figure 4.1. The wash with 0.2 NaCl did indeed remove some nonspecifically bound proteins. Typically out of 10 grams of crude β-casein loaded onto the mercuro agarose column, only 50-100mg of cysteine-containing proteins was obtained.

Alkaline gels (See Fig. 4.2), acid gels (See Figure 4.3), as well as isoelectric focusing (See Figure 4.4) indicated that the mercuro agarose cysteine-containing protein peak is heterogeneous and does not contain only candidates for cys-β-casein.

Fig. 4.1 Mercuro agarose chromotography of a crude β-casein sample.

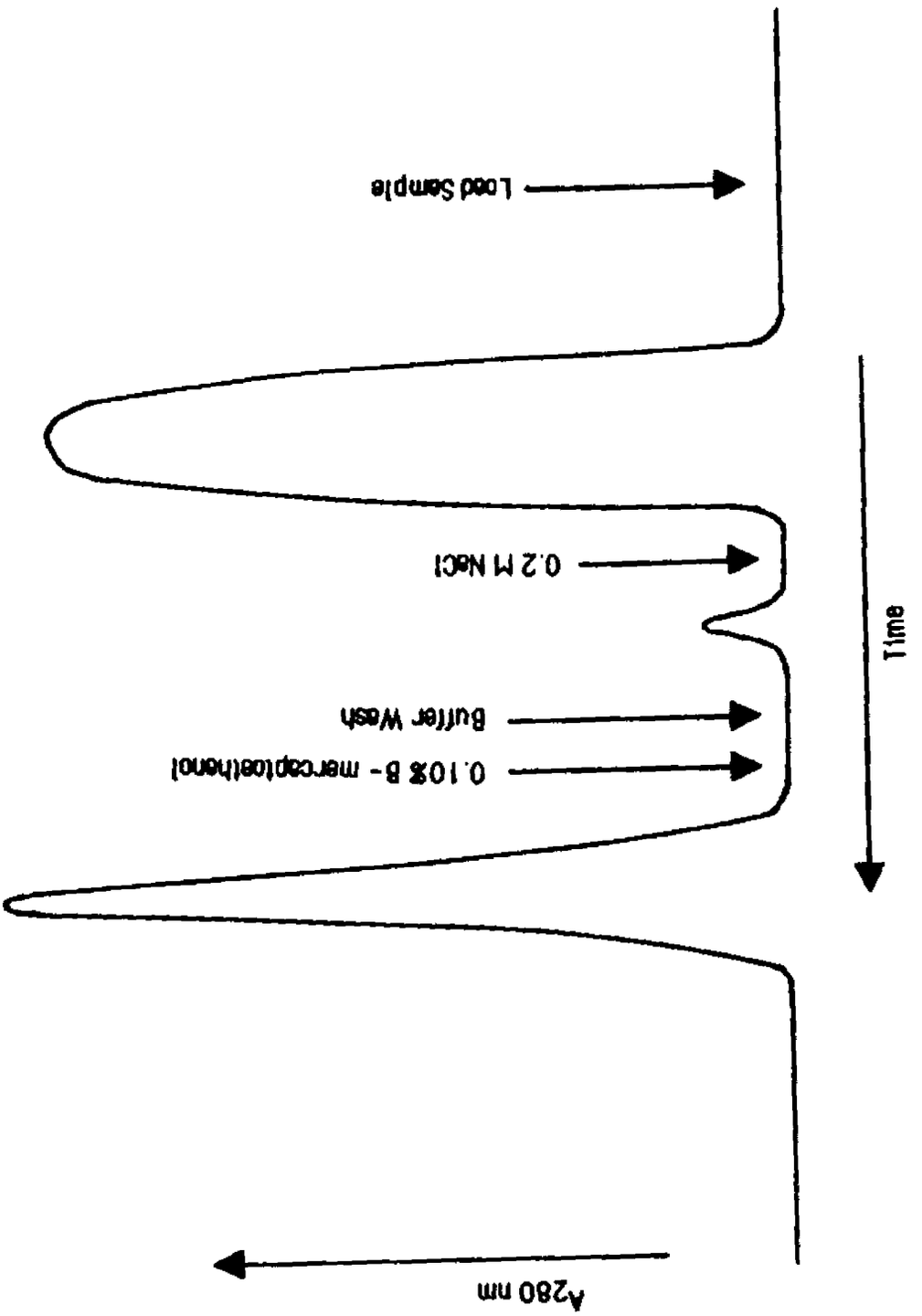

Load Sample
0.2 M NaCl
Buffer Wash
0.10% B- mercaptoethanol
Time
A280 nm

Fig. 4.1

Fig. 4.2 Alkaline gels:
a) crude β -casein not bound to mercuro agarose,
b) cysteine-containing proteins eluted from mercuro agarose.

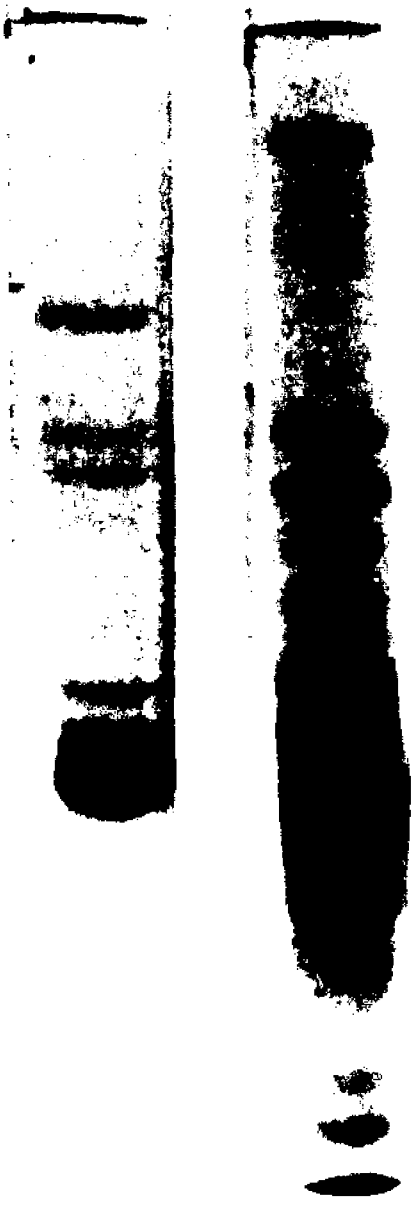


Fig. 4.2

Fig. 4.3 Acid gels:
a) crude β -casein not bound to mercuro agarose,
b) cysteine-containing proteins eluted from mercuro agarose.

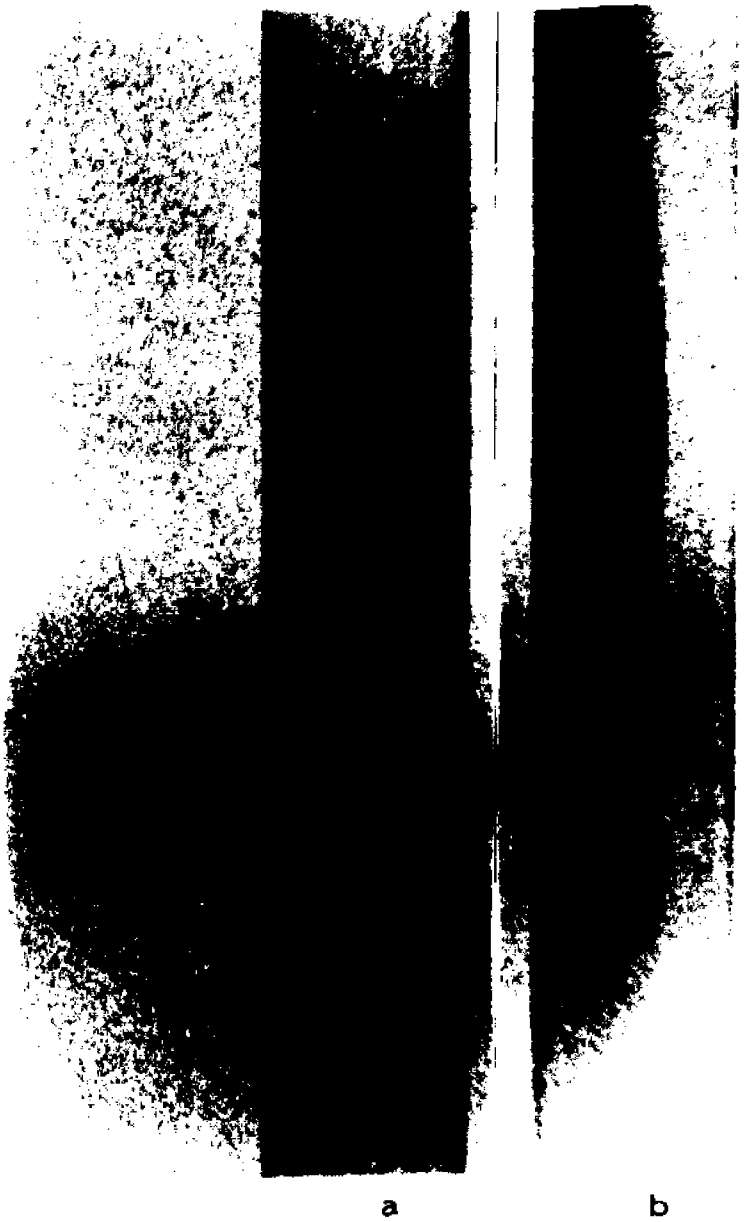


Fig. 4.3

Fig. 4.4 Isoelectric focusing, pH 4-6:
a) crude β -casein not bound to mercuro agarose,
b) cysteine-containing proteins eluted from mercuro agarose.

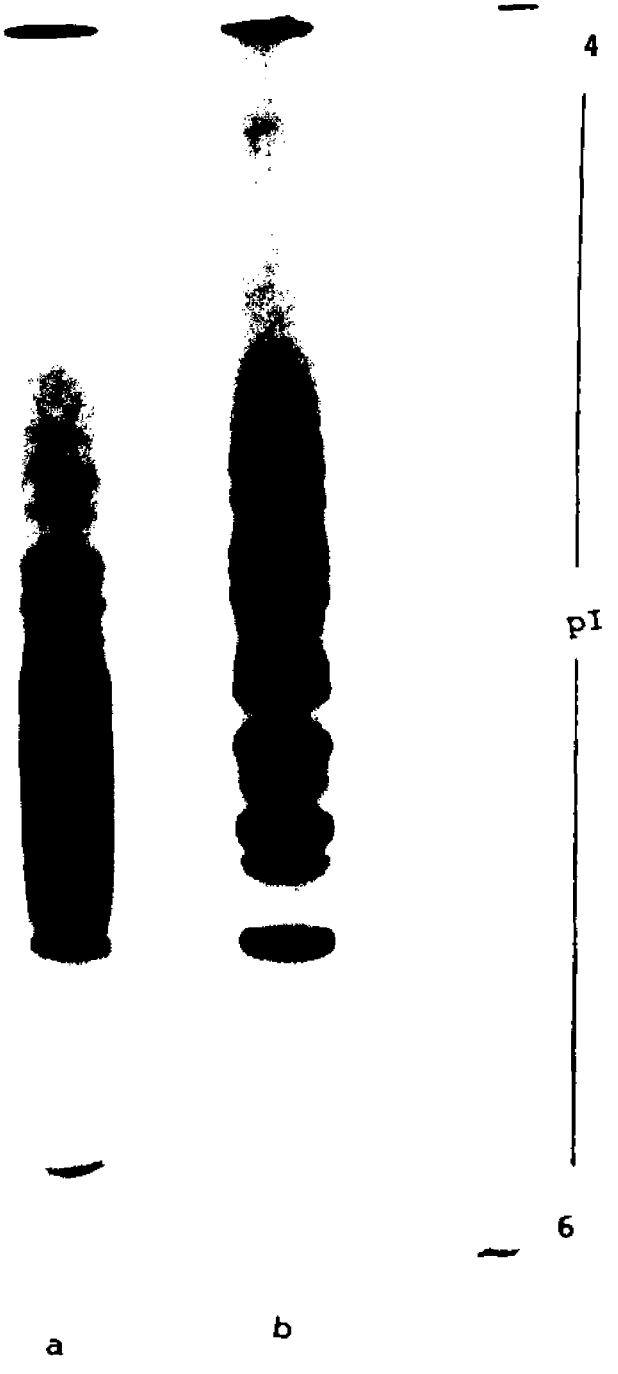


Fig. 4.4

V.

RESULTS AND DISCUSSION

ω- NH_2 - HEXYL AGAROSE CHROMATOGRAPHY OF CYSTEINE-CONTAINING PROTEINS

The heterogeneous peak of cysteine-containing proteins was chromatographed on ω-NH_2-hexyl agarose as described previously. A chromatograph of such a run is shown in Figure 5.1. Control runs of normal crude β-casein as well as whole casein were done on the ω-NH_2-hexyl agarose column to determine the location of β-casein in the chromatograph. It should be noted that the majority of proteins don't bind to this column and are easily washed off with plain buffer ($> 90\%$). Both alkaline gels (See Figure 5.2) and isoelectric focusing (See Figure 5.3) of ω-NH_2-hexyl agarose peak III (containing protein X or cys-β-casein?) showed this peak was not homogeneous either. Thus two-dimensional electrophoresis was performed on the proteins from ω-NH_2-hexyl agarose peak III (protein X here?). S-carboxymethyl derivatives[69] of this peak had to be prepared first because unless cysteine was so derivitized it was hidden in the glutamic acid peak (as cysteic acid) when amino acid analysis was performed.

Fig. 5.1 ω-NH_2-hexyl agarose chromatography of cysteine proteins eluted from mercuro agarose column.

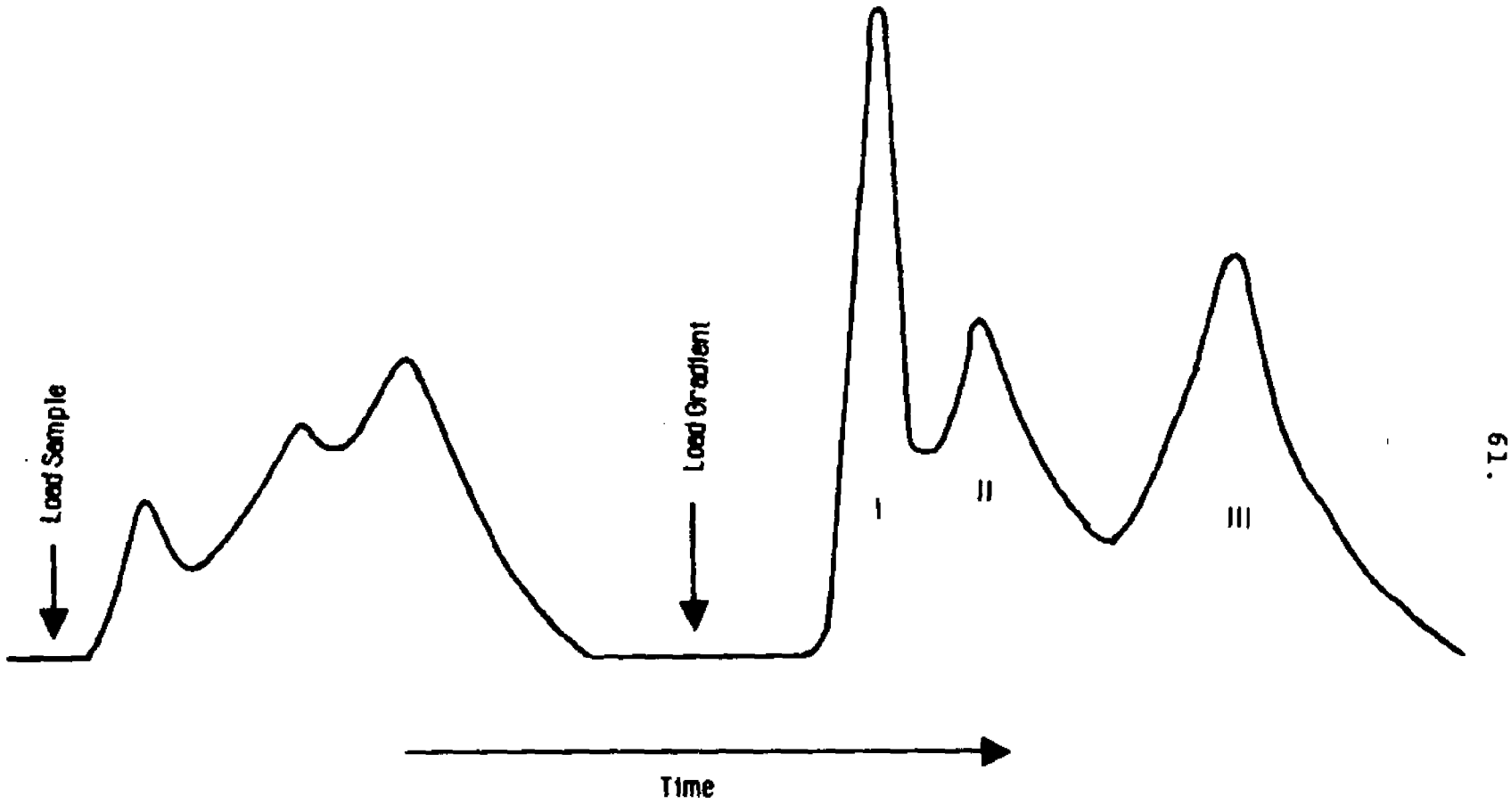
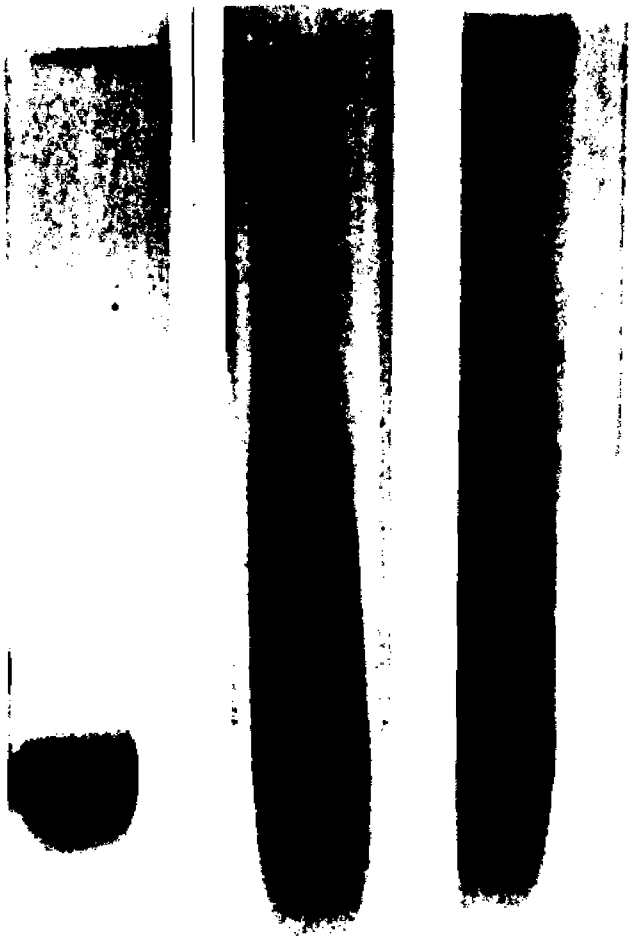


Fig. 5.1

Fig. 5.2 Alkaline gels:
a) crude β-casein,
b) ω-NH_2-hexyl agarose peak III,
c) ω-NH_2-hexyl agarose peak III + β-casein.



a b c

Fig. 5.2

Fig. 5.3 Isoelectric focusing, pH 4-6:
a) β-casein,
b) ω-NH_2-hexyl agarose peak III,
c) ω-NH_2-hexyl agarose peak III + β casein.

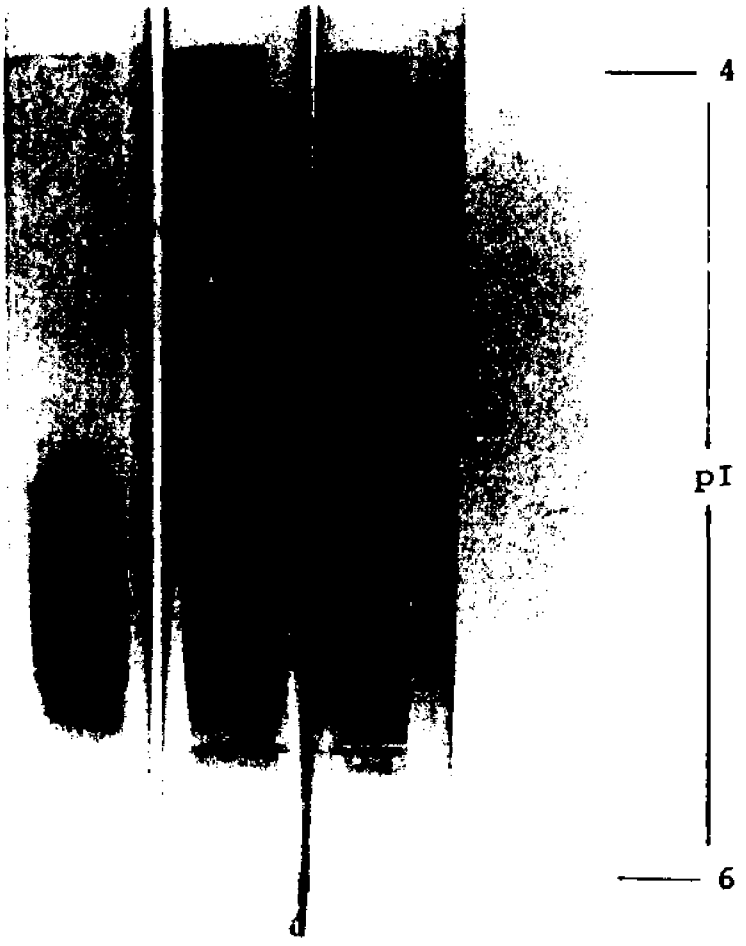


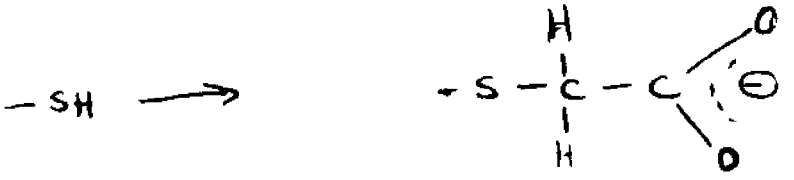
Fig. 5.3

VI.

RESULTS AND DISCUSSION

S-CARBOXYMETHYLATION OF ω-NH_2-HEXYL AGAROSE PROTEINS

S-carboxymethyl derivatives of the ω-NH_2-hexyl agarose peak containing protein -X were made.[69] The degree of S-carboxymethylation was assessed by isoelectric focusing. Each -SH when alkylated yields:



Thus -SH containing proteins would acquire more negative charge and their pI's would become more negative (lower). This is readily seen in the isoelectric focusing gels shown in Fig. 6.1.

Fig. 6.1 Isoelectric Focusing, pH 4-6:
a) β-casein,
b) β-casein -S carboxymethyl control (negative),
c) ω -NH_2-hexyl agarose peak III,
d) S-carboxymethyl derivatives of ω-NH_2-hexyl agarose peak III.

Fig. 6.1

VII.

RESULTS AND DISCUSSION

TWO-DIMENSIONAL ELECTROPHORESIS

Two-dimensional gel electrophoresis was performed, as described previously, on the S-carboxymethyl derivatives of the ω-NH_2-hexyl agarose peak containing protein -X. Typical first-dimensional gels are shown in Figure 7.1. Typical two-dimensional gels are shown in Figures 7.2, 7.3, 7.4. Figures 7.2 and 7.3 are from individual cows; Figure 7.4 is a two-dimensional gel of a pooled commercial sample. The gel pattern of this pooled sample was used to determine the position of protein -X (cysteine-containing β-casein) based on the position of normal β-casein. Two possibilities existed but once amino acid analysis was performed, there was only one candidate (spot #1) for cystein -β-casein.

Control samples of crude β-casein (normal) were run on 2-D gels to establish the position and pI of normal β-casein. Figures 7.5a and 7.5b show this for a pooled commercial sample. Similar runs were done for each individual cow sample, the control gels were compared with the run of cysteine-containing proteins and the spot most nearly corresponding to that of normal β-casein was cut out and assayed for amino acid composition.

Fig. 7.1 Isoelectric focusing, pH 4-6:
a) Jeannie-3 crude β-casein,
b) Jeannie-3 S-carboxymethyl derivatives of ω-NH_2-hexyl agarose peak III,
c) Japonica-5 crude β-casein,
d) Japonica-5 S-carboxymethyl derivatives of ω-NH_2-hexyl agarose peak III.

Fig. 7.1

Fig. 7.2 Two-dimensional electrophoresis of S-carboxymethyl derivatives of ω-NH_2-hexyl agarose peak III from Valma-13.

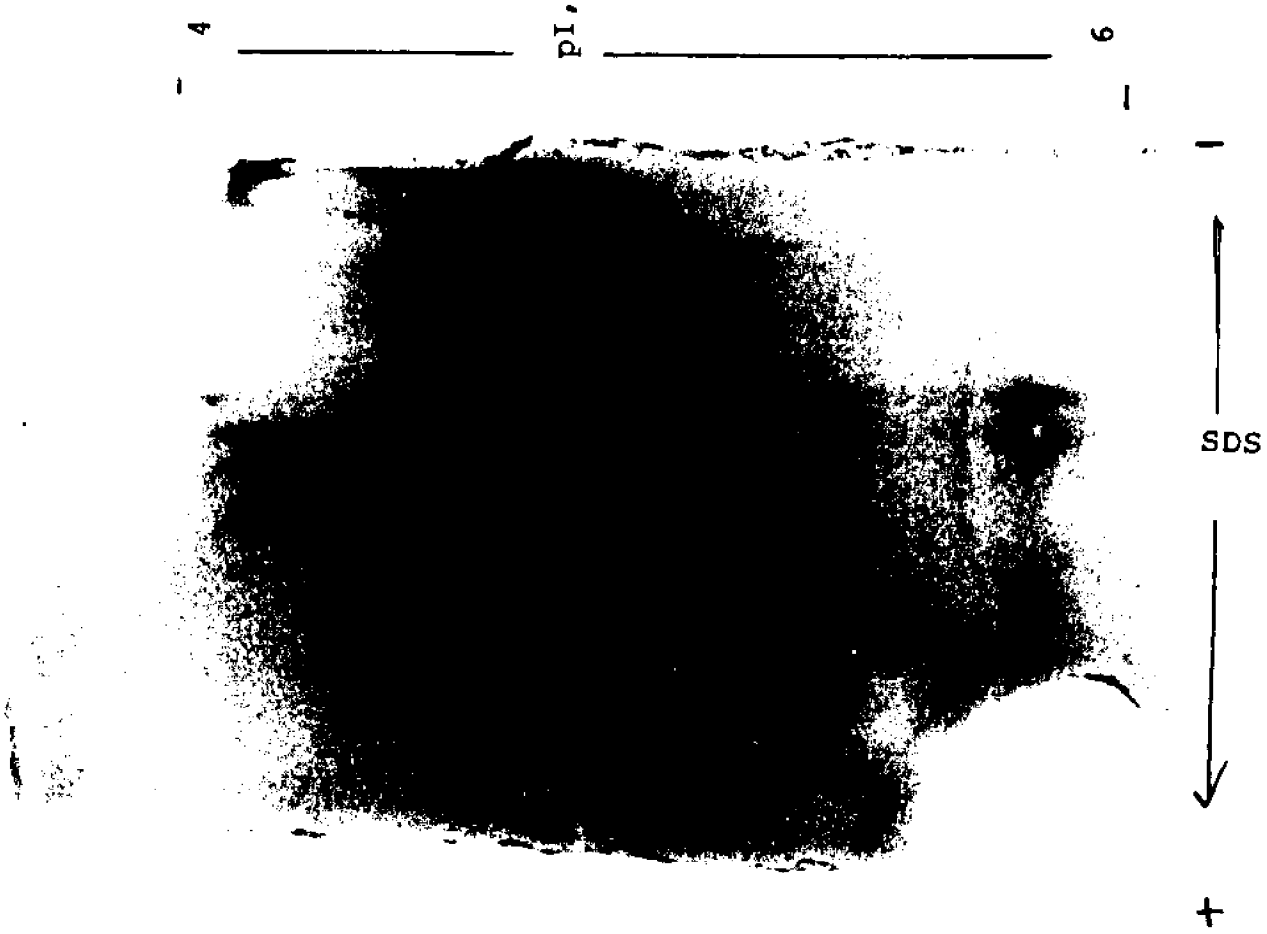


Fig. 7.2

Fig. 7.3 Two-dimensional electrophoresis of S-carboxymethyl derivatives of ω-NH_2-hexyl agarose peak III from Japonica-5.

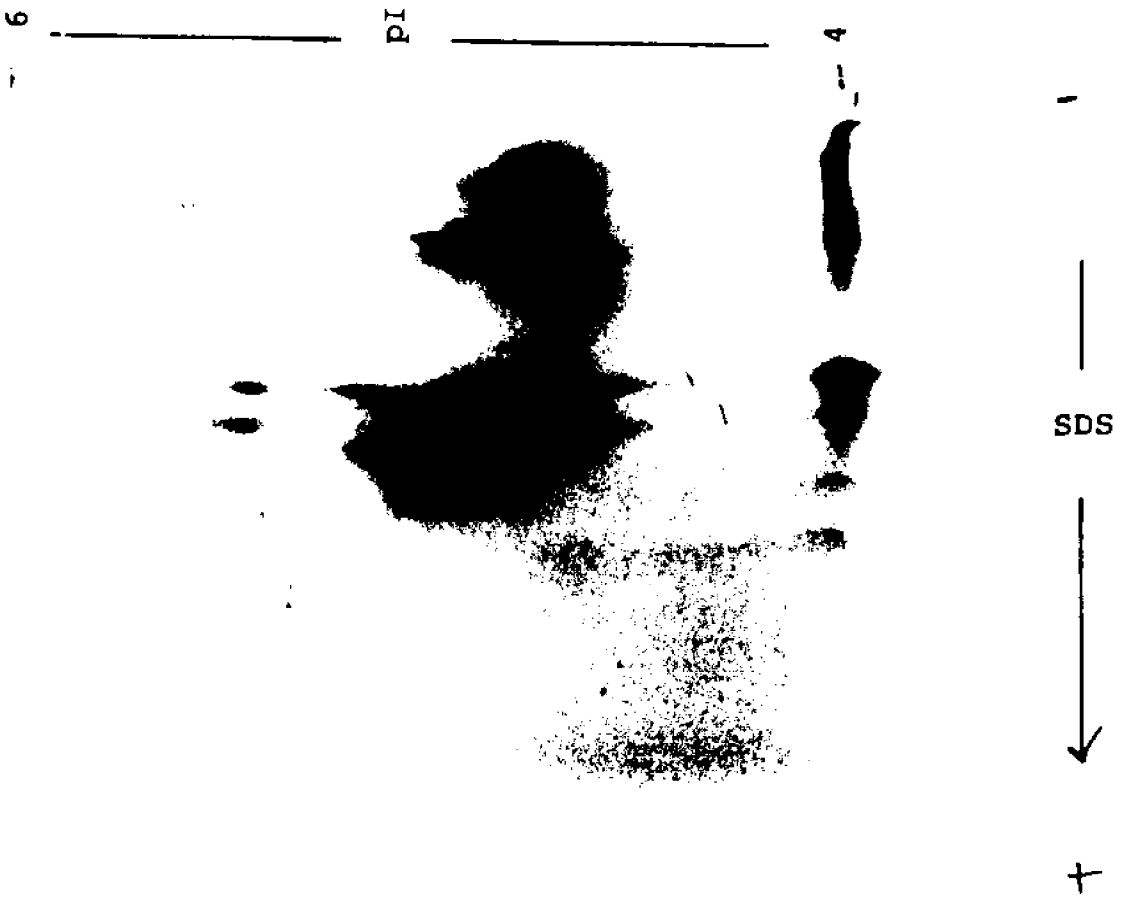


Fig. 7.3

Fig. 7.4 Two-dimensional electrophoresis of S-carboxymethyl derivatives of ω-NH_2-hexyl agarose peak III from a pooled commercial sample.

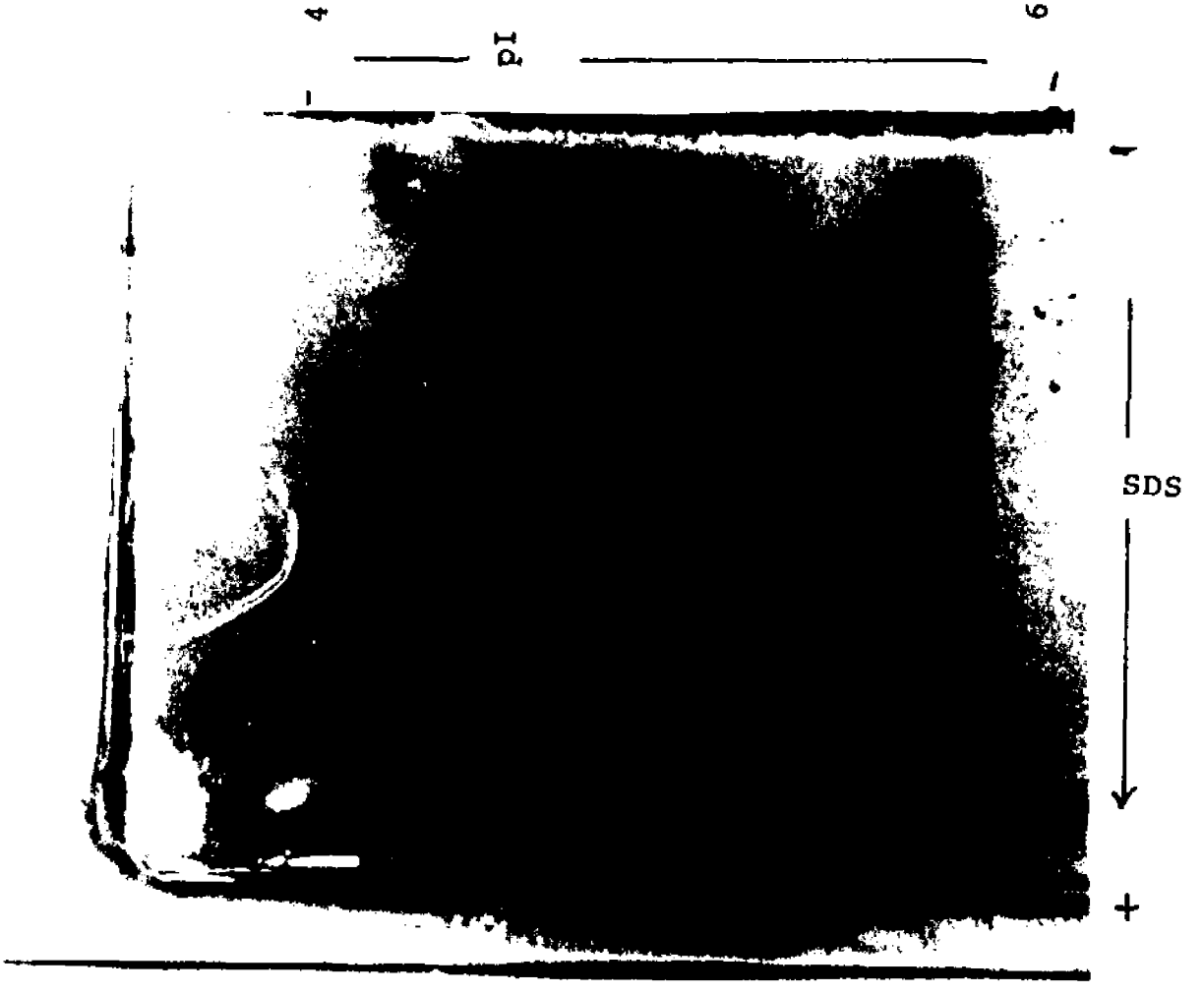


Fig. 7.4

Fig. 7.5a Two-dimensional electrophones of cysteine-containing proteins with putative cys-β-casein (pooled commercial sample).

Fig. 7.5b Two-dimensional electrophoresis of control crude β-casein (pooled commercial sample).

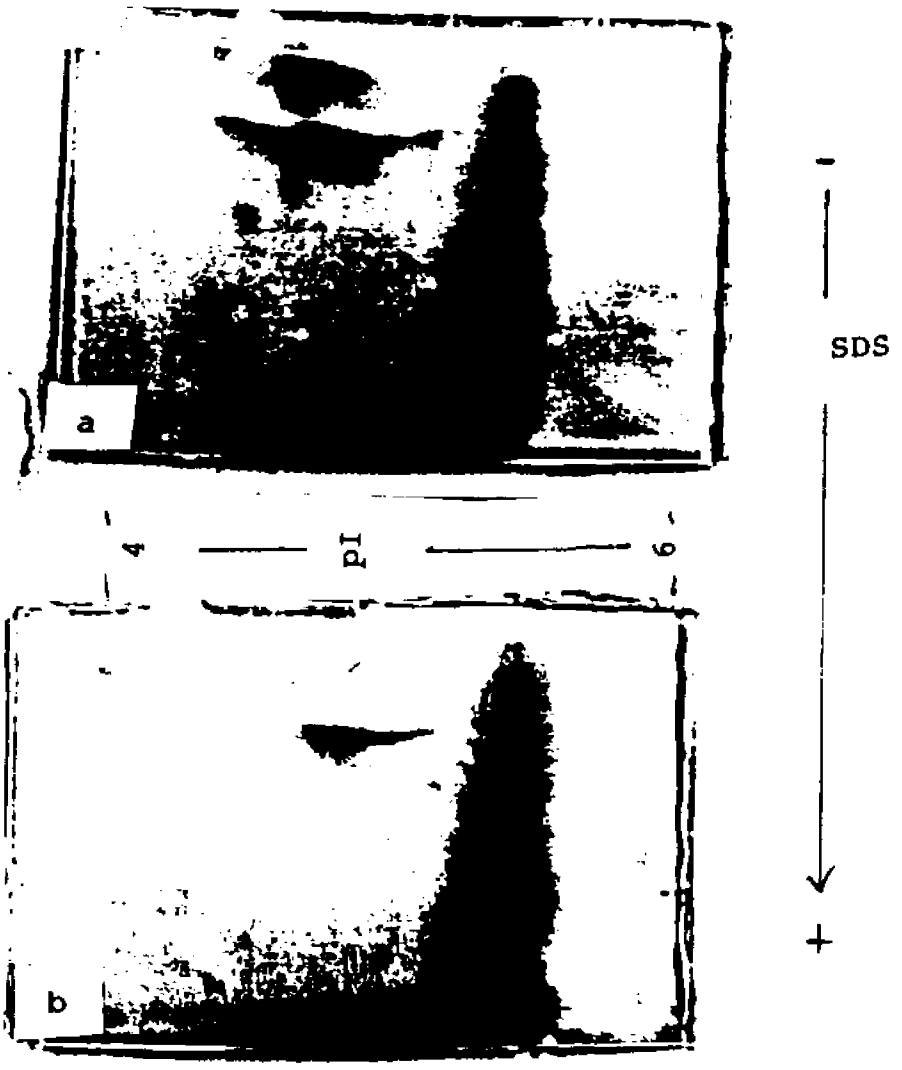


Fig. 7.5

VIII.

RESULTS AND DISCUSSION

PROTEIN ASSAYS

A typical standard curve for the Bradford Assay[78] is shown in Figure 8.1. This assay was used throughout the purification procedures rather than using A_{280nm} because the molar absorbtivities of α & χ caseine (the other main components of milk) are about twice that of β-casein. It was very important to use purified β-casein as the standard in the Bradford assay because it had a much lower binding affinity for the Coomassie G-250 dye than the BSA (Bovine Serum Albumin) often used as a protein standard. See Figure 8.2.

Fig. 8.1 Standard curve for Bradford protein assay, using β -casein as standard.

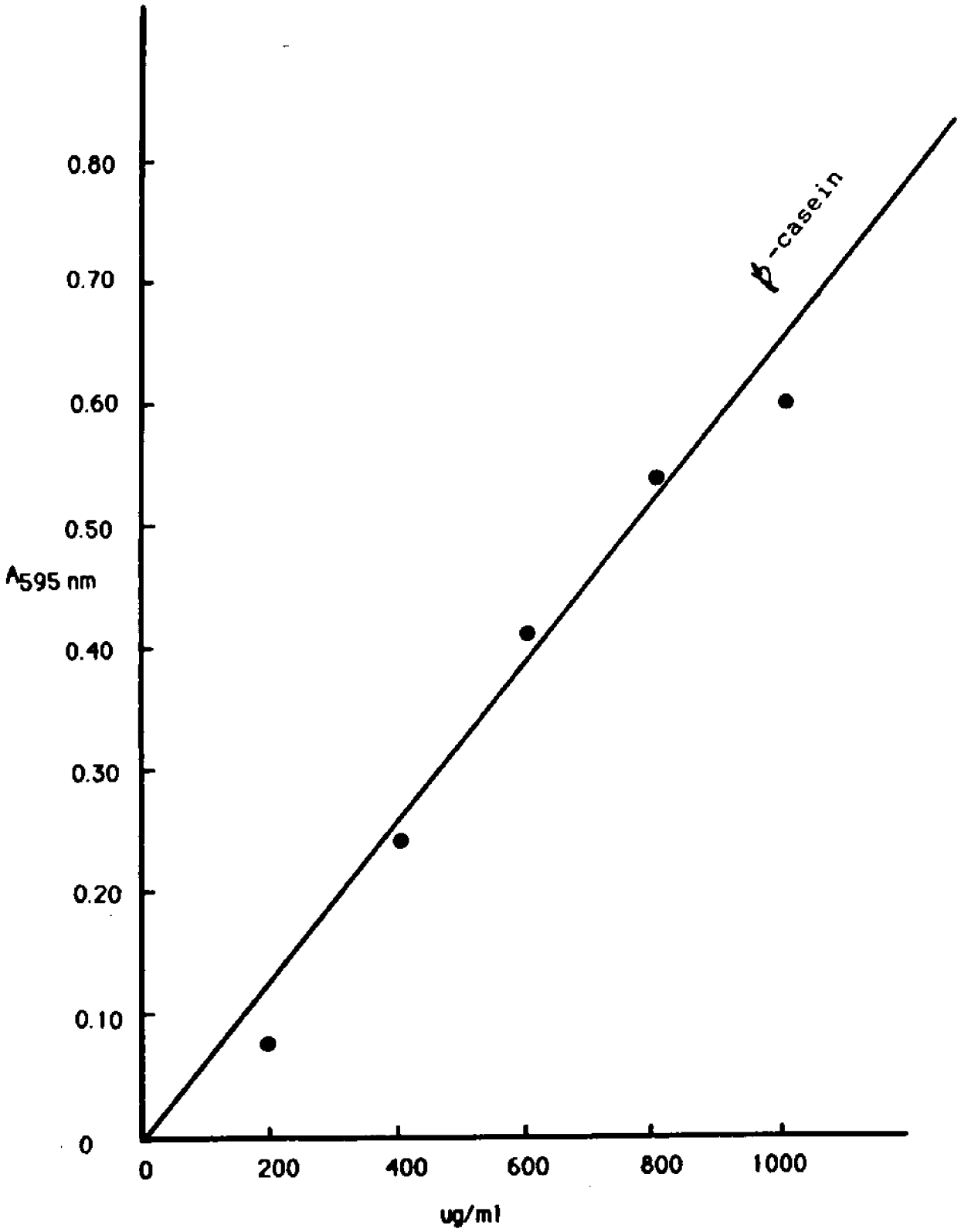

0.80
0.70
0.60
0.50
A595 nm
0.40
0.30
0.20
0.10
0
0
200
400
600
800
1000
ug/ml
β-casein

Fig. 8.1

Fig. 8.2 Comparison of β-casein and BSA as standards for Bradford protein assay.

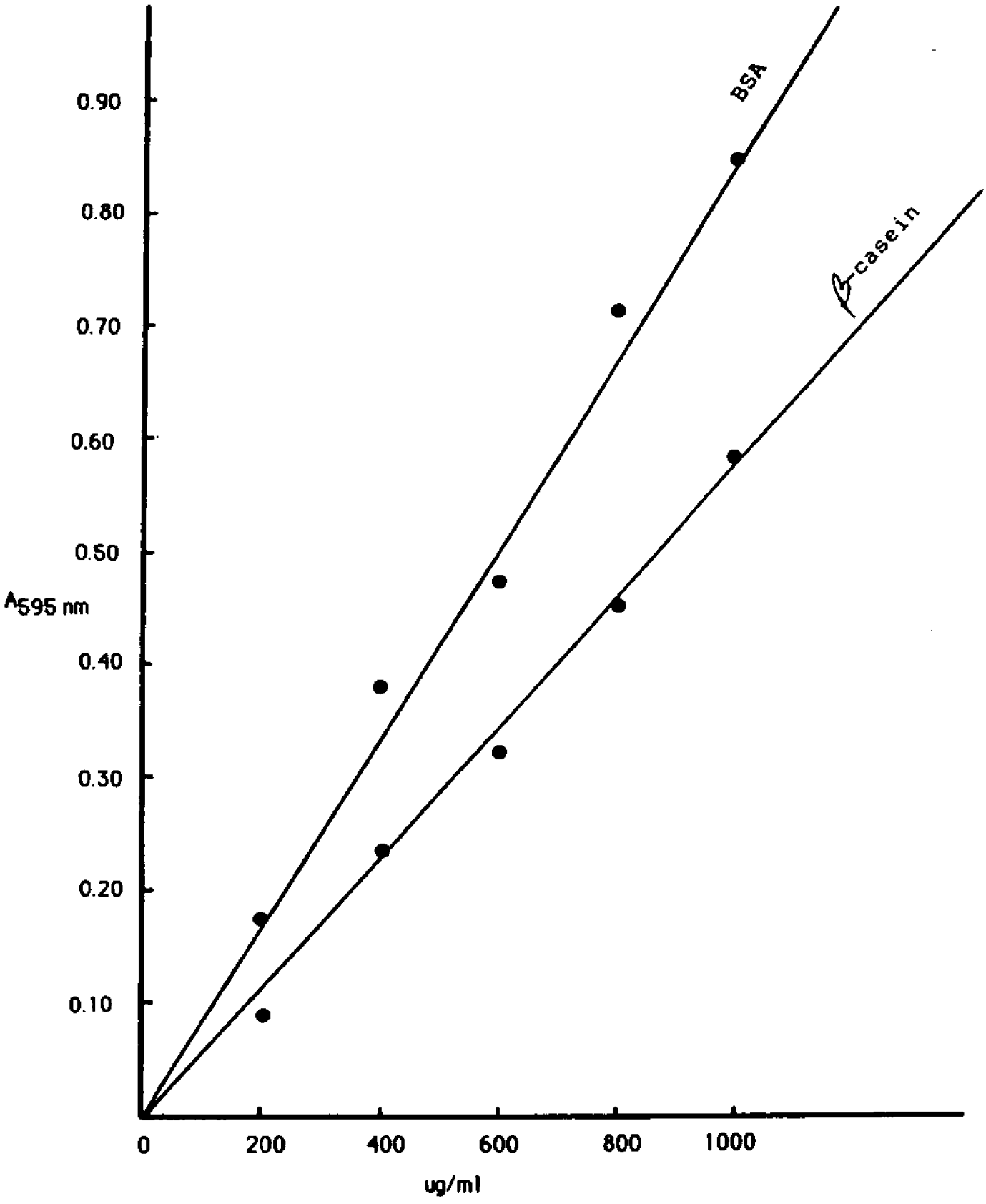

0.90
0.80
0.70
0.60
0.50
$A_{595\ nm}$
0.40
0.30
0.20
0.10
BSA
β-casein
0
200
400
600
800
1000
ug/ml

Fig. 8.2

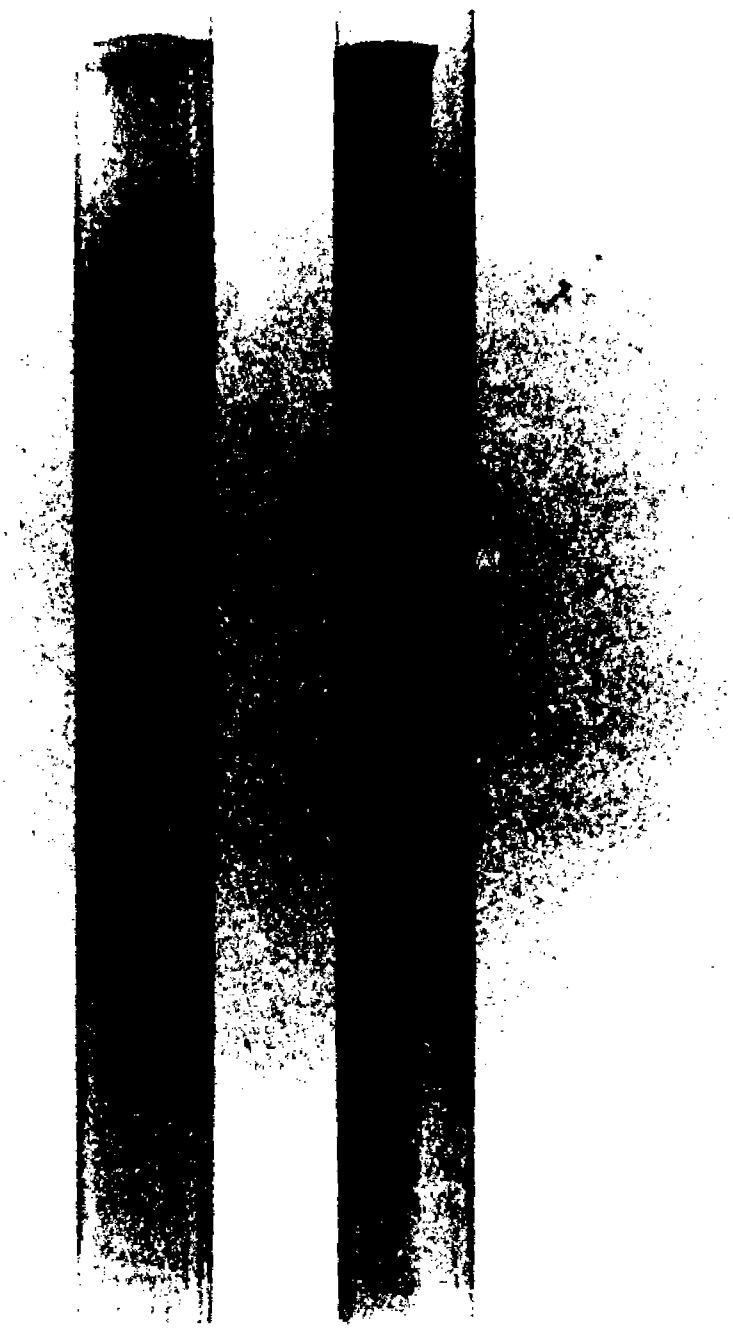
IX.

RESULTS AND DISCUSSION

PAS STAINING

Results from the PAS staining[80] were negative for the protein -X containing peak from ω-NH_2-hexyl agarose chromatography. The procedure worked because positive results were obtained for the positive control, Ovalbumin. See Figure 9.1. It was concluded that there were no carbohydrate-containing proteins in the ω-NH_2-hexyl agarose peak with protein -X.

Fig. 9.1 Periodic Schiff (PAS) Staining:
a) Ovalbumin with PAS stain,
b) Ovalbumin with Coomassie R-250 stain.



a b

Fig. 9.1

X.

RESULTS AND DISCUSSION

AMINO ACID ANALYSIS

As can be seen in Fig. 10.1, there are several amino acids present in normal β-casein which, with a single base change, could become cysteine. Note that there are four codes for Ser, two each for Gly, Arg and Phe and one code for Tyr.

Amino acid analysis of 2 spots from commercial sample two-dimensional gel which had been purified as described previously showed one protein to be very similar to β-casein but not identical in amino acid composition. Pro was too low, Arg was too high. Gly was questionable because it was also high in the control, probably due to the use of a Tris-Gly buffer system. See Table 10.1. Based on the criteria that amino acides analysis shold be within 90% of the published (or control sample) values, for analysis done on spots cut out of polyacrylamide gels[84], it was concluded that spot #1 was not β-casein containing cysteine.

Amino acid analysis of the samples from Rosebud - 2 years, Japonica - 5 years, Lucy - 12 years, and Ruby - 13 years (different aged Ayrshire cows) revealed that protein -X was not cysteine containing β-casein. That is, the protein spot most nearly corresponding to the location of β-casein was not β-casein containing misincorporated cysteine. See Table 10.2

An estimate of the error rate can be made based on the limits of detection of our system as follows:

1) 1 μg protein is about the minimum amount that can be detected on a two-dimensional gel in this system.
2) If about 250 μg protein was loaded per two-dimensional gel and
3) There was about 5mg of protein in the ω-NH_2-hexyl agarose peak containing protein -x, from a total of about 5g of β-casein, then:

$$\frac{1\ \mu g}{250\ \mu g} \times 5000\ g = 20\ \mu g\ \text{protein -x}$$

$$\frac{20\ \mu g\ (\text{protein -x})}{5\times10^{6}\ g\ (\text{total } \beta\text{-casein})} = 4\times10^{-6}$$

Or, 4 out of 1,000,000 parts β-casein can be detected as protein -x.

Therefore, the error rate of cysteine misincorporation in β-casein must be lower than 4×10^{-6}. Others who have measured misincorporation rates have found values of between 10^{-4} and 10^{-5} or at least one order of magnitude higher. Loftfield and Panderjaet[85] analyzed peptides of human and rabbit hemoglobin α chains for the misincorporation of Val for Ile. They found an error rate of 2-6 parts per 10,000 in an in vitro system. Harley et al.[86] used cultured human fibroblasts from fetal, young and old donors as well as cells from subjects with Hutchinson-Gilford and Werner

Syndromes, to study missynthesis of proteins with and without histidine starvation. They examined two-dimensional gels of the fibroblast proteins and looked for satellite spots trailing the native protein spots. These satellite spots represented proteins with amino acid substitutions caused by histidine starvation. Harley et al. found error rates of about 1 per 10,000 for all cells. There was no correlation with age of donor or maximal lifespan in vitro.

Popp et al.[87] measured the error rates for misincorporation of isoleucine into human hemoglobin A_1, which does not normally contain isoleucine. They used conventional purification techniques such as molecular selving and ion exchange to purify the hemoglobin A_1. They did not, however, use a two dimensional gel system to verify the purity of their samples. They estimated an error rate of $3x10^{-5}$ for isoleucine misincorporation for adults.

Hirsch et al.[88] used the amino acid analog AIBA (α - aminoisobutyric acid) to measure the misincorporation rate of this analog for leucine in mice. They found no statistical differences in young and old mice for misincorporation of AIBA. Their overall error rate was $3x10^{-5}$.

Even though the misincorporation studies with β-casein could not be correlated with age because no cysteine was detected at any age, a limit has been set on error rates for misincorporation. If cysteine is misincorporated at less than $4x10^{-6}$ and if we take misincorporation to be a random phenomena, affecting all amino acids equally, then 20x $4x10^{-6}$ or $8x10^{-5}$ or about 10^{-4} would be the total error rate for all amino acids. This would mean that in any one protein about 1 part per 10,000 would have some kind of amino acid error. This total error rate seems more reasonable than the two mentioned above since $10^{-4}x20 \approx 2x10^{-3}$ or 2 parts out of 1000 in every protein would have an amino acid error. It can also be stated that the position of a protein on a two-dimensional gel is not absolute proof of the identity of the protein. Only after amino acid analysis is done on the spot can the identity of the protein be positively made.

Fig. 10.1 Genetic code. Circled amino acids can become Cysteine with one base change.

	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Non2	Non3	A
	Leu	Ser	Non1	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Fig. 10.1

TABLE 10.1

AMINO ACID ANALYSIS OF POOLED COMMERCIAL SAMPLE (Two-Dimensional Spot)

Amino Acid	Sample Spot #1	Sample Spot #2	Sample β-casein control	Sample β-casein Published values
CM Cys	3	3	0	0
Asp	10	12	9	9
Thr	10	12	9	9
Ser	17	16	16	16
Glu	40	37	39	39
Pro	33	28	35	35
Gly	10	10	5	5
Ala	5	10	5	5
Val	13	10	19	19
Met	4	3	6	6
Ile	9	11	10	10
Leu	22	22	22	22
Tyr	3	6	4	4
Phe	8	7	8	9
His	5	5	5	5
Lys	12	12	11	11
Arg	6	7	4	4
Trp	ND	ND	ND	1

TABLE 10.2

AMINO ACID ANALYSIS OF INDIVIDUAL COW SAMPLE (Two-dimensional Spot)

Amino Acid	Sample Rosebud-2	Sample Japonica-5	Sample Lucy-12	Sample Ruby-13	Sample β-casein Control	β-casein Atlas Values
CM Cys	3	3	3	2	0	0
Asp	18	19	13	15	9	9
Thr	10	12	8	11	9	9
Ser	19	21	15	12	16	16
Glu	36	43	34	39	46	39
Pro	ND	ND	ND	ND	ND	35
Gly	34	26	18	4	7	5
Ala	13	14	11	7	5	5
Val	16	17	11	13	17	19
Met	3	3	2	2	5	6
Ile	12	12	9	10	11	10
Leu	22	22	15	17	22	22
Tyr	7	10	6	9	4	4
Phe	14	10	6	8	8	9
His	6	7	8	3	6	5
Lys	13	14	9	16	12	11
Arg	8	8	5	6	3	4
Trp	ND	ND	ND	ND	ND	1

XI.

RESULTS AND DISCUSSION

PHOSPHORYLATION STUDIES

Wet digestion[82] of ω-NH_2-hexyl agarose purified β-casein samples from Rosebud-2, Jeannie-3, Ruby-13 and Japetta-14 showed no differences in the amount of phosphate attached to the protein. Each sample had the normal 5 phosphates per molecule. See Table 11.1.

Phosphate was determined by the method of Kirsten & Carlson[83]. A typical standard curve is shown in Figure 11.1. The theoretical yield of phosphate was calculated as follows:

$$\text{mg/mL (curve of } \beta\text{-casein)} \times \frac{1 \text{ m mole}}{24000 \text{ mg}} \times \frac{1000 \ \mu\text{mole}}{\text{m mole}}$$

$$= \mu\text{moles/mL } \beta\text{-casein} \times 5 \text{ (theoretical \# phosphates/mole)}$$

$$= \mu\text{moles/mL phosphate} \times \frac{30.97 \ \mu\text{g}}{\mu\text{mole}} = \mu\text{g/ml phosphate}$$

Enzymatic dephosphorylation of β-casein was initially done to determine if the numerous bands obtained on isoelectric focusing of β-casein were due to different numbers of phosphates on individual molecules or to artifacts or other post-translational modifications. Focusing of the dephosphorylation of β-casein by alkaline phosphatase (See Figures 11.2, 11.3) revealed that the numerous bands obtained from β-casein were not due to different numbers of phosphates since the control β-casein

and dephosphorylated β-casein had about the same number of bands. If the control focusing pattern were due to differences in phosphate content, the number of bands would decrease upon dephosphorylation since the proteins would then have identical charges. Focusing revealed that β-casein was indeed losing phosphates since the bands became more and more positive as the alkaline phosphatase treatment proceeded.

The numerous bands may be caused by deamidation of Gln and Asn. Random deamidation would cause an increase in negative charge on the bands and change the isoelectric points slightly relative to the native β-casein.

The number of phosphates released per mole was determined kinetically by measuring the phosphate released over various time periods from a certain concentration of β-casein. Inorganic phosphate released was measured by the method of Lin and Morales[81]. A typical standard curve is shown in Figure 11.4. The theoretical phosphate released was calculated as follows:

$$8.7\ \text{mg/mL (conc. of } \beta\text{-casein)} \times \frac{1\ \text{mole}}{24000\ \text{mg}} = 3.75\times10^{-4}\ \frac{\text{m moles}}{\text{mL}}$$

$$3.75\times10^{-4}\ \frac{\text{m moles}}{\text{mL}} \times \frac{1000\ \text{mL}}{\text{L}} = 0.375\ \frac{\text{m moles}}{\text{L}} = 375\ \frac{\mu\text{moles}}{\text{L}}$$

OR

375 μM (β-casein conc.) x 5 (phosphates/mole) =

1875 μM (phosphate conc.) — 2.445 (dilution assay factor) =

767 μM phosphate = theoretical maximum value

The experimentally determined maximum phosphate released was 715 μM phosphate or a recovery of about 93%. See Fig. 11.5. These studies were done on a commercial pooled sample of β-casein. Since wet digestion phosphate determination showed no difference in the individual cow samples, the phosphate in these samples was not determined enzymatically.

Fig. 11.1 Standard curve for Kirsten-Carlsson phosphate determination used for wet digestion procedure.

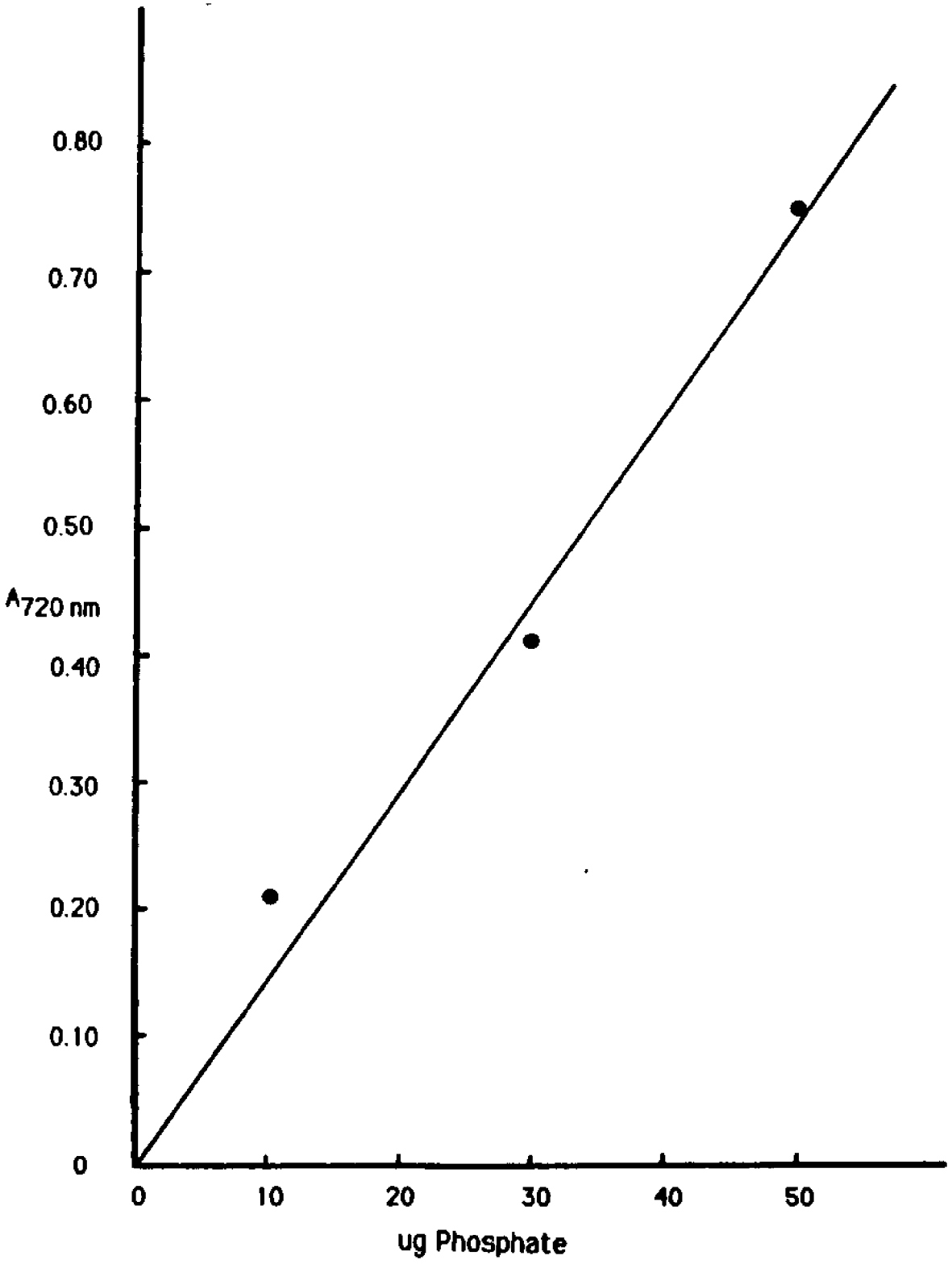

0.80
0.70
0.60
0.50
A720 nm
0.40
0.30
0.20
0.10
0
0
10
20
30
40
50
ug Phosphate

Fig. 11.1

Fig. 11.2 Enzymatic dephosphorylation of β-casein:
a) β-casein-3 hr. control,
b) β-casein-3 hr. alkaline phosphatase treatment,
c) β-casein-6 hr. alkaline phosphatase treatment,
d) β-casein-9 hr. alkaline phosphatase treatment,
e) β-casein-24hr. alkaline phosphatase treatment,
f) β-casein-24hr. control.

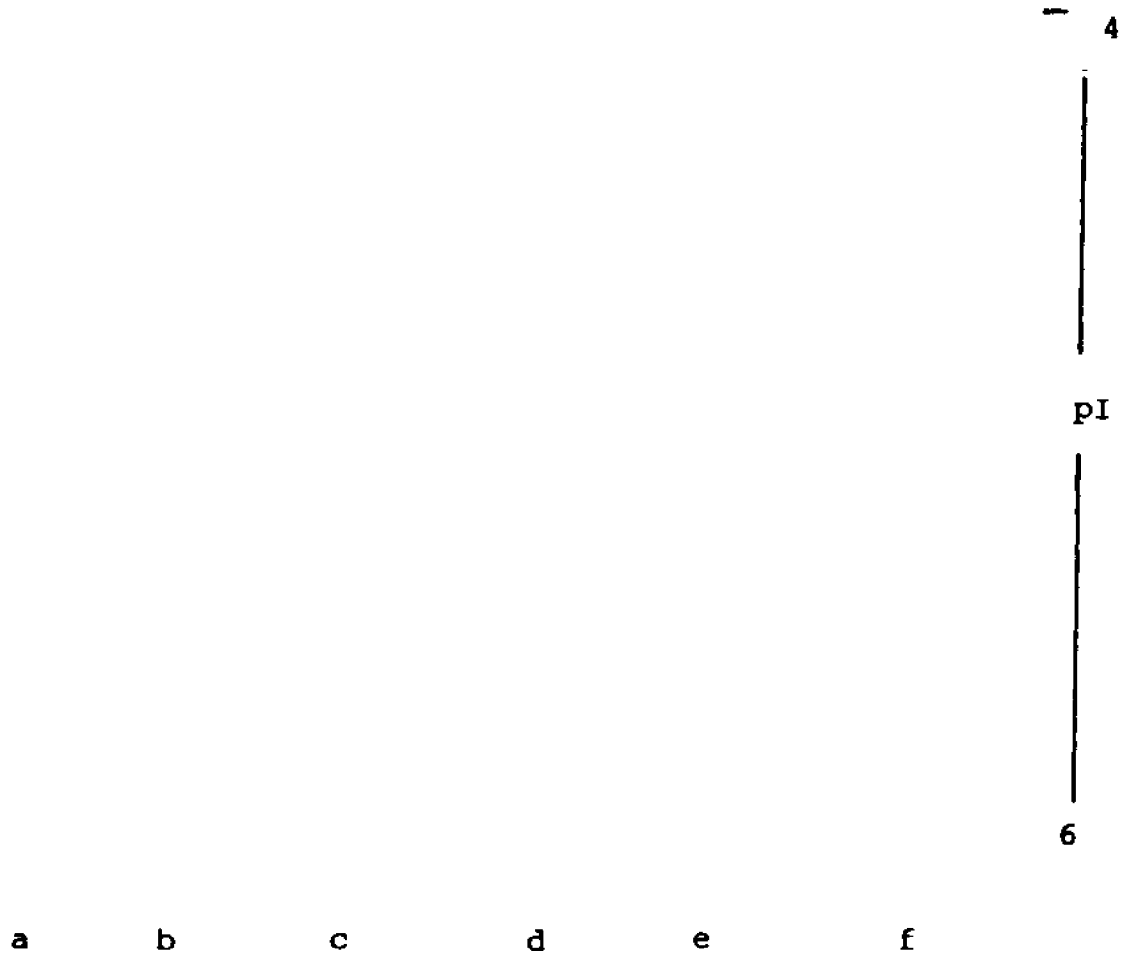


Fig. 11.2

Fig. 11.3 Enzymatic dephosphorylation of β-casein:
a) β-casein-1 day alkaline phosphatase treatment,
b) β-casein-1 day control,
c) β-casein-2 day alkaline phosphatase treatment,
d) β-casein-2 day control,
e) β-casein-3 day alkaline phosphatase treatment,
f) β-casein-3 day control,
g) β-casein-6 day alkaline phosphatase treatment,
h) β-casein-6 day control.

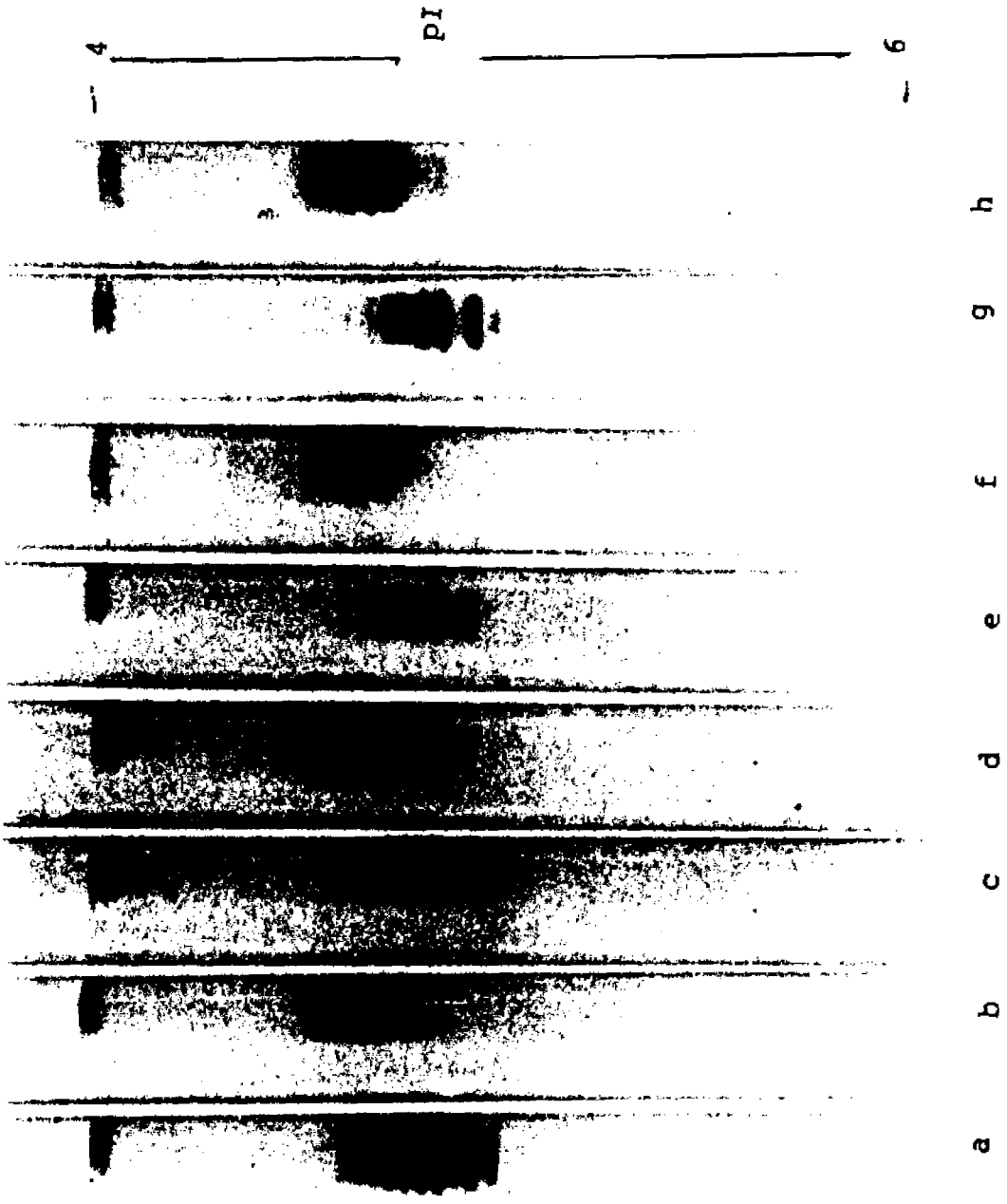


Fig. 11.3

Fig. 11.4 Standard curve for Lin-Morales phosphate assay used for enzymatic dephosphorylation.

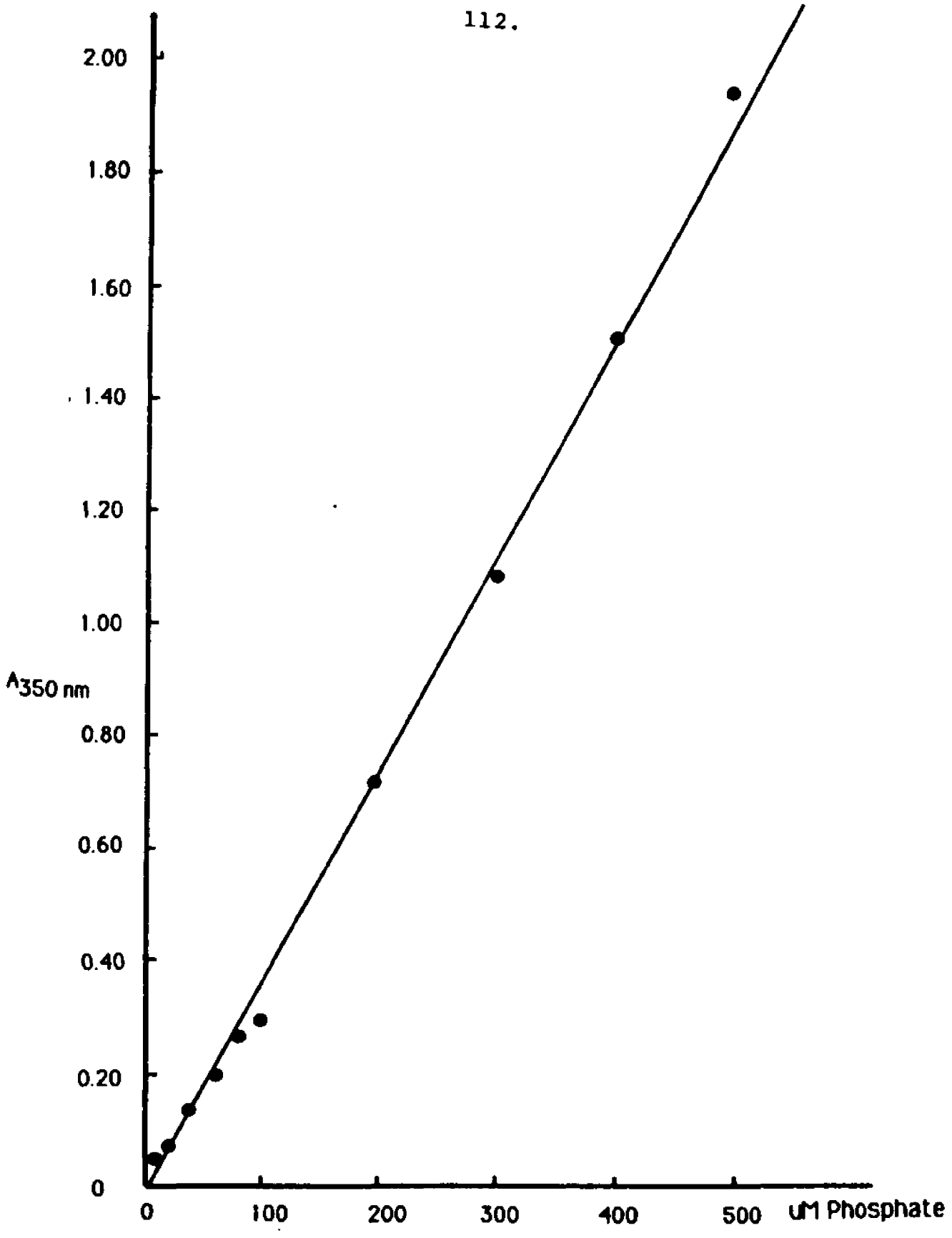

2.00
1.80
1.60
1.40
1.20
1.00
A350 nm
0.80
0.60
0.40
0.20
0
0
100
200
300
400
500
uM Phosphate

Fig. 11.4

Fig. 11.5 Kinetic curve showing phosphate released from β-casein by alkaline phosphatase versus time.

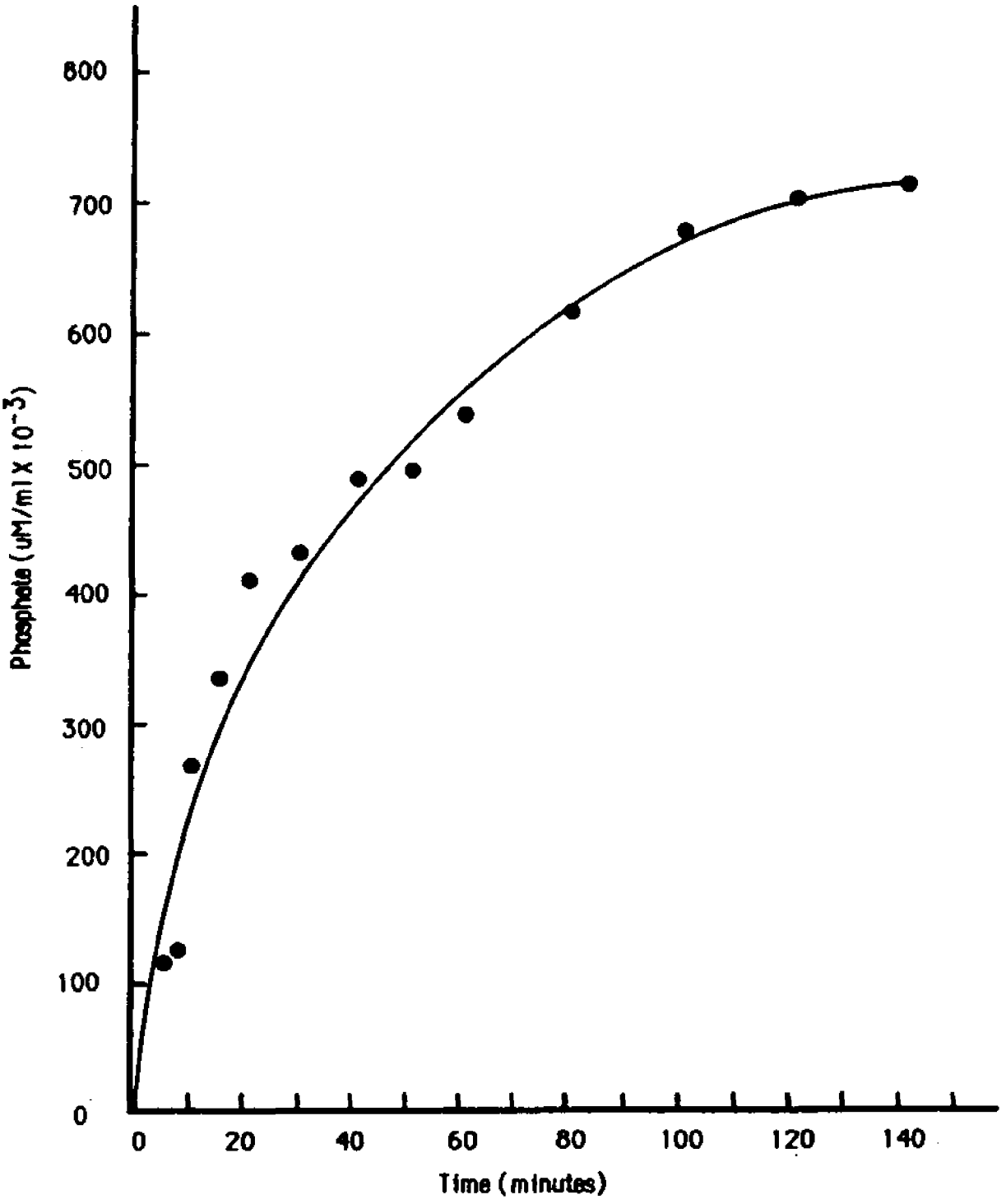

800
700
600
500
400
300
200
100
0
Phosphate (uM/ml X 10⁻³)
0
20
40
60
80
100
120
140
Time (minutes)

Fig. 11.5

TABLE 11.1

PHOSPHATE CONTENT OF β-CASEIN -- WET DIGESTION

Sample	Experimental Phosphate μg/mL	Theoretical Phosphate μg/mL	% Recovery
Rosebud-2	56.0	55.4	101%
Jeannie-3	60.3	62.0	97%
Ruby-13	55.3	56.5	98%
Japetta-14	47.1	48.5	97%
Control (Commercial pooled β-casein)	66.5	67.6	98%

CONCLUSIONS

An error rate for cysteine misincorporation and total amino acid misincorporation in β-casein has been estimated. This rate of about 10^{-6} is significantly lower than error rates determined in other in vitro systems (for 1 amino acid = 10^{-4}-10^{-5}). The system used to measure these error rates in β-casein is essentially an in vitro system since the protein is not manipulated or changed in any way.

Since no cysteine error containing β-casein was isolated from any aged cow this is direct evidence against Orgel's Error Hypothesis. Other researchers[85,86,87,88] using in vitro systems and indirect methods have measured error rates and found no differences with increased age.

There was no change in phosphate content of β-casein with increased age of the cow. However, other post-translational changes have not been ruled out as being the cause of aging. Indeed, others[9.10,18,19,20,22,23,24] have implicated post-translational modifications as being correlated with aging.

Useful techniques have been developed during these studies. Isoelectric focusing, using a narrow range of ampholytes, can be used to monitor both phosphorylation-dephosphorylation and S-carboxymethylation. Amino acid analysis of samples cut out of two-dimensional gels and electroeluted has been accomplished.

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