STUDIES ON BILIVERDIN ASSOCIATED PROTEINS IN RUMINANT MILK

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By

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This is to certify that T.Rajeshwar Rao, M.Sc., has carried out research work described in this thesis entitled, "Studies on biliverdin associated protein in ruminant milk" under my guidance and direct supervision at this Institute. The thesis describes results of original research work carried out solely by the candidate. The results have not been submitted to any other University. The thesis is recommended to be worthy of consideration by the Kurukshetra University for the degree of Doctor of Philosophy in the faculty of Dairying, Animal Husbandry and Agriculture.

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(T.RAJESHWAR RAO)

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CHAPTER I

INTRODUCTION

1.0 INTRODUCTION

1.1 Milk from the ruminants is a recognised source of valuable proteins required in human nutrition. Cows and buffaloes form the main source of market milk in India, their respective contributions being nearly 34 per cent and 63 per cent, the remaining 3 per cent being accounted by goats, sheep and camels milk. India ranks as the highest producer of buffaloes' milk, accounting for nearly 69 per cent of the total world production (F.A.O., 1979). Till the recent years much emphasis was placed on the fat content of milk in judging its quality based on which payment for milk is regulated. The current trend in many countries is to shift this emphasis to the protein content and in this context rapid automatic methods which compare well in accuracy with the standard Kjeldahl method, have been standardised (Booy et al., 1962). It has also been established that the protein content of cows' milk bears a close relation to its solids-not-fat and fat content. Such correlations are helpful in evolving judicious breeding policies for dairy herds (Vanschoubroek, 1963).

1.2 Buffaloes' milk is characterised by a high fat content, a high percentage of protein and absence of carotene, the predominant pigment of cows' milk. The

crude protein content in normal buffaloes' milk varies from 3.2 to 4.0 per cent and true protein from 2.8 to 3.6 per cent (Kay, 1974). Milk proteins contain 19 amino acids from amongst 20 found in foods and satisfy to a great extent the requirement for essential amino acids formulated by the Food and Agricultural Organisation of the United Nations as illustrated in Table I.

Table I Essential amino acids content of casein and whey proteins as compared with F.A.O. Standards*

Amino acid	Grams per 100 g of protein		
	FAO standard	Casein	Whey proteins
Methionine	4.2	3.4	4.3
Leucine	9.0	16.4	15.5
Lysine	4.2	8.2	8.2
Phenylalanine	2.8	5.5	4.0
Threonine	2.8	4.5	5.5
Valine	4.2	7.3	5.5
Tyrosine	2.8	6.2	3.7
Tryptophan	1.4	1.4	2.5

*US Dept.of Agri., Whey Products Conference, 14-15 June, 1972, page 97.

The amino acids of milk serum and whey supplement the low sulphur containing amino acids of casein, thereby emphasising the need for employing such milk processing

treatments to ensure the retention of the natural characteristics of the entire protein complex present in milk, rather than the individual components.

1.3 Buffaloes' milk proteins exhibit certain characteristics differing from cows' milk. It has been found that in cows' milk, there is a close parallel between percentages of fat and protein (Vanschourbroek, 1963). Such a relationship in the milks of Indian cows and buffaloes was first examined by Schneider et al (1948) who found that the percentage of butter fat had a distinct linear association with the percentage of crude protein and casein. The coefficient of correlation between fat and crude protein varied from 0.54 to 0.57 in cows' milk and from 0.21 to 0.46 in milk from village buffaloes. All values were significant at 1 per cent level of probability. Such a relationship was also examined by Ghosh and Anantakrishnan (1965) who found a significant correlation between fat and protein of milk of Red Sindhi and cross-breed cows. In the milk of Murrah buffaloes, the above correlation was not significant. For cows' milk, the regression equation was

$$\text{Protein} = 2.900 + 0.086 \text{ fat},$$

and for Murrah buffaloes' milk it was

$$\text{Protein} = 3.66 + 0.036 \text{ fat}.$$

Milk of Surti buffaloes contains higher protein than Murrah buffaloes' milk so that the relationship between protein and fat formulated by Vyas and Patel (1974) was as follows:

Protein = 0.036 + 0.45 fat % + 0.30.

1.4 Cows' milk proteins have been the subject of a number of investigations from a long time. The continued interest in the subject is evident even today as would be evident by the number of publications on different aspects of milk proteins facilitated through advances in analytical techniques such as electron-microscopy, ultra-centrifugation, electrophoresis, gel-filtration, ion-exchange chromatography, immunological tests, X-ray crystallography, amino acid sequence analysis, affinity labelling, spectroscopy, fluorinated titration, amongst others (Galesloot, 1973; Davies and Holt, 1979). Apart from the major component casein which is present in a colloidal form, milk contains α-lactalbumin, serum albumin, β-lactoglobulin, transferrin and lactoferrin, a number of immunoglobulins soluble in whey (Whitney et al., 1976), over 30 enzymes (Shahani et al., 1973) besides the milk fat globule membrane proteins (Groves, 1971). Casein which precipitates at pH 4.6, forms 76 to 80 per cent of the total proteins of cows' and buffaloes' milk and is useful in

the manufacture of several industrial products. Casein is present in milk as sphere shaped micelles of 120 nm (avg.) diameter and has a flexible structure varying in the proportion of its components with the change in environment (Morr, 1975). Buffaloes' milk contains a higher amount of casein and soluble whey proteins. Buffaloes' milk casein has been shown to differ markedly from the cows' milk casein both in the size of the micelles and composition. The former is poorer in β-, and k-caseins (Ganguli, 1968, 1974). Inspite of the above differences, proteins from both the species have been equally well adapted to the manufacture of all the important dairy products like fermented milks, condensed and dried milks, cheese and frozen desserts.

1.5 The heterogeneity of casein is now well established. Earlier it was shown by many, notably among them by Osborne and Wakeman (1918), and Linderstrøm-Lang and Kodama (1925) that casein could be fractionated using several chemicals like ethyl alcohol, alcoholic hydrochloric acid, sodium chloride, urea, carbolic acid etc. The fractions differed in their nitrogen:phosphorous ratio, phosphorous and sulphur contents as well as in tyrosine and tryptophan contents. Using electrophoretic technique, casein has now been shown to contain three independent components namely, α_s-, β-, and

k-casein, each one of which has several sub-units differing in electrophoretic mobility, iso-electric point and molecular weight. The above components, in turn, show further sub-divisions in the form of genetic variants and minor components. γ-casein though closely associated with the casein micelle is formed by the proteolysis of β-casein, since milk has a natural trypain like proteolytic enzyme (Eigel, 1977). The basic blocks α_s-, β-, and k-caseins of the hollow permeable micellar casein complex are stabilized by colloidal calcium phosphate - $Ca_3(PO_4)_2$ and citrate (Holt et al., 1979). According to Downey (1973), casein micelles are composed of at least 50 to 100 sub-micelles complexed together by colloidal calcium phosphate to yield a porous micelle structure. Nearly 90 per cent of the calcium in skim milk is associated with casein micelles (Thompson and Farrell, 1973). Hydrophobic interactions are mainly responsible for maintaining most (about 80 per cent) of the β-casein within the hydrophobic core of the micelles.

1.6 Casein from milks of different species though precipitated between pH 4.0 to 5.0 show wide differences in their α_s-, β-, k-, and γ-casein contents. Recently Davies and Law (1980) have indicated the distribution of different casein components and their relationship to

each other in milk received by creameries in Scotland and the data are reproduced in Table II.

Table II Major protein fractions of milk, casein and serum proteins and their relationships**

Item	Skim milk		Total casein	Total milk serum protein
	g / l	Total protein %	%	
Total protein	32.71			
Total casein	26.92	82.2^{+++}		
Total serum proteins	5.79	17.8^{+++}		
α_{s1}-casein	10.25	31.3^{+++}	38.1^{+++}	+++
β-casein	9.60	29.3^{+++}	35.7^{+++}	+++
k- casein	3.45	10.5^{+++}	12.8^{+++}	+++
α_{s2}-casein	2.74	8.4^{++}	10.2^{+++}	
γ-casein	0.88	2.7^{+++}	3.2^{++}	+++
β-lactoglobulin	3.14	9.6^{+++}	+++	54.2^{+++}
α-lactalbumin	1.23	3.8^{+}		21.2^{++}
IPL*	0.97	3.0^{+++}	+++	16.8^{+++}
Bovine serum albumin	0.45	1.4		7.8^{++}

*Mixture of immunoglobulins, protease-peptone component 3, and lactoferrin

Significant levels +++ $P < 0.001$
++ $P < 0.01$
+ $P < 0.05$

** D.T.Davies and A.J.R. Law, J.Dairy Res., 1980, 47, 83.

β-casein is the predominant component of human milk casein and is present in milk of a number of species including that from cows and buffaloes. The calcium sensitive α_s-casein while being present in cows and buffaloes milk, is scanty in human milk casein. The calcium insensitive k-casein has been identified in milks from several animal species and has been assigned a key role by Waugh and von Hippel (1956) since k-casein protects the casein micelles and stabilizes milk, until it is hydrolysed by proteolytic enzymes like rennein. k-casein is a glyco-protein rich in sialic acid and forms 8 to 15 per cent of skim milk proteins, this large variation depending on the method used for its isolation and estimation. Jenness (1973) has given a high average value of 22.5 per cent for k-casein in skim milk and the above value was obtained by the determination of disulfide groups colorimetrically by reduction with sodium borohydride ($NaBH_4$) and reaction with 5,5'-dithiobis (2)-nitrobenzoate.

1.7 Cows' and buffaloes' milk caseins

1.7.1 The interest in the study of the physical and chemical properties of casein in milk is further highlighted by a comparative study of the components isolated from the milk of different species as illustrated by cows'

and buffaloes' milk caseins. Most of the casein in buffaloes' milk is present as micellar casein and hardly 1 per cent occurs as soluble-casein so that the ratio of micellar to soluble-casein exceeds 91, as compared to the value of only 21 in the case of cows' milk casein. Nearly 82 per cent of the casein micelles in buffaloes' milk settle at a speed of 10 000 r.p.m., as compared to 33 per cent in case of cows' milk casein. With the increase in the centrifugal speed to 20 000 r.p.m., a further 16 per cent of micellar casein settled in buffaloes' milk as compared to 54 per cent in cows' milk. At 30 000 r.p.m. the average number of micelles settling was hardly 2 per cent for buffaloes' milk as against 13 per cent in the case of cows' milk. Smaller micelles which are known to contain smaller amounts of calcium and phosphate but more k-casein, show higher solubility than larger micelles, the difference being greater in buffaloes' milk (Sood et al., 1976). The inorganic constituents in cows' and buffaloes' milk casein differ as shown by the values (Table III) given by Ganguli (1973).

The sialic acid in the micellar caseins of cows' and buffaloes' milk known to increase with the decrease in the size of the micelles. However, there is no difference between cows' and buffaloes' milk in regard

Table III Composition of Micellar and acid casein of cows' and buffaloes' milk*

Casein	Components	Micellar casein (avg.%)	Acid casein (avg.%)
Cow milk casein	Casein	2.3	
	Nitrogen	12.7	15.2
	Calcium	0.028	0.01
	Phosphorus	0.12	1.5
	Casein: Ca: P	1:0.012:0.04	
	Sialic acid	1.2	
Buffalo milk casein	Casein	2.9	
	Nitrogen	12.0	14.8
	Calcium	0.035	0.2
	Phosphorus	0.015	1.5
	Casein: Ca: P	1:0.012:0.01	
	Sialic acid	0.63	

*Ganguli, N.C. (1973) Neth.Milk & Dairy J., 27, 258

to the pattern of release of sialopeptide from the micellar caseins of different particle sizes by rennet. Buffalo casein gave a higher opacity value with phosphate, malate and citrate buffers (pH range 5.0 and 7.8) than cows' milk casein presumably due to larger amount of calcium bound to buffalo casein micelles.

1.7.2 The constituents of buffaloes' milk casein were examined in detail by Nagasawa et al (1973) using

polyacrylamide gel electrophoresis and diethylaminoethyl (DEAE) cellulose column chromatography. The α_s-casein was found to have a slightly slower mobility than the corresponding bovine fraction and revealed four accompanying bands. Buffalo and bovine β-caseins exhibited the same mobility and were homogeneous, while buffalo milk k-casein revealed two bands. The amino acid composition of buffalo whole casein was similar to bovine whole casein, but some differences were noticed amongst α_s-, β-, and k-caseins.

1.7.3 While examining colouration in buffalo butterfat prepared from sour milk, Daniel and Dastur (1975) observed another interesting property of buffaloes' milk is that it was found to have a green pigment as normal constituent. Buffaloes' butterfat being devoid of carotene is white in appearance and in view of this the extract of annatto (Bixa orelana) is always added to table butter made from buffaloes' milk. However, when the butter fat is isolated from sour buffaloes' milk, it acquires an appealing greenish-yellow tint that has much significance in the marketing of buffaloes' butter fat (ghee). Chandravedana and Dastur (1976) identified the green pigment as biliverdin (BV) which is conjugated to protein(s) and precipitated at pH 4.6. From the

coagulum, the pigment could be extracted with acetone or diethyl ether. The fat insoluble biliverdin when dissociated from the binding protein, is reduced to bilirubin (BLR) which being fat-soluble is taken up by the butterfat and imparts a greenish-yellow colour to it. The nature of the biliverdin binding protein has not been established but on the basis of a preliminary analysis it is presumed to be a new phosphoprotein containing iron and having S_w^{20} of 13.90 (Chandravadana, 1976).

1.8 The above briefly outlined introduction illustrates interesting similarities and differences between cow and buffaloes' milk caseins. Other proteins from buffaloes' milk have not been examined in greater detail. Both cows' and buffaloes' blood serum contain bilirubin which is present as a normal constituent, of which the precursor is biliverdin. The fact that only buffaloes' milk contains biliverdin and is conjugated to a protein, indicates the possibility of the presence of an hitherto unidentified protein that merits a detailed investigation. Such a study would also help in our understanding of phenomenon of milk secretion.

CHAPTER II

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

CONJUGATION OF PROTEINS AND PROTEIN CONJUGATES IN MILK

Conjugation of proteins with different constituents in the media in which they occur is of common occurrence. Such associations help to increase solubility and mobility of compounds enabling them to pass through a separating membrane. Thus, transport of essential materials is facilitated from one tissue to the other and at the same time toxic materials are removed with ease from the system. In the human system, albumin and globulins along with other proteins fulfil this role. The well-known examples are the retinol binding protein (Sreekrishna and Sams, 1979), thyroxine binding prealbumin (Branch et al., 1971) ceruleplasmin which binds copper (Henson et al., 1967) cortico steroid binding transcortin (Van Baalen and Demarr, 1974), hemopexin which binds heme (Geery et al., 1972) and others. This type of conjugation is equally common in other animal systems including the ruminants. Bilirubin in the blood of the cow and the buffalo is conjugated to both albumin and globulin (Kapoor, 1978; McDonagh, 1979). In special systems like milk of the ruminants, a number of

constituents are conjugated to proteins in the natural state. In addition, under the stress of processing treatments and added substances, new associations are established.

2.1 CONJUGATED PROTEINS

Proteins combine with non-protein substances to form more or less stable complexes which when they occur in nature have been designated as conjugated proteins. The combination of proteins with non-proteins is brought about by intermolecular forces which operate between pairs of (a) ionic groups; (b) uncharged polar groups; and (c) non-polar groups or by combinations of such groups. The same forces are responsible for the formation of intramolecular cross-links in globular protein molecules. Glycoproteins, lipoproteins, metalloproteins, chromoproteins, nucleoproteins and phosphoproteins form the most important groups of conjugated proteins. Most of these complexes are formed by the combination of one molecule of a prosthetic group with one molecule of the protein. The phosphoproteins, however, contain numerous phosphoric acid residues bound to ester bonds of the hydroxyl groups of serine and threonine. They can be considered as phosphoric acid esters of simple proteins. One such example is well-known among the milk proteins, the

principle milk protein casein forms phosphoester links with serine.

2.1.1 Nucleo proteins

The nucleo proteins are composed of simple basic proteins (protamine or histone) in a salt combination with nucleic acids as the prosthetic groups. They are most abundant in tissues of both plant and animals, such as yeast, asparagustips, thymus and other glandular organs and sperm are having a large proportion of nuclear material.

2.1.2 Glyco proteins or muco proteins

The muco proteins are composed of simple proteins combined with mucopolysaccharides such as hyaluronic acid and the chendroition sulfates. They generally contain rather large amounts of N-acetylated hexosamine and in addition, more or less such substances as uronic acid, sialic acid and monosaccharides. Mucoproteins are important constituents of the ground substances of connective tissues. They are present as tendomucoid, osseomucoid and chondroproteins in tendons, bones and cartilage respectively. Several gonadotrophic harmones, such as interstitial cell-stimulating hormone, follicle-stimulating hormone, pregnant mares' serum gonadotropin and human chorionic gonadotropin are muco proteins.

Casein in milk is a glycoprotein, the main carbohydrate present being sialic acid.

2.1.3 Lipo proteins

Lipo proteins are formed by combination of protein with a lipid. These complexes are widely distributed in animal and plant materials. They occur in milk, blood, cell nuclei, egg yolk, cell membranes and chloroplasts of plants. They are also found in bacterial antigens and viruses. The lipo proteins are distinguished from proteolipids in that the proteolipids are soluble in organic solvents and insoluble in water. They contain lesser lipid component, than proteolipids. Milk fat globule membrane proteins are also lipo proteins.

2.1.4 Metallo proteins

A large group of enzyme proteins contain metallic elements, such as Fe, Co, Mn, Zn, Cu, Mg etc. which are parts of their essential structures. The heme proteins, which contain iron and classed as chromoproteins are also metallo proteins. Milk contains lactoperoxidase, xanthine oxidase, lactoferrin, lactollin and these are all ferroproteins. Similarly, milk also contains copper binding ceruloplasmin.

2.1.5 Chromoproteins

2.1.5.1 The chromoproteins are composed of simple proteins united with a coloured prosthetic group. Many proteins of important biological functions belong to this group. Hemoglobin, the respiratory protein in which the prosthetic group is the iron containing phorphyrin complex, heme chromoproteins similar to the hemoglobin in function and in chemical constitution are found in certain molluscs (helicorubin), marine worms (chlorocruorin) and anemones (actino hematin), cytochromes, cellular oxidation reduction proteins in which the prosthetic group is a carotenoid pigment. Catalase, the enzyme that decomposes hydrogen peroxide into water and oxygen, is a chromoprotein in which the prosthetic group is heme. The protein precipitate obtained from the blood serum of patients suffering from jaundice has an intense yellow colour caused by bilirubin. This bile pigment is firmly bound to serum albumin. Little is known about the melanins, brown and black pigments of the hair, the skin and certain pathological tumors. Tyrosinase, dihydroxy phenylalanine oxidase and other similar enzymes catalyse the oxidation of tyrosine tryptophan, dihydroxy phenylalanine and their derivatives to red, brown or dark quinoid substances (Mason, 1948 and Sizer, 1953). However,

it is not yet clear whether this oxidation takes place while these amino acids are still bound in the peptide chain or whether the melanins are true conjugated proteins in which colourless proteins are bound to the polymerized quinoid oxidation products of aromatic amino acids likely by means of -SH groups (Burton and Stoves, 1950).

2.1.5.2 Bile pigments associated proteins of blood

Bilirubin in blood is bound to a protein. Bernheld (1932) was the first to study the electrophoretic behaviour of human serum bilirubin. He showed that bilirubin migrated with the albumin fraction in both direct and indirect reacting sera. Coolidge (1942) demonstrated that practically the entire bilirubin of icteric sera was precipitated by ammonium sulphate. The precipitate contains bilirubin associated with albumin fraction and to a small extent with α-globulins and other proteins (McDonagh, 1978).

2.1.6 Plasma and associated proteins

Plasma contains a large number of proteins in varying amounts. Albumin, α-, β-, and γ-globulins and fibrinogen form the major components, while the minor components include hormones, carrier binding proteins for

variety of substance (for example, vitamin binding proteins) blood clotting factors, enzymes etc. Albumins α-, and β - globulins and fibrinogen are synthesised in the liver, while the γ-globulins are synthesized mostly in the lymphoid system.

2.1.6.1 Albumins

They are rich in essential amino acids and serve as a carrier for free fatty acids, adrenal and thyroid hormones and other substances, to the tissues requiring them and for the removal of toxic cations, bilirubin and other phenolic end products to the sites of excretion.

2.1.6.2 Globulins

Globulins are closely involved in giving immune properties to young ones through colostrum. Cow colostrum contains nearly 17 per cent globulins which accounts for about 78 per cent of proteins in colostrum. The carbohydrate rich orosomucoid and α_1-globulin are likely carriers for hexosamine complexes to the tissues. The α_2-globulins contain coagulation factors like prothrombin and Stuart factor, copper carrying protein ceruleoplasmin and hapto-globulins, which form complexes with any haemoglobin that spills in to plasma and these also form part of the α-globulins. β-globulins contain transferrin concerned with iron transport, coagulation factors V, VII,

VIII, IX and XI and fibrinolytic proenzyme plasminogen. Both α-, and β-globulins are lipoproteins. γ-globulins contain the antibodies which constitute the defence mechanism against infection.

2.2 ASSOCIATION AMONGST MILK PROTEINS AND CONSTITUENTS

2.2.1 Combining capacity of casein

Casein has the well known property of combining qualitatively and quantitatively with a number of substances like acids, alkalies, dyes, formaldehyde, halogens etc. The above reactions with casein are utilized in analytical work and in preparing industrial products. The apparent acidity of fresh milk is due to the binding of added alkali with casein. Van der Have et al (1979) mention that 1 g casein binds 0.26 meq NaOH or 0.22 meq $Ca(OH)_2$ at pH 6.7 to 8.3. Casein along with serum proteins react with the orange G dye (Niklsen and Shukri, 1975) and amido black (O'Connell, 1970), so that these reactions provide rapid sensitive methods. One of the well-known products of reaction of casein with iodine is the formation of thyroxine. Majumder and Ganguli (1968) and Chakrabarthi and Ganguli (1977) found that radio active amino acids added to skim milk or casein micelles resulted in the incorporation of these acids to varying degrees in the micelles. The reaction was heat sensitive and freezing of casein micelles resulted 16 fold increase

in the rate of incorporation. On the other hand, under the influence of low temperatures, it has been found that β-, and k-casein and to some extent α_{s1}-casein diffuses out of the casein micelles (Downey and Murphy, 1970). Waugh (1971) demonstrated that α_{s1}-, and k-casein complexes could be reformed as revealed by the sedimentation values.

The individual casein components also show remarkable properties of association and dessociation depending on the environmental conditions as elaborated below.

2.2.1.1 α_s-Casein

The association of α_s-casein has been examined by a number of physico-chemical methods, such as sedimentation analysis, intrinsic viscosity and optical rotary dispersion measurements and others. Examining the nature of the forces involved in aggregation processes, Waugh and his coworkers (1971) and, Ho and Waugh (1965), have examined the general aspects of association reactions and conclude that hydrophobic pressure of water is the force involved in these reactions. Payens and Schmidt (1965) reported a molecular weight of 113,000 ± 3,000 for the associated unit of α_s-casein at

pH 6.4 after ultracentrifugation analysis and suggested that the stable polymeric unit is a tetramer, the molecular weight of monomer being 30,000. They also suggested that the tendency of α_s-casein to associate was enhanced by increasing the concentration of proteins. The dessociation of α_s-casein between pH 6 and 12 and the sedimentation coefficient of α_s-casein at pH 6 is around S^w_{20} 6 as revealed by sedimentation analysis, while at pH 10.5 the sedimentation coefficient is S^w_{20} 35. Based on this transition in sedimentation coefficient, it has been suggested that the dissociation of α_s-casein is governed by acid base equilibrium of the hydroxy groups of tyrosine and the epsilon-amino group of lysine (Schmidt et al., 1967). The dissociation of α_s-casein in the pH range between 7 and 12 was examined using ultra centrifugation and viscosity charges by Swaisgood and Timasheff (1968) and by Schmidt (1970) at various ionic strengths in the presence and absence of organic solvents. These studies showed that dissociation is favoured by an increase in pH and a decrease in ionic strength. Polymerisation of α_{s1}-casein B under different experimental conditions of ionic strength and temperature showed that at neutral pH, polymerization of α_{s1}-casein is dependent much more on ionic strength than on temperature. The average molecular weight of

α_s-casein B in 0.01 M KCl, pH 7.0, at 20°C was found to be 29,400 which corresponds to the monomer, whereas the value in 0.1 M KCl corresponds to that of a trimer (He and Chen, 1967). α_s-, and β-caseins are rich in amino acids with a polar side chain, resulting in inter and intramolecular hydrophobic interactions. The association process is also accompanied by an increase in both of enthalpy and entropy (Payens and Schmidt, 1965). Nemethy and Scheraga (1962) demonstrated that thermodynamic features are required for hydrophobic bonding. Another evidence in favour of hydrophobic bonding as an important source of intra molecular stabilisation energy is the fact that the casein association decreased in the cold or at high pressure (Bloomfield and Mead, 1975). Recently, Dalgleish and Parker (1979) have formulated a model for the coagulation of α_{s1}-caseinate by calcium wherein the casein molecules is considered to aggregate as poly-functional units. The functionalities on the protein molecules are produced as a consequence of the binding of calcium ions and the multiple equilibria which are established during this binding produce a distribution in which not all casein molecules have the same functionality. This has been established by experimental evidence.

2.2.1.2 *β*-casein

β-casein is the most interesting of all the caseins from a thermodynamic point of view. Sullivan *et al* (1955) were the first to consider seriously the physical behaviour of *β*-casein. They showed that this protein forms aggregates at room temperature. The tendency of *β*-casein to associate with raising temperature was also observed by Von Hippel and Waugh (1955). The molecular weight given by sedimentation is in agreement with that obtained with viscosity measured at low temperatures. Extensive investigations on the temperature dependent association behaviour of *β*-casein by Payens and Van Markwijk (1963) showed that the ultracentrifugal patterns at 8.5°C and a concentration dependent change in sedimentation coefficient of *β*-casein monomers and polymers could be determined. The rate of association appears to be low since at 8.5°C the degree of polymerization was about 22, while at 13.5°C it is appreciably higher. Thompson (1971) observed a similar behaviour of the three genetic variants of *β*-casein A, B and C. However, *β*-casein showed tendency to associate more than A and B. Each variant, however, had an S^{w}_{20} of about 1.50 at 4°C. The removal of C-terminal valine and Penultimate isoleucine residues resulted in the virtual

inability of β-casein to associate at 8.5°C, thereby showing that these hydrophobic amino acids are essential in the polymerization reaction (Thompson et al., 1967). Interesting observations on the aggregation behaviour of β-casein have been recorded by Hogland (1966). Under normal conditions in phosphate buffer (pH 6.86, I=0.20), β-casein sediments with a major components of S^{w}_{20} 9.3. However, succinylated β-casein did not form the fast-sedimenting peak generally associated with this protein. Acetylated β-casein also had a lower sedimentation value than untreated β-casein. Hogland (1966) interpreted these findings to mean that an increase in net negative charge of the modified protein affected aggregation behaviour of β-casein. He further observed that succinylated and acetylated β-casein is not as sensitive to calcium as the untreated β-casein. The temperature and concentration dependent polymerisation of β-casein has been studied by Arima et al (1979) by means of viscometry, gel filtration chromatography, electronmicroscopy, analytical ultra centrifugation and ultraviolet (UV) difference spectrometry. It has been shown that the degrees of polymerization are 12, 22 and 49 and free energies of association of -21, -23 and -25 KJ/mole monomer are found at temperatures of 10°, 15°

and 20°C, respectively, in 0.2 M sodium-phosphate buffer of pH 6.7. Monomeric β-casein is not completely a random coil but becomes more compact with increasing temperature due to hydrophobic interactions.

2.2.1.3 k-casein

Cows' milk k-casein has been studied intensively since its discovery by Waugh and van Hippel (1956). It is the most important single factor responsible for micellar stabilization. Swaisgood et al (1964) showed that the polymers have molecular weight of 650 000 and they are formed through interaction of sub units, having a maximum molecular weight of 56 000 and an average molecular weight of 100 000. These subunits are formed by covalent S-S-linkages between k-casein peptide chains. Talbot and Waugh (1970) found that the sub units are not necessary for polymer formation, since SH-k-casein (i.e., k-casein with reduced S-S-bonds) also forms polymers, although with a lower sedimentation coefficient; for example for non-treated k-casein $S^{w}_{20} = 19.6 \pm 4.8$, SH-k-casein $S^{w}_{20} = 11.0 \pm 1.2$. The standard deviations illustrate the poor reproducibility usually observed in experiments with k-casein. In order to explain the non-covalent association of SH-k-casein and its interaction with other caseins, attention has been focussed on the hydrophobic nature of

the peptide chains, in particular the para-k-casein which consists of the first 105 amino acid residues of k-casein (Hill and Wake 1969). k-casein is found to polymerize in solution (S^{w}_{20} 13.5) and this polymerization is insensitive to Ca ion concentration and temperature (Vreeman 1979).

2.2.2 Association of casein with lactoferrin and iron

2.2.2.1 In 1939, Sørensen and Sørensen described a red protein fraction from bovine milk which was later shown to consist of two iron binding components; a protein homologous to serum transferrin and a component absent in plasma designated as lactoferrin by Blane and Isliker (1961a). Lactoferrin which occurs in several body secretions besides milk was examined in detail by Groves (1960, 1965). The red protein fraction precipitated along with casein from which it was separated by adjusting the pH to 6.0 and then treating with ammonium sulphate (40 to 65 per cent). The average nitrogen content of the red protein fraction was 15.02 per cent, P 0.26, Fe 0.11 while carbohydrates was 7.20 per cent. The carbohydrates were identified as hexose, hexosamine and sialic acid. The M.W. assigned was 87,100. The protein gave absorption bands at 280 nm and broad maxima around 470 nm. It was possible to remove iron from the protein and restore

the original complex under optimal conditions. The red protein fraction exhibited mild phosphatase activity. A minor colourless crystalizable protein was also associated but its removal did not affect the characteristics of the red protein fraction. Lactoferrin constitutes the major part of the red protein fractions isolated from human milk. The metalchelating properties of transferrins and lactoferrins are very similar. Both bind two atoms of iron with two molecules of bicarbonate being involved in the reaction (Masson and Heremans, 1968). From studies on optical and magnetic spectra (Windle et al., 1963), as well as by titration experiments (Masson and Heremans, 1971) it has been demonstrated that the metal binding sites are similar in both the proteins. However, the affinity of lactoferrin for iron is maintained at pH 4, whereas transferrin releases all the iron under such conditions (Blanc and Isliker, 1961b). Furthermore, except for the M.W., two proteins differ widely in physico-chemical properties such as solubility amino acid and carbohydrate composition (Montreuil et al., 1965), tryptic maps (Spik and Montreuil, 1966), antigenic determinations (Blanc and Isliker, 1961b) and ionic charge (Masson and Heremans, 1971). Lactoferrin has been identified in milks from a variety of mammals such as

guinea pigs, cows, buffaloes, goats, mare and mice (Masson and Heremans, 1971; Bhatia, 1978). On the basis of electrophoretic mobility, stability at acid pH of the iron complex and immunological properties, milks from rats, rabbits and dog are devoid of lactoferrin. The concentration of lactoferrin is not related to the iron content of milk and it has been shown that lactoferrin does not play a major role in the excretion of iron in milk. Another interesting property of lactoferrin is that like serum transferrin it serves as a bacteriostatic angent in milk by depriving the cultural medium from its ionised iron (Oram and Reiter, 1968).

2.2.2.2 Association of iron with casein

Iron was the first among the trace elements to be recognised as essential to life functions. It is always present in milk, the range varying from 100 to 500 µg/l. The feeding of supplemental iron to cows does not increase the iron content of milk (Johnson, 1974). Consideration of addition of iron directly to milk for supplementing iron deficiency, leads to the question of the status of such added iron. Early workers like Allan (1950) and King et al (1959) thought that both natural and added iron are nonionic and are bound to the fat globule membrane. Subsequently many workers (Vaughan

and Knauff, 1961; Basch et al., 1974 and Demott and Park, 1974) found that the added iron is associated with proteins, primarily with casein. During the course of a study on the influence of processing of milk on the binding of iron to various milk proteins, Park (1973) has shown that most of the added iron can be recovered in the skim milk portion. Pasteurization reduced the amount of iron bound to whey proteins, while homogenization increased the binding capacity of all milk proteins. The extent of binding capacity in homogenized milk is in the decreasing order as follows: rennin coagulated casein, fat globule membrane protein, acid casein, proteose-peptone and whey proteins. Basch et al (1974) studied the distribution of iron and polyphosphate phosphorous added to cows' milk by both analytical and radiochemical techniques. Whole milk is separated isoelectrically or centrifugally into three major fractions namely, cream, casein and whey after the addition of ferripolyphosphate and other iron or polyphosphate compounds. Casein phosphoprotein has a greater affinity for the iron than polyphosphate, since 85-95 per cent of the iron and 50-55 per cent of polyphosphate is bound to acid precipitated casein. When casein is obtained by centrifugation 60-70 per cent of the iron and 50-55 per cent polyphosphate is bound to the micelles.

Demott and Dinner (1976) studied the distribution of added iron to skim milk. It was shown that about 85 per cent of the added iron was bound to casein and out of this 72, 21 and 4 per cent were associated with α_s-, β-, and k-caseins, respectively. There was a loss of only 1.7 per cent of added iron during dialysis of casein. When ^{59}Fe was added to raw cows' milk, 85 per cent of the activity was found with skim milk fraction (Dincer, 1976). Unnikrishnan and Rao (1977a) have also shown that more than 80 per cent of the added iron is associated with skim milk and the added iron was mostly bound to the casein in both cows' and buffaloes' milk.

Manson and Cannon (1978) have put forward a hypothesis that the phosphoseryl residue present in α_s-, and β-caseins rapidly catalyse the oxidation of iron from the Fe^{2+} to Fe^{3+} state with the formation of stable ferri/phospho protein complex involving consumption of the theoretical amount of oxygen. During their studies on the ability to iron binding by wheat glutein, soyisolate, zein, albumin and casein, Nelson and Poller (1979) indicated that the source of iron influenced factors like pH, temperature and time of reaction. Similarly Hegenauer et al (1979) have studied the behaviour of iron supplemented in cows' milk. The studies showed that the different forms of iron as well as their source influenced

the nutritional and organoleptic properties of iron fortified milk.

2.2.3 Association of copper with casein and other milk proteins

The presence of copper in milk has received much attention because of its catalytic effect on the development of oxidised flavours in milk and milk products. The normal concentration of Cu varies from 20 to 200 µg/l. Thompson et al (1948) found that when 1 per cent solution of casein was allowed to react with an excess of L-ascorbic acid in the presence of a small quantity of finely ground copper oxide at 30°C and pH 6.5 to 7.0, Cu was bound to the protein and the complex that was formed underwent a series of colour changes beginning with light yellow to brick-red within 48 h. The complex exhibited an isoelectric point almost identical with that of casein. Acid hydrolysate of casein did not yield the complex. Menzer and Mulder (1956) found that the added Cu was primarily associated with the skim milk proteins whereas the natural Cu in milk was associated with fat globule membrane. In experiments using radio active Cu, King et al (1959) found that 97-98 per cent of the added Cu to milk was uniformly associated with skim milk proteins. The affinity of proteins for Cu at pH 6.8 decreased in the following order: Fat globule membrane, sodium caseinate,

α-lactalbumin, β-lactoglobulin (Koops, 1963). Minato and Ogiso (1964) allowed casein to react with copper sulphate at pH 9.5 for 12 h and then after precipitating it three times at pH 4.8, found that it contained 0.74 per cent Cu. Casein containing 0.54 per cent Cu was also prepared by electrolysis. Only a small proportion of added Cu was found in washed cream obtained from normal milk. Both cows' and buffaloes' milk caseins exhibited similar characteristics (Unnikrishnan, 1976). Casein at its isoelectric point was found to bind more Cu when it was added in the cupric state. In centrifuged casein, Cu was approximately 10 per cent more than in isoelectric casein.

2.2.4 Association of casein with calcium

The close association of calcium in milk with the casein micelles has been described in para 1.5. Using whole casein, Zittle et al (1958) found a maximum binding of calcium with protein. In studies using different ionic solutions, Dickson and Perkins (1969) reported the relative order of ion binding for α_s-, β-, and k-casein as follows: Ca > Mg > Sr, and the relative order of binding capacities was α_s- > β- > k-casein. Waugh et al (1971) also examined in detail the binding of cations to α_s-, and β-casein and a unit weight ratio mixture at

pH 6.6. Casein is known to bind calcium ions and it has been suggested that the binding of calcium ions to the casein inside the golgivesicle leads to micelle formation. *in vitro* experiments by Holt and West (1977) could not substantiate this as larger amounts of calcium were required than that found in milk.

According to Rose (1968) α_s-, β-, and γ-, k-casein fractions are solubilised in the serum when milk is cooled to 3°-4°C with β-casein accounting for about 55 per cent. Serum casein content decreased by addition of Ca, but increased by addition of phosphate, thereby suggesting that at fixed levels of calcium caseinate, the calcium phosphate content of the micelles and the degree of polymerization of temperature-sensitive β-casein are major factors controlling the properties of casein present in micellar form. Sabarwal and Ganguli (1973) have shown that pasteurization, boiling and sterilization of milk resulted in an increase in Ca content in the buffalo milk casein micelles by 1.75, 4.75 and 7.25 per cent, respectively, while in the cow milk casein micelles, the increase in the Ca content was 3.54, 6.02 and 14.53 per cent, respectively. An increase in the size of casein micelles was observed due to the influence of Ca ions and heat treatment during pasteurization of milk. Addition of 3.6 mM $CaCl_2$ to milk before pasteurization considerably

increased the micelle size than in case of raw milk (Langley, 1978).

2.2.5 Association of casein and milk proteins during processing and storage

Heating skim milk is said to cause interaction between β-lactoglobulin and k-casein (Sawyer, 1969). The small casein micelles in skim milk, which also has highest k-casein content also contains the highest concentration of the heat-induced complex. When the concentration of casein was reduced, there was an increase in the amount of α-lactalbumin and β-lactoglobulin recovered after heating, thereby showing that casein facilitates the interaction of α-lactalbumin with β-lactoglobulin (Elfagm and Wheelock, 1978). A progressive decrease in the amount of carbohydrates attached to k-casein during the aging of heat-sterilized milk was noticed by Wheelock and Hindle (1971). The authors surmise that the above may be a factor in the development of gelatinous condition in heat-sterilized milk during storage. In recent studies, Ludwig (1979) noted interaction of lactose with milk proteins as a result of ultra-high-temperature (UHT) processing at 143.5° ± 2°C. The reaction involved Amadori rearrangement of Schiff's base to give enol and keto forms. Nearly two-thirds of the lactose got incorporated into fractions rich in k-casein.

It was also found that the available lysine was reduced during the freeze-drying of β-lactoglobulin mixture. During the handling of raw bulk milk, β -lactoglobulin reacted with lactose by the Maillard reaction so nitrogen and available lysine are reduced.

2.2.6 Association between protein and vitamins in milk

Hartman and Dryden (1974) have reviewed literature on the association of most of the vitamins with carrier proteins in milk.

2.2.6.1 Association of carotene and vitamin A

It is known that on centrifugation of milk at different speeds, the smaller fat globules showed progressive richness in carotenoids and vitamin A. McGillivary (1957) examined this aspect in detail and showed that the differences in concentrations of the above two were due to the presence in milk of protein bound forms of these two constituents but not due to the size of fat globules.

2.2.6.2 Association of thiamine (Vit.B_1)

In milk, thiamine occurs both in free as well as phosphorylated form and is complexed with protein. Dejong (1942) found that in cows' milk, the level of free thiamine was 50 to 70 per cent, thiamine monophosphate was 18 to 45 per cent and protein bound thiamine was 5 to 17

per cent. The values varied however, with the stage of lactation of the animals.

2.2.6.3 Association of Riboflavin (Vit.B_2)

A small quantity of riboflavin in milk occurs in bound form, mostly attached to the proteins as a part of enzymes systems namely, xanthine oxidase and diaphrase (Leviton and Pallensch, 1960). The site of binding between the vitamin and casein are the tyrosine residues of protein.

2.2.6.4 Association of folic acid (Folacin)

The folic acid in milk is strongly and specifically bound to a minor whey protein, forming a complex of primary molecular weight of about 38,000, but exhibiting concentration-dependant reversible aggregation (Ford et al., 1969). The binding protein is present in excess and the cows' milk has the capacity to bind about 50 µg of added folic acid per litre. The binding is influenced by pH levels between 6.0 to 8.6. The folate was found to be bound entirely to the protein.

2.2.6.5 Association of cynocobalamin (Vit.B_{12})

This vitamin is bound to the protein in milk of various species. The cows' milk has a high proportion

which occurs in whey proteins (Qizis et al., 1965).

2.2.7 Association of milk proteins with bile pigments

Recently, Daniel and Dastur (1975) reported that buffaloes' milk contained a green pigment as a normal constituent. This finding came to light as a result of the differences in appearance between the butter fat from fresh buffaloes' milk cream and that obtained from sour milk, both samples from the same batch. While the former has a white appearance when solidified, the latter exhibited a greenish-yellow tint (Lalitha and Dastur, 1956). The pigment responsible for such colouration has been identified as one of the bile pigments biliverdin which on reduction is converted to bilirubin (Chandravadana and Dastur, 1976). Biliverdin obtained from buffaloes' milk after direct extraction using acetone in the presence of saturated ammonium sulphate system was found to be associated with the protein moiety. The green pigment extract in 80 per cent aqueous acetone after filtering through Whatman No.42 filter paper exhibited a single absorption maximum at 660 nm, unlike the pigment isolated from casein coagulum which showed the absorption maxima at both 380 and 660 nm. Biliverdin either directly extracted from milk using acetone in the presence of saturated ammonium sulphate system, or extracted from acid precipitated coagulum (pH 4.6), with 80 per cent acetone was always

associated with protein that was green in colour and from which biliverdin could be easily extracted by treatment with a suitable solvent like methanol, petroleum ether, etc. Polyacrylamide gel electrophoresis (PAGE) of the protein revealed its heterogeneity. The protein fraction obtained from the elutent of the Sephadex G-200 (from peak I) contained two bands, while the other fraction (from peak II) was heterogeneous and contained several bands.

2.3 OBJECTIVES OF THE PRESENT STUDY

The above review amply illustrates the interesting property of association of milk proteins with a number of components present in milk itself. This property is further influenced by chilling or heat treatments of milk. While establishing the presence of biliverdin in buffaloes' milk, it was found to be associated with the protein. On the other hand, cows' milk and goats' milk do not exhibit this behaviour. It is therefore likely that the buffaloes' milk contains a special mechanism for the conversion of blood bilirubin to biliverdin in the mammary gland, thereby facilitating the excretion of the pigment along with a associated protein in milk. It was decided to examine the above aspect in detail and establish the identity of the biliverdin associated protein. With the above object in view, the following aspects have been studied:

2.3.1 The nature of the biliverdin associated protein in milk

2.3.2 Relationship between the protein content and biliverdin concentration in milk

2.3.3 Evaluate the precursor of biliverdin found in milk; and

2.3.4 Study the behaviour of biliverdin when added to cows' and buffaloes' milk.

Results of these studies have been described in the following chapters.

CHAPTER XII

RELATIONSHIP BETWEEN BILIVERDIN ASSOCIATED PROTEIN AND BILIVERDIN CONTENT IN BUFFALOES' MILK

3.0 RELATIONSHIP BETWEEN BILIVERDIN ASSOCIATED PROTEIN AND BILIVERDIN CONTENT IN BUFFALOES' MILK

While isolating biliverdin (BLV) from buffaloes' milk it has been noticed that a proteineous material is always associated with the 80 per cent acetone extract of the pigment. Further, the concentration of biliverdin in milk varied from day to day, both in bulk samples and in samples collected from individual animals. It was, therefore, necessary to assess if the concentration of biliverdin in milk is related to the amount of binding protein extracted simultaneously. For the estimation of protein it was considered to have a rapid method for its determination. The method described by Lowry et al (1951) was examined for its suitability and adapted using Folin-Ciocalteu-phenol reagent in the studies described below. In addition, the effect of green and dry feeding on total protein levels of milk and biliverdin associated protein (BLVAP) was also examined.

3.1 MILK SAMPLES AND REAGENTS

Milk obtained from Murti and Murrah breeds of buffaloes was used for the present study. Samples were collected soon after milking from the Institute's herd, generally during morning milking. Samples of milk were

stored in a refrigerator and used within 3-4 h after milking. Milk was skimmed by using a mechanical cream separator.

All the reagents used were of analytical grade and glass distilled water was used.

3.2 METHODS USED

3.2.1 Isolation of biliverdin associated protein from buffaloes' milk

The biliverdin associated protein (BLVAP) was isolated by taking 100 ml skimmed milk, and 60 ml of acetone were added with constant stirring, followed by 60 g of $(NH_4)_2SO_4$. The green acetone layer that separated from the aqueous ammonium sulphate layer was filtered through Whatman 42 filter paper. The extract was kept at about -5°C for 2-4 h so as to solidify the lipids soluble in acetone. Later, it was filtered and left in a shallow wide diameter glass dish at room temperature under a fan in order to remove the solvent at low temperatures. The green protein fraction that precipitated was collected by centrifugation of the residual solution at 2500 r.p.m., and washed several times with water so as to free it from $(NH_4)_2SO_4$.

3.2.2 Estimation of biliverdin associated protein

3.2.2.1 The green BLVAP isolated as above was dissolved in 1.5 ml of 1N NaOH and diluted to 100 ml with distilled water. One ml of aliquot was taken for the estimation of protein by the method described by Lowry et al (1951) using Folin-Ciocalteu-phenol reagent. The optical density was measured at 750 nm in a spectrophotometer with 1 cm path length cells. The protein values was read on a standard curve (Fig 1) and the values were expressed as mg protein per 100 ml of skim milk.

3.2.2.2 Calibration curve

Bovine serum albumin (BSA-Sigma) was taken as the standard protein for constructing a calibration curve. Ten mg of powdered BSA were dissolved in 100 ml of water and stored in a refrigerator with toluene. Suitable aliquots of this stock solution were taken for preparation of the standard graph. The optical density of the graded dilutions of the protein solution after the development of the colour with Folin-Ciocalteu-phenol reagent were read against similarly treated reagent blank at 750 nm in a spectrophotometer (Fig.1).

3.2.2.3 Recovery of added protein

The percentage recovery was estimated by adding known amounts of purified protein (BLVAP purified on

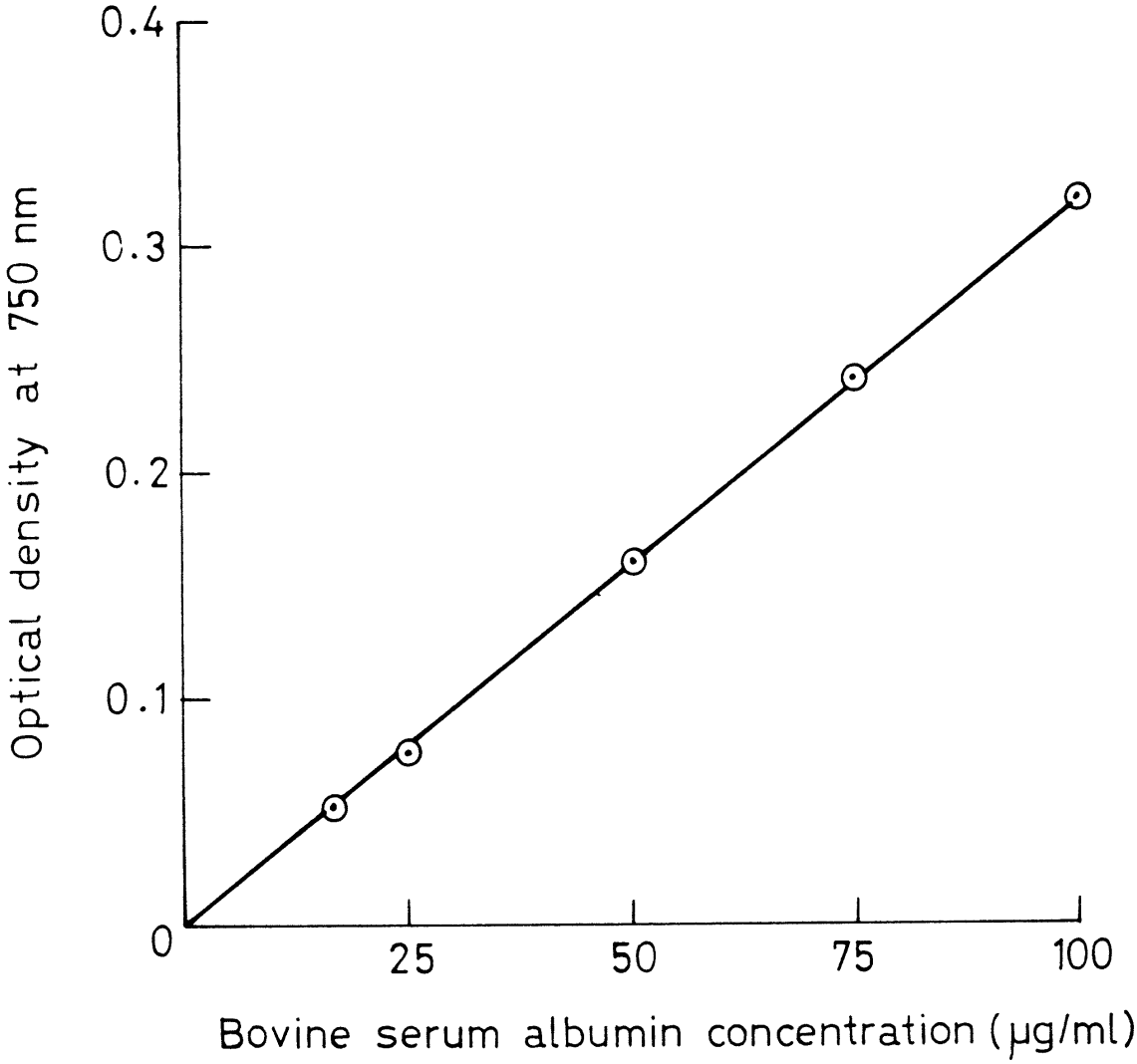


FIG.1 STANDARD CURVE, CONCENTRATION VS. OPTICAL DENSITY.

Sephadex G-200, fraction 1) and BSA, to the prepared sample solution (para 3.2.2.1) and estimating the total protein in the sample.

3.2.2.4 Interference of biliverdin with the estimation of biliverdin associated protein

To check whether BLV was interfering with the estimation of BLVAP, protein was estimated separately in cows', buffaloes' and in the mixture of the two types (50:50) of milks, since cows' milk does not contain any BLV. It was found that the recovery was satisfactory, thereby indicating that BLV does not interfere in the estimation.

In order to find out whether the BLVAP estimation follows the Beer's law over a wide range of concentrations, various dilutions of BLVAP were prepared from the stock solution and protein was estimated. The values thus obtained were plotted on a graph to examine the relationship between optical density vs protein concentration (Fig.2).

3.2.3 Estimation of biliverdin

3.2.3.1 Isolation of biliverdin

The BLV in buffaloes' milk was estimated following the method developed by Chandravedana and Daniel (1977). Twenty five ml of skim milk was taken in a

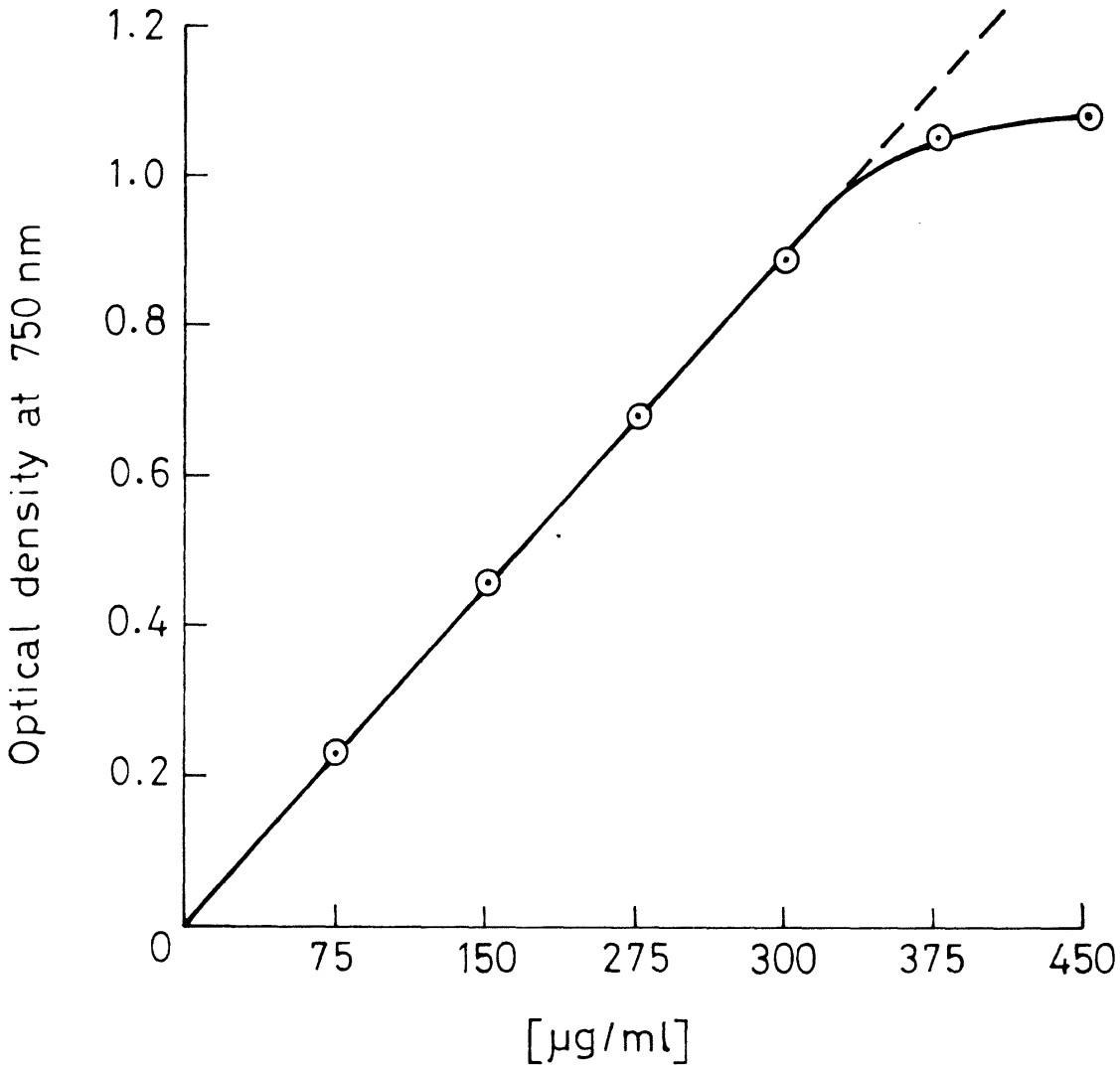


FIG.2 RELATIONSHIP BETWEEN CONCENTRATION OF BILIVERDIN ASSOCIATED PROTEIN AND OPTICAL DENSITY.

beaker and 15 ml of acetone was added with constant stirring and this was continued for 2-3 min. Ammonium sulphate at 60 per cent w/v of milk (15 g) was added next to saturate the system. When the two phases separated with precipitated protein in the lower aqueous layer, the upper green acetone layer was separated, filtered through Whatman 42 filter paper and the optical density was immediately measured at 660 nm against the blank (para 3.2.3.2).

3.2.3.2 Preparation of blank

Whey was obtained by acidifying skim milk warmed to 50°C by addition of 0.6 per cent w/v of citric acid and the acetone extract was prepared as described above (3.2.3.1).

3.2.3.3 Standard curve

Standard solution of N.N-dihydrochloride (Sigma 80 per cent purity) was prepared by dissolving 5.0 mg of crystalline material in 100 ml of acetone diluent. The acetone diluent was prepared by adding 60 volumes of acetone to 100 volumes of fresh skim milk at ambient temperature. The acetone layer was separated by saturating the system with solid ammonium sulphate and filtering the acetone layer through Whatman 42 filter paper. Suitable aliquots of the above stock solution were then diluted with acetone diluent to attain

s concentrations varying from 10, 20, 30, 40 and 50 µg of BLV per 100 ml. The optical density values of the graded dilutions were read against the acetone diluent as blank to compensate for the absorbance due to the biliverdin present in the diluent itself (Fig.3).

3.2.3.4 Estimation of biliverdin in the test solution

Trials showed that BLV present in 100 ml of milk was extracted into 56 ml acetone solution so as to achieve a 1.7 fold concentration of BLV (Table 1). From the standard graph (Fig.3A) it was necessary to multiply the values by 0.56 to obtain the true concentration (Fig.3B). The concentration of biliverdin is expressed as µg/100 ml of skim milk.

3.2.4 Effect of green and dry feed on the levels of biliverdin associated protein and total milk proteins

To study the influence of feeds on the concentration of BLVAP and total milk protein, six Murti buffaloes' of similar age and lactational stage were selected and fed individually as per the following schedule.

3.2.4.1 Details of feed given

(a) Control period (3 weeks) Normal ration consisting of green fodder, ragi straw and concentrate

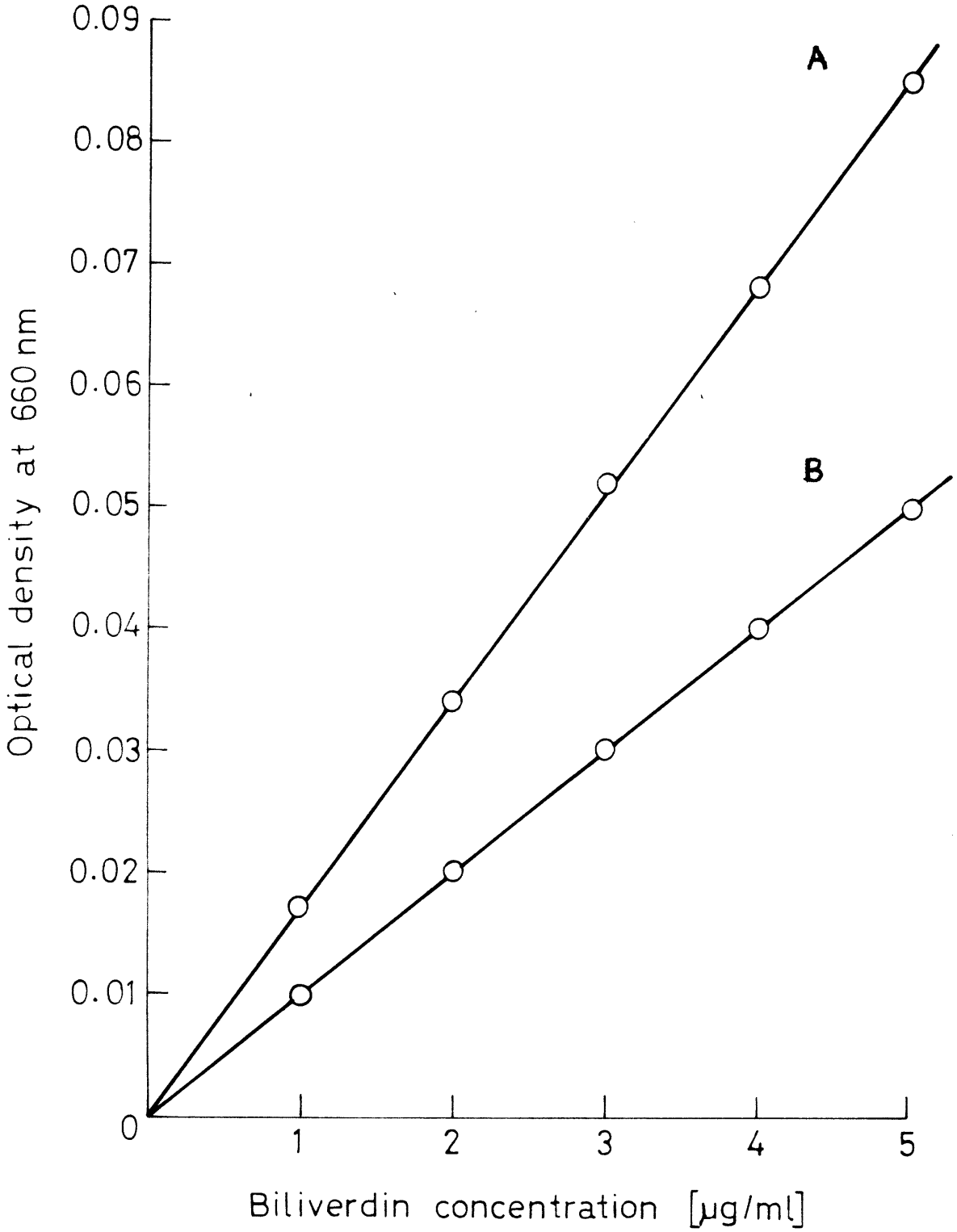


FIG. 3 STANDARD CURVE, CONCENTRATION VS. OPTICAL DENSITY.

Table 1

Separation of acetone phase in the buffalone'milk after addition of 15 ml of acetone and 15 g of $(NH_4)_2SO_4$ to 25 ml of skim milk*

Trial Number	Acetone phase separated (ml)
1	14.20
2	14.00
3	14.00
4	14.00
5	14.00
6	14.50
7	14.70
8	13.90
9	14.00
10	13.90
11	13.80
12	13.90
13	14.00
14	13.75
15	13.80
Average	14.01 ± 0.25

* Samples cover all the stages of lactation.

mixture was continued, although the animals were fed individually. (b) Dry fodder feeding (4 weeks) Ration consisting of ragi straw and concentrate mixture only. (c) Intense green fodder feeding (4 weeks): Ration of green fodder and concentrate mixture only. Under the different schedules of feeding the same total digestible nutrients (TDN) and digestible crude protein (DCP) were ensured. The basis for the feeding was according to Sen and Ray (1971), namely:

	Dry matter	DCP	TDN
Maintenance ration per 100 kg body weight	3.0 kg	0.07 kg	0.80 kg
Production ration per kg milk with 8% fat	-	0.7 kg	0.51 kg

The body weights of six buffaloes varied from 357 to 390 kg, the milk yield at the beginning of the experiment was 5.0 to 7.0 kg/day and the fat content of milk was 6 to 9%. On the above basis detail rations were prepared consisting of green fodder maize (Zea mays DCP 1.02%, TDN 18.4%) concentrate mixture (DCP 16.00%, TDN 70.00%) and ragi straw (Eleusine coracana DCP 0.0 TDN 30.00%). Biliverdin associated protein and total milk protein were estimated at intervals of 2 days from the pooled milk samples drawn from the experimental animals.

3.3 RESULTS

3.3.1 Estimation of Biliverdin Associated Protein

Before finding out the correlation between the BLV and BLVAP and examining the effect of feed on BLVAP and total milk protein concentration, a method for the estimation of BLVAP was standardized and used in the following studies. The method was first tested for its reproducibility and feasibility over wide range of protein concentrations and interference from BLV if any.

3.3.1.1 Reproducibility

BLVAP was estimated in replicate samples and reproducibility of values was determined. In trials with a series of five estimations on the same sample, the results were found to be reproducible. This is illustrated by the following results in one series; 9.60, 9.50, 9.50, 9.50 and 9.50, average 9.52 ± 0.15 mg protein/100 ml of skim milk.

The range of absorption curve of the colour developed due to Folin-Ciocalteu-phenol reagent at 750 nm varies with the nature of protein. This point was therefore examined. Results of four trials indicate that the BLVAP obeys the Beer's law over a wide range of concentrations upto 335 µg/ml (Fig.2).

3.3.1.2 Recovery

The percentage recovery was determined by adding known amounts of BLVAP and BSA to the prepared protein sample and the total protein was estimated. The recoveries are given in Tables 2 and 3. With the BLVAP the average recovery was 99.79 ± 1.78 (Table 2) and with BSA it was 98.00 per cent (Table 3).

3.3.1.3 Interference from bilivardin

In order to find out if the presence of BLV with protein influenced the development of colour with Folin-Ciocalteu-phenol reagent, protein was estimated in mixed milks of cows and buffaloes (50:50) as well as in milk samples of individual animals. Cows' milk does not contain BLV but has similar protein extracted by 80 per cent acetone in the presence of saturated $(NH_4)_2SO_4$ system. From the results presented in Table 4 it may be seen that the BLV does not interfere with the development of colour and estimation of protein.

The method standardised for the estimation of BLVAP is thus dependable and has been adopted for further studies.

Table 2

Recovery of added purified biliverdin associated protein to biliverdin associated protein solution

BLVAP concentration in sample µg	Purified BLVAP added to samples µg	Total protein found µg	Added protein recovered µg	Percentage of recovery
95	324	425	330	101.85
190	243	430	240	98.76
285	162	445	160	98.76
			Average recovery percentage	99.79 ± 1.78

Table 3

Recovery of added BSA to bilivernin associated protein solution

BLVAP Concentration in sample µg	BSA added µg	Total protein found µg	Added protein recovered µg	Percentage of recovery
95	400	480	385	96.25
190	300	480	290	96.66
285	200	475	190	95.00
110	400	490	380	95.00
220	300	507	280	95.66
330	200	522	192	95.60
		Average recovery percentage		95.59* ± 0.66

*BSA (Sigma) used was about 97% pure. Therefore the recovery after correction for the purity of BSA comes to about 98.00%.

Table 4

Estimation of biliverdin associated protein in mixed milk of cows and buffaloes (50:50) its recovery compared to individual samples (mg/100 ml)

BVAP in buffalo milk sample	80% acetone soluble $(NH_4)_2SO_4$ treated protein from cow milk sample	Total protein expected in mixed milk sample	Total protein found in mixed milk sample	Percentage recovery based on the expected value
7.21	7.75	7.48	7.25	97.47
6.15	6.28	6.21	6.18	99.47
6.15	6.28	6.21	6.20	99.71
6.15	6.28	6.21	6.17	98.50
		Average recovery percentage		98.78 ± 1.02

3.3.2 Biliverdin and biliverdin associated protein content in Murrah and Surti buffaloes' milk

Tables 5 and 6 give the BLV and BLVAP content of pooled milk samples drawn from Murrah and Surti buffaloes. The average content of BLV and BLVAP in two breeds were:

	Murrah	Surti
BLV (µg/100 ml)	43.94±24.28	50.64±21.36
BLVAP (mg/100 ml)	10.24±3.15	5.91± 1.41

The data show wide variation in the levels of pigment. The variation was less in the case of BLVAP and the coefficient of correlation between the binding protein with the pigment was negative, the values for Murrah buffaloes' milk being 0.44 and for Surti buffaloes' milk 0.26, the values not being significant at 5 and 1 per cent levels.

3.3.3 Effect of feeds on the biliverdin associated protein and the protein content of milk

Six Surti buffaloes selected in mid-stage of lactation (about 15-20 weeks) were first maintained on the normal ration for a fortnight but animals were fed individually. Milk samples were collected on alternate days during the second week after which animals were

Table 3

Estimation of bilivardin and bilivardin associated protein content from Surtee buffaloes' milk samples (Collected between 12.1.79 to 28.6.79)

BLV (µg/100 ml)	BLVAP (Mg/100 ml)
16.80	9.52
44.80	12.52
50.00	7.00
28.00	16.50
89.00	8.75
11.20	10.86
28.00	14.35
72.80	5.58
67.20	13.72
22.20	10.40
28.00	9.10
22.40	11.30
44.80	7.80
56.00	10.50
78.00	5.58
Avg. 43.94 ± 24.28	10.24 ± 3.15

Table 6

Estimation of the content of biliverdin and biliverdin associated protein from Surti buffaloes milk samples (Collected between 28.8.1979 and 16.5.1980)

BLV (µg/100 ml)	BLVAP (mg/100 ml)
50.40	3.50
56.00	5.20
51.00	5.00
96.00	5.60
63.00	6.90
84.00	5.30
44.80	7.80
39.00	5.00
33.60	8.30
30.00	5.50
Avg. 54.64±21.36	5.91± 1.41

changed over to intense dry feeding (about 10 kg ragi strew per animal per day) but supplemented with concentrate mixture. Milk samples were collected during the last week of the feeding interval. The same practice was repeated when the animals were changed over to complete green fodder consisting green maize (fed about 35 kg/day/animal) supplemented with concentrate mixture. The results of total protein content and M.V.P are shown in Table 7. It may be seen from the Table 7 that neither dry nor green fodder had any effect on protein of milk and M.V.P contents in milk.

Table 7

Effect of feeding on the content of biliverdin associated protein and total milk protein in Sikalogs' milk

Control		Dry fodder feeding		Green fodder feeding	
Percentage of total milk protein	BLVAP mg/100 ml	Percentage of total milk protein	BLVAP mg/100 ml	Percentage of total milk protein	BLVAP mg/100 ml
3.87	4.06	3.98	7.57	3.63	3.22
3.75	4.45	3.87	3.25	3.75	3.56
3.63	5.90	3.75	3.59	3.75	3.75
3.90	5.15	3.62	2.80	3.87	4.53
Avg.3.80	4.80	3.80	4.30	3.75	4.27

3.4 DISCUSSION

3.4.1 The conjugation of different classes of constituents like lipids, carbohydrates, pigments, metals etc., with proteins is well recognised as a part of the normal metabolic process in plants and animals. In a similar manner, recently bile pigments like biliverdin and bilirubin are found associated with protein in buffaloes' milk. The conjugated protein is soluble in 60 per cent aqueous acetone in the presence of saturated $(NH_4)_2SO_4$ system. It was, therefore of interest to study the correlation between the amounts of pigment and the amount of binding protein and also the effect of feeds and the relationship between the BLVAP and total milk proteins with a view to examine a number of samples rapidly. Hence, the method for the estimation of BLVAP was standardised (Rao and Dastur, 1980). The method is simple and accurate and involves the extraction of milk with acetone after saturating the system with ammonium sulphate to obtain the BLVAP into acetone layer. The solvent is evaporated at room temperature and the residual protein is dissolved in 1.0 N NaOH and estimated according to Lowry *et al* (1951) using Folin-Ciocalteu-phenol reagent.

3.4.2 In trials involving five estimations on the same sample protein solution, the results are reproducible as illustrated by following results: 9.60, 9.50, 9.50, 9.50 and 9.50, average 9.52 ± 0.157 mg protein/ 100 ml of skim milk. The percentage recovery of added protein (BLVAP) to the sample under study, and BSA, gave average recoveries of 99.79 and 98.00 per cent, respectively (Table 2 and 3).

3.4.3 The method of Lowry et al (1951) has been widely used for the estimation of proteins owing to its high sensitivity and simplicity. The colour formation has been attributed to the reduction of Folin's reagent by the copper complexes formed by the specific peptides and proteins, tyrosine and tryptophan residues (Layne, 1957; Wu et al., 1978). The reaction is influenced by a number of substances. The non-interference of biliverdin with the estimation was verified (Table 4) by comparing the results obtained from the individual cows' and buffaloes' skim milk and their mixture. The colour formed by the BLVAP was found to be directly proportional to the extent of protein present in sample (Fig.2), the range being 0 to 335 µg/ml.

3.4.4 In a similar study with BLV content, Daniel (1977) has also observed wide variations in the content

of BLV, with an average values of 62.53 ± 21.47 µg/100 ml the range being 23-155 µg/100 ml. The content of BLV in 220 samples not only varied in the pooled samples but also in samples from individual animals. There was a marked difference in the day to day concentration of BLV in individuals. The average BLV content of 15 samples of morning milks of Murrah buffaloes was 43.94 ± 24.28, the range of values being 11.20 to 89.00 µg/100 ml (Table 5). Similarly, the average BLV content of ten samples of Surti buffaloes was 54.64 ± 21.36 the range of values being 30.00-90.00 µg/100 ml. (Table 6). The wide standard deviation shows that there was considerable day to day variation in the values. The variations in BLVAP was comparatively less for the Murrah and Surti animals, the average values being 10.24 ± 3.15 and 5.91 ± 1.41 mg/100 ml, respectively. The values of the two breeds differed significantly.

3.4.5 It was further observed that there was no relationship between the BLV concentration in milk and the amount of binding protein present. The highest protein value of 16.50 mg/100 ml was obtained in the milk from Murrah buffaloes, when the BLV concentration was only 28.00 µg/100 ml. Similarly, with lowest value for protein was 5.58 mg/100 ml, the BLV value was 78.00 µg/100 ml.

A similar trend was observed in milk samples from Murti buffaloes. The highest M.V value was 96.00 µg/100 ml corresponding with 5.60 mg/100 ml protein; with the highest protein content of 8.30 mg/100 ml, the M.V concentration was only 33.60 µg/100 ml.

3.4.6 The results on the study of the effect of green and dry feeds on the content of total protein and M.V.P are shown in Table 7. Samples of bulk milk from six buffaloes were analysed during the last stage of the particular feeding regime. The average M.V.P content and the total milk protein content did not show any significant difference during different feeding regimes. However, green and dry feed rations slightly decreased the M.V.P content but the total milk protein content was not affected. Thus, neither the M.V.P nor the total protein content of milk appears to be related to the diet of the animals.

3.4.7 In the results of the studies described above, the M.V.P content in buffaloes' milk shows day to day variations, similar to the quantity of M.V present in milk and the two bear no relation to each other. No relationship was also observed between the total protein content and the quantity of the M.V.P in buffaloes' milk. Feeding regimes comprising intensive green and dry fodder

feeding appears to have no influence. This is in marked contrast to the carotene present in cows' milk which is highly dependant and closely correlated to the green feed injested by the animals. The results seem to indicate that V.A in buffaloes' milk does not appear to be bound to specific protein and the concentration of V.A in milk is not determined by the quantity of any special protein that is precipitated at pH 4.6 along with casein. This point has been further investigated in the following chapters which deal with the compositional characteristics of the associated protein.

CHAPTER IV

STUDY OF THE ELEMENTARY COMPOSITION OF SILIVERSKIN ASSOCIATED PROTEIN FROM BUFFALOES' MILK

4.0 STUDY OF THE ELEMENTARY COMPOSITION OF BILIVERDIN ASSOCIATED PROTEIN FROM BUFFALOES' MILK

In order to ascertain the elementary composition of the BLVAP, the latter was isolated from fresh buffaloes' skim milk treated with acetone and ammonium sulphate and examined for presence of P, Fe, Cu and Zn. The iron binding capacity of the BLVAP was also ascertained.

4.1 MATERIALS AND REAGENTS

Fresh skim milk was used in all cases. Glassware used were soaked in 1:1 nitric acid overnight, washed with water, allowed to soak in water and finally rinsed with glass distilled water. The glassware were dried in a hot air oven. Glass distilled water was used for all purposes and the chemicals used were of analytical grade.

4.2 METHODS USED

4.2.1 Isolation of biliverdin associated protein

4.2.1.1 Isolation of biliverdin associated protein at room temperature

BLVAP was isolated at room temperature using 1000 ml fresh skim milk according to the procedure outlined in para 3.2.1 by adding acetone with stirring,

followed by ammonium sulphate. The recovered protein was dried in a vacuum desiccator over sulphuric acid.

4.2.1.2 Isolation of bilivardin associated protein at 4°C

In order to isolate the BLVAP under mild conditions of treatment, the isolation was carried out at 4°C. The fresh skim milk was chilled in a refrigerator for 4 h and the protein was isolated at 4°C using ice cold water bath and chilled acetone. The rest of the procedure was same as described earlier.

Before analysing for the elementary composition, the BLVAP samples were dialysed for 48 h at 4°C against glass distilled water in order to free them from ammonium sulphate and other contaminants, if any.

4.2.2 Estimation of nitrogen in bilivardin associated protein

Nitrogen in BLVAP was estimated using micro Kjeldahl method. On digestion with sulphuric acid, protein degrades into ammonia with the formation of ammonium sulphate. The hydrolysate on treatment with alkali solution liberates ammonia quantitatively. The liberated ammonia is estimated by titration using standard sulphuric acid. Nitrogen content can then be calculated from liberated ammonia.

4.2.2.1 Reagents

(a) Sulphuric acid Sp.gr.1.84
(b) Potassium sulphate
(c) Copper sulphate
(d) NaOH solution: 50 per cent
(e) Standard NaOH solution:0.10 N
(f) Standard H_2SO_4 solution: 0.10 N
(g) Indicator: Methylene blue + methylene red

4.2.2.2 Procedure

Twenty mg of HLVAP was transferred to a 30 ml Kjeldahl flask. To this, 2 ml of conc. sulphuric acid, 1.9 to 2.0 g potassium sulphate and 70-80 mg of copper sulphate were added. The mixture was digested. After cooling a small quantity of water was added to the digest and then transferred quantitatively to a distillation assembly. About 15 ml of 50 per cent NaOH solution was added to the distillation flask and ammonia was distilled. The liberated ammonia was collected in 20 ml 0.1N H_2SO_4 containing 2 drops of indicator. About 30-40 ml of distillate was collected and the unused acid was estimated by titrating with 0.1 N NaOH.

4.2.3 Ashing and preparation of protein sample for analysis

To test the presence of Cu, Zn and to estimate Fe and P content of the HLVAP, 20 mg of the protein was weighed into a silica crucible and ignited in a muffle

furnace at 550°C for 90 min. The crucible was cooled in a dessicator and the dry ash was dissolved in 4 ml of hot 6N HCl and made up to 25 ml with water. The acidic ash solution was used as such for all tests and estimations, except for Cu where it was neutralised before testing.

4.2.4 Estimation of iron

Iron was estimated using 1, 10 phenonthroline reagent (Sandell, 1959). It has been reported that as much as 50 mg of phosphorous in 25 ml of solution at pH 3.5 does not interfere with the determination of 0.2 mg iron if the mixture was allowed to stand at least for half an hour after adding O-phenonthroline and hydroquinone reagents. The same procedure was followed.

4.2.4.1 Reagents

(a) Hydroquinone: 1 per cent solution in water

(b) Sodium citrate: 25 g dissolved in 100ml water

(c) 1, 10 phenonthroline: 0.25 per cent in water

(d) Standard iron: A solution of ferrous sulphate ($FeSO_4.7H_2O$) in water (50 µg/ml) was used as standard stock solution. From this various graded dilutions were prepared for constructing the standard curve.

4.2.4.2 Procedure

Two ml aliquot of the sample (4.2.3) were pipetted into a test tube. The reagent blank consisted of 2 ml solution of HCl water mixture. One ml of hydroquinone, followed by 2 ml of phenonthroline solution and 2 ml of sodium citrate (to bring the pH to 3.5) were added to each tube and mixed well. The tubes were allowed to stand for 1 h at room temperature and absorbance was measured at 508 nm in a spectrophotometer against the blank in 1 cm path length cuvettes. Estimations were carried out in duplicate and average values have been reported.

4.2.5 Estimation of phosphorous

The phosphorous content of the BLVAP was estimated according to the method of Fiske and Subba Row (1925). The protein sample was treated with an acid molybdate solution, which forms phophomolybolic acid with the phosphate present in the sample. Phospho molybdic acid is further reduced by the addition of 1,2,4 amino napthol-sulphonic acid reagent to produce a blue colour whose intensity is proportional to the amount of phosphate present in the sample.

4.2.5.1 Reagents

(a) Sulphuric acid: 10 N

(b) Ammonium molybdate: 2.5 g in 100 ml of water

(c) Sodium bisulphate: 15 g in 100 ml of water

(d) Sodium sulphate: 20 g in 100 ml of water

(e) Fiske and Subba Row reagent: 195 ml of 15 per cent sodium bisulphate solution is placed in a stoppered measuring cylinder. To this, 0.5 g of 1, 2, 4 amino naptholsulphonic acid was added, followed by 5 ml of 20 per cent sodium sulphate solution and the mixture was shaken well. Twenty per cent sodium sulphite was added at the rate of 1 ml at a time.

4.2.5.2 Procedure

Two ml of aliquot of the sample (4.2.3) was transferred to a test tube and contents mixed well with 0.2 ml of 10 N sulphuric acid, 0.4 ml of 2.5 per cent ammonium molybdate and 0.2 ml of Fiske and Subba Row reagent which were added subsequently. The final volume was made upto 4.2 ml with distilled water. The absorbancy of the blue colour was measured in a spectrophotometer against reagent blank at 720 nm, 10 min after the addition of Fiske and Subba Row reagent. The estimations were carried out in duplicate and the average values have been reported.

The phosphorous standard consists of a solution of potassium dihydrogen phosphate in distilled water (30 µg/ml). Different graded dilutions such as 3, 6, 9, 12 and 15 µg/ml were withdrawn from the stock solution for the purpose of constructing the standard curve.

4.2.6 Analysis of copper

The presence of copper in H.V.P was tested by colorimetric method using diphenylthiocarbazone (dithizone) as suggested by Mulder et al (1964).

4.2.6.1 Reagents

(a) Sulphuric acid: 10 per cent

(b) Dithizone solution: 20 mg of dithizone in 100 ml of carbon tetrachloride

(c) Carbon tetrachloride

(d) Dilute ammonia: 1:200 with water

(e) Sulphuric acid: 1 per cent

4.2.6.2 Procedures

The neutralised sample obtained by ashing (4.2.3) was acidified with 1 ml of 10 per cent sulphuric acid. The liquid was extracted four times by gentle shaking, each time with 5 ml of dithizone solution and then with 5 ml of carbontetrachloride. The combined extracts were shaken four times with 5 ml of dithizone

solution and then with 5 ml of carbontetrachloride. The pooled extracts were shaken four times with dilute ammonia in order to remove excess dithizone. The violet colour solution in carbon tetrachloride was then washed with 5 ml of 1 per cent sulphuric acid and made up to 25 ml. The colour intensity was measured in a Hilger colorimeter using 52 filter against reagent blank.

For preparing the standard sample, copper sulphate solution (1 µg/ml) was used.

4.2.7 Analysis for zinc

Zinc was analysed using dithizone reagent (Sandell, 1959). Zn reacts readily with dithizone in weakly alkaline medium to form the primary dithizonate which is soluble in chloroform or carbon tetrachloride giving a solution of bright red colour. Since many other metals react with dithizone under the same conditions as Zn, it was necessary to use a complex forming agent to prevent such interference. At pH 4.0-5.5 sodium thiosulphate prevents the reaction of copper, mercury, silver, gold, bismuth, lead and cadmium, while permitting with Zn to proceed.

4.2.7.1 Reagents

(a) Dithizone solutions: 0.01, 0.001 and 0.005 percents in carbontetra chloride

(b) Sodium thiosulphate: 25 g in 100 ml water

(c) Hydrochloric acid: 6 N

(d) Hydrochloric acid: 0.02N

(e) Ammonia solution sp.gr. 0.91

(f) Sodium citrate: 10 g in 100 ml water, made faintly ammonical was extracted with small portions of 0.01 per cent dithizone to remove contaminant heavy metals. The solution was filtered through a small filter paper previously washed with dilute HCl to remove droplets of carbon tetrachloride.

(g) Acetate buffer: mixed equal volumes of 2.0 N sodium acetate and 2N acetic acid. The contaminating heavy metals were removed by shaking with 0.005 per cent dithizone solution. Droplets of carbon tetrachloride were removed by filtering through Whatman No.42 filter paper.

4.2.7.2 Procedure

To an aliquot of 10 ml of ash solution (4.2.3), 5 ml of sodium citrate was added and made ammonical. An

excess of 0.2-0.3 ml of ammonia was added and extracted with 0.01 per cent dithizone in a separating funnel. The extraction with more dithizone solution was repeated until the last portion remained green after shaking for atleast one and half minute. The combined carbon tetrachloride extract was washed with a small quantity of water and then shaken twice with 10 ml of 0.02N HCl to ensure the removal of all the Zn and made upto 25 ml with water.

Ten ml of aqueous acidic solution obtained as above were transferred to a small separating funnel. To this, 5.0 ml of acetate buffer (pH 4.75), followed by 10 ml sodium thiosulphate, were added and shaken vigorously for 2 min with 500 ml of 0.001 per cent dithizone. The stem of the funnel was dried with filter paper and a clear carbon tetrachloride extract was obtained. The absorbance was read in a spectrophotometer at 620 nm having 1 cm path length taking a similarly prepared reagent blank. For preparing standard sample solution, zinc acetate solution (5 µg/ml) was used and the colour developed exactly as mentioned in the case of test sample.

4.2.8 Addition of iron to see the formation of iron-protein complex

4.2.8.1 In order to constitute the complex of iron-protein with added iron, the procedure followed for

reconstitution of iron-lactoferrin complex by Groves (1960) was employed. BLVAP was dissolved in vernol buffer of 0.1 ionic strength (pH 8.4). Ferrous ammonium sulphate was dissolved in 0.001 N acetic acid. An aliquot was added to the protein solution equivalent to 0.2 per cent iron, based on the protein content. The final concentration of the protein was 1 per cent. BLVAP without the addition of iron was run together as control. After standing overnight at 2°C, the absorption spectra was determined in the visible region (360-750 nm) using 1 cm path length cells. The same procedure was followed for 80 per cent acetone soluble cows' milk protein and the presence of iron was tested in the control protein.

4.3 RESULTS

4.3.1 Nitrogen content of the biliverdin associated protein

Seven samples of BLVAP were examined by the Kjeldahl method. The results are shown in Table 8. The average nitrogen content found was 15.55 ± 0.29 per cent.

4.3.2 Phosphorous in biliverdin associated protein

Phosphorous content of the BLVAP was determined in the protein isolated at room temperature as well as in the protein isolated at 4°C. The average phosphorous content in the BLVAP of the former was 354.60 ± 21.93 µg/100 mg the range being 331.00-380.00 µg, while in the latter case, the average phosphorous content of the BLVAP was 272.20 ± 15.57 µg/100 mg of the protein, the range being 256.00-287.00 µg. The values of different trials are shown in Table 9.

4.3.3 Iron in biliverdin associated protein

In order to remove the contaminant and adhering minerals, if any from BLVAP, after dialysing the same against the water, it was subjected to further dialysis against 0.5 M EDTA solution, pH 4.5, at 4°C for 24 h, followed by glass distilled water for another 24 h.

Table 8

Nitrogen content of bilivertin associated protein

Sample Number	Percentage of nitrogen
1	15.86
2	15.86
3	15.13
4	15.40
5	15.86
6	15.40
7	15.40
Average	15.55 ± 0.29

Table 9

Phosphorous content of biliverdin associated protein (µg/100 mg of the protein)

BLVAP isolated at room temperature	BLVAP Isolated at 4°C
340.00	256.00
375.00	256.00
380.00	287.00
331.00	287.00
358.00	275.00
Average 354.60±21.93	272.20±15.97

4.3.3.1 The average values obtained for the iron content of BLVAP isolated at room temperature was 103.10±64.05 µg/100 mg of the protein, whereas for the protein isolated at 4°C from chilled milk, the average value of iron was 96.20 ±33.46 µg/100 mg of the protein, the ranges being 50.00 - 187.00 and 62.5-135.00 µg respectively. The results obtained from individual samples are presented in Table 10. These data in both cases indicate wide variations among the samples which gives rise to a doubt whether the iron present with BLVAP is forming a true complex or it is an association due to the chelating property of the protein. To find out whether there was no contamination from EDTA used, the iron content in BLVAP was estimated without subjecting it to dialysis against the EDTA. The results are shown in Table 11. In these trials, the values are varied but are significantly lower than those shown in Table 10.

4.3.3.2 In order to examine whether iron formed a complex with the protein, BLVAP was subjected to addition of iron under optimal pH 8.4 and at a low temperature (2°C). The observed spectra in visible region, namely 360-750 nm, did not indicate any adsorption maxima. Slightly higher values were observed in the case of BLVAP solution with added iron as compared to BLVAP solution without added iron. The optical density at various wave lengths are shown in Table 12. A similar trend was observed with 80 per cent acetone soluble $(NH_4)_2SO_4$ treated cows' milk protein as it can be seen from the same table in the last column.

Table 10

Iron content of bilivardin associated protein(EDTA dialysed) (µg/100 mg of the protein)

BLVAP isolated at room temperature	BLVAP isolated at 4°C
50.00	125.00
50.50	135.00
72.00	94.00
187.00	62.50
156.00	64.50
Average 103.10±64.05	96.20±33.46

Table 11

Iron content of bilivergin associated protein isolated 4°C without EDTA dialysed (µg/100 mg of the protein)

Sample Number	Iron content
1	34.25
2	93.75
3	51.25
Average	59.78±30.64

Table 12

Absorptivity of biliverdin associated protein and 80 per cent acetone soluble $(NH_4)_2SO_4$ treated cows milk protein in the presence of added iron against control protein blank*

Wave length (nm)	Optical density	
	BLVAP	80% acetone soluble $(NH_4)_2SO_4$ treated cows milk protein
360	0.324	0.238
380	0.208	0.230
400	0.153	0.136
420	0.127	0.112
430	0.116	0.109
440	0.108	0.103
450	0.103	0.099
460	0.101	0.097
465	0.098	0.092
470	0.097	0.088
480	0.086	0.082
490	0.081	0.079
500	0.080	0.079
540	0.067	0.054
580	0.055	0.046
620	0.045	0.038
660	0.045	0.038
700	0.030	0.020
750	0.022	0.018

*Concentration of iron was 0.2% based on the protein and protein concentration was 1.0% in vernol buffer (pH 8.4) in both the cases.

4.4 DISCUSSION

In order to establish the identity of BLVP, it was decided to study its nitrogen and phosphorous content along with trace elements like iron, copper and zinc, since these are associated with some of the proteins in milk.

4.4.1 The nitrogen content of biliverdin associated protein isolated from buffaloes' milk shows an average nitrogen content 15.55 per cent (Table 8). McKenzie (1971) has quoted the values for casein N as 15.9 and 15.4 per cent for acid caseins prepared at 30°C and 20°C, respectively. The value for $(NH_4)_2SO_4$ precipitated casein is 15.5 per cent (2°C) and for calcium chloride precipitated casein is 15.2 per cent (37°C). Earlier, Gorden et al (1949, 1953) found that the average N per cent in cows' milk was 15.63 while in α_s-, β-, and γ-casein fractions, the values were 15.53, 15.33 and 15.40 per cent, respectively. In a recent review, Woodward (1976) has quoted the values for N content of cows' milk casein which is said to vary from 13.2 to 15.7 per cent. From a study on Italian buffaloes' milk, Albonico et al (1968) found that the N content of casein was 15.1 to 15.3 per cent. In contrast, Nagasawa et al (1973) from an analysis of Murrah buffaloes' milk casein,

found the values for N content of whole casein as 15.4 per cent and for α_s-, β-, and k-caseins as 13.2, 13.6 and 14.3 per cent respectively. The values obtained for BLVAP were also close to those obtained for cows and buffaloes' milk caseins. BLVAP isolated at room temperature and at 4°C gave similar results.

4.4.2 Phosphorous in biliverdin associated protein

The phosphorous content in BLVAP was 354.60 ± 21.93 and 272.20 ± 15.57 µg/100 mg protein (Table 9) for the proteins isolated at room temperature and 4°C, respectively. There was a marked difference in the phosphorous content of the protein isolated at the two temperatures. As already shown by Reimerdes and Herlitz (1979), these differences could be due to structural changes in protein molecule at low temperatures. Similar changes seem to occur resulting in the solubalization of some phosphoprotein components in milk serum during chilling. It has been established that chilling of milk activates the proteinase system which in turn acts on β-casein cleaving it into γ-caseins and proteose-peptone 5 and 8 F. PP5 and 8F are soluble in milk serum. During the extraction of BLVAP from chilled milk, only γ-caseins go along with other casein fractions while PP5 and 8F are not present (Rao et al., 1981)

and this aspect is elaborated further in Chapter V, para 5.3.3 and 5.4.5. It has been shown that there is a time dependent transfer of β-casein and the milk serine proteinase system from micelles to milk serum with the change in temperature from 38 to 4°C. It has also been established that γ-casein can be formed by proteolytic degradation of β-casein. Two constitutents of the proteose-peptone fractions of bovine milk have been isolated and characterized. Component 5 (PP5) represents residues 1-105 and 1-107 of the β-casein amino acid sequence, while component 8 Fast (PP8F) corresponds to residues 1-28 of β-casein. Thus, proteose-peptone represent N terminal portions of the β-casein molecule produced by proteolytic cleavages at low temperatures, while γ_1-, γ_2-, and γ_3-caseins form C terminal portion of β-casein (Andrews, 1979). The behaviour of the BLVAP appears very much like that noticed in the case of bovine milk casein.

4.4.3 Iron in biliverdin associated protein

The iron content of BLVAP isolated at room temperature and at 4°C dialysed against EDTA and isolated at 4°C without EDTA dialysis were 103.10±64.05, 96.20 ± 33.46 and 59.78 ± 30.64 µg/100 mg of the protein, respectively (Table 10, 11). These data indicate

wide variations in the samples and also a high standard deviation from the mean values. Similar variations have been noted in the work reported earlier for the iron content of milk. Prafulla and Anantakrishnan (1959) found that the iron content in cows' milk produced in Bangalore farm, varied from 70 to 155 µg/100 ml and in buffaloes' milk, the variations were from 75 to 133 µg per cent. In a subsequent study Gosh et al (1965) found that the average value of iron to be a 127 µg/100 ml in cows' milk and 131 µg/100 ml in buffaloes' milk. In a recent study, Unnikrishnan and Rao (1977b) found the iron content in the milk of both the species ranged from 50-55 µg/100 ml. The above authors also found that nearly 7.5 µg natural iron was associated per g of casein. Two kinds of iron binding proteins have been detected in milk. One of these is identical to the circulating transferrin of blood, while the other, a very different protein, designated as lactoferrin is specific to milk (Derechin and Johnson, 1962; Groves, 1965; Masson and Heremans, 1971). The iron complexed lactoferrin is salmon red in colour with a broad absorption maximum near 470 nm. The iron complex of the red protein is similar in many respects to those of the Fe^{+3}-metal combining proteins, namely, siderophilin and

transferrin (Koechlin, 1952) obtained from blood plasma and Conalbumin (Warner, 1951). These proteins show maximum absorbance at 460 to 470 nm. BLVAP even though associated with iron did not show any absorption maximum in the region 360-700 nm, either in native form or when iron was added at the optimal pH 8.4 at 2°C. When iron was added to the metal free lactoferrin, it reconstituted the iron complex and showed an absorption maximum near 470 nm. In the case of BLVAP, the formation of any such complex with iron was not noticed. However, it was observed that BLVAP solution with added iron, indicated higher absorption than control protein blank. This may be due to a slight change in the colour of protein solution from green to yellowish-green (Table 12). The behaviour of 80 per cent acetone soluble $(NH_4)_2SO_4$ treated cows' milk protein also exhibited similar behaviour as shown in the last column of the same table. The protein without added iron gave a positive reaction for presence of iron.

4.4.4 The association of iron with BLVAP appears to be more stable in presence of EDTA. Normally, EDTA rapidly chelates iron and other metals. A similar observation has been made by Groves (1960) while studying with lactoferrin. The results of the present study indicate that the presence of iron in BLVAP may not be

due to any complex formation, but only due to association. It is very likely that the presence of iron may be due to post milking contamination during separation, etc., in the dairy plant. Several studies have shown that casein has a high tendency to bind with added or contaminant iron. Consideration of addition of iron to milk as a deterrant against development of iron deficiency in anemia leads to the question of binding of added iron to various milk components. Vaughan and Knauff (1961), Basch et al (1974) and Demott and Park (1974) found that the added iron is largely associated with protein, primarily casein. Basch et al (1974) studied the distribution of iron and polyphosphate phosphorous added to cows' milk by both analytical and radio chemical techniques. Whole milk was separated isoelectrically or centrifugally into 3 major fractions, nasely, cream, casein and whey, after the addition of ferri-polyphosphate and other iron or polyphosphate compounds. Casein, a phosphoprotein had a greater affinity for the iron and the polyphosphate; 80-95 per cent of iron and 50-55 per cent of the polyphosphate was bound to acid precipitated casein. In studies with ^{59}Fe added to skim milk Demott and Dimeer (1976) has shown that about 85 per cent of the added iron was bound to casein and out of this amount, 72, 21, 4 per cents were associated with

α_s-, β-, and k-caseins, respectively. There was a loss of 1.77 per cent only of added iron due to dialysis of casein. Unnikrishnan and Rao (1977a) also found that more than 90 per cent of the added iron was associated with skim milk, mostly bound to casein in cows' as well as in buffaloes' milk. It is likely that BLVAP also behaves in a similar manner while associating with available contaminant iron.

4.4.5 BLVAP was tested for the copper and zinc, since these trace elements are essential in nutrition and also associated with the protein in bovine milk. However, in both cases these metals could not be detected.

4.4.6 The above study has not brought out any special characteristics of BLVAP except that its nitrogen content is in the same range as that found in cows' and buffaloes' milk caseins. Similarly, like casein BLVAP associated with iron without forming a complex, which clearly indicates that though BLVAP is associated with iron it is different from lactoferrin.

CHAPTER V

STUDIES WITH BUFFALO MILK CASEIN AND MILK FAT GLOBULE MEMBRANE PROTEINS FOR THEIR ASSOCIATION WITH BILIVERDIN

5.0 STUDIES WITH BUFFALO MILK CASEIN AND MILK FAT GLOBULE MEMBRANE PROTEINS FOR THEIR ASSOCIATION WITH BILIVERDIN

As already mentioned earlier, BLV was isolated from buffaloes' milk casein fraction which is soluble in 80 per cent acetone. It appears that BLV may be associated with either some of the casein fractions or with proteins that coprecipitates with the caseins of buffaloes' milk at pH 4.6. To elucidate this aspect and to examine the nature of BLVAP, the present study on the micellar caseins along with casein fractions were undertaken.

5.1 MATERIALS AND REAGENTS

Fresh skim milk was used for all purposes.

Cynogum 41 was procured from BDH (England) and N,N, N'N' tetra methyl ethylene diamine (TEMED) 2-mercapto ethanol, ammonium persulphate and biliverdin dihydrochloride were obtained from Sigma (USA).

5.2 METHODS USED

5.2.1 Preparation and extraction of biliverdin from micellar casein fractions

The micellar casein fractions of varying sizes were prepared from five batches of skimmed milk according

to the procedure of Majumdar and Ganguli (1967) by centrifuging at different speeds namely, 12,000, 23,000 and 37,000 r.p.m. for 30 min. Milk was first centrifuged at 12,000 r.p.m. (11,789xg) using rotar No.50 in a Beckman Model L ultracentrifuge by which the larger micelles of casein settled at the bottom and were subsequently collected. Then the supernatant was centrifuged at 23,000 r.p.m. (40.956xg) when it was possible to obtain medium sized casein particles settling between 12000-23000 r.p.m. The last fraction comprising smaller casein micelles was obtained by centrifuging the above supernatant at 37,000 r.p.m. (105.651xg). The fractions thus obtained were soaked in 10 ml acetone for 4 to 6 h for complete extraction of H.V. The extract was filtered through Whatman No 42 filter paper and examined for spectral characteristics between 360-800 nm.

5.2.2 Preparation and extraction of biliverdin from α_s- and k-casein

5.2.2.1 To ensure adequate amount of pigment, milk was enriched with H.V. The pigment was dissolved in sodium phosphate buffer (pH 7.6) and added at the rate of 5 mg/l. Casein was then precipitated by slowly adding

with constant stirring ten per cent acetic acid to adjust the pH to 4.6, was washed free from whey and dried in a vacuum dessicator.

5.2.2.2 From the casein prepared as above, α_s-, and k-casein fractions were subsequently prepared according to the method suggested by Singhal and Ganguli (1972), for the simultaneous preparation of these fractions based on the methods of Hipp et al (1952) and Zittle and Custer (1963). About 50 g of dry acid casein prepared as above was dissolved in 1 litre of 6.6 M urea. This solution was acidified with 200 ml of 7N H_2SO_4. After acidification, 2 liters of distilled water were added. The pH of the mixture was maintained at 1.3 to 1.5. The mixture was then left standing for 2 h and filtered through a fluted Whatman No 1 filter paper. The precipitate was used for preparation of α_s-casein. The supernatant which contains k-casein was precipitated by the addition of ammonium sulphate to the extent of 1 M (132 g) to each litre of filtrate. The precipitated k-casein obtained was washed repeatedly to free it from acid and ammonium sulphate. The precipitate which contains α_s-casein was washed thoroughly with a 10:20:20 mixture of 6.6 M urea:7N H_2SO_4:distilled water, till free from k-casein. Finally, it was washed with distilled

water to make free from sulphate ions. The washed precipitate containing α_s- and β-caseins was then dispersed in 5.0 M urea solution at the rate of 150 ml for every 30 g of the precipitate. The pH was adjusted to 4.6. The precipitate was collected by centrifugation and the supernatent was discarded. The precipitate was washed three times with urea solution and finally with distilled water. The α_s-, and k-casein fractions thus obtained were soaked in 25 ml of acetone and kept in the refrigerator for 10 h for complete extraction of any pigment, if any. Each extract was examined for spectral characteristics and tested for the presence of bile pigments.

5.2.3 Preparation and extraction of biliverdin from γ-casein

Gamma casein was prepared according to the method of Heimsrdes and Herlitz (1979). The BLV enriched milk was allowed to stay at the refrigerator temperatures for 4 h. After incubation of BLV enriched milk at the refrigerator temperature, casein was precipitated by adjusting the pH to 4.6. The casein thus obtained was soaked in 1-propanol and diethyl ether mixture (2:1 v/v) for 30 min, filtered and the 1-propanol-diethyl ether extract (PDEK) was examined for its spectral properties.

It was also tested for presence of bile pigments. γ-casein was obtained by evaporating the solvent and after recovering the soluble fraction.

5.2.4 Preparation and extraction of biliverdin from proteose-peptone

Proteose-peptone components were isolated according to the method given by Andrews (1979) from B.V enriched and chilled milk. The chilled and B.V enriched skim milk was brought at room temperature and the casein was precipitated by two methods. In one of the methods, acetic acid was added and in the other rennet was added. The casein was freed from whey. The whey was then heated to 95°C for 30 min and the precipitated proteins were removed by filtration. The filtrate obtained as above was used for preparation of proteose-peptone. To the filtrate, trichloro acetic acid was added to a final concentration of 12% (w/v). The precipitate was collected by centrifugation and washed free from acid. The fractions thus obtained were soaked in acetone and the acetone extracts were examined for spectral properties and tested for presence of bile pigments.

5.2.5 Isolation and examination of biliverdin associated protein from skim milk

Biliverdin associated protein was isolated according to the procedure described earlier in para 4.2.1.2. A separate protein fraction was also isolated

by soaking the citric acid precipitated casein in 80 per cent acetone and the residue was collected after evaporating acetone.

5.2.6 Starch gel electrophoresis of biliverdin associated protein and casein

Starch gel electrophoresis of BLVAP and casein was carried out following the procedure outlined by Ganguli and Majumder (1967) using the petri dish technique.

5.2.6.1 Preparation of filter paper strips

Seventy mg of BLVAP and casein were dissolved in 1.0 ml of urea vernol buffer (pH 8.6). The solution was applied on to a strip of Whatman filter paper (3 mm) of 1.0 x 15.0 cm size after leaving 1x3.0 cm space on the top for writing the particulars of the samples. The sample strips thus prepared were preserved in a dessicator over NaOH.

5.2.6.2 Preparation of starch gel plate

The following composition was used for the preparation of starch gel:

Starch 4.17 g, water 17.04 ml, Tris-citrate buffer 4.57 ml (pH 8.6), urea 9.7 g and 2 mercapto ethanol 0.12 ml. This quantity was used for a petri dish of 13.8 cm diameter.

5.2.6.3 Application of the samples

1.5 mm x 1.0 cm bits of the protein soaked filter paper strips were cut and gently inserted in the gels with the help of a master marker. The gel plate was kept for 30 min after insertion of the samples before the electrophoretic run.

5.2.6.4 The electrophoretic run

The electrophoretic run was carried out using boric acid buffer (pH 8.0) and enriched with 0.2 per cent 2-mercapto ethanol. The proteins were allowed to migrate in the electrophoretic field for 3-4 h from cathode to anode. The voltage was kept constant at 300 v, whereas the flow of current varied from 5-7 mA. After completion of the run, the filter paper bridges were gently separated from the gel and the latter was stained with 0.1% amido black in methanol:acetic acid: water (5:1:5) in the plate itself, for 10 min. The excess of the dye was removed by giving 4-5 successive washings of with methanol:acetic acid:water mixture (5:1:5). The pattern of resolution was traced with the help of a transparent paper.

5.2.7 Polyacrylamide gel electrophoresis

Discontinuous polyacrylamide gel electrophoresis (PAGE) with 8 per cent cynogum gels was performed

according to Tembe and Akroyd (1967). For preparing 8 per cent acrylamide gel, 2.0 g of cynogum 41 were transferred to a 25 ml measuring cylinder and Tris-citrate buffer (pH 8.6) having 28 per cent urea was added and the volume was made to 24 ml. Twenty five µl of TEMED was then added, mixed and solution was filtered. When everything is ready, polymerization was initiated by adding 0.1 ml of freshly prepared 10% ammonium persulphate to cynogum. This solution was quickly transferred to the bottom closed gel tubes leaving 1.0 cm space at the top and air was excluded by layering a few drops of distilled water on to the surface of the solution with a syringe. The upper and lower buffer tanks were filled with boric acid buffer (pH 8.0) and a pre-run for 10 min was given with a current of 2 mA per tube. The protein sample dissolved in vernol buffer (pH 8.6), having 6.6 M urea was applied on to each gel tube with a micropipette. Then, a constant current of 4 mA per tube was applied until the bromophenol dye migrated to 0.5 cm above the bottom of the gel tube. Gels were removed by injecting water through the sides of the tube and stained with 0.1% amido black in 10 per cent acetic acid for 10 h and destained repeatedly with 10 per cent acetic acid until the bands are clear. The pattern of resolution was photographed.

5.2.8 Phosphoprotein staining of polyacrylamide gel electrophoresis gels

The polyacrylamide gels of casein and BLVAP were stained for phosphoproteins with methyl green according to the procedure of Cutting and Roth (1973). After the termination of electrophoresis the gels were fixed in 10 per cent sulfosalicylic acid (SSA) for 10 h to facilitate free phosphate and low molecular weight phospho-compounds present in the initial sample to diffuse out of the gels. The method depends on hydrolysis of the phospho-protein phosphoester linkages using dilute base in the presence of calcium ions. The gels containing newly formed insoluble calcium phosphate were then treated with ammonium molybdate in dilute nitric acid. The resultant insoluble nitrophospho-molybdate complex was finally stained with basic dye (methyl green dissolved in dilute acetic acid). After destaining with 10% SSA, green bands are observed on the gel at the locus of the phosphoprotein.

5.2.9 Preparation and isolation of the biliverdin from milk fat globule membrane proteins (MFGMP)

For the preparation of MFGMP, the procedure adopted was essentially that of Herald and Brunner (1957). The required volume of freshly collected milk was warmed to a temperature of 30°C and separated

through a mechanical cream separator. The fat percentage of the cream sample was adjusted to approximately 30 per cent and re-separated after mixing with four volumes of distilled water. Washing of the cream was repeated another four times in order to exclude plasma constituents. The washed cream thus obtained was adjusted to 35 per cent fat, cooled to about 10°C in a refrigerator and subsequently churned in a laboratory glass churn.

5.2.9.1 Milk fat globule membrane proteins from butter milk

Butter milk was passed through several layers of cheese cloth and centrifuged at low speed to remove the unchurned fat globules. Ammonium sulphate was gradually added to the butter milk to a final concentration of 2.2 M and stored at 5°C for 1 h. The concentrated material was collected by centrifugation and washed free from ammonium sulphate.

5.2.9.2 Milk fat globule membrane proteins from butter serum

The butter obtained by churning the cream was maintained in a water bath at 40°C. The MFGMP materials which settled at the bottom was collected by careful pipetting. The rest of the procedure was as that described in the preceding para. The MFGMP fractions thus obtained were pooled and soaked in acetone. The acetone extract was examined for spectral properties in the regions of 360-800 nm and tested for presence of bile pigments.

5.3 RESULTS

5.3.1 Spectral study of the biliverdin extract from micellar casein fractions

The green pigment, BLV, of buffaloes' milk could be readily extracted with acetone from micellar casein fractions as shown in Fig.4. The spectral study of the acetone extracts of micellar casein fractions of varying sizes revealed an uniform absorption maximum at 370-380 and 660 nm, corresponding to that of free biliverdin isolated from casein (Chandravadana and Dastur, 1976) thereby indicating that BLV is associated with all the three fractions. The concentration of the pigment and yield of micelles decreased with the size of micelles. Fraction I yielded 1.345 g, fraction II yielded 0.830 g and fraction III yielded 0.370 g.

5.3.2 Characteristics of the pigment extracts of α_s-, β-, and γ-caseins and protease-peptone

The acetone extracts of α_s-, and k-caseins and protease-peptone and PDEE of casein containing γ-caseins showed positive Gmelin reaction (Hamberg and Legge, 1949) and zinc fluorescence test (Gray et al., 1961 and Siedel, 1957), similar to free BLV extracted from casein. The spectral properties of acetone extracts of k-casein and PDEE of casein (γ-casein) have similar absorption maximum at 370-380 nm and 660 nm regions

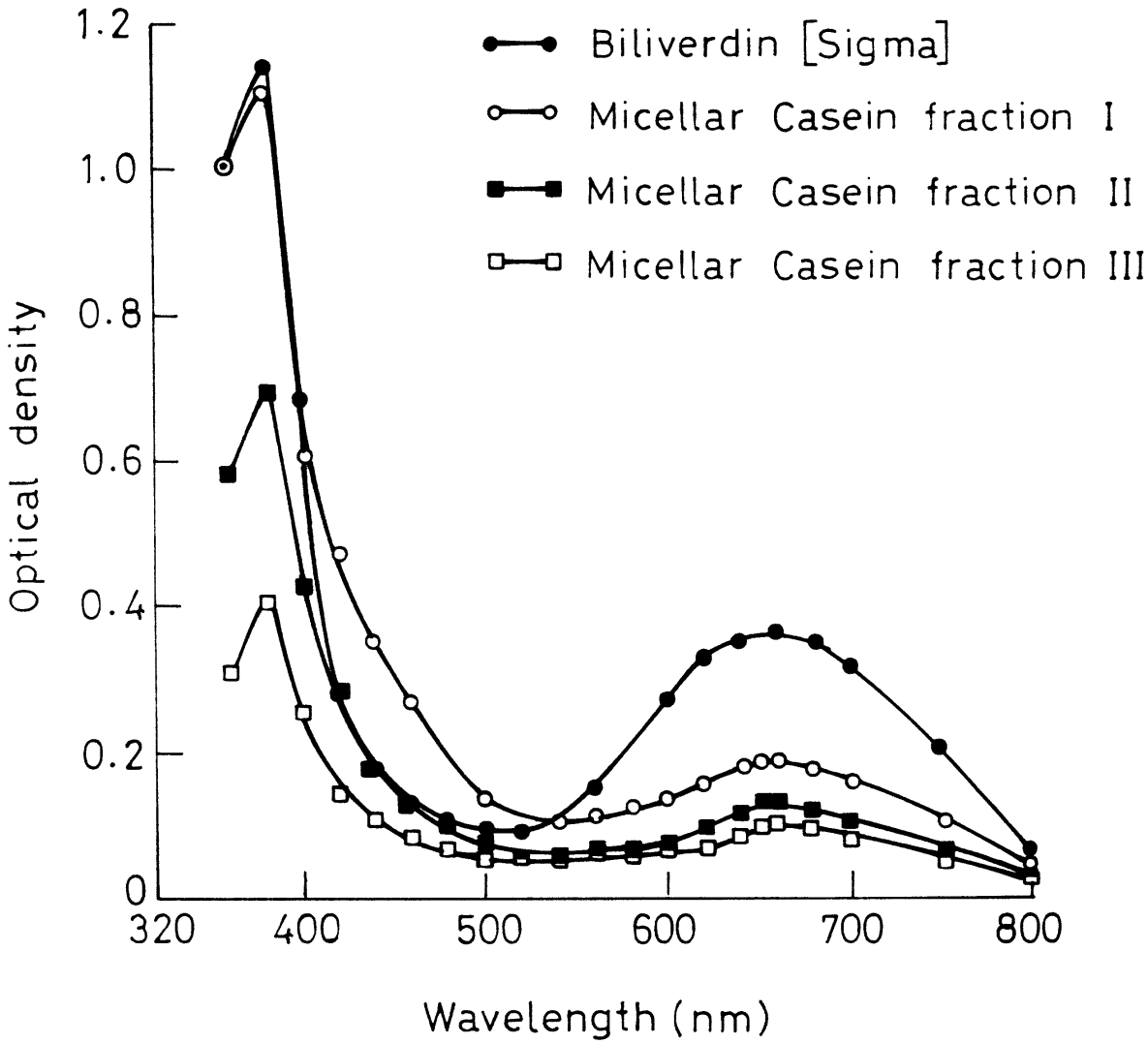


FIG.4 ABSORPTION SPECTRA OF THE PIGMENT EXTRACT FROM MICELLAR CASEIN FRACTIONS.

characteristic of free B.V (Fig. 5 and 6), except for a minor drift towards the short wave length (from 370-380 to 360) as in the case of k-casein. This may be due to the presence of traces of mineral acid and ammonium sulphate used during the isolation. Immediate extraction of the pigment without dialysis was necessary to avoid prolonged exposure of pigment to acid and ammonium sulphate. The spectrum of α_s-casein in acetone (Fig.5) shows absence of absorption in the regions of 370-380 and 660 nm which are characteristic of free B.V. Instead, there was a peak at 430 nm for mesobilirubin, similar to that extracted from sour milk casein (Chandravadana and Bastur, 1976) thereby indicating the conversion of the pigment B.V to B.R due to exposure to urea and acid during its isolation.

The absorption spectrum of the acetone extract (Fig.7) from proteose-peptone obtained from either rennet whey or acid whey, exhibited an absorption maximum at 660 nm only. The reason for quinching of the peak at 370-380 may be due to the effect of heat on milk B.V and agrees with the earlier results of Chandravadana (1976). It may be seen from the Fig.7 that the proteose-peptone obtained from rennet whey is having more pigment as compared to that obtained from

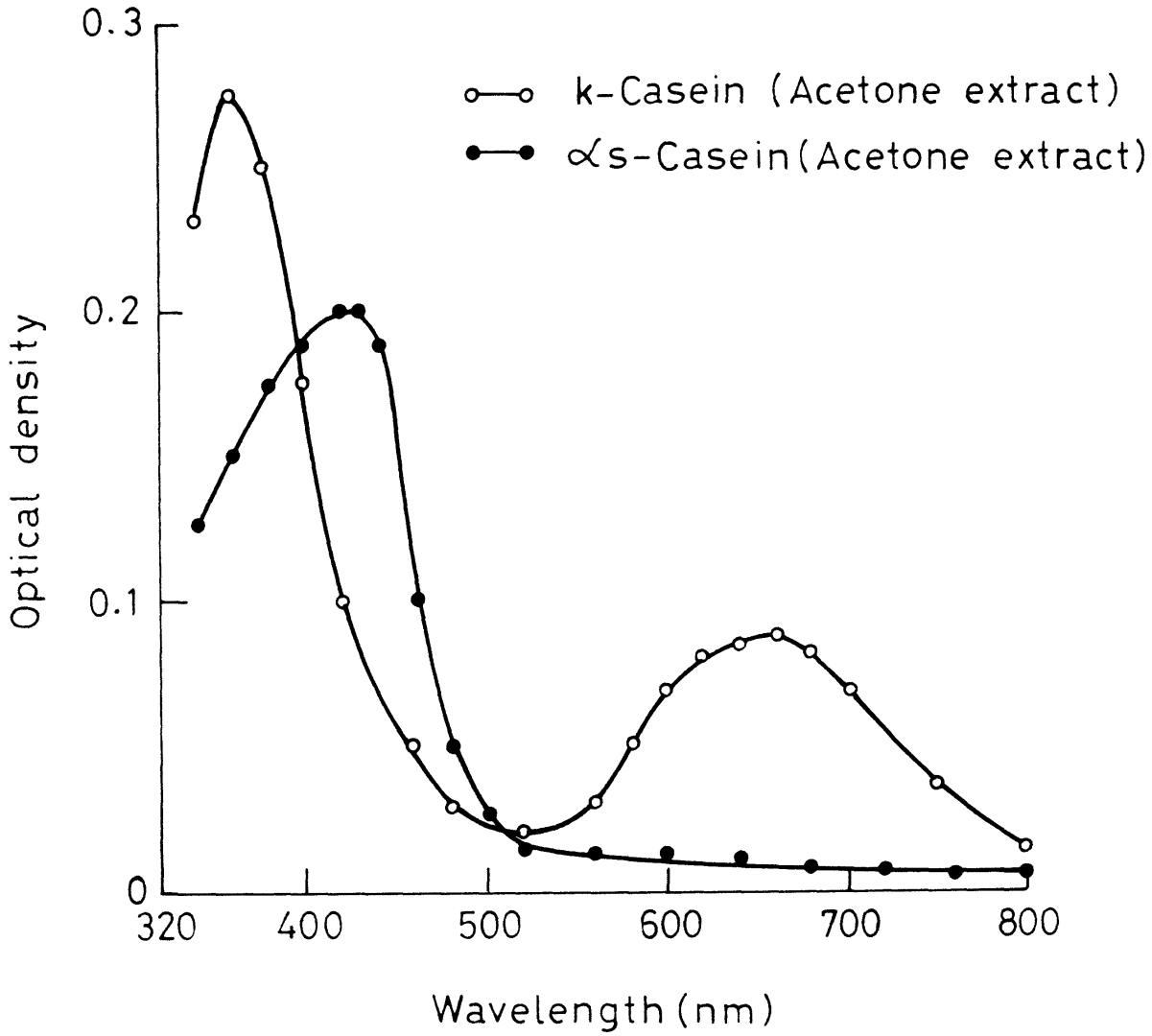


FIG.5 ABSORPTION SPECTRA OF THE PIGMENT EXTRACT FROM α_s-, AND k-CASEINS.

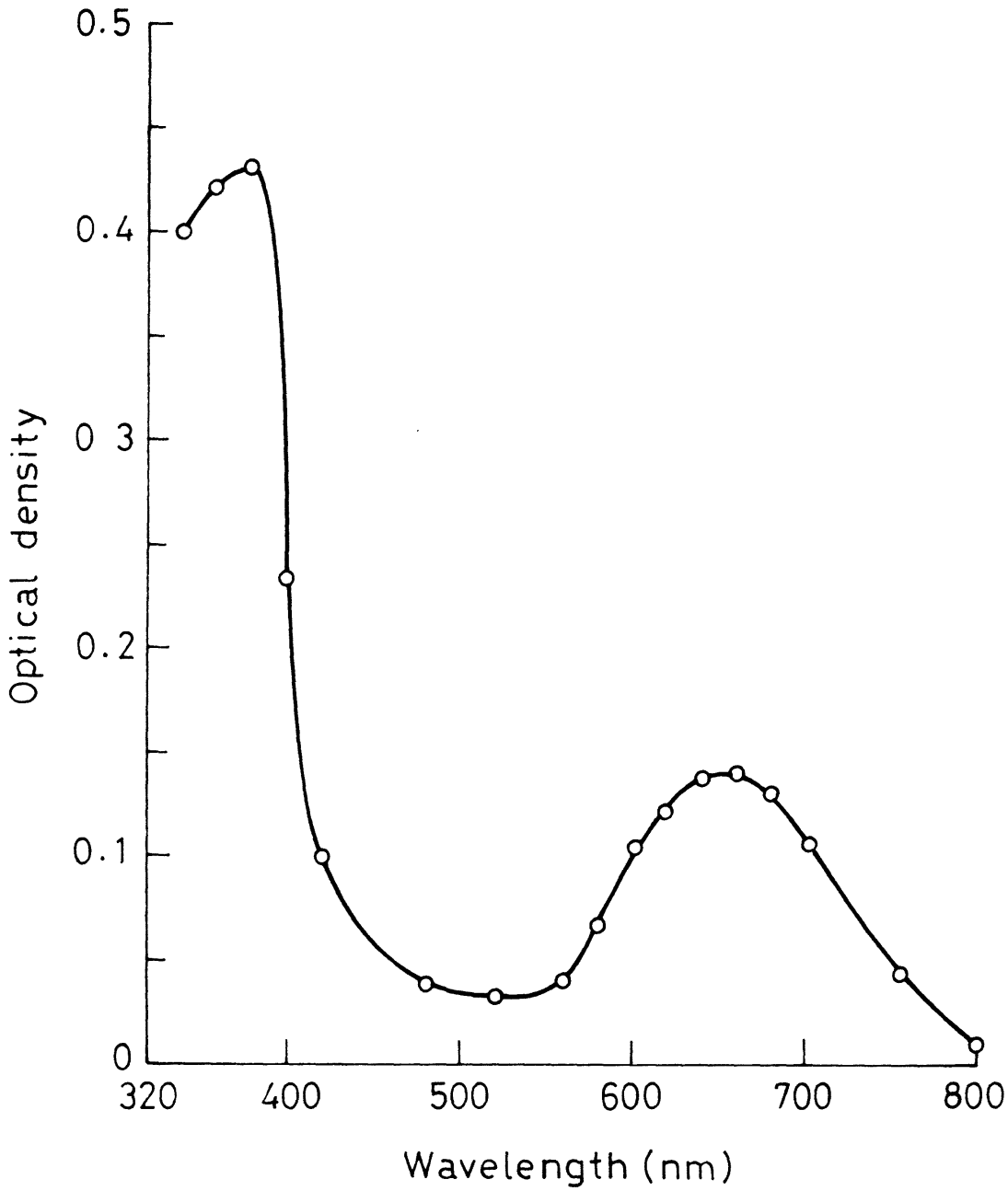


FIG.6 ABSORPTION SPECTRA OF THE PDEE FROM CASEIN [Containing γ-Caseins]

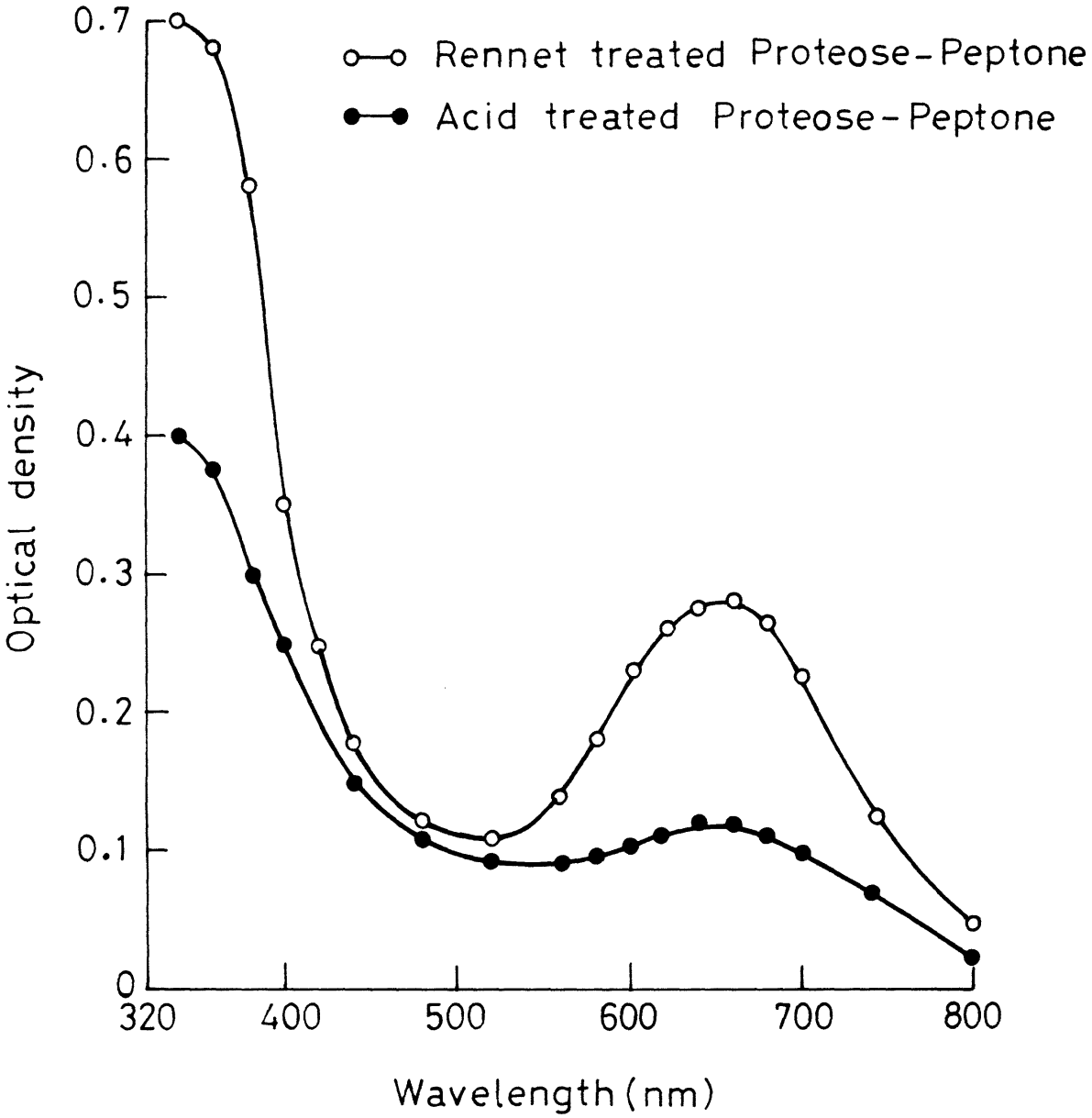


FIG.7 ABSORPTION SPECTRA OF THE PIGMENT EXTRACT FROM PROTEOSE-PEPTONE.

acid whey. This may be due to coprecipitation of macropeptide released by rennet action on k-casein along with protease-peptone. Similarly, the yield of protease-peptone from rennet whey was 0.118 g/100 ml of skim milk and that from acid whey it was only 0.033 g/100 ml of skim milk. The optical density at 660 nm for protease-peptone obtained from rennet whey was 0.31, whereas that obtained from acid whey was only 0.068. Thus, the differences exhibited by rennet whey clearly confirms the observation in regard to the association of BLV with k-casein.

5.3.3 Electrophoretic analysis

The resolution pattern depicted in Fig.8 shows that both BLVAP and casein have similar resolutions and mobilities on starch gel thereby indicating similarity in nature.

PAGE patterns of BLVAP and casein show similar patterns of resolution on 8 per cent cynogum gels in alkaline pH range at 8.6 (Fig.9A). They also have similar phosphoprotein staining patterns (Fig.10). The protein fraction obtained by soaking the citric acid precipitated casein in 80 per cent acetone exhibited only one band corresponding with the first

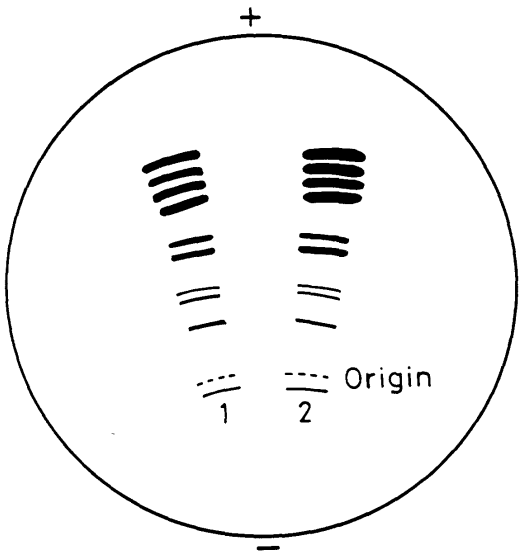


Fig.8 Starch gel electrophoresis patterns of
(1) biliverdin associated protein
(2) casein

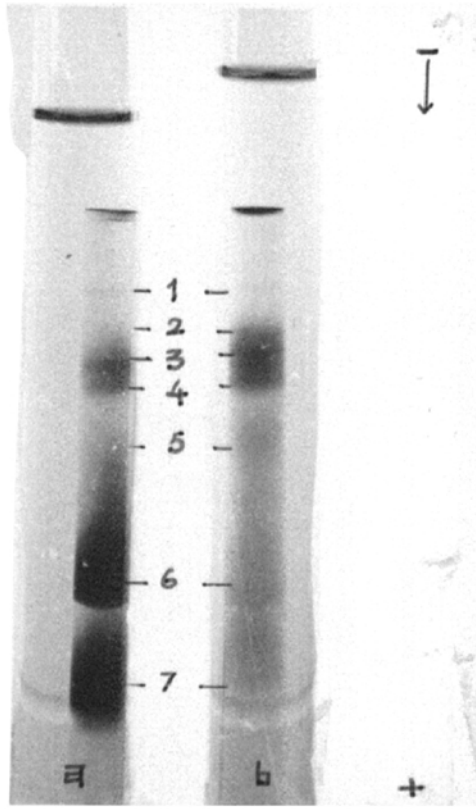


Fig.9A PAGE patterns of casein(a), biliverdin associated protein (b).

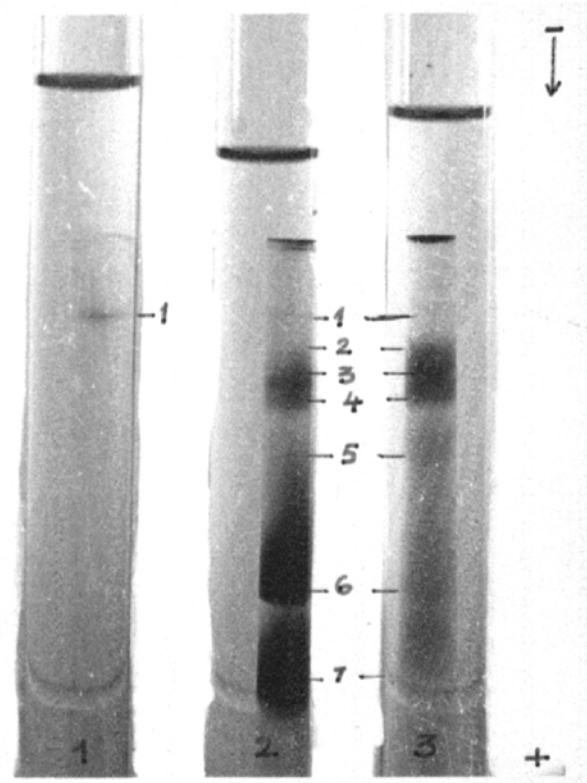


Fig.9B PAGE patterns of 80 per cent acetone soluble protein fraction (1) casein (2) biliverdin associated protein (3)

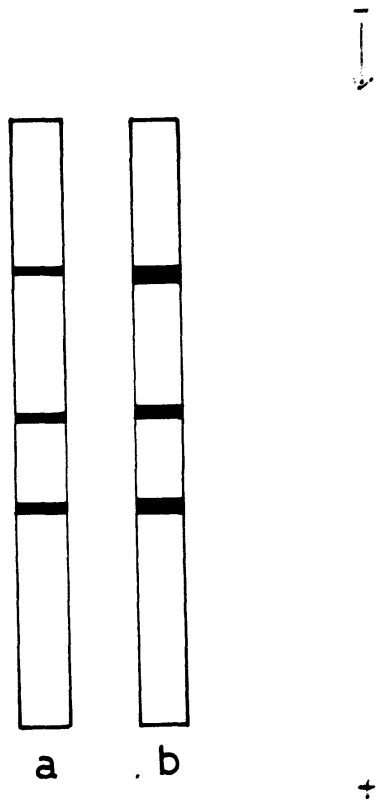


Fig.10 Phosphoprotein staining patterns of
(a) biliverdin associated protein
(b) casein

band of MLVAP and casein, indicating thereby that by direct extraction of casein with 80 per cent acetone, only one fraction in casein is soluble (Fig.9B). The MLVAP and γ-casein prepared from chilled milk again show similar mobilities in the bands 2, 3, 4 and 5 of MLVAP with 1, 2, 3 and 4 (trace) bands of γ-casein, thus establishing the presence of γ-casein in MLVAP moiety (Fig.11).

5.3.4 Association of biliverdin with milk fat globule membrane proteins

The acetone extracts of FGMP did not answer the tests for bile pigments and there was no adsorption maxima in 360-800 nm region unlike free biliverdin extracted from casein, indicating that MLV was not associated with FGMP.

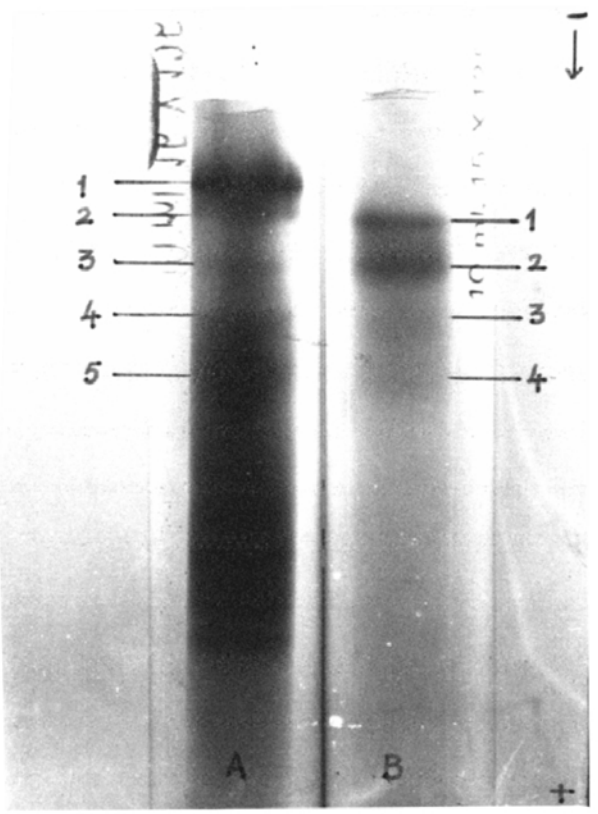


Fig.11 PAGE patterns of (A) biliverdin associated protein (B) γ-casein isolated from chilled milk

5.4 DISCUSSION

5.4.1 The pigment BLV in buffaloes' milk occurs as a conjugate. That the pigment is protein bound is shown by the fact that it precipitated along with casein and can be extracted from the coagulum by aqueous acetone. Further, BLV in buffaloes' milk can be readily extracted with acetone from micellar casein fractions, thereby clearly indicating that the BLV is associated with all the three fractions isolated. Absorption at 370-380 and 660 nm is similar to that shown by free BLV isolated from casein. It is seen from the Fig.4 that the concentration of the pigment and yield of micelles decreased with size of micelles. The values agree with the results of Saharwarl and Gangali (1971) on the yield of micellar caseins from buffaloes' milk. In buffaloes' milk micellar caseins I, II and III, respectively, account for 81.7, 16.6 and 1.7 per cent of the total micellar casein. In the present study also the yield patterns are similar.

5.4.2 The acetone extracts of α_s-, k-caseins, protease-peptone and PDEE of casein containing γ-caseins, show a positive Gmelin and zinc fluorescent test which clearly shows that the bile pigment is associated with

all casein fractions. The presence of BLV with k-, and γ-caseins (C-terminal, portion of β-casein) is definitely established, while in the case of α_s-casein, the reduced form, mesobilirubin is found. Spectral properties of acetone extracts of k-casein and PDEE of casein (containing γ-caseins) have shown similar absorption maxima with that of free BLV. In the case of α_s-casein, there is absorption maximum only at 430 nm instead of at 370-380 and 660 nm as shown by BLV. The absorption peak at 430 nm for acetone extract of α_s-casein corresponds with absorption maxima of meso-bilirubin (λ max 433 in $CHCl_3$, MacDonagh, 1979). This shows that meso-bilirubin is present along with normal BLR formed due to acidity developed by microbial activity or by added acids.

5.4.3 The data from the spectral studies of pigment from proteose-peptone are interesting. Pigment concentration and the yield of proteose-peptone from rennet whey are higher as compared to acid whey fraction, thus indicating the coprecipitation of macro peptide of k-casein released due to rennet action and precipitated along with proteose-peptone by 12 per cent (w/v) TCA concentration, from the above results, it can be concluded that the parent compound, k-casein is associated with BLV.

From the appearance of pigment BLV with γ-caseins which is in the C-terminal portion of β-casein as well as from proteose-peptone i.e., N-terminal portion of β-caseins, it can be established that the BLV is associated with β-casein. These results clearly show that BLV in buffaloes' milk is associated with all the casein fractions namely, α_s-, β-, k-, and γ-caseins and that no special protein is involved.

5.4.4 The absorption spectrum of the acetone extract from proteose-peptone exhibited a maximum at 660 nm only. The reason for quenching of the peak at 370-380 nm may be due to the effect of heat on milk BLV and agrees with the results of earlier study by Chandravadana (1976) on the effect of heat treatment on milk BLV of buffaloes' milk. When milk is subjected to pasteurization and boiling temperature for very short period, no changes were noticed in the spectral properties of BLV. However, when milk is subjected to steaming for 1 h and autoclaving, the peak at 370-380 nm is completely quenched and the intensity of absorption at 660 nm is diminished considerably. Milk subjected to higher temperatures and lower temperatures (65°C) for long periods, resulted in the conversion of BLV to the colourless leuco compound, urobilinogen and the latter can be detected by Ehrlich

aldehyde reagent (Para dimethyl amino benzaldehyde in concentrated HCl).

5.4.5 Starch gel electrophoretic patterns and PAGE phosphoprotein staining patterns of ELVAP and casein show a close similarity in their resolution which clearly brings out their identity. The ELVAP and γ-caseins prepared from chilled milk again exhibit similar mobilities in the bands 2, 3, 4 and 5 of ELVAP with 1, 2, 3 and 4 of γ-caseins, thereby establishing the presence of γ-caseins in ELVAP moiety.

5.4.6 The presence of ELV with α_s-, k-casein, N terminal end of β-casein (proteose-peptone 5 and 8F) and with C terminal end of β-casein (1, 2 and 3 γ-caseins) thus establishes that ELV is associated with all the fractions of casein and with micelles of different sizes. The similarity of electrophoretic mobilities and patterns of resolution of ELVAP and casein on starch gel, PAGE and phosphoprotein staining patterns on PAGE gels have established that the ELVAP extracted from chilled milk at 4°C with acetone and ammonium sulphate is casein and its individual components.

CHAPTER VI

BEHAVIOUR ON SEPHADEX AND DEAE-CELLULOSE COLUMNS AND OTHER PROPERTIES OF BILIVERDIN ASSOCIATED PROTEINS

6.0 BEHAVIOUR ON SEPHADEX AND DEAE-CELLULOSE COLUMNS AND OTHER PROPERTIES OF BLUE VEIN ASSOCIATED PROTEIN

Studies on the association of BLV with casein fractions using techniques such as the electrophoresis, spectral analysis, binding properties of added BLV with casein fractions have established that BLVAP as casein (consisting all the components) which is soluble in 80 per cent acetone in the presence of saturated $(NH_4)_2SO_4$ system at 4°C. In the present Chapter, a further attempt has been made to analyse some of the properties of BLVAP, such as behaviour on gel filtration, ion exchange chromatography and amino acid pattern, proteolytic behaviour, molecular weights of different components.

6.1 MATERIALS AND REAGENTS

Fresh skim milk was used for all purposes.

Sephadex G-200 (Pharmacia) DEAE-cellulose, Acrylamide, N,N' methylene bisacrylamide, Ammonium persulphate, DL amino acids, sodium dodecyl sulphate, trypsin, coomassie brilliant blue R and standard proteins (Haemoglobin crosslinked) were obtained from Sigma (USA).

6.2 METHODS USED

6.2.1 Preparation of Biliverdin associated protein

6.2.1.1 BLVAP was prepared according to the procedure outlined in para 4.2.1.1 and 4.2.1.2.

6.2.2 Preparation of casein

Acid casein was prepared from chilled buffaloes' milk at pH 4.6 using 10 per cent acetic acid and the precipitate was thoroughly washed.

6.2.3 Elution of biliverdin associated protein on Sephadex G 200 column

Sephadex G-200 of 40-120 μ mesh (Pharmacia) was allowed to swell in 0.05 M Tris-HCl buffer at pH 9.0 containing 0.02 per cent sodium azide as preservative to avoid microbial growth. The gel was degassed under vacuum and poured as a thick slurry to form a uniform gel bed. The latter was equilibrated with 3 columns volumes of buffer before use. The homogeneity and void volume were checked using Blue dextran. The flow rate was 18-20 ml/h. Seventy five milligrams of the protein in 4.5 ml buffer were applied with the help of a bent pipette. The sample applied constituted 3 per cent of the bed volume. Protein fractions were eluted in 300 ml of the buffer. The absorbance of the 3 ml fractions was

measured at 280 nm in a spectrophotometer with 1 cm path length cells, or the colour developed by Folin reagent was measured at 750 nm against the reagent blank.

The same procedure was followed for the both types of samples namely, BLVAP isolated at room temperature and BLVAP isolated at 4°C from chilled milk.

6.2.4 Elution of biliverdin associated protein on DEAE cellulose column

BLVAP was chromotagraphed on DEAE-cellulose in 0.01 M imidazole-HCl buffer (pH 8.0), containing 3.3 M urea and 0.01 M 2-mercapto ethanol. The column (1.5 x 30 cm) was equilibrated with the same buffer. One hundred and fifty mg of the BLVAP were dissolved in 5.0 ml of buffer and applied to the column. Elution with linear gradiant was performed by placing 200 ml of 0.01 M imidazole - HCl buffer in the mixing chamber and 200 ml of same buffer containing 0.4 M sodium chloride in reservoir. The flow rate was 60 ml/h. The effluent was collected in 5 ml fractions and the protein in each fraction was estimated by the method of Lowry et al (1951).

6.2.5 Enzymatic hydrolysis of biliverdin associated protein and casein

The rate of proteolysis of BLVAP and casein with trypsin was determined by the method of Kunitz (1947).

Both spectrophotometric assay by reading the absorption at 280 nm and the colour development due to Folin's reagent at 750 nm were used for the measurement of the activity of trypsin using 1 cm path length cells.

The procedure followed for tryptic hydrolysis of BLVAP and casein was according to Anson (1938) as described by Harriet (1955). The complete system contained 5 ml of a 1 per cent solution of BLVAP and casein in 0.05 M Tris-HCl buffer pH 7.9 and 0.5 ml of trypsin solution containing 5 mg of the enzyme. The mixture was incubated at 39-40°C for different time intervals such as 45, 90, 135, 180 and 225 min. The reaction was stopped after each time interval by adding 5 ml of 12 per cent TCA and the precipitated protein was removed by filtration through Whatman 42 paper. A blank was run by stopping the reaction of a similar mixture at zero minute period. Readings were corrected for blank values.

6.2.6 Acid hydrolysis of biliverdin associated protein and casein

6.2.6.1 Acid hydrolysis of BLVAP and casein was done by refluxing 20 mg of the material with 2 ml of 6N HCl in degassed and sealed tubes for a period of 48 h at 110°C. After hydrolysis, the casein and BLVAP hydrolysates

were rendered free of the excess of HCl by repeated evaporation of the hydrolysate under vacuum over NaOH pellets. Finally, the acid-free dried material was dissolved in 1 ml of 10 per cent isopropanol. A few drops of toluene were added in the tubes to prevent bacterial contamination.

6.2.6.2 Separation and identification of amino acids in hydrolysate of biliverdin associated protein and casein

The separation and identification of individual amino acids in BLVAP and in casein hydrolysate was carried out by single dimensional descending paper chromatography using n-butanol:Acetic acid:water (B:A:W 4:1:5) as solvent system for 16 h in a saturated environment of the same solvent. Amino acids were detected by spraying 0.1 per cent ninhydrin in acetone and heating the chromatographic paper at 80°C for 15 min.

Twenty DL-individual amino acids were categorised into four groups and used as standard reference amino acids along with samples.

6.2.7 UV absorption spectra, molar and specific extinction coefficients of biliverdin associated protein and casein

The UV-absorption spectra of BLVAP and casein were recorded in Gilford spectrophotometer, Model No. 1084 K 260, with 1 cm path length cuvellets in the

regions of 250-320, nm by taking 0.015 per cent protein solution in 0.05 N NaOH.

Specific extinction coefficient A1% 1cm and molar extinction coefficient of BLVAP and casein were calculated taking their absorption at 281 nm.

6.2.8 Molecular weight determination of biliverdin associated protein and casein components using SDS-polyacrylamide gel electrophoresis method

Electrophoresis in polyacrylamide in the presence of anionic sodium dodecyl sulfate (SDS) proved to be useful tool for the separation of protein subunits and for determination of molecular weights. The molecular weight can be determined by comparing the electrophoretic mobility of a given protein with known protein markers. A linear relationship is obtained if the electrophoretic mobilities (Rf) are plotted against the known polypeptide chain molecular weights. The procedure for SDS-PAGE molecular weight determination was according to the modified method of Weber and Osborn (1969) and Davis and Stark (1970) described in Sigma Tech-Bulletin MWS-87.

6.2.8.1 Reagents

(a) Sample buffer: prepared by combining 0.441 g of $NaH_2PO_4.2H_2O$, 2.511 g of $Na_2HPO_4.12\ H_2O$, 1.0 g of SDS, 1 ml of 2-mercaptoethanol, 0.015 g bromophenol blue and 36.00 g of urea, dissolved and made up to 100 ml with distilled water. The pH of buffer is 7.0 at 25°C.

(b) Gel buffer: Prepared by combining 8.84 g of NaH_2PO_4. 2 H_2O, 51.55 g of Na_2HPO_4.12 H_2O and 2.0 g of SDS, dissolved and made upto 1 liter with distilled water. The pH is 7.0 at 25°C.

(c) Acrylamide gel: To prepare 10 per cent gel 22.20 g of acrylamide and 0.6 g N,N' methylene bis-acrylamide were dissolved in 100 ml distilled water. The insoluble material was removed by filtration.

(d) N,N,N',N' - tetramethyl ethylenediamine (TEMED)

(e) Ammonium persulphate solution: Prepared freshly before use by dissolving 100 mg of the ammonium per sulphate in 15 ml of distilled water.

(f) Fixative solution: Fixative solution was prepared by combining 40 ml of methanol, 10 ml acetic acid and 50 ml of distilled water.

(g) Staining reagent: Prepared by taking 4.50 ml of 50 per cent methanol (v/v), 50 ml acetic acid and 1.25 g Coomassaie brilliant blue R (CBB).

(h) Destaining reagent: Destaining solution contained 10 ml of acetic acid, 50 ml of methanol made to 1 l with distilled water.

6.2.8.2 Procedure

(i) Preparation of sample: BLVAP and casein samples were prepared by taking 1 mg of each sample and dissolving the

same in 1 ml of sample buffer.

(ii) Preparation of SDS molecular weight marker proteins

The marker protein sample was prepared by taking Hemoglobin-crosslinked vial and contents of each vial was reconstituted with 1 ml of the sample buffer. The final concentration of protein mix was 3 mg/ml. Hemoglobin crosslinked on SDS PAGE stained with CBB gives 4 bands with molecular weights of 16,000, 32,000, 48,000 and 64,000 daltons, respectively.

(iii) Preparation of electrophoretic gels

To prepare 12 gels: 15 ml of gel buffer (b), 13.5 ml of acrylamide gel (c), were mixed and degerated for 1 min with a water aspirator. To this 1.5 ml of freshly prepared ammonium persulphate (e), followed by 0.090 ml of TEMED, were added. This mixture was carefully added to the bottom closed gel tubes upto the height of 9.0 cm leaving 1 cm space at the top and allowed to polymerise. Before the gel hardened a few drops of water were layered on top of the gel in order to get a smooth surface.

(iv) Electrophoresis:

Five hundred ml of gel buffer (reagent b) was diluted to 1000 ml with distilled water before both the compartments were filled with gel buffer. Water was

decanted from the top of the gels and fixed in the electrophoretic apparatus. Ten µl of the sample was applied on each gel along with SDS-marker protein sample and a constant current 8 ma/gel was applied until the marker dye reached 1 cm above from the anodic end of the gel.

(v) Staining and destaining

The gels were carefully removed by injecting water through sides, the bromophenol blue dye front was marked with a piece of fine wire and fixed for 10 h in the fixative solution. Gels were stained with coomassie brilliant blue solution for overnight and destained with reagent (f) with several changes, until the bands are clearly seen.

(vi) Calculation of Rf

The relative mobility of each protein band was calculated as follows:

$$Rf = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

The Rf values of known marker proteins are plotted against its molecular weight on a semi log graph, while the molecular weights for ELVAP and casein components are calculated from calibrated curve (Fig.18).

6.3 RESULTS

6.3.1 Elution of biliverdin associated protein on Sephadex

The gel filtration pattern of BLVAP isolated at room temperature and at 4°C are shown in Fig 12 and 13, respectively. It is seen from Fig.12 that BLVAP isolated at room temperature resolved in to three discrete peaks, one of the protein fraction was eluted immediately after the void volume. Fig.13 indicates that BLVAP isolated at 4°C resolved into only one peak and it was eluted immediately after the void volume indicating that the former has undergone disaggregation due to isolation at room temperature and latter is present as single entity.

6.3.2 Elution of biliverdin associated protein on DEAE-cellulose

The elution profile of BLVAP on DEAE cellulose column chromatography (Fig.14) using imidazole buffer having 3.3 M urea in the presence of NaCl gradiant indicates three major protein fractions. The typical pattern of this elution profile with NaCl gradiant closely agrees with published literature on buffalo casein (Nagasawa et al., 1973) indicating similarities.

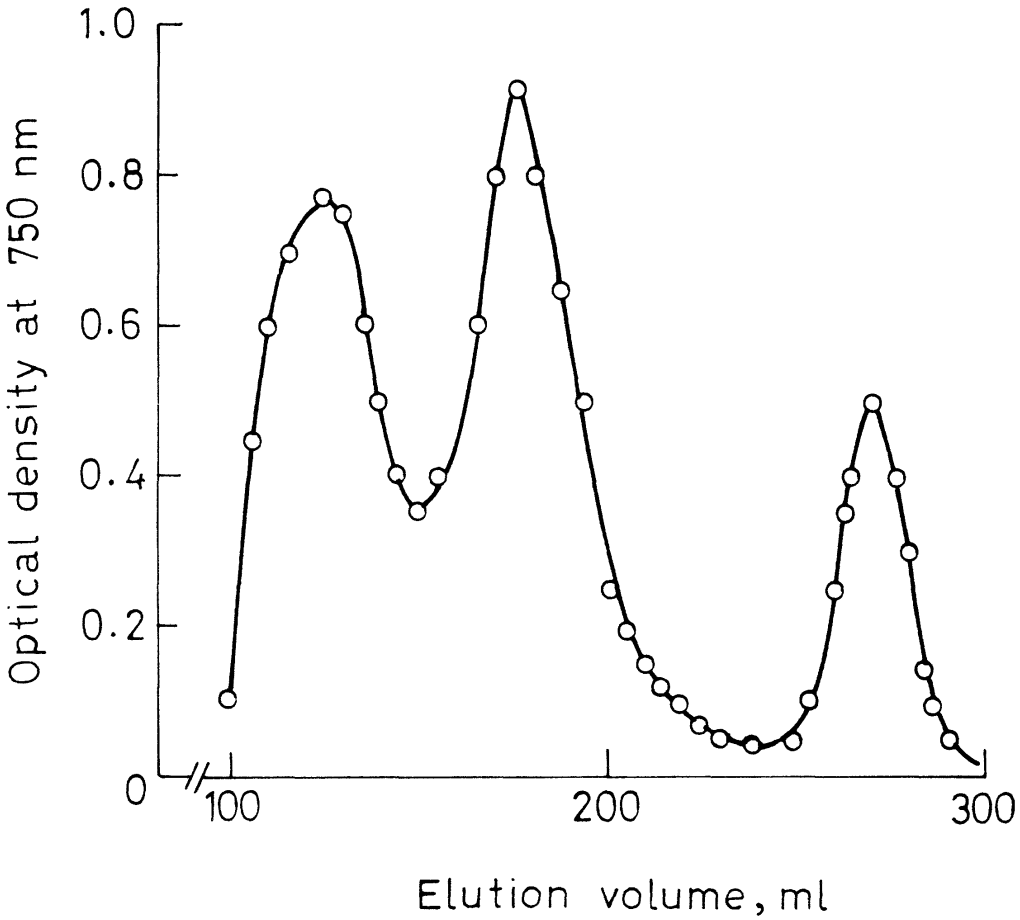


FIG.12 ELUTION PATTERN OF THE BILIVERDIN ASSOCIATED PROTEIN ON SEPHADEX G-200 (Isolated at room temperature)

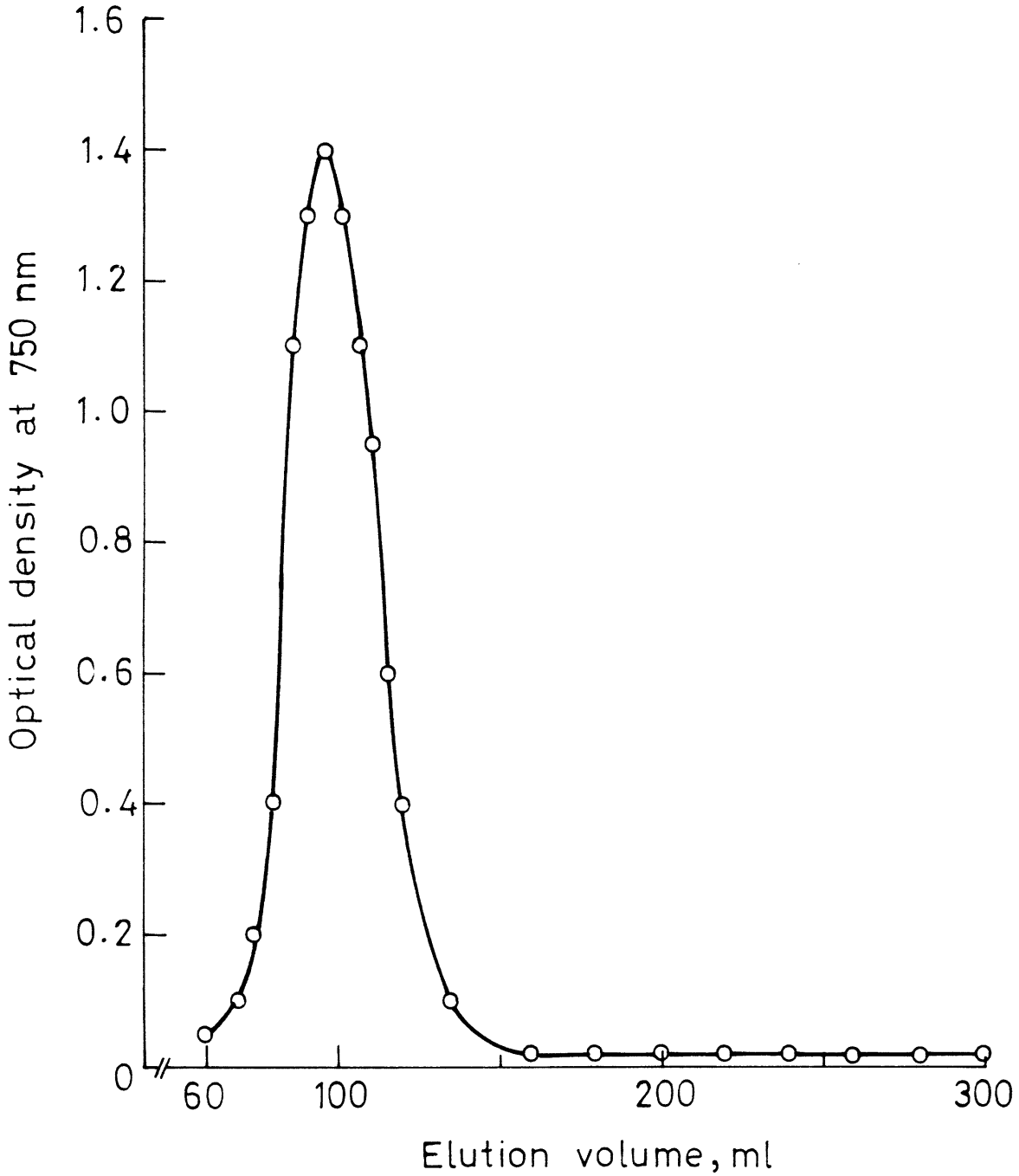


FIG.13 ELUTION PATTERN OF BILIVERDIN ASSOCIATED PROTEIN ON SEPHADEX G-200 (Isolated at 4°C)

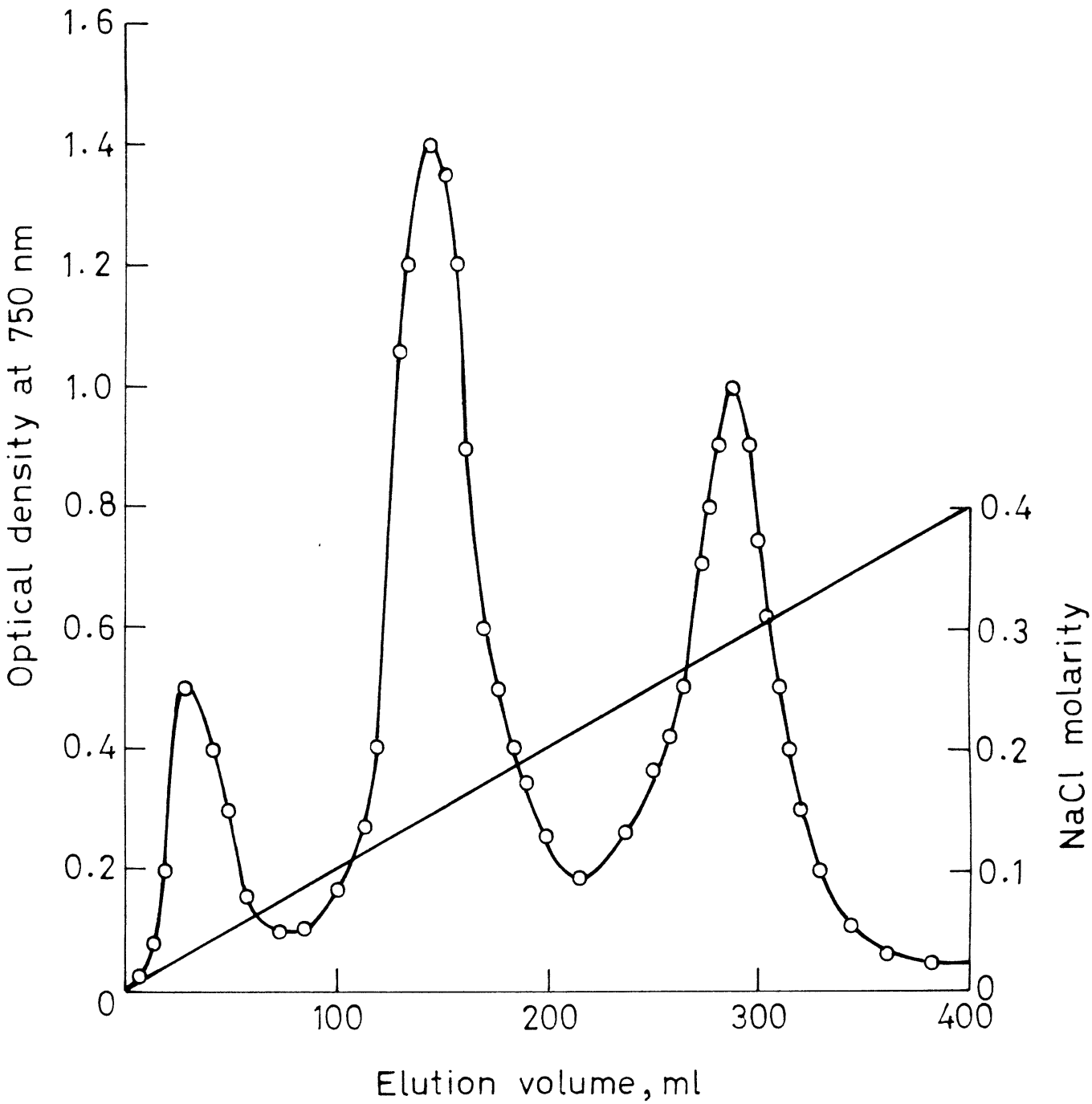


FIG.14 ELUTION PROFILE OF BILIVERDIN ASSOCIATED PROTEIN ON DEAE-CELLULOSE COLUMN.

6.3.3 Amino acid composition of bilivardin associated protein and casein

The Rf values of known amino acids in n-butanol: acetic acid:water (BAW) solvent system as carried out by single dimensional descending paper chromotography are shown in Table 13 along with amino acid spots of hydrolysed samples of BLVAP and caseins and their patterns are illustrated in Fig.15. The Rf values of amino acids in hydrolysed test samples of BLVAP and casein in BAW solvent system were compared with the Rf values of known amino acids spotted in the same run. It may be seen from Table 13 and Fig.15 that BLVAP and casein comprised of 18 amino acids with same Rf values, indicating that both are identical in their amino acid composition which in turn gives an idea of similarity in their primary structure.

6.3.4 Tryptic hydrolysis of bilivardin associated protein and casein

Fig.16 shows the comparative hydrolysis of BLVAP and casein using proteolytic enzyme trypsine. It may be seen from the Fig.16 that BLVAP is having a slower proteolytic rate as compared to casein regardless of period of incubation, substrate and concentration of the enzyme. There is a marked change in the content of tyrosine released from the BLVAP and casein due to

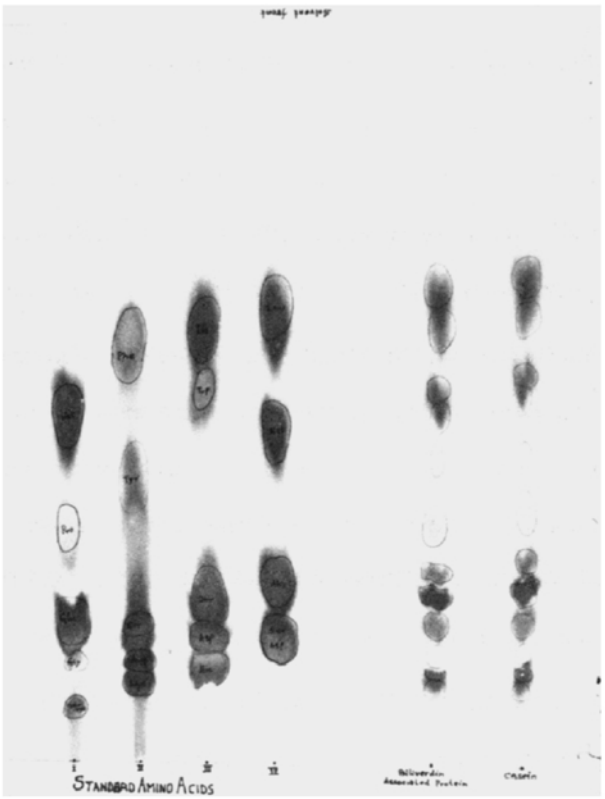


Fig.15 Amino acid separation patterns of biliverdin associated protein and casein on single dimensional descending paper chromatography

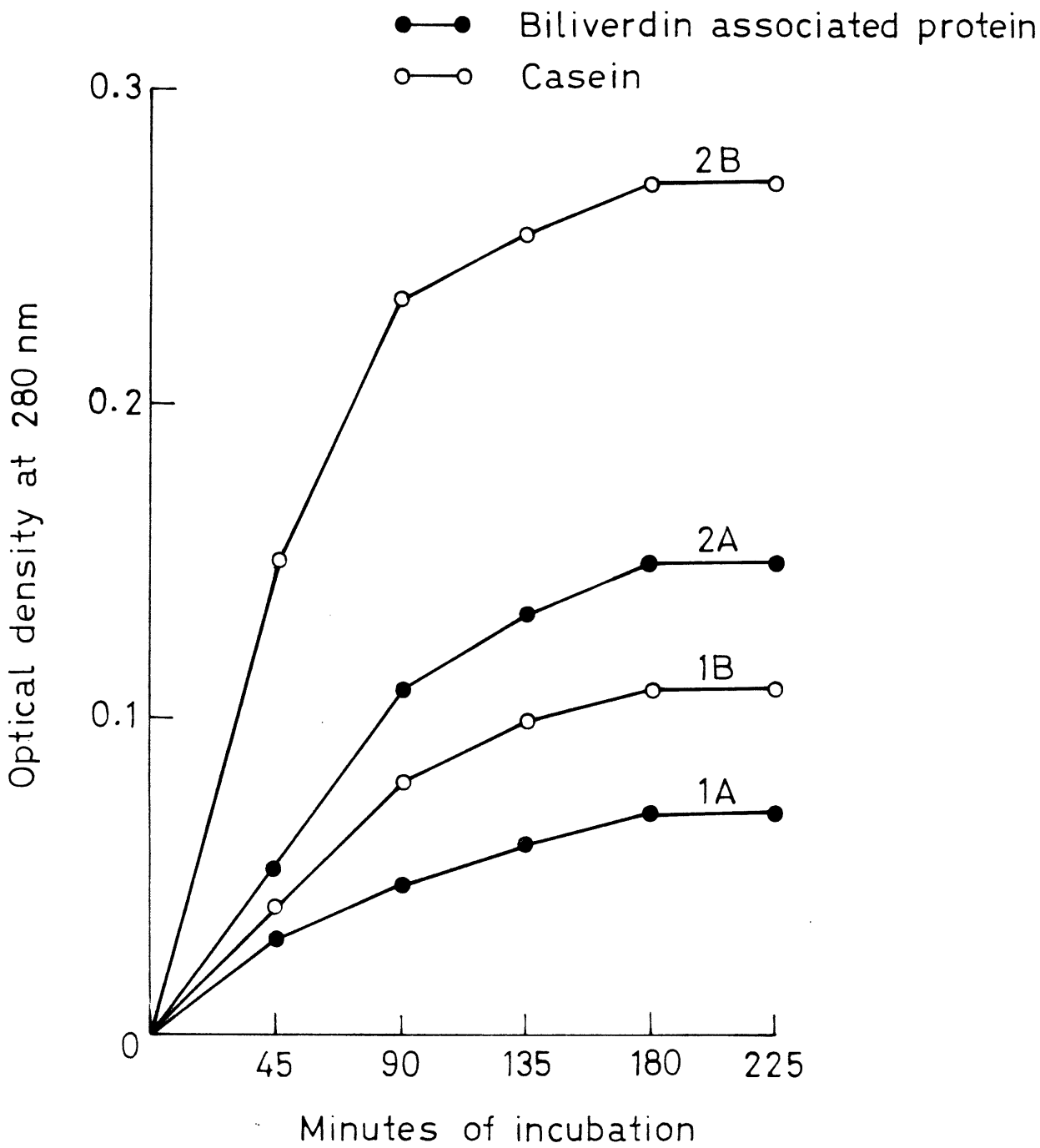


FIG.16 PROTEOLYTIC RATE OF BILIVERDIN ASSOCIATED PROTEIN AND CASEIN [1A, 1B ENZ: PROTEIN 2:40 ; 2A, 2B ENZ: PROTEIN 5:50 mg] IN THE PRESENCE OF TRYPSIN AT 39 °C.

Table 13

Identification of amino acids in acid hydrolysate of BLVAP and casein of buffalo milk by single dimensional descending paper chromatography

Amino acids	Rf values in BAW system			Amino acids identified in BLVAP and casein
	Standard amino acids	BLVAP	Casein	
Alanine	0.25	0.26	0.26	Alanine
Arginine	0.14	0.15	0.15	Arginine
Aspartic acid	0.17	0.18	0.18	Aspartic acid
Cystine	0.07	0.08	0.08	Cystine
Glutamic acid	0.18	0.19	0.19	Glutamic acid
Glycine	0.14	0.13	0.13	Glycine
Histidine	0.13	0.13	0.13	Histidine
Isoleucine	0.59	0.58	0.59	Isoleucine
Leucine	0.62	0.64	0.64	Leucine
Lysine	0.11	0.11	0.11	Lysine
Methionine	0.46	0.47	0.47	Methionine
Phenylalanine	0.56	*	*	Phenylalanine
Proline	0.32	0.32	0.32	Proline
Serine	0.17	0.18	0.18	Serine
Threonine	0.23	0.23	0.23	Threonine
Tryptophan	0.50	-	-	-
Tyrosine	0.40	0.40	0.40	Tyrosine
Valine	0.47	0.47	0.47	Valine

*Combined with isoleucine.

proteolytic action of trypsin under similar conditions (Table 14). From the Fig.16 it is seen that in both the cases, the release of tyrosine due to trypsin action attained a stationary phase after 180 min and remained stationary even after further incubation. The slower proteolytic rate and lesser release of tyrosine may be due to presence of high concentration of BLV in BLVAP as similar pigments are known for their inhibitory action on enzymes.

6.3.5 Molar absorptivity

The UV spectra of BLVAP and casein are shown in Fig.17. The absorption in the region 250-300 nm arises primarily from tyrosyl and tryptophenyl side chain groups. It may be seen from Fig.17 that the absorption of the BLVAP is less in this region than casein. This may be due to the presence of lesser number of tyrosine groups as also due to the differences in proportional distribution of components in BLVAP as compared to casein. The molar extinction coefficient at 281 nm for BLVAP and for casein based on the approximate aggregate molecular weight 70 000 are 1014 and 1056, while the corresponding specific extinction coefficients are $A^{281\ nm}_{1\%\ 1cm}$ 14.60 and $A^{281\ nm}_{1\%\ cm}$ 15.10 respectively. A close similarity of these values indicate identical nature of their primary structure as well as their components.

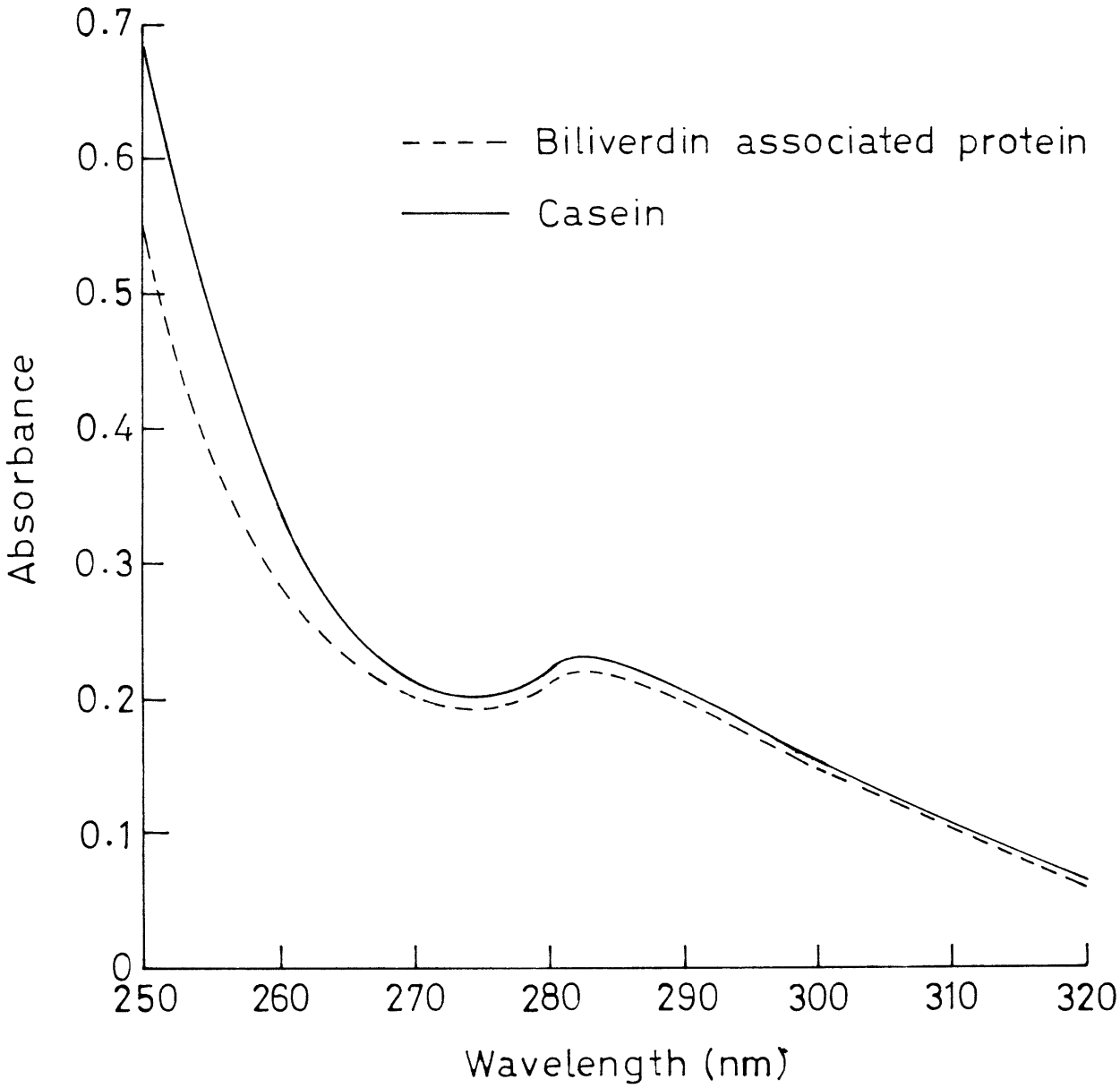


FIG.17 ULTRAVIOLET ABSORPTION SPECTRA OF BILIVERDIN ASSOCIATED PROTEIN AND CASEIN.

Table 14

Amount of tyrosine released from bilivardin asso-ciated protein,casein due to proteolytic action of trypsin (mg/100 mg of the protein, average of three trials

Period of incubation at 37°C	BLVAP	Buffaloes' milk casein
30	7.50	18.75
100	12.18	20.62
180	13.12	23.43
280	13.12	23.43

6.3.6 Molecular weights

The molecular weights obtained on SDS-PAGE for BLVAP and casein electrophoretic components are shown in Table 15. The molecular weights of BLVAP and casein range from 15,000 to 60,000. As it can be seen from Table 15 there are minor differences in their molecular weights but they are within the limits of deviation allowable due to technical difficulties. The close similarity in molecular weights between BLVAP with casein components as well as their agreement with the values of published literature for α_s-, β-, and k-caseins indicates that BLVAP and casein have identical polypeptide units. The molecular weights ranging from 41,000 to 60,000 of the trace components is likely due to aggregates of these components. The separation patterns of electrophoretic components of BLVAP and casein are shown in Fig.19.

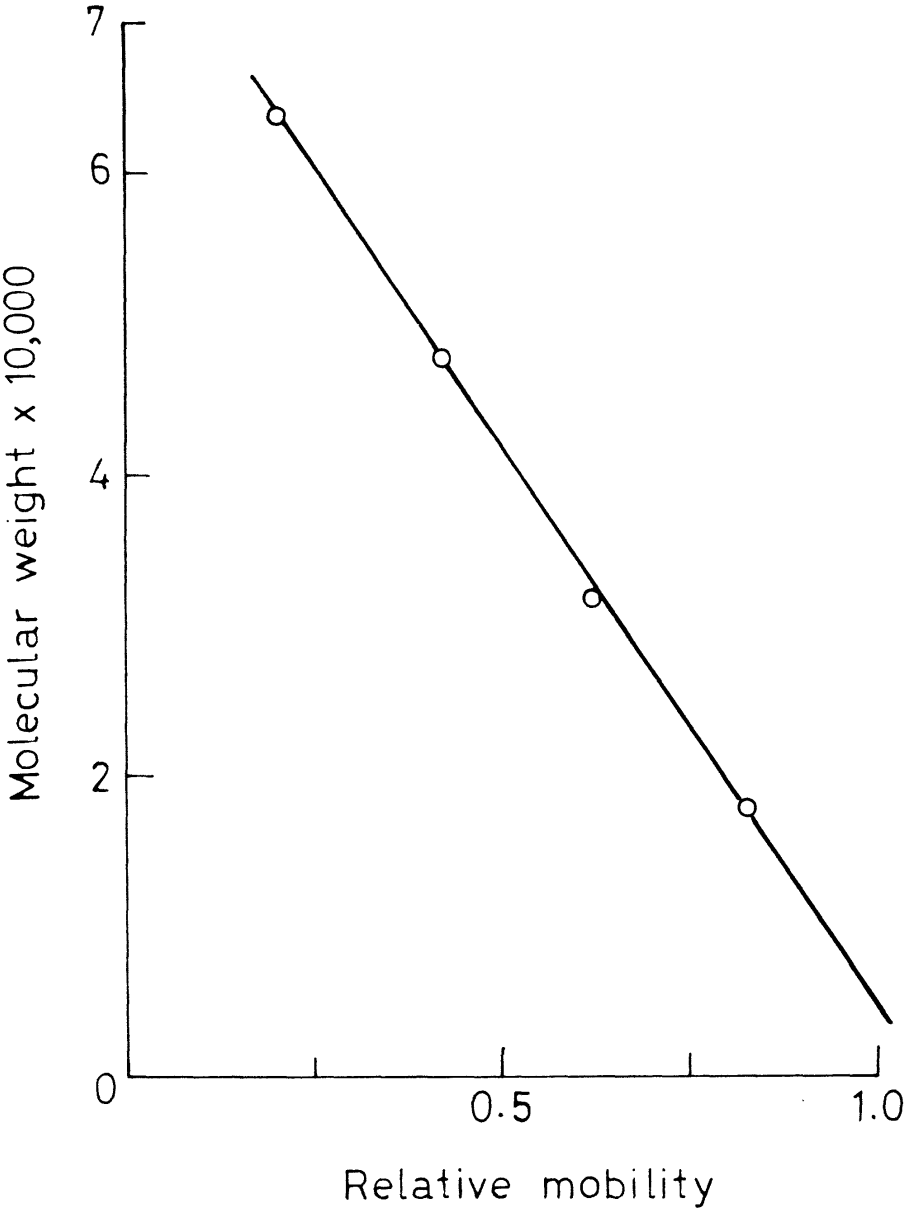


FIG.18 CALIBRATION CURVE, RELATIVE MOBILITY OF STANDARD PROTEINS VS. MOLECULAR WEIGHT.

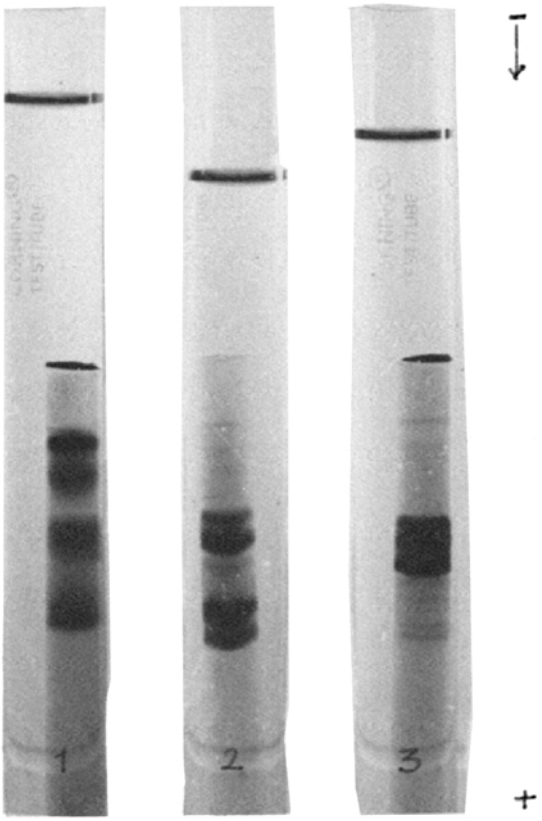


Fig.19 SDS-PAGE patterns (1) Standard molecular weight proteins (2) biliverdin associated protein and (3) casein

Table 15

Molecular weights for electrophoretic components of biliverdin associated protein and casein determined on SDS-PAGE (average of three trials)

Electrophoretic components		Values from literature for casein fractions	
BLVAP	Casein		
60000*	60000*		
49000*	49000*		
43000*	43000*		
41000*	41000*		
34000*	35000	34000 for α_s-casein	Mullin and Wolfe (1974)
30000	32000	28000-30800 for β-casein	Doretta et al (1975)
28000	28000	26000-32000 for k-casein	El-Negoumey (1980)
20000	21000		
15000	18000		

*Trace bands

6.4 DISCUSSION

6.4.1 It may be seen from the Fig.13 and 14 that BLVAP isolated at room temperature and at 4°C eluted on sephadex resolved in to 3 peaks and 1 peak, respectively. The greater number of protein fractions in the case of BLVAP isolated at room temperature seems to be due to isolation of the protein at higher temperature using acetone, which has disintegrated the protein resulting in resolution into more fractions. On the other hand, the protein isolated at 4°C from chilled milk using chilled acetone resolved into single peak, thereby indicating that the protein isolated at 4°C is not disaggregated and present as single entity. Further, this behaviour is in close agreement with that of casein. Ommen (1973) obtained a single peak, when micellar casein was eluted on Sephadex. Morr et al (1964) and Hill and Hansen (1964) observed that when cows' skim milk was eluted on sephadex the casein was eluted in the first fraction having higher filtration rate. In present study also the BLVAP elutes as a single peak with high elution rate immediately after void volume.

6.4.2 Fig.14 depicts the elution profile of BLVAP on DEAE-cellulose column. Three major protein fractions

are resolved due to different concentrations of NaCl gradient. This typical pattern is very similar to buffalo casein eluted on DEAE-cellulose column observed by Nagasawa et al.(1973)

6.4.3 The present studies show that BLVAP and casein hydrolysate are having similar number of amino acids (Table 13). The absence of tryptophan in both cases may be due to acid hydrolysis before analysis, since its presence has been reported earlier in buffaloes' milk casein by Ganguli (1974). These data clearly indicate that BLVAP and casein are identical in their amino acid patterns which, in turn, indicates similarity in the primary structure of both the proteins.

6.4.4 The BLVAP is having slower proteolytic activity as compared to casein is shown by the data in Table 14 and illustrated in Fig.16. In both cases, the stationary phase is attained after 180 min and the values remained stationary even on further incubation. The amount of tyrosine released from BLVAP is much less than in case of casein and may be due to its association with high concentration of BLV, which is inhibiting the enzyme activity. BLR is known to inhibit several enzymes, for example, alcohol dehydrogenase (Flitman and Worth, 1966), glutamate dehydrogenase (Yamagoshi,1970),

trypain, chymotrypain (Strumia, 1969) and cholesterol esterase (Natti et al., 1962-1963). As shown by the above studies inhibition of alcohol and glutamate dehydrogenase is due to chelatin with Zn^{+2} by ELR, whereas inhibition of malate dehydrogenase is related to the effect of ELR on the enzyme protein. Inhibition of hepatic cholesterol esterase is due to a possible relationship between cholesterol esterification and ELR glucoronide formation. In the present study since ELV and ELR are structurally very similar (except that in ELR the central methene ring is reduced), the inhibition of trypsin can be related to the effect of ELV on the enzyme protein which would be lower the activity of trypsin, resulting in lesser release of tyrosine from ELVAP.

6.4.5 The double bond systems in proteins are insulated from one another, the peptide groups being separated from one another by the α-carbon atom and the side chain chromopheric groups being insulated by the polypeptide nature of proteins. The absorption in the region 250-300 nm arises from the tyrosyl and tryptophenyl side chain groups. It is seen from the Fig.17 that the absorption of the ELVAP is slightly lower in this region than in case of casein. This may be due to

lesser tyrosine or tryptophan groups per mole in the BLVAP than in casein and the change in proportional distribution of components (individual components of protein) leading to lower absorption. The absorption peak in the 280 nm region for most of the milk proteins occur in the range of 278 to 281 nm and in the present case both BLVAP and casein have shown maximum absorption at 281 nm. At the characteristic values for the specific extinction coefficient and molar extinction coefficient based on the approximate molecular weight of 7000 daltons both BLVAP and casein show close similarity to one another. The continued absorption, after 310 nm is due to the association with BLV, which is imparting green colour to both BLVAP and casein resulting in continued absorption even after 310 nm, while in the case of colourless proteins the absorption is negligible after 310 nm.

6.4.6 PAGE in the presence of SDS has been applied satisfactorily to the analysis of milk proteins. According to Mullin and Wolfe (1974), Peretta et al (1974) and El-McGoumy (1980) molecular weights were 34,000 for α_s-, 28,000-30000 for β-, and 2600-32000 for k-casein. The data from the present study on the relative mobilities (Rf) of the standard proteins plotted on x axis on a semi logorithemic graph paper against

their known molecular weights on Y axis show a linear relationship between Rf value and molecular weight (Fig.18). The molecular weights derived on standard graph (constructed with known molecular weights of standard proteins) for B.V.A. and casein were from the average of three trials. The values closely agree with those published in literature for casein (Table 15). The trace bands in the high molecular weight range of 41,000-60 000 daltons, are very likely due to aggregates formation among the components. In a similar study, the electrophoretic pattern of calcium caseinate on SDS-PAGE by Mullin and Wolfe (1974) gave multiple bands ranging from 9000-80000 daltons, the three major bands corresponding to α_s-, β-, and k-caseins. k-casein was however, masked by β-casein, due to the appearance in the β-casein molecular weight range under their experimental conditions. The bands which have molecular weights 65000-80000 indicate that they are possibly aggregates.

6.4.7 The study described in this Chapter has brought out the fact that the amino acid composition, patterns after tryptic hydrolysis, the U.V. spectra, behaviour on gel filtration, ion exchange column, electrophoresis and molecular weight of electrophoretic components of the

BLVAP and buffaloes milk casein are alike, thus establishing that the BLV in buffalo milk is conjugated to the casein moiety only and to no other specific protein.

CHAPTER VII

IMMUNOLOGICAL BEHAVIOUR OF HELIVERGAIN ASSOCIATED PROTEIN

7.0 IMMUNOLOGICAL BEHAVIOUR OF BLUEGILL ASSOCIATED PROTEIN

For characterization of proteins, immuno chemical methods are commonly used in view of their sensitivity and specificity. Having known BLVAP as casein based on electrophoretic behaviour, binding with BLV and other properties, it was of interest to examine its immunological behaviour. An attempt was therefore made to raise antisera against the BLVAP in rabbits and study its specificity in comparison with casein using immuno-double diffusion analysis.

7.1 MATERIALS AND REAGENTS

Albino rabbits aged about six months of same age group were selected from the Institute's small animal house and maintained separately during the experimental period.

Freund's adjuvent (complete) and noble agar were procured from Difco (USA).

7.2 METHODS USED

7.2.1 Preparation of antisera

The antisera against BLVAP was obtained from rabbits after a series of fifteen subcutaneous or intra-peretoneal injections of 175 mg of protein (total) in

complete Freund's adjuvant, given on alternate days over a period of 30 days. The animals were bled from the marginal ear vein 15 days after the last injection. Blood was allowed to clot at 25°C for 4 h, kept overnight in refrigerator and the serum was collected by centrifugation at 2000 r.p.m. for 10 min. Antisera was stored in deep freeze in the presence of 0.0001 per cent merthiolate which acts as a preservative.

7.2.2 Immuno-double diffusion analysis

Immuno-double diffusion analysis was carried following modified Ouchterlony plate method (Robbins et al., 1974). Plates (50x12 mm) were prepared by pouring 3 ml of 1.2 per cent noble agar in borate saline solution. A total of three wells were made as shown in Fig.20A. The antiserum was placed in the central well and outer two wells contained BLVAP and buffaloes' milk casein solutions in different concentrations. In control plate, the outer two wells contained borate saline solution. The experimental and control petri plates were incubated at 37°C for 28-48 h in a humidified box. Precipitation line in the form of semi arch shape formed between the antiserum well and antigen wells, was considered as a positive test.

Borate-saline solution and borate saline buffer were prepared as follows:

Borate saline solution

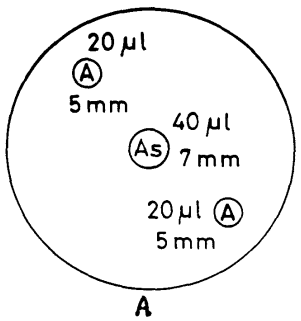
Five parts of borate saline buffer and 95 parts of saline (9g NaCl/1) were mixed.

Borate saline buffer

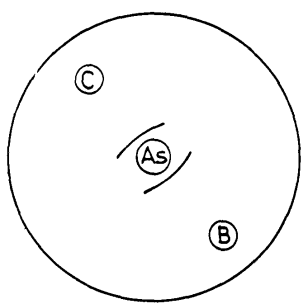
Boric acid 6.184 g, borax ($Na_2B_4O_7$ $10H_2O$) 9.536 g and NaCl 4.384 g were dissolved in distilled water and made to 1 liter with distilled water.

7.3 RESULTS

The immuno-double diffusion patterns of BLVAP and casein after incubation for 48 h at 37°C in humidified conditions are shown in Fig.20B. In both cases, only one sharp precipitin line in the form of semi arch was obtained. In control, no such arch was formed. This results indicate that BLVAP and buffaloes' milk casein are immunologically similar. Data presented in Table 16 show positive reactions between the different concentrations of BLVAP and casein with antibody (antisera raised against BLVAP).



A



B

A = Antigen As = Antiserum
B = BLVAP C = Casein

Fig.20 A Immuno-double diffusion agar gel model plate

B Immuno-double diffusion patterns of biliverdin associated protein and casein

Table 16

Reactions at different concentrations of biliverdin associated protein and casein against the antisera of biliverdin associated protein*

Concentration of protein (mg/100 ml)	Buffaloes' milk casein	BLVAP
5	+++	+++
15	+++	+++
20	+++	+++

*Sera used was without dilution.

7.4 DISCUSSION

Results from immuno-double diffusion test provide a sensitive technique to study the immunological relationships of closely related proteins and to ascertain their purity. The gel diffusion test involves the precipitation reaction in a semi solid medium rather than in a fluid medium. The object of immuno-double diffusion test is to bring together through diffusion of optimal concentrations of antigen and antibody to form visible bands of precipitation. In the present investigation the test has been used to find out the immunological relationship between BLVAP and casein in order to establish the identity of the BLVAP. BLVAP and casein showed precipitation lines against each well without any heterogeneity when they were placed in two different wells against the central well which contained the antisera (raised against BLVAP). This helps to prove that they are immunologically identical (Fig.20B). The results of this investigation establish a close structural and functional relationship between the BLVAP isolated from chilled milk by acetone in the presence of saturated ammonium sulphate at 4°C and casein.

CHAPTER VIII

BEHAVIOUR OF COWS' AND BUFFALOES' MILK TO ADDED BILIVERDIN

8.0 BEHAVIOUR OF COWS' AND BUFFALOES' MILK TO ADDED BILIVERDIN

When cows' skim milk is treated with acetone and ammonium sulphate as described for the buffaloes' milk, a green pigment is also liberated. In a preliminary study, Daniel (1977) reported that this green pigment differs in its spectral characteristics from BLV and it does not give reactions for bile pigments. The pigment occurs in trace amounts and it is also associated with a protein moiety. The protein sample was therefore examined to find out differences, if any from the samples of protein isolated from buffaloes' milk.

8.1 MATERIALS

Fresh cows' and buffaloes' milk from the Institute herd were used for experimental work after skimming.

8.2 METHODS USED

8.2.1 Binding of added biliverdin with casein in milk system and with isolated casein

8.2.1.1 In order to find out whether added BLV is bound to casein in cows' milk or to casein isolated from milk, the following experiments were designed.

Two hundred ml of cows' and buffaloes' skim milk were enriched with pigment H.V by adding 400 µg of H.V dissolved in one ml sodium phosphate buffer (pH 7.8) the casein was precipitated from this milk by adding 0.6 g of citric acid dissolved in about 20 ml of distilled water. Biliverdin was extracted using 25 ml acetone from the casein thus obtained. O.D. was measured at 660 nm and this quantity recovered was calculated. A control was run with milk without addition of H.V.

8.2.1.2 In another set of experiment, casein was isolated from skim milk of both the species directly by adding citric acid solution. Casein was dissolved in 200 ml of 0.02 N NaOH solution and the pH of the solution was adjusted to that of normal milk (6.8). To this solution, 400 µg of H.V dissolved in one ml of sodium phosphate buffer was added and allowed to stand for 30 min for binding to take place. Casein was once again precipitated from this solution by adding 10 per cent acetic acid to pH 4.6. H.V was extracted from the casein coagulum using 25 ml acetone. Recovery was calculated as described in earlier para.

8.2.2 Isolation of added biliverdin from cows' skim milk

8.2.2.1 Isolation from citric acid precipitated casein

To find out whether the BLV added to cows' milk binds with the casein and whether cows' milk brings about any changes in the pigment, BLV was dissolved in sodium phosphate buffer and added to cows' milk. Casein was extracted by adding 0.6 per cent (w/v) citric acid to milk. Casein thus obtained was soaked in acetone and examined for spectral characteristics and tested for the presence of bile pigments.

8.2.2.2 Isolation from acid casein prepared from chilled milk

Two hundred ml of BLV enriched skimmed milk were allowed to stand for 4 h at refrigerator temperature. Casein was isolated from this milk by adjusting the pH to 4.6 by adding 10 per cent acetic acid. Casein thus obtained was soaked in 1-propanol:diethyl ether mixture (2:1). This extract contains γ-caseins only and was examined for spectral properties and tested for the presence of bile pigments.

8.2.3 Recovery of added biliverdin from cows' milk

To find out quantitatively whether the added BLV could be recovered from cows' milk, 240 µg of BLV

dissolved in sodium phosphate buffer was added to 100 ml skim milk and the BLV was estimated following the procedure of Chandrasvadana and Daniel (1977) mentioned in 3.2.3. A control was also run with buffaloes' milk. The acetone extract from respective samples of milks without added BLV served as blanks.

3.2.4 Preparation of 80 per cent acetone soluble ammonium sulphate saturated protein from cows' milk

The pigment associated protein from cows' milk was isolated following the procedure similar to that used for buffaloes' milk as described in para 4.2.1.2. A separate protein fraction was also collected by soaking the citric acid precipitated curd in 80 per cent acetone and the residue from the acetone extract was collected after removing the acetone and water.

3.2.5 Preparation of casein from cows' milk

Casein was prepared from chilled milk by adjusting the pH of milk to 4.6 by adding 10 per cent acetic acid. The coagulum was freed from whey after repeated washings with distilled water (pH 4.6) and dried in a vacuum desiccator.

3.2.6 Electrophoretic analysis of pigment associated protein and casein from cows' milk

The electrophoretic pattern was obtained on 8 percent cynogum gels in accordance with the procedure outlined in para 5.2.7.

8.3 RESULTS

8.3.1 Binding of added biliverdin with casein in milk system and with isolated casein

As shown in Table 17, BLV added to cows' milk casein in the milk system and also when added to isolated casein, was bound with ease in both cases. This behaviour was identical with buffaloes' milk casein used for confirmation since BLV is a normal constituent of buffaloes' milk casein. From these studies it can be concluded that the BLV has a tendency to bind with casein, irrespective of the state in which it is present and there is no difference in the behaviour of caseins isolated from cows' and buffaloes' milk.

8.3.2 Isolation of added biliverdin from cows' milk

Fig.21 shows the absorption spectra of acetone extract obtained from citric acid precipitated casein and propanol-diethyl ether extract of acid casein. In both the cases, an absorption maxima at 375 and 660 nm region were present similar to free BLV isolated from buffaloes' milk casein, thereby indicating that cows' milk system has not brought about any changes in added BLV. The results also confirm the association of BLV with casein and its fractions (as evidenced by its association with γ-casein). Both the acetone extract and

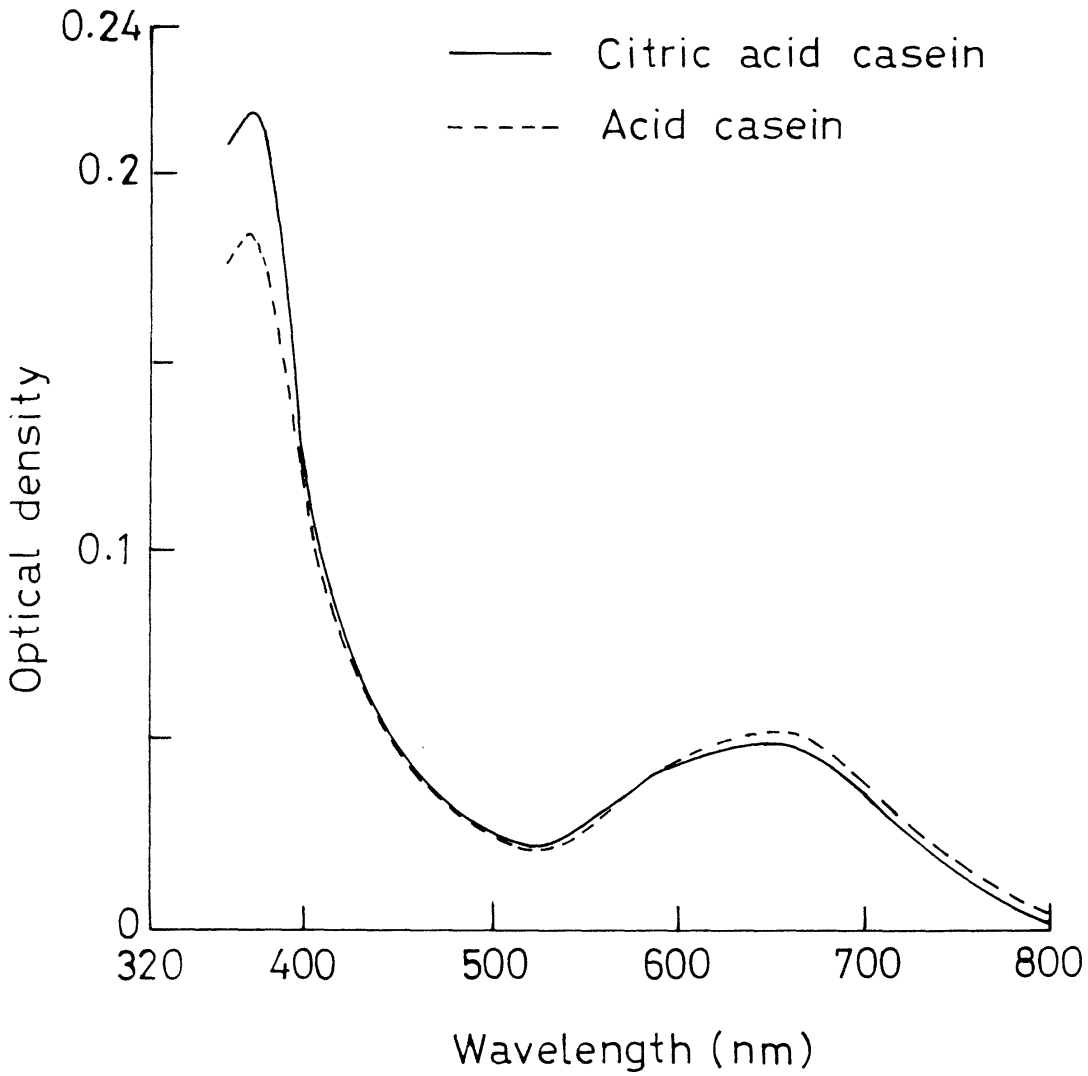


FIG. 21 ABSORPTION SPECTRA OF ACETONE EXTRACT AND PROPANOL : DIETHYLETHER EXTRACT OF PIGMENT FROM COWS' MILK CASEIN.

Table 17

Recovery of bilivardin added to cow and buffalo casein in milk and isolated casein

Nature of the sample	B.V in acetone extract from (200 µg B.V/100 ml of skim milk)			
	Recovery of B.V from 200 ml skim milk*			
	B.V enriched casein		B.V added to isolated casein	
	O.D. at 660 nm	Concentration µg	O.D. at 660 nm	Concentration µg
Cow	0.287	390	0.292	390
Buffalo	0.290	390	0.286	390

*Percentage of recovery in all cases were 97.5 per cent.

1-propanol diethyl ether extract answered the tests for presence of bile pigments (Table 18), which shows that cows' milk system did not influence in any way the added BLV. Cows' milk behaves in a manner similar to buffaloes' milk towards added BLV.

8.3.3 Recovery of added biliverdin

In four trials, the average content of BLV recovered from cows' milk was 94.12 ± 1.09 and that from control buffaloes' milk, 97.41 ± 1.17 (Table 19). The data show good recovery for added BLV from milk in both types of milks. Though the study with cows' milk has given a slightly lower recovery than the buffaloes' milk, the difference was not significant.

8.3.4 Electrophoretic analysis

The PAGE patterns of ammonium sulphate saturated 80 percent acetone soluble protein of cows' milk and casein isolated from chilled milk are shown in Fig.22. It may be noted that protein and casein resolved into four bands and resolution pattern is identical except for the third component of the cows' milk pigment protein exhibited faster movement than casein, whereas the electrophoretic pattern of protein fraction isolated from casein using 80 per cent acetone alone has only one major band corresponding to the

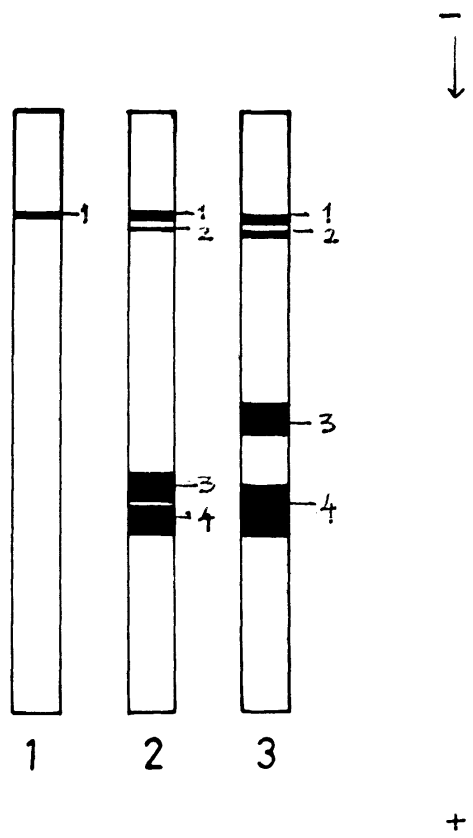


Fig.22 PAGE patterns of (1) 60 per cent acetone soluble protein fraction (2) ammonium sulphate saturated 60 per cent acetone soluble protein and (3) casein of cows' milk.

Table 18

Tests on acetone extract from citric acid casein and propanol diethyl ether extract of acid casein from cows' milk for bile pigments

Nature of extract	Gmelin reaction	Zinc fluorescence test
Acetone extract from citric acid precipitated casein	+++	++
1-proponal diethyl ether extract from acid casein	+++	++

Table 19

Recovery of added bilivardin from cows' and buffaloes' milk

Cows' milk			Buffaloes' milk		
BLV added µg	BLV estimated µg	Percent recovery	BLV added µg	BLV estimated µg	Percent recovery
240	230	95.66	240	235.2	98.00
240	224	93.33	240	235.2	98.00
240	224	93.33	240	230.0	95.66
240	226	94.16	240	235.2	98.00
Average percent recovery		94.12±1.09			97.41±1.17

second band of the two other samples. These results show that the protein isolated from chilled cows' milk (4°C) by direct acetone extraction in the presence of saturated $(NH_4)_2SO_4$ system has protein components similar to casein except for the some what faster movement of third band compared to casein.

8.4 DISCUSSION

8.4.1 The results show that cows' milk casein has high susceptibility to bind BLV and its behaviour is identical to buffaloes' milk casein. BLV is shown to bind casein in buffaloes' milk. In comparison, cows' milk casein also shows high tendency to bind with BLV whether in natural state represented by milk itself or separately as casein when isolated by acid precipitation at pH 4.6 (Table 17).

8.4.2 Buffaloes' milk has BLV as a normal constituent similar green pigment was isolated from cows' milk which shows absorption maximum at 660 nm but it differs from BLV of buffaloes' milk by not having absorption at 370-380 nm in addition to 660 nm, nor does this pigment answers the tests for presence of bile pigments (Gmelin and Zinc flourescence tests). The results show that even though cows' milk does not contain BLV, it behaves similar manner to buffaloes' milk. When BLV was added to cows' milk, it did not undergo any changes due to its exposure to cows' milk constituents as it is illustrated in Fig.22. Daniel (1977) has also shown conversion of added BLV in cows' milk to BLR, when milk was allowed to sour at 37°C, thus duplicating the pattern with buffaloes' milk.

both with natural and added BLV. The two types of milk behaved similarly even though cows' milk was devoid of BLV. The absorption spectra of 1-propanol-diethyl ether extract of casein comprising of γ-casein show that BLV is associated with casein and its components.

8.4.3 BLV is estimated in buffaloes' milk by extracting with acetone in presence of $(NH_4)_2SO_4$ saturated system. This method is highly reproducible (Chandravadana and Daniel, 1977). The observed data in Table 19 also indicates that BLV added to cows' milk can be estimated using the same method and the cows' milk system does not interfere during estimation of added BLV, eventhough BLV is not present originally in cows' milk.

8.4.4 Electrophoretic analysis of the cows' milk protein soluble in 80 per cent acetone saturated with ammonium sulphate when compared with casein, shows that two are having similar characteristics (Fig.22) except for the faster movement of third component. Electrophoresis of 12 samples of cows' milk casein have shown identical results having four bands on 8 per cent cynogum gels at pH 8.6, according to the method of Tombs and Akroyd (1967). This limited number of bands is in

contrast to seven bands resolved with buffaloes' casein (Fig.9 A&B) under identical conditions (para 5.2.7). Using calcium caseinate on SDS-PAGE gels, Mullin and Wolfe (1974) observed nine bands varying in molecular weight from 9000 to 60000. Similarly, Greenberg and Groves (1979) also observed eight bands on PAGE at pH 9.6, using buffer with 4 M urea. No special reason could be assigned for the behaviour of cows' casein in the present study.

8.4.5 Cows' milk and cows' milk casein behaved in a manner similar to that noted in the case of the buffaloes' milk. In both the cases, the added S.V could be recovered quantitatively. Further, the protein(s) solubulized in 80 per cent acetone saturated with $(NH_4)_2SO_4$ were found to behave in a manner similar to casein and casein fractions.

CHAPTER IX

ORIGIN OF RIBOFLAVIN IN BUFFALOES' MILK

9.0 ORIGIN OF BILIVERDIN IN BUFFALOES' MILK

In the animal system, the heme of hemoglobin is converted to biliverdin and the subsequent reduction of BLV in reticulo endothelial cells gives rise to bilirubin (Kapoor, 1978). The overall reaction involved is an oxidative cleavage of the ferri protoporphyrin ring at the methane bridge, resulting in the formation of a linear tetrapyrrole biliverdin IXα which is further reduced to BLR by soluble NADH and NADPH dependent BLV reductase as described by Singleton and Laster (1965), and Tenhunen et al (1970). Having established the presence of BLV as a normal constituent of buffaloes' milk associated with casein moiety soluble in 80 per cent acetone saturated with $(NH_4)_2SO_4$ it was of interest to ascertain its origin. The only component of blood from which BLV could be derived readily is BLR, since BLV is not present in the normal blood serum. In the present chapter an attempt has been made to establish the origin of the BLV by infusing C^{14}-BLR and examining the milk and blood samples.

9.1 MATERIALS AND REAGENTS

9.1.1 Selection of experimental animal

A Murrah buffalo in first lactation, aged about 5½ years, was selected from the Institute's herd. During

the period of experiment, the animal was given sufficient time to acclimatise to the experimental conditions.

9.1.1.1 Before injecting C^{14}-BLR, the animal was given 5 mg BLR without label and examined for changes if any, in the physiological activities due to increase in the circulating BLR levels.

9.1.1.2 The experimental animal was trained to milk three times a day, i.e., during morning, noon and evening with the help of oxytocin (5 IU) injection.

9.1.2 C^{14}-BLR was obtained from Amersham Buchler, GmbH, Braunschweig, FRG with atoms at 1, 2, 3, 4, 5, 6, 7, 8 with 15.9 µci/mM specific activity.

9.2 METHODS USED

9.2.1 Mode of injection of C^{14}-bilirubin into buffalo and collection of blood samples

9.2.1.1 Ten µci of C^{14}-BLR having 15.9 µci/mM specific activity was dissolved in 2 ml of 1 N NaOH and made upto 10 ml with sterile distilled water. C^{14}-BLR solution was infused into the jugular vein at constant rate over a period of 10 min and the animal was allowed to rest.

9.2.1.2 Blood samples were plotted at 6 h intervals starting with 2 h after the injection of C^{14}-BLR. Blood was tapped from the animal by bleeding the jugular

vein with the help of a sterile needle, collected into a wide mouth glass tube and protected from light. It was allowed to coagulate at room temperature and left overnight in a refrigerator so as to obtain clear sera. The latter was separated from the clot in a centrifuge tube and subjected to centrifugation for 10 min at 1500 r.p.m. to remove RBC, if any.

9.2.3 Determination of C^{14}-bilirubin in blood serum

One ml of the serum sample was taken in the counting vial and 10 ml of toluene-triton scintillation fluid (2:1) were added. The toluene-triton scintillation fluid consisted the following:

(a) Toluene-triton mixture 2:1 ratio

(b) 2.5 - diphenyl oxazole (PPO) 1:2 g for 200 ml

(c) 1,4 bis 2 (5-phenyloxozolyl) benzene (Popop) 20 mg for 200 ml of a.

The radio activity of the serum-scintillation fluid mixture was measured in a liquid scintillation spectrometer and the values are expressed as cpm.

9.2.3 Determination of total C^{14}-bilirubin in circulating blood

Total C^{14}-BLR in circulation was calculated by ascertaining the values obtained for C^{14}-BLR from different

serum samples and converting them to total blood volume of the body on the basis of body weight by taking 7.7% per cent of body weight as the blood volume (Hansard et al., 1953).

9.2.4 Collection and processing milk samples

Milk samples were collected from the animal two hours after the infusion of C^{14}-BLR and continued for period of 140 h. In the beginning, milk samples were collected at 2, 6, and 14 h intervals, while for the remaining period, the experimental samples were withdrawn during morning and evening milkings. Skim milk was obtained by subjecting whole milk to centrifugation and allowing the fat layer to harden and keeping it in the refrigerator for 1 h. The solidified fat layer was carefully separated out and milk was filtered through a layer of muslin cloth to remove the fat particles, if any.

9.2.5 Isolation of biliverdin from skim milk

Biliverdin from skim milk was extracted by taking 25 ml of milk sample and processing as mentioned in para 3.2.3.1. The acetone layer was examined for radio activity.

9.2.6 Isolation of biliverdin from citric acid precipitated casein

Citric acid casein was prepared by taking skim milk and adding citric acid at the rate of 0.6 per cent

w/v to skim milk. The casein thus obtained was freed from whey, by washing it with distilled water and soaking it in acetone. The acetone extract was examined for radio activity.

9.2.7 Isolation of Riboflavin from micellar casein

Micellar casein and fractions were prepared according to the method described by Majumder and Ganguli (1967) by taking freshly prepared skim milk.

9.2.7.1 Preparation of whole micellar casein

Skim milk was centrifuged at 37,000 r.p.m. (105,651xg) for a period of 30 min. The supernatant was drained off as far as possible. The sedimented micellar casein was then removed from the tubes with the help of a spatula, blended and soaked in acetone. One ml aliquots of the acetone extract and ultracentrifugal whey were examined for radio activity.

9.2.7.2 Preparation of micellar casein fractions of varying sizes at different speeds

The micellar casein fractions of varying sizes were prepared by centrifuging milk at different speeds as mentioned in para 5.2.1. The various casein fractions thus obtained were soaked in 10 ml of acetone. The acetone extracts were measured for radio activity.

9.3 RESULTS

9.3.1 Presence of C^{14}-bilirubin in blood

The presence of C^{14}-BLR was noticed in all the serum samples collected at 2, 8 and 14 h after injection of C^{14}-BLR. The total C^{14}-BLR in circulation was calculated on the basis of body weight by taking 7.71 per cent of the body weight as the blood volume. The observed data indicate that the total amount of C^{14}-BLR found in blood for a period of 14 h was only 9.37 per cent of the amount injected (Table 20).

The blood samples collected after 2 h contained 5.74 per cent of the total activity (cpm), the 8 h sample 2.58 per cent and 14 h sample 1.05 per cent after the injection of C^{14}-BLR.

9.3.2 Presence of C^{14}-pigment in milk

9.3.2.1 C^{14}-pigment in skim milk and acetone extract of the same

Measurement of radio activity of skim milk and acetone extract clearly show the appearance of C^{14}-pigment in the milk sample drawn 2 h after C^{14}-BLR injection. The activity continued for 140 h. The data are summarised in Table 21. Skim milk samples covering a period of 140 h accounted for only 2.03 per cent of the total C^{14} BLR

Table 20

Distribution of C^{14}-bilirubin in circulating blood

Period after the injection of C^{14}-BLR	Percentage of C^{14}-BLR in circulating blood
2 h	5.74
8 h	2.58
16 h	1.05
Total	9.37

Table 21

Amount of C^{14}-pigment in skim milk and acetone extract of skim milk

Period after administration of C^{14}-BLR (in h)	Milk yield (ml)	Total counts accounted to 1000 ml of acetone extract of pigment**	Total counts accounted to 1000 ml of skim milk**
2	250	15120	23000*
8	900	12320	18000*
14	300	10080	12000*
23	1200	5040	12000
32	200	11200	20000
44	1500	10640	15000
71	1600	5600	14000
79	900	6160	11000
94	2900	6160	16000
117	1900	7480	15000
125	1700	6160	10000
140	1800	8400	42000

* Milk collected after injection of oxytocin (5/IU)

** The percentage of the C^{14}-pigment found in acetone extract of skim milk is 1.11 and in skim milk it is 2.03 based on the C^{14}-BLR injected and accounted for 140 h.

injected into the animal. The amount of labelled pigment in acetone extract was 1.11 per cent which constitutes 54.61 per cent of the total pigment appearing in milk.

9.3.2.2 C^{14}-pigment in casein and whey fraction

The acetone extract obtained from citric acid precipitated casein and as well as micellar casein fractions contained the radio active pigment. Similarly, radio activity was also present in ultracentrifugal whey and in acid whey. The data on the relative distribution of C^{14}-pigment in milk, in the acetone extract of skim milk, in citric acid precipitated casein and in citric acid whey are recorded in Table 22.

Table 22

Relative distribution of C^{14}-pigment in mixed milk sample, acetone extract of the pigment from skim milk, acetone extract of the citric acid casein and whey

Nature of the sample	C^{14}-pigment in 100 ml (cpm)
1. Skim milk	18,000
2. Acetone extract of the pigment from skim milk	12,320
3. Citric acid precipitated casein	
(a) Acetone extract	8,400
(b) Whey	5,500

9.4 DISCUSSION

9.4.1 The major components of milk are synthesized within the mammary gland from simple metabolities extracted from blood, while others are transferred directly from the blood into milk. Since bilirubin happens to be a normal constituent of blood, it is natural to expect biliverdin, the immediate precursor of bilirubin to be derived from the direct uptake of blood bilirubin by the mammary gland and its subsequent oxidation to BV. The results obtained in the present study indicate bilirubin of blood as the direct precursor for biliverdin in buffaloes' milk.

9.4.2 The observed data indicate that the total amount of C^{14}-BLR accounted for a period of 14 h in circulating blood appears to be low as compared to the amount of C^{14}-BLR injected into the animal. The gradual decrease in the amount of C^{14}-BLR in the circulating blood (Table 20) indicates that apart from the BLR of blood taken up by the mammary gland, the rest of the BLR in the blood system follows a different pathway. Earlier work by Brown et al (1964) and Bernstein et al (1966) showed that liver was able to transfer BLR from blood plasma to parenchymal cells, within 5 min after the intravenous injection of H^3-BLR. About 60 per cent of the injected material was recovered in the liver, out of which 85 per cent was

associated with the non-particulate supernatant fraction. After the injection of unconjugated H^3-BLR into normal rats and subsequent subcellular fractioning, Bernstein and associates (1966) were unable to recover unconjugated H^3-BLR from the cell free fraction, although conjugated H^3-BLR could be readily recovered after injection of this material. The failure to recover conjugated H^3-BLR or the corresponding azo-derivatives, either from the supernatant or from the microsomal fractions of rat liver after injection of unconjugated H^3-BLR, suggests that conjugated BLR may be more strongly bound to cytoplasmic proteins than in unconjugated BLR, so that BLR is not extracted during recovery procedure. It is likely that the conjugated BLR rapidly leaves the cells or that it may be further degraded during cell fractionation. In the present study the rapid appearance of C^{14}-pigment suggests the source of milk BLV is BLR of the blood.

9.4.3 The acetone extract obtained from citric acid-precipitated casein and micellar caseins contained the radio active pigment. Similarly, radio activity was also present in the ultracentrifugal whey and in acid whey. The high counts obtained in milk as compared in acetone extract of skim milk (Table 21) as well as the occurrence of radio activity in whey suggests that the BLV is

associated with the whey constitutents (Table 22). This is in full agreement with the earlier study conducted with the help of BLV enriched milk and has shown that the whey constituent proteose-peptone is also associated with BLV (reported in Chapter V in para 5.2.4 and 5.3.2).

9.4.4 The bilirubin of blood as the source of biliverdin in buffaloes' milk has been established by infusing C^{14}-BLR and examining the milk, along with casein samples and whey.

CHAPTER X

SUMMARY

10.0 SUMMARY

Milk proteins which have been the subject of study over many decades covering physical, chemical and nutritional aspects continues to provide an inexhaustible source of material for further studies that are facilitated by the developments in analytical techniques. Studies described here have revealed yet another property of conjugation of biliverdin in buffaloes' milk and hitherto unidentified green pigments in cows' milk, to proteins isolated at pH 4.6.

10.1 As the concentration of biliverdin in buffaloes' milk varies from day to day, it was of interest to study the correlation between the content of biliverdin and binding protein to find out whether this fluctuation was governed by the amount of conjugating protein that is soluble in $(NH_4)_2SO_4$ saturated 80 per cent acetone. To enable a large number of samples to be examined, a method for the estimation of biliverdin associated protein was standardised. The isolated protein is dissolved in 1N NaOH, the solution is suitably diluted and protein is estimated using Folin-Ciocalteu-phenol reagent. The method is found to give reproducible results, the recovery of added proteins was of the order of 98-99 per cent and enabled rapid estimation of the samples. No relationship

was found between the biliverdin concentration of milk and the amount of binding protein. As in the case of biliverdin, the quantity of binding proteins also varied considerably. Buffaloes were maintained on intense dry and green feeding regimes to examine the effect of feed on the concentrations of total milk protein and biliverdin associated protein. Results indicate that these feeds did not have any influence on the concentration of biliverdin associated protein as well as total milk protein (Chapter III).

10.2 To examine the nature of biliverdin associated protein, its composition was analysed. The nitrogen content of biliverdin associated protein from buffaloes' milk gave average values of 15.55 per cent. The phosphorous content of the biliverdin associated protein sample isolated at room temperature and biliverdin associated protein sample isolated from chilled milk at 4°C were 354.60 ± 21.93 and 272.20 ± 15.57 µg/100 mg of protein, respectively. There was a marked difference in phosphorous content of the proteins isolated at two temperatures due to solubalisation of some of the phosphoprotein components during chilling of the milk, thereby indicating that biliverdin is bound to a large number of components. The iron content of the biliverdin associated protein isolated at room temperature and at 4°C dialysed against EDTA

and the sample isolated at 4°C without EDTA dialysis were, 103 ± 64.05, 96.20 ± 33.46 and 99.78 ± 30.64 µg/100 mg of the protein, respectively. These data indicate wide fluctuations within the samples. Biliverdin associated protein in its association with iron did not show any absorption maximum in the region 360-700 nm either in native form or when iron was added at the optimal pH 8.4 at 2°C. The association of iron with biliverdin associated protein is stable and remained stable in the presence of EDTA also. These results indicate that the presence of iron in protein may not be due to any complex formation, but the association occurred due to post-milking contamination from the cream separator and other equipments used in the dairy. Though biliverdin associated protein is also associated with iron as shown by spectrophotometric examination it differed from lactoferrin by not forming a complex with iron (Chapter IV).

10.3 The association of biliverdin with micellar casein and casein fractions has been established using spectral and electrophoretic analysis. Biliverdin can be readily extracted with acetone from micellar casein and casein fractions. This established that biliverdin is associated with all the casein fractions namely α_s-, β-, γ-, and k-caseins and protease-peptone. The biliverdin

associated protein is found to resemble casein in electrophoretic behaviour on PAGE, starch gel and phosphoprotein staining patterns on PAGE gels. It can therefore be concluded that biliverdin associated protein extracted from chilled milk using chilled acetone and $(NH_4)_2SO_4$ at 4°C is a casein and its components. Biliverdin is absent in the fat globule membrane protein (Chapter V).

10.4 The properties of biliverdin associated protein isolated at 4°C is similar to casein in its amino acid composition, pattern of tryptic hydrolysis, U.V.spectra, behaviour on gel filtration and ion exchange columns and in molecular weights of its electrophoretic components on SDS-PAGE. These results establish that the biliverdin in buffaloes' milk is conjugated to the casein moiety only and to no other specific protein. (Chapter VI).

10.5 The immunological behaviour of biliverdin associated protein and casein has been examined against antisera of the biliverdin associated protein. The results establish a close structural and functional relationship between casein and biliverdin associated protein isolated from chilled milk (Chapter VII).

10.6 Cows' milk casein exhibited high susceptibility to bind with added biliverdin and its behaviour was

identical to buffaloes' milk casein. The biliverdin added to cows' milk did not undergo any change. The added biliverdin could be quantitatively recovered from cows' milk and estimated using the same method used in the case of buffaloes' milk. The protein soluble in ammonium sulphate saturated 80 per cent acetone from cows' milk is found to be similar in nature to casein and its components as judged by electrophoresis (Chapter VIII).

10.7 To establish the precursor of biliverdin in milk, the studies conducted using of C^{14}-bilirubin have established that serum bilirubin is the direct source of the buffaloes' milk biliverdin. The possible conversion of bilirubin to biliverdin before excretion into milk must have therefore taken place in the mammary gland (Chapter IX).

The present study has brought out another interesting property of casein namely, that it binds with biliverdin in buffaloes' milk using bilirubin drawn from the blood serum, which helped to throw more light on the mechanism of milk secretion and also help in our understanding of phenomenon of milk secretion.

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APPENDIX

PUBLISHED PAPERS

1. RAJESHWAR RAO, T. and DASTUR, N.N.
Estimation of biliverdin associated protein in milk
Indian J.Biochem.Biophys., 17, suppl.p.45
(1980)

2. RAJESHWAR RAO, T., AJIT SINGH and GANGULI, N.C.
Source of milk biliverdin
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3. RAJESHWAR RAO, T., AJIT SINGH, GANGULI, N.C. and
DASTUR, N.N.
Studies on the biochemical changes in Milk: III.
origin of biliverdin in buffaloes' milk
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4. RAJESHWAR RAO, T., GANGULI, N.C. and DASTUR, N.N.
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