REVIEW OF LITERATURE
GENETIC POLYMORPHISM

A. CASEIN

\(\alpha_{s_1}\)-Casein

Using first paper electrophoresis, then starch gel electrophoresis, Thompson et al. (254) discovered the \(\alpha_{s_1}\)-A and \(\alpha_{s_1}\)-B variants, and, with the collaboration of Aschaffenburg, the \(\alpha_{s_1}\)-C variant (254, 254). Kiddy et al. (124) showed that these variants were determined by three alleles of the \(\alpha_{s_1}\)-casein locus. In addition to the originally discovered variants, \(\alpha_{s_1}\)-A, \(\alpha_{s_1}\)-B, and \(\alpha_{s_1}\)-C, only one new variant of \(\alpha_{s_1}\)-casein has been found, \(\alpha_{s_1}\)-D (87). The most widely distributed \(\alpha_{s_1}\)-casein variants found in bovine as well as in Zebu cattle are \(\alpha_{s_1}\)-B and \(\alpha_{s_1}\)-C, respectively. Juneja and Chaudhary (129) also reported a high predominance of \(\alpha_{s_1}\)-casein C variant in Sahiwal and Rathi breed of Indian Cattle. However, the proportion of heterozygotes was more in the Jersey cross-bred cattle.

It is thus reasonable to consider that these variants represent the original type, though the situation is unclear in this respect since \(\alpha_{s_1}\)-B is predominant in bovine, and \(\alpha_{s_1}\)-C in Zebu cattle (17). Aschaffenburg et al. (17) and Thompson et al. (258) have reported that \(\beta\)-casein \(\kappa^2\) and \(\alpha_{s_1}\)-caseins B and C obtained from Zebu milks were identical in peptide mapping to their counter-parts in Western milks.
Grosclaude et al. (91) have established that in Zebu the $\lambda_2$-C/B and $\beta A^1/A^2$ amino acid substitutions are exactly the same as in bovine. Thus it is very likely that for each locus, one single mutation event is responsible for the presence of genetic variants in bovine and Zebu cattle. These mutations must have occurred perhaps at the beginning of quaternary era.

$\beta$-Casein

$\beta$-casein is the first example of a casein polymorphism discovered by using paper electrophoresis (12). The variants detected were termed A, B and C. Aschaffenburg concluded that synthesis of $\beta$-casein in the mammary gland of the cow was under the control of three alleles of a gene, $\beta$-Ca, capable of causing three electrophoretically distinguishable variants, $\beta A$, $\beta B$ and $\beta C$ (11,12). Ever since the discovery of three $\beta$-casein variants situation has changed considerably. First, after the observation of Nauman et al. (136) of the variation in histidine content of a larger peptide of $\beta$-casein, Peterson and colleagues (194,195) showed that in acid gel (pH 2.8), $\beta A$ split into three different variants, finally termed $\beta A^1$, $\beta A^2$ and $\beta A^3$ (136). Aschaffenburg et al. (17) found in Zebu cattle a sixth electrophoretic variant, designated as $\beta D$. It has been shown that the amino acid composition of $\beta B$ is not the same in Zebu as in bovine (258). The $\beta B$ variant of Zebu was thus
termed as $\beta B_3$. Finally Voglino (274) found recently an eighth variant, $\beta E$. All these variants, except $\beta B_3$, are detected by electrophoresis.

Zebu $\beta$-casein D, observed on the subcontinent of India and also East Africa, is more similar to $\beta C$ than to either $\beta$ or $\lambda$ variant. It is likely that $\beta C$ is a mutation of $\beta D$ involving multiple substitutions.

The polymorphism of the classical $\gamma$-casein fraction, first mentioned by Aschaffenburg (11), was thoroughly analysed by Groves and colleagues (92,93,94,95,96) who identified in the group four species, $\gamma^-$, TS-, $R^-$, and $S^-$, and noted a striking interrelationship between the polymorphism of these minor caseins and that of $\beta$-casein. From the results of amino acid analysis, peptide mapping, molecular weight determinations and from studies of terminal residues, Gordon et al. (82) recently suggested that $\gamma^-$, $R^-$, $S^-$, and TS- caseins might be fragments of $\beta$-casein. Definite proofs were obtained by studying the amino terminal sequences of approximately 12 residues for each of them. It can now be stated that $\gamma^-$, $T3A^2$- and $S^-$, $T3B$, and $R$-caseins represent fragments 29-209, 106 - 209 and 108 - 209, respectively, of the $\beta$-casein peptide chain, and that these fragments originate from a tryptic-like hydrolysis of $\beta$-casein.

The localization of amino-acid substitutions which differentiate the known variants of $\beta$-casein (89)
explains the observed relationship between the polymorphism of $\beta_c$, $\gamma$, $R$, $S$, and TS-caseins, but not the absence of the $\kappa C$ variant in milks containing $\beta$-casein C variant (2).

Aschaffenburg et al. (17) have reported a high predominance of $\beta$-casein $\kappa$ in the milk of Zebu breeds, $\beta$-casein $\beta$ was also typed with a low gene frequency of 0.13 compared to 0.35 for $\beta$-casein $\kappa$. In acid pH, $\beta$-casein $\kappa^1$ and $\kappa^2$ could be detected though $\beta$-casein $\kappa^2$ was highly predominant. Juneja and Chaudhary (129) also confirmed the results of Aschaffenburg et al. (17) from Sahiwal and Rathi breeds in alkaline pH. One Rathi cow was observed to have $\beta$-casein D. No $\beta$-casein $\beta$ could be detected in any of the breed studied.

$\kappa$-Casein

The genetic polymorphism demonstration of $\kappa$-casein has taken somewhat longer time compared to $\kappa_{s1}$- and $\beta$-caseins. This is so because in the electrophoretic conditions described by Wake and Baldwin (276), $\kappa$-casein fails to form bands and instead appears as a blurred zone in the gel bed. But by incorporating $\beta$-mercaptoethanol in the gel, Swaisgood and Brunner (248) were able to dissociate $\kappa$-casein aggregates. Neelin (137), Schmidt (226) and Woychik (291b) could as well resolve $\kappa$-casein into one major and several minor bands, and finally disclosed a polymorphism at least
in the major component. The genetic variants of this polymorphism (locus $k$-$Cn$ with two alleles $K$-$Cn^A$ and $K$-$Cn^B$) has been established, independently, by Grosclaude et al. (86) and Larsen and Thymann (149). Since both $k$-caseins $A$ and $B$ give the same para-$k$-caseins, it can be concluded that the amino acid substitutions associated with genetic variants occur in the macropeptide section of $k$-casein. This has now been verified (58, 5). Much more comprehensive data for the occurrence of $k$-casein in various breeds of cattle in several countries have been obtained.

Aschaffenburg (150) has prepared a very comprehensive summary of the gene frequencies of the milk protein variants. The $A$ allele tends to be predominant in the majority of the breeds.

Aschaffenburg et al. (17) have examined the caseins of Indian and African Zebu cattle ($Bos indicus$) for casein polymorphism. They observed a high frequency of $k$-casein $A$ in all the Indian breeds and reported that it is inherited in a straightforward Mendelian manner as shown by daughter-dam comparison and Hardy-Weinburg expectation.

Majumder and Ganguli (161) also determined the $k$-casein polymorphism in Tharparkar, Sahiwal and Red Sindhi breeds. The frequency of occurrence of homozygous $k$-casein $A$ variant ($0.62-0.78$) was much higher than the heterozygous $k$-casein $AB$ ($0.22-0.38$). Homozygous $k$-casein $B$ could not be detected in any of the milk samples analysed.
The gene frequency of k-casein A was highest in Sahiwal (0.78) as compared to other breeds whereas the genetic make-up of k-casein in Tharparkar and Red Sindhi was similar.

Recently, Juneja and Chaudhary (12,9) typed milk proteins in Indian cattle namely Sahiwal and Rathi and Jersey x Sahiwal crossbred. K-casein B could not be detected in Sahiwal and Rathi cattle except the two phenotypes k-casein A and k-casein AB. Jersey cross-bred had an intermediate gene frequencies between Jersey and Zebu cattle. The proportion of k-AB was more as expected in these cross-breds.

B. WHEY PROTEINS

\(\alpha\)-Lactalbumin

Aschaffenburg et al. (9b), Blumberg et al. (27) and Plowman et al. (196) have reported the absence of the A gene of \(\alpha\)-lactalbumin in the breeds of European cows. According to Bhattacharya et al. (24), \(\alpha\)-lactalbumin polymorphism occurs in Indian Zebu cattle of Hariana, Sahiwal and Deshi breeds. Their work showed the presence of variants A and B, with the frequency of A gene much lower (0.219) than that of the B gene(0.781). Because of the absence of \(\alpha\)-lactalbumin A in British breeds (24), a relatively lower gene frequency for \(\alpha\)-lactalbumin A was observed in the Haryana x Jersey crosses. Maval (164) followed up the study and
surveyed two other Indian breeds, namely, Gir and Gavathi (164) and reported the existence of polymorphic \( \alpha \)-lactalbumin A and B.

**\( \beta \)-Lactoglobulin**

Aschaffenburg and Drewry (95) demonstrated the presence of two genetic variants \( \beta \)-lactoglobulin A and \( \beta \)-lactoglobulin B in cow's milk. Bell (22a) reported the discovery of a genetically determined third variant \( \beta \)-lactoglobulin C having a still lower mobility on the starch gel electropherogram than either of the two. In 1966, two other variants were announced. Grosclaude et al. (87) found a variant, designated as \( \beta \)-lactoglobulin D. The second new variant was isolated from Droughtmaster beef cattle by Bell et al. (22b). This later variant had the same amino acid composition as the bovine A variant but with a carbohydrate moiety attached to it.

Bhattacharya et al. (24) and Mawal (164) have surveyed the distribution of \( \beta \)-lactoglobulin polymorphism in Indian breeds. The frequency of the A-gene of \( \beta \)-lactoglobulin followed a reverse order compared to \( \alpha \)-lactalbumin, with the Indian Zebu cattle having the minimum and the European breeds the maximum value. In the case of \( \beta \)-lactoglobulin A, the gene frequency in British breeds was shown (9b) to be 28 percent, which was a significantly higher figure than 9.4 percent obtained by Bhattacharya et al. (24).
MILK ENZYMES

(a) Alkaline phosphatase

This class of enzyme hydrolyzes phosphoric acid esters. Graham and Kay (246) first of all established its identity in milk. Milk essentially contains alkaline phosphatase as a phosphomonoesterase with a pH optimum of 37°C. Zittle and associates (296, 297, 299) and Kitchen et al. (140) have done extensive work on its purification and characterization. It has been purified from both buttermilk and skimmilk, achieving 5,000-fold purification. It is activated by Mg²⁺ and Mn²⁺ (182).

Variation in milk:

Alkaline phosphatase has been studied in the milk of various species. The alkaline phosphatase content of milk is rather variable; in one study (107) a variation of nearly 40-fold was found among individual milkings and a nearly 2-fold variation among bulked milks taken at different seasons of the year.

Folley and Kay (73) found that the high level of phosphatase diminished to a minimum at 15–25 days post-partum. Aschaffenburg and Neave (6) observed low phosphatase activity during first few weeks of calving. Kannan and Basu (130) studied the alkaline phosphatase in colostrum of various species and reported that the level of this enzyme is high in colostrum of cows, buffaloes and...
ewes which gradually dropped to a minimum after 4-15 days post partum with a subsequent rise until the end of lactation. They also reported that buffalo milk contained lower alkaline phosphatase than cow. Low level of alkaline phosphatase in early lactation and high in advanced lactation has been observed by Morton (181) in pooled milk samples of commercial herds. Jacquet and Villette (125) reported high level of phosphatase in secretions like colostrum and mastitis milk. Haab and Smith (167) found that in case of cow, the phosphatase values decreased sharply after the first milking, reached to minimum before the 10th day and then began to increase gradually. Variation due to season was maximum during October to December. No effect of feeding was noticed on the activity of this enzyme in milk. Mohamed and El-Rafey (176) studied the alkaline phosphatase of Egyptian buffaloes milk and compared with Brown Swiss and local breeds of cow. They reported that the buffalo milk has lower alkaline phosphatase activity than the cow breeds studied. The cow and buffalo milk showed a similar trend of fluctuation throughout lactation.

Sharma and Ganguli (235) found both individual and species variation in the level of alkaline phosphatase in buffalo and cow milk. Buffalo milk on an average contained one-third alkaline phosphatase compared to the cow milk. Fractionation of milk by various methods like rennet coagulation ultracentrifugation and skimming revealed that alkaline phosphatase of both buffalo and cow
milk was distributed throughout the milk in all the major fractions in varying concentrations (235).

Isozymes of alkaline phosphatase:

Coupius Peereboom (51) using agar gel electrophoresis found three isozymes, namely $\alpha$-, $\beta$- and $\gamma$-isozymes. The $\gamma$-isozyme characteristically moved towards the anode side. Through densitometric determinations the average percentages of $\alpha$-, $\beta$- and $\gamma$-forms were reported to be 65, 25 and 10 percent, respectively. The isozyme pattern of raw and reactivated alkaline phosphatase was found to be different. Subsequently, Coupius Peereboom (52) using agar gel electrophoresis, polyacrylamide disc electrophoresis and Sephadex thin layer chromatography, found that the reactivated cream gave exclusively $\beta$-isozyme. This $\beta$-isozyme was found to be located only on the fat globule membrane complex and was never observed in the skim milk and the skim milk phase of water washed cream, which contained mainly $\alpha$-alkaline phosphatase. By studying the isozyme patterns of several samples of reactivated cream it was found that these renatured $\beta$-alkaline phosphatase patterns may vary considerably. Some samples contained exclusively $\beta$-isozyme, other $\beta_2$ or a combination of both. A special $\beta_3$-isozyme was identified in reactivated cream, but could not be detected in normal "native" isozyme patterns. This $\beta_3$-isozyme had greatest mobility on agar gels. Depending upon their mobilities on Sephadex thin layer chromatograms with respect
to the proteins of known molecular weight, it was found that \( \alpha \)- and \( \beta \)-isozymes had 140,000 and 570,000 molecular weights, respectively.

Lefrane and Han (152) reported that the alkaline phosphatase isolated from butter milk resolved into two enzymes, A and B, on DEAE-cellulose chromatography. Fraction A was found to be precipitated at 70-90 percent saturation with ammonium sulphate and had a molecular weight about 140,000. Fraction B was precipitated at 40-65 percent ammonium sulphate saturation. Such component had a molecular weight of about 200,000.

Buruiana and Marin (32) studied the isozyme pattern of 4 breeds of cow in Romania and their crosses on Sephadex G-200. The alkaline phosphatase was found to be polymorphic and modified by crossing. Milk from Holstein-Friesian cow's contained 4 isozymes and milk from other breeds contained less.

Buruiana and Dema (31) separated alkaline phosphatase from buttermilk and skim milk by Sephadex G-200 and both were found to be similar. However, a number of isozymes were detected. The number of isoenzymes present in milk depended on the breed of the cow, Red Danish cows having more isoenzymes of phosphatase present in milk than had Holstein-Friesian cows.

(b) Xanthine Oxidase

Xanthine oxidase has been implicated in the development of undesirable oxidized flavour in market milk and
other dairy products (18,20,21). It was first observed in milk in 1902 by Schardinger. Besides being present in relatively large amounts in bovine milk, xanthine oxidase is also present in milk of other ruminants such as sheep and goats but is devoid in human, sow and mare milk (175). The fact that there are no reports on mammary tumors in cows, sheep, and goats may be correlated with xanthine oxidase.

Xanthine oxidase is a metallo-protein containing molybdenum and iron. Its molecular weight is rather high about 300,000. It is moderately stable to heat and, in contrast to other enzymes, its activity in milk increases with several heat treatments, homogenization, and by protease and lipase action (251).

Xanthine oxidase activity has been shown to be distributed into three closely grouped population (99,294). Polarographic studies of Zikakis and Treece (295) showed recently that it exists in two polymorphic - low and high active forms within a breed. These activities are controlled by high activity allele xanthine oxidaseA and low activity allele xanthine oxidaseB.

Krishna Iyengar and Laxminarayana (143) and Sharma and Ganguli (234) have observed lower xanthine oxidase activity in buffalo milk compared to that of cow milk. The distribution studies of Sharma (255) revealed that 60 percent of this enzyme is present in fat fraction of cow and buffalo milk during ultracentrifugation. Also 20-30 percent of the enzyme was found in the ultracentrifugal whey.
(c) Lipase

Milk contain lipases which are of great importance because they release volatile short-chain fatty acids which can produce undesirable flavours in milk and milk products; yet they produce desirable flavour in certain cheeses. Milk lipases review have been published by Jensen (126), Chandan and Shahani (46), Shahani (231) and Shahani et al. (232).

Lipase is an enzyme which catalyzes the hydrolysis of glycerolesters (fats and oils) in emulsion. In 1922, its presence in milk was conclusively shown by Rice and Markley (207), although as early as 1904, Rogers (241) observed an increase in the acidity of canned butter. During recent years, several workers (61, 62, 74, 76, 112, 220) have isolated many lipases, each differing from the other in many respects. Chandan and Shahani (46) and Richter and Randolph (209) purified lipase from clarifier slime. Recently, Rout (214) observed that lipase chromatographs with casein on Sephadex G-100, with water as the eluant but with phosphate buffer the lipase fraction spreads, yielding a broad band or several peaks.

Normal milk contains lipase which is associated with casein (250, 251, 63, 64). Lipase activity has been shown to be associated with k-casein (292) and can be dissociated from this fraction by dimethylformamide (74). Milk lipase appears to be a true lipase because it hydrolyzes only esters of glycerol in a two-phase
heterogeneous emulsified system and does not hydrolyze simple esters in solution (47,112,190). It exhibits a pH optimum of 9.0 and temperature optimum of 37°C. Various milk constituents have a profound effect upon its activity - salts and casein being inhibitory, lactose having no effect, and albumin and globulins being stimulatory. Therefore, the observed lipase activity in a complete milk system may be the net result of the inhibitory and stimulatory action of various milk constituents upon the lipase (232). With a molecular weight of 7,000 to 8,000, it is probably the smallest enzyme ever isolated (47). Rout (214) also observed that one of two lipase fractions isolated had a molecular weight of less than 10,000.

Kannan and Basu (130) and Sharma and Ganguli (235) studied lipase content in Indian cow and buffalo milk, and reported that buffalo milk contained slightly lower lipase activity than cow milk. The distribution studies of Sharma and Ganguli (235) revealed that most of the enzyme was present in varying concentration in all the ultracentrifugal fractions like micellar casein, opalescent layer, ultracentrifugal serum and fat.
HEAT TREATMENT OF MILK

Knowledge on the heat-induced changes on proteins of milk is desirable, since it may help in the formulation of suitable time-temperature combinations for manufacturing milk products of certain desirable qualities. Many investigators have made concerted efforts to elucidate the heat-induced changes on the constituents of milk. These changes may be reversible or irreversible. Although heat treatments bring about changes on the constituents of milk, it manifests finally phenomena like cooked flavour, development of antioxidative properties, prolongation of rennet clotting time, inhibition of gelation of evaporated milk on sterilization, and finally in the heat stability of milk. Rose (212) has reviewed the factors which affect the heat stability of milk. A number of changes namely aggregation, denaturation, coagulation and interaction between the various proteins takes place, as a result of heat treatments. In view of the great volume of data in literature over the past 30 years on heat stability of milk, an attempt has been made here to review only the work concerning the changes occurring in milk proteins as a result of heat treatments.

Effect of heat treatment on milk proteins
(a) Distribution of nitrogen in milk

As a result of heat treatment especially at
temperatures higher than 65°C, there occurs considerable changes in the distribution of nitrogen in milk. Rowland (215) ascertained the extent of heat denaturation of albumin and globulin in cow's milk at various temperatures from 63°C to 80°C for periods ranging from 2.5 to 60 minutes. An average of 10.4 percent of the total soluble protein was denatured after 30 minutes of heating at as low a temperature of 63°C. Later, Rowland (217) studied the heat denaturation of albumin and globulin in cow's milk at temperatures varying from 75°C to 120°C. The denaturation of albumin and globulin was rapid in samples of milk heated at temperatures of 75°C and above. Non-protein nitrogen content of milk remained the same irrespective of the heating temperature up to 100°C was a result of continued heat treatments at 95°C and 100°C; very small amount of proteose-peptone was produced by hydrolysis of the protein. Further increase in the heating temperature resulted in considerable increase in the proteose-peptone content of milk.

The coagulation of albumin and globulin in milk by heat treatment are dependent upon the pH of the serum. While studying the effect of pH on heat coagulation of albumin and globulin fractions of milk, Rowland (216) observed that the maximum coagulation of albumin and globulin occurred at pH 4.75 - 4.80, on boiling the casein-free filtrate of milk. As such as 70 percent of total soluble protein nitrogen was rendered coagulable at boiling conditions. In order to determine the
distribution of nitrogen into the various fractions of milk, a fractionation procedure was suggested by Rowland (217).

Menefee et al. (171) investigated the distribution of nitrogen in evaporated milk. The most significant change observed was the coagulation of albumin and globulin especially albumin. Hetrick and Tracy (114) studied the effect of high temperature short time heat treatment on the distribution of nitrogen in milk. These investigators did not observe any denaturation of albumin and globulin when milk was heated to minimum conditions for inactivation of phosphatase enzyme. The extent of denaturation of albumin and globulin with time at constant temperature of 170°F followed closely the first order kinetics. But at 230°F the denaturation of these proteins did not follow the first order kinetics. Pasteurization of milk at 155°F/30 minutes did not cause any change in the nitrogen distribution of milk according to Shahani and Sommer (230). However, about 9 percent of globulin and 5 percent albumin coagulated.

Although the fractionation procedure suggested by Rowland (217) remained undisputed over two decades, it lacked the ability to partition the globulins and even proteose-peptone was not fractionated without the aid of heat treatment. Procedure to crystallize α-lactalbumin and β-lactoglobulin suggested by Aschaffenburg and Drewry (9a) brought further refinement in the fractionation of whey proteins of bovine milk.
Based on this and the electrophoretic patterns, an almost complete fractionation procedure for measuring the distribution of nitrogen in milk have been achieved by Aschaffenburg and Drewry (10). Adopting the nitrogen fractionation procedure of Rowland (217), Dill et al. (59) studied the denaturation of serum proteins in skim milk heated by direct steam injection, over a wide range of time (8 seconds - 200 seconds) at temperatures ranging from 160°F - 300°F. The amount of serum proteins denatured varied from about 10 percent for the minimum and over 80 percent for the most severe heat treatment.

Melachouris and Tuckey (170) using the Aschaffenburg-Drewry nitrogen distribution method and Harland-Ashworth (111) test for whey protein, investigated the effect of heat treatment on the whey proteins of bovine milk for temperatures ranging from 61.7°C to 143.3°C. It was observed that Harland-Ashworth test gave lower results for whey protein denaturation than that obtained by Aschaffenburg-Drewry nitrogen fractionation procedure. Total albumin showed low degree of denaturation at the lower temperatures of heating, but at higher temperature the denaturation was similar to that of whey protein fraction. The β-lactoglobulin fraction showed a high degree of sensitivity to heat (70.03 percent denaturation at 143.3°C), whereas residual albumin exhibited an appreciable degree of denaturation when milk was heated at 110°C or higher. The
proteose-peptone fraction decreased as temperature of heating increased.

The same observation was reported by Davies and White (55) and Hostettler et al. (121) for proteose-peptone fraction. Globulin showed rapid increase in denaturation even at lower temperatures of heating. Non-casein nitrogen decreased as temperature of heating increased. However, the non-protein nitrogen content was not affected by heat treatment.

Zhadanova and Sergeeva (293) studied the nitrogen distribution in milk subjected to UHT treatment. About 60 percent of the whey proteins was denatured in bovine milk.

While all these investigations were carried out with bovine milk, detailed studies were not carried out with buffalo milk. However, Venkattappaih and Basu (273) did not observe any change in the non-protein content of buffalo milk when heated to boiling.

Using the nitrogen fractionation procedures of Rowland (217) and Aschaffenburg and Drewry (10), Ismail et al. (124) reported their findings on the distribution of nitrogen in heat treated buffalo and cow milk for temperatures varying from 75°C - 120°C for periods of 15 minutes. The albulins and globulins disappeared in both samples of milk at 95°C. Proteose-peptone nitrogen was considerably increased on sterilization.

Lyster et al. (155) investigated the effect of direct
and indirect methods of ultra high temperature treatment on the distribution of nitrogen in milk. Their results indicated considerable increase in casein nitrogen. Non-protein nitrogen was unchanged. Proteose-peptone nitrogen showed slight decrease, whereas all other whey protein nitrogen showed greater extent of decrease. The greatest decrease was of globulin nitrogen.

Recently, Tandon and Ganguli (265) reported their findings on the distribution of nitrogen in buffalo milk subjected to graded sterilization. According to them, the non-protein nitrogen increased marginally on sterilization. Total albumin and $\beta$-lactoglobulin nitrogen decreased more in buffalo milk than in cow milk. Proteose-peptone decreased more in cow milk than in buffalo milk. According to Garnier (77) heating goat milk at 95°C/15 minutes, did not significantly increase the non-protein content.

Gupta et al. (102,103) observed that proteose-peptone content of buffalo and cow milk decreased to the extent of 50 percent during heat sterilization of milk at 115°C - 120°C for 10-20 minutes. The presence of calcium and citric acid facilitated the reduction of proteose-peptone during the heat sterilization of milk. Reducing sugars protected the decrease of proteose-peptone to certain extent during heat sterilization of milk.

(b) Rennet Clotting Time

It is now established that the primary phase of
Rennin action in the coagulation of milk involves the hydrolysis of k-casein (275, 278) with the release of peptides and glycopeptides, which are soluble in trichloroacetic acid (5). This action of rennin destroys the micelle stabilizing power of k-casein so that in the presence of Ca++ ions the casein coagulates. Under standard conditions, there is a wide variation between milk samples in the rennin coagulation time (68, 78, 283). These differences may be partly due to differences between milks in pH (285) or in the soluble Ca++ concentration (203) which are known to affect the coagulation phase. The pH itself may also affect the primary phase (78) which can be an important contributing factor (283). Polymorphic combination (68), and amount and composition of k-casein are also important contributory factors for rennin action (285).

Heat induced changes in rennet coagulation of milk were reported first of all by Mattick and Hellett (165a). These studies showed that rennet coagulation time was considerably increased in milk heated above 65°C. Later, this was also confirmed by the work of Powell (260), Powell and Palmer (201) and Pyne (202). Sen and Dastur (229) studied the effect of heat treatment on cow and buffalo milk. They showed that heated buffalo milk clotted faster than cow milk. Similar observations were also substantiated by Krishnamurthy and Subramanyam (244).

The coagulability of milk by rennin, subjected to heat treatment at temperature from 0°C - 62°C appears to be
reversible. Pyne (202) concluded that on heating milk there was a temporary transfer of some of the soluble calcium and phosphate to the colloidal calcium phosphate casein complex. This caused an increased sensitivity of the para-caseinate to calcium ions which became responsible for reduced rennin coagulation time. Subsequent lowering in temperature caused a reverse effect as calcium and phosphate shifted from the colloidal to the soluble state (54). According to Pyne (202) dissolution of the colloidal calcium phosphate decreased the sensitivity of the para-caseinate to precipitation by calcium ions. The pH of milk would be expected to alter as a result of the change in the state of calcium and phosphate caused by heat treatment. An intermediate increase in the rennin clotting time has been observed when milk was heated at temperatures of 65°C - 100°C. This was followed by a further increase in clotting time when milk was 'cool aged' after heat treatments. The immediate increase in the rennin coagulation time of heated milk suggests that milk proteins were altered as a result of heat treatment.

The degree of alteration of rennet clotting time of heated milk is more as a result of heat treatment than changes in the case of calcium phosphate. Kannan and Jenness (131) observed no change in the rennin coagulation time upon heating (85°C/30 minutes) and subsequent holding at reduced temperatures of whey protein free caseinate solutions. When β-lactoglobulin was added to this caseinate solution before heat treatment, an immediate
increase in clotting time and subsequent hysteresis, which was typical of heated skim milk was observed. On this basis, they suggested that heat treatments at 85°C/30 minutes induced changes involving β-lactoglobulin which interfered with the primary action.

Long (158) proposed a heat induced interaction between β-lactoglobulin and k-casein. Such an interaction considerably reduced the rennin coagulation of k-casein (300). It is now well established that heat treatment of milk at high temperature prolongs its rennet coagulation time due to interaction between β-lactoglobulin and k-casein. These proteins must be present together during heating if the increase in rennet coagulation time to occur (131, 178, 300). The heat induced complex is formed only above 65°C (225). Heating of a 1:1 mixture of β-lactoglobulin and k-casein for 30 minutes at pH 6.5 produces the following amounts of β-lactoglobulin reacting with k-casein: 3.4 percent at 65°C, 83 percent at 85°C and 76 percent at 99°C (142). Though such complexes have not been isolated from heated milk (253) corresponding to isolated β-lactoglobulin and k-casein, it has been established that there is a reduction in the total amounts of peptides released by rennin when the whole milk is heated (178, 116, 288). This indirectly indicates that k-casein is affected due to heat treatment and thus affects rennet action in releasing the peptides. Wilson and Wheelock (288) while studying the factors affecting rennin action in heated milk, observed that there was an
inhibition in primary phase of rennin action due to complex formation between k-casein and \( \beta \)-lactoglobulin involving sulphydryl groups (222, 223, 300) due to the rearrangement of disulphide cross-linkages (289).

(c) Effect of heat treatments on electrophoretic patterns of milk proteins

The effect of heat treatments on milk proteins has been studied by a number of investigators. Although the techniques employed varied in terms of the type of electrophoresis and the pH, an important outcome of such a study was the revelation of the interaction between k-casein and \( \beta \)-lactoglobulin.

Bosticco and Ubertalle (28) analysed milk by paper electrophoresis before and after heat treatments. Raw milk was resolved into three protein fractions. As a result of boiling the first two fractions showed decrease and the third increased proportionately. In the case of pasteurised milk a fourth fraction was observed, and was in all probability attributed to the degradation of proteins. In electrophoretic studies of evaporated milk, Hostettler and Stein (121) observed a number of changes in the protein fractions. Compared with unheated skim milk proteins, the H.T.S.T. evaporated milk showed a slight rise in the case of \( \alpha \)-casein. In the case of autoclaved evaporated milk, a more marked rise in the apparent proportion of \( \alpha \)-casein was observed. There was a corresponding fall in \( \beta \)-lactoglobulin. It was, therefore, suggested that heat induced complex between \( \alpha \)-casein and \( \beta \)-lactoglobulin as a
result of heat treatments. Such a complex was believed to be a stabilizing factor in the stability of evaporated milk.

Brown et al. (30) examined the electrophoretic pattern of whey proteins of skim milk subjected to a number of heat treatments including steam injection method. It was observed that the rate of whey protein denaturation increased with both increasing pre-heat treatment and increasing steam injection. Changes in the serum proteins of milk on heat treatments ranging from 63°C for 30 minutes to 100°C for 10 minutes, H.T.S.T. pasteurized sterilized and uperised milk were demonstrated by Benassi (22c) electrophoretically. Pasteurization at 63°C for 30 minutes or 73°C/8 seconds did not affect the whey proteins. All other heat treatments caused profound changes in the serum proteins. Heating milk at 100°C/10 minutes and sterilization completely denatured the whey proteins. It was observed that amongst the whey proteins, α-lactalbumin was the most thermoresistant.

Murand et al. (19) by means of gel and immunoelectrophoretic techniques demonstrated the stability of the serum proteins in milk on heating in the order of immunoglobulin, α-lactalbumin, serum albumin, β-lactoglobulin and casein. A complex between β-lactoglobulin and casein was demonstrated in heat treated samples.
Molecular Weight

In recent years, gel filtration and sodium dodecyl sulphate (SDS)-gel electrophoresis has been successfully used to determine the molecular weight of proteins (4,233, 280, 284). Both the techniques are simple, rapid and reliable and a consistent and reasonable estimation of molecular weight is possible. Estimates of comparative molecular weights of milk protein have thus been obtained by determining mobilities in gels or passage through swollen gels (4,233, 280, 284).

A. Casein

Numerous values for the molecular weight of casein fractions have been reported in literature using several techniques. The following is a summarized account for the same.

(a) $\alpha_{s_1}$-casein

Ribadeu-Dumas et al. (206) calculated recently from the primary structure of $\alpha_{s_1}$-casein the molecular weight of 23,615. Measurements of the molecular weight by sedimentation and diffusion give a value of 24,800 ± 1,000 and 27,600 ± 1,000 at pH 11 and 7.3, respectively (64). The average molecular weight of $\alpha_{s_1}$-casein determined by different physical methods is around 27,000 (279); 31,000 (163); 30,000 (56); and 27,300 (65). Gel filtration studies of Richardson et al. (208) showed that the molecular weight of bovine $\alpha_{s_1}$-casein
was 25,400 compared with 25,700 for the caprine 
$\alpha$-casein or 47,500 for ovine $\alpha_1$-casein (208).
Recent studies of Mullin and Wolfe (185) from mobilities 
in SDS gels determined a value of 34,000 daltons.

(b) $\beta$-casein

Following the determination of primary structure of 
$\beta$-casein, Grosclaude et al. (90) calculated the molecular weight as 23,983. Other physico-chemical studies 
for $\beta$-casein give the values of 17,300 ± 300 at pH 11 
and 19,800 ± 1,000 at pH 7 (165); 25,000 (192);
24,200 (31) and 24,100 (242). When SDS-disc 
electrophoresis was used in molecular weight determination 
Groves et al. (98) and Mullin and Wolfe (185) 
obtained the figures of 27,000 and 28,000, respectively.

(c) $\kappa$-casein

There are wide variations in the literature for the 
molecular weight of $\kappa$-casein which seems to be due to the 
formation of $-S-S-$ bonded polymers ranging in size from 
trimers (248, 249), to decamers or larger (250). 
Measurements by Archibald method give a value of 26,000 ± 
3,000 at pH 12 (165, 246). Beeby (22) suggested a 
molecular weight of 50,000 and Swaisgood and Brunner (248) 
of about 60,000. Payens (191) gave a value of 28,000 and 
Gibbons and Cheeseman (80) indicated a value of 
160,000 - 200,000. Mullin and Wolfe (185) calculated 
the molecular weight of 26,000 daltons in SDS-gels for 
the monomeric $\kappa$-casein.
B. Whey Proteins

(a) \( \alpha \)-Lactalbumin

\( \alpha \)-lactalbumin has a molecular weight of about 16,200 \( \text{(169)} \). It is characterized by a zone of insolubility in the pH region of 4 to 5.5 \( \text{(169)} \). On both sides of the zone of insolubility, \( \alpha \)-lactalbumin undergoes a set of interaction leading to various states of polymerization. Detailed studies of the behaviour of \( \alpha \)-lactalbumin were undertaken by Kronman and co-workers \( \text{(144, 169)} \). It seems that on the alkaline side, there are weak intermolecular association, while on the acid side, a slow polymerization of the molecule results.

(b) \( \beta \)-Lactoglobulin

At neutral pH and at moderate concentrations, \( \beta \)-lactoglobulin exists as a dimer of the 18,000 molecular weight monomer unit \( \text{(166b)} \). Positive evidence of the dissociation of \( \beta \)-lactoglobulin into units of molecular weights of 17,000 daltons was obtained by Bull \( \text{(40)} \). As the pH of \( \beta \)-lactoglobulin is raised above 6.5, the protein undergo conformational transitions, dissociation and aggregation \( \text{(169)} \).

When \( \beta \)-lactoglobulin solutions in the pH range of
3.7 to 5.1 are cooled below room temperature, the 36,000 molecular weight species enters into a rapidly reversible equilibrium to form a tetramer of 144,000 molecular weight (260,261,267,291). The tetramerization is maximal at pH 4.65 and the extent of reaction increases with a decrease in solution temperature.

Below pH 3.5 \( \beta \)-lactoglobulin \( \alpha \) and \( \beta \)-lactoglobulin \( \beta \) have been shown to dissociate progressively into two identical sub-units of about 18,000 molecular weight as shown by others (168,169,260,261,267,297).
BOVINE IMMUNOGLOBULINS

The recognition of immunoglobulin classes and recent advances in the biological and chemical investigations of their properties have led to the characterization of three antigenically distinct classes of immunoglobulins in the bovidae family. Bovine serum and lacteal secretions have three major antigenically distinct classes of immunoglobulins, IgG, IgM and IgA (33, 34, 213).

The IgG class contains at least two sub-classes, IgG1 and IgG2, and likely to contain other classes (213, 132, 135). Moreover, there are recent reports (25, 39) of genetic variants within IgG. Also recently, complexes between lactoferrin - IgG2 in bovine colostrum and milk whey have been shown and detected (25, 38, 128).

In a recent symposium on Bovine Immune System (35) held at Maryland, U.S.A., a nomenclature scheme was drawn up for bovine immune system (35) so as to cover the recent advances in this field and reach agreement on the nomenclature of bovine immunoglobulins.

Despite the amount of physiological work done on cattle, detailed investigations into the biological activity, physico-chemical characteristics and significance of each bovine immunoglobulin are just beginning. A summarized account on the physico-chemical characteristics of bovine immunoglobulins from the data of several workers has been tabulated recently by Butler (34, 39).
Immunoglobulin Preparation

The first attempts to isolate a globulin whose presence in milk was foreseen by Hammarsten (108) was made in 1885 by Sebelien (223) by precipitation through MgSO₄. Since then many methods have been used for the separation and purification of immunoglobulins, utilizing the solubility under different conditions, the molecular size, and the electric charge.

Chromatographic techniques for protein fractionation have contributed greatly to advances made in bovine immunology. During the last decade or so, there are several reports on the preparation and purification of serum and lacteal immunoglobulins (25, 75, 101, 109, 110, 133, 146, 147, 157, 183a, 184, 185, 198, 199, 263, 281).

Recently, Butler and Maxwell (36) have reported the most precise technique for the preparation of bovine immunoglobulins and their specific antisera. These separation and purification of immunoglobulins have primarily employed ion exchange and gel filtration (exclusion) chromatography, ultracentrifugation, zone electrophoresis and other methods such as isoelectric-focussing.

Quantitation of Immunoglobulins

The quantitation of bovine immunoglobulins has been mostly carried out by single radial diffusion (70, 162, 286). This is so because the technique is rapid, sensitive
and accurate to about 10 percent, but is dependent upon the diffusion coefficient of the immunoglobulin to be measured and the ability of immunoglobulin to form a measurable precipitate with its antiserum. Because, polymers, fragments, and low concentrations of immunoglobulins are often encountered in secretions, the technique of radial immunogel filtration (110), and radioimmunoassay may be employed to overcome these problems. Electro-immunodiffusion may be more sensitive and accurate than single radial diffusion (39) and two dimensional immunoelectrophoresis provides semi-quantitative data on several proteins simultaneously (150).

\textbf{IgG}

Earlier work on the quantitation of IgG reported in literature has been primarily on human milk IgG, where it has been reported to be a minor fraction (168). This is supported by the quantitative studies of Gugler \textit{et al.}(100); Gugler and von Muralt (101); Schwick \textit{et al.}(227); Chodirker and Tomasi (50) and Blanc(26). Schwick \textit{et al.}(227) using the quantitative immunoprecipitation technique of Schultze and Schwick (226a), found mean values of 36 mg/100 ml 3 to 8 days post partum, and 9 mg/100 ml, 9 to 26 days post partum. Ammann and Stiehm(3) found 43 mg/100 ml of IgG-reactive material in initial colostrum and 4 mg/100 ml in 4 day colostrum using the Mancini method.

Recent work of Brandon \textit{et al.}(29) shows that IgG1
is the predominant immunoglobulin in normal milk. They observed an IgG1: IgG2 ratio in whey of 4.7:1 compared to 1:1 in blood serum. The mean IgG1 and IgG2 contents were found to be 0.33 and 0.07 mg/ml in whey.

Quantitative studies of Mach and Pahud (156) showed that while relative concentration of IgA was low, the level of IgG1 was extremely high. IgG1 represented as much as 70 to 80 percent of colostrum whey proteins, 48 to 54 percent of colostrum total proteins, while IgG2 was 40 times less abundant. On the contrary, a sharp drop in immunoglobulin concentration was observed which represented 3 percent of milk whey proteins and 0.8 percent of total milk proteins.

Kiddy et al. (137) and Butler et al. (37) determined the total IgG, IgG1 and IgA (mg/ml) on weekly samples of serum, lacteal secretions and other body fluids from 6 Holstein-Friesian cows six weeks before and four weeks after calving. A marked drop in the mean of all immunoglobulins occurred at parturition. 90 percent of the total IgG in preparatum reported lacteal samples was IgG1. The following values have been reported: the concentration of total IgG (mg/ml) was 42.6 to 0.62; IgG1, 40.8 to 0.69; serum total IgG, 24.8 to 31.0; and IgG1, 14.5 to 16.3, respectively, from 6th week prepartum to 4th week post partum. (137).

Butler et al. (unpublished data 35), Penhale and Christie (193) and Klaus et al. (141) have also estimated quantitatively the concentration of different
immunoglobulins. The respective values reported by these three workers for serum and colostrum total IgG (mg/ml) are: 22.0, 13.0 and 28.4 for serum and 37.4, 34.0 and 42.3 for colostrum.

Moll (177) studied the serum and colostrum IgG from Holstein, Guernsey and Jersey breeds. The observed colostrum IgG value reported were 52.2, 95.3 and 71.5 mg/ml for the above breeds compared to 14.5, 17.8 and 10.5 mg/ml level in serum.

The distribution of fast IgG: slow IgG ratio was calculated from involuted glands by Smith et al. (238). For colostrum milk a ratio of 11.5 : 1.0; 1.35 : 1.0 for blood serum and 3.0 : 1.0 to 5.0 : 1.0 for the whey of non-lactating quarters was determined. These ratios indicated that there is three to five times as much fast-IgG (IgG1) as slow-IgG (IgG2) in the secretion from non-lactating quarters.

Recently, Wilson et al. (237) measured the IgG1 and IgG2 concentrations in bovine colostrum and milk from 3 vaccinated and non-vaccinated mammary glands. The 1st day colostrum of non-vaccinated mammary gland gave the average values of 34.04 and 3.67 mg/ml for IgG1 and IgG2 compared to 0.29 and 0.03 mg/ml on 28th day showing thereby a sharp decline.

IgM

A protein in human colostrum corresponding to serum IgM was described by Gugler et al. (100), Hanson (165),
Tech-Huidobro (252) and Vivell (274). Using the comparative immunoelectrophoretic method, it was also observed in pre-colostrum and mature milk. Ammann and Steihm (3) found 259 mg/100 ml of IgM in initial colostrum and 10 mg/100 ml in 4 day colostrum.

The mean IgM level in sera and colostrum of 10 cows reported by Klaus et al. (141) was 2.8 ± 0.8 and 3.2 ± 1.7 mg/ml. Penhale and Christie (193) results showed an average content of 2.8 and 4.9 mg/ml for serum and colostrum, respectively. However, serum IgM (mg/ml) reported animals ranged between 3.61 - 5.06 when serum samples were analysed from 4 animals (66).

Quantiation of IgM fraction in blood serum and calf's milk has also been reported by Brandon et al. (29). The mean levels (computed from the results of the nine cows studied) were 3.87 mg/ml for blood serum compared to 0.08 mg/ml in milk. The level of all the immunoglobulin fractions was higher in mastitis milk. Thus, the concentration of IgM was 58 percent higher in whey from mastitis quarters.

Mach and Pahud (156) have estimated IgM globulin in milk, colostrum and blood sera. They observed that the level of IgM in milk was much lower (0.04 mg/ml) as compared to colostrum (4.9 mg/ml) and serum (2.5 mg/ml).

Recently, IgM concentration have also been measured in colostrum and milk by Wilson et al. (287). E.coli vaccinated glands showed a higher value of 2.18 and 0.65 mg/ml 2 and 3 days calving compared to 0.85 and 0.22 mg/ml from non-vaccinated glands. The average IgM content in milk were found to be 0.18 and 0.06 mg/ml on the 14th and 28th day after calving.