CHAPTER 2
REVIEW OF LITERATURE
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2.1 Carotenoid pigments

2.1.1 The orange yellow colour of cow milk fat is due to fat soluble carotenoids of which carotene makes by far the largest fraction (Palmer & Eckles, 1914). If the feed of the cow contains carotenoids other than carotene, some of them also appear in milk in greater or lesser amounts, since the carotenoid pigments are derived solely from the feed of the cow. The average ratio of carotene to xanthophyll in milk fat was found to be 14:1 by weight (Gillam, 1934). Nearly 94% of the total yellow colour of butterfat is thus due to carotene.

2.1.2 Gillam and Heilbron (1935), by employing chromatographic and spectroscopic methods showed that carotene was present as a mixture of \( \alpha \) (a) and \( \beta \) (B) forms, the relative amounts varying in different samples. Cryptoxanthin and lycopene were detected in small amounts in several of the more pigmented butters. Later experiments by Gillam and El Ridi (1937), in which crystalline pigment was isolated, showed that butter carotene is normally composed of \( \beta \) isomer with only minute amounts of \( \alpha \)-carotene. Although \( \alpha \)-carotene is generally absent from butterfat, if cows are fed carrots which contain about 25% of carotenoids in the form of \( \alpha \)-carotene it passes readily into the milk and butter fat (Hauge, 1942). To investigate whether lycopene,
the tomato pigment, can pass from feed into milk Gillam and Kon (1940), fed three Shorthorn cows with tomato puree and examined the resulting milk fats separately for lycopene. Lycopene was not detected in the unsaponifiable matter of butter fat when this was subjected to careful chromatographic absorption on a column of activated alumina. However, Thomson et al (1942), identified lycopene in milk fat of Guernsey cows by application of chromatography and showed that B-carotene in Guernsey milk forms a smaller fraction of the total carotenoid pigments than in milk of Shorthorn cows.

2.1.3 When cows were fed certain types of silage, pigments formed as breakdown products of xanthophyll or carotene, especially by the action of mineral acids on the silage, may also appear in milk in small amounts. It was shown by Garrett and Bosshardt (1944) that the yellow colour of milk improved by feeding alfalfa silage preserved either with molasses or phosphoric acid. Bosshardt and Garrett (1943) studied the relation of the feed of the cows to the types of carotenoid pigments in milk. Oxidative degradation products of carotene and possibly xanthophyll fraction were isolated and partially identified. Eight different bands of pigments were found chromatographically in milks produced on molasses, green oat silage and corn
silage. Seven bands were isolated from milk fat produced on green pasture. The results showed that although, xanthophyll was a predominant carotenoid in the feeds, only a small portion of this was secreted into milk.

2.1.4 As with feeds, the carotenoid content in milk varies with individual cows, season and breeds (Sharp and Hand, 1939). The average carotenoid content of milk fat from 551 samples of New York state cows was 3.4 mg per litre of melted fat in winter and 10.1 in summer. The corresponding average values for Holstein milk was 3.49 and 5.78, for Jersey 5.89 and 10.5, and for Guernsey milk 8.5 and 16.4. Individual samples of Holstein milk in June ranged from 2.72 and 10.6 and Guernsey milk from 10.4 to 20.8 mg per litre of melted fat. Singh et al (1962) estimated the carotene content of milk samples from 78 cows. The values ranged from 2.25 to 9.75 µg/50 ml of milk in 74% of the samples. The extensive literature on carotenoids in food has been ably reviewed by Borenstein and Bunnell (1967), Isler (1971) and Bauernfeind (1972).

2.1.5 Substantial amounts of carotene in milk is protein bound, the protein probably being globulin (McGillivray, 1957b). Palmer (1915) had postulated earlier a carotene albumin complex in cattle sera.

2.1.6 Though buffalo milk is richer in vitamin A than cow milk, there is no definite evidence for the presence of carotene in buffalo milk. Vermesanu et al (1958) reported a value of 500 I.U./liter in June and 340 I.U./liter
in November-December for milk of 13 buffaloes analysed, while Singh et al (1962) estimating the carotene content in the milk of 91 buffaloes reported a value of 0.48 ug/100 ml in 80% of the samples. In reporting the presence of the carotene in buffalo milk fat, the workers concerned have used colorimetric or tintometric methods to match the observed colour assuming that the yellow colour is due to carotene on analogy with cows milk fat. Sirry and El Said Saleh (1962) studied the carotene content of buffaloes milk throughout the lactation period and reported that no carotene was detectable in milk. Roy (1966) also reported the absence of carotene in buffalo milk. Carotene has not been detected even in buffalo colostrum (Narayanan & Anantakrishnan, 1959; Narayanan et al, 1952).

2.1.7 Only traces of carotene have been reported in the milk of goat and sheep (Hoidsten et al, 1948; Ray Sarkar, 1948). Chanda and Owen (1952) in experiments with lactating goats noted that the colostrum of the animals varied from orange to yellow in colour. In view of the well known whiteness of goats milk and the absence of carotenoids from it, the pigment of the colostrum was examined. It was found that the yellow pigment was B-carotene accompanied by yellow lipochrome lutein. The amount of B-carotene varied from 12 to 46 ug/100 ml of colostrum in six goats examined. The persistancy of the secretion of B-carotene in goats colostrum varied widely
in different goats. In one animal it persisted upto 11th milking. B-carotene was invariably accompanied by lutein (Chanda, 1953). However, Dikshit and Ranganathan (1955) found no carotene in goats colostrum but it was richer in vitamin A than milk. They reported that the pigments that imparted strong yellow colour to colostrum were non-carotenoid. Tests showed that these were water soluble. No carotene was found in the milk of goats following the administration of large doses of carotene by oral, intramuscular and intravenous routes. This result is in marked contrast to that reported by Chanda and Owen (1952) and is very likely due to the differences in breeds of goats used for studies.

2.1.8 The reported literature indicates wide difference in the carotene content of milk of cow and other herbivores viz., goat, buffalo and sheep. This difference is apparently due to the different levels of carotenoids in the blood plasma of cattle and other herbivora. Pandey and Roy (1966) found no carotene in the blood serum of buffaloes and goats, whereas in zebu cow the value was 1228.33 ug per 100 ml. The highest concentration of carotene recorded for Egyptian sheep was 10 ug/100 ml (Elmoty & El Malla, 1967).

2.1.9 Goodwin and Gregory (1948) demonstrated that carotene is converted to vitamin A in the gut wall and transported via the lymph to the liver where it is stored. Thus, if carotene is transported by the lymphatic route to
the systemic circulation, so by-passing the liver, its accumu-
mulation in the systemic blood would depend on: (a) the rate of absorption from the gut, (b) the rate of lymphatic transport and (c) the rate of uptake by the liver. The difference in the relative rates of these processes perhaps would explain the different levels of carotene in the blood plasma of cow and other herbivora.

2.2 Riboflavin in milk

2.2.1 Normal fresh milk emits greenish yellow fluorescence which is due principally to riboflavin (Supplee et al., 1936). The water soluble riboflavin produces a slight yellow or greenish tint in milk which becomes a distinct green-yellow fluorescence in whey after the removal of light reflecting components. The concentration of riboflavin in milk varies from species to species. Venkateswara Rao and Basu (1951) estimated the riboflavin content of Indian cows, buffaloes, goats and sheep. Sheep milk had the highest riboflavin content with an average value of $1.85 \pm 0.192 \text{ug/ml}$, the poorest being goat milk with $0.75 \pm 0.043 \text{ug/ml}$. Cow milk with a range of $1.69 \pm 0.025$ to $1.32 \pm 0.093 \text{ug/ml}$ was richer in riboflavin than buffalo milk with an average of $1.07 \pm 0.023 \text{ug/ml}$. The colostrum of all species of animals was shown to be richer in riboflavin as compared with their respective normal milk. The values, respectively, being $1.48 - 2.49 \text{ug/ml}$, $1.87 - 1.72 \text{ug/ml}$, $1.73 \text{ug/ml}$, $2.82 \text{ug/ml}$ for cows, buffaloes, goats and sheep colostrum.
Ration does not have very significant effect on the riboflavin content of milk. McElroy and Goss (1939) found that cows on a ration very poor in riboflavin had about as much riboflavin in their milk as did cows receiving normal winter rations. They further found the rumen contents of sheep and cows were much richer in riboflavin than the diet. This provided the first evidence that the rumen was an important site of synthesis of riboflavin. This was further confirmed by Hunt et al. (1941) and Crossland et al. (1958). 3-11% of the quantity of riboflavin synthesised in the rumen passes into milk (Singh & Merilan, 1959). Greater riboflavin production in the rumen does not always lead to a higher riboflavin content in milk. It appears that total riboflavin excretion in milk is limited at top by a negative correlation between quantity of milk and riboflavin content per unit volume.

Riboflavin occurs in milk in the free form and also in the bound form as flavin mononucleotide and flavine adenine dinucleotide. Nagasawa et al. (1961) found that the average total riboflavin content of mid-lactation milk was 170.2 ug/100 ml consisting of 53.5% free riboflavin, 28.5% flavin mononucleotide and 18% flavin adenine dinucleotide. The free riboflavin content of mid-lactation milk was greater than that of colostrum and late lactation milk.
2.3 Red Proteins in milk

Cows milk has been shown to contain proteins that chelate iron (red proteins). These proteins have been isolated both from whey and casein. One of the proteins transferrin has been found to be identical in blood and milk of the same animal, whilst lactoferrin is specific to milk (Groves 1960, 1965). Lactoferrin and transferrin have been found to occur in the milk of a number of species in varying concentrations. The amount of protein in milk is not related to the total iron content of the sample. (Derechin & Johnson, 1962; Masson & Heremans, 1971). There is no data available on the lactoferrin and transferrin in buffaloes' milk.

2.4 Colour fermentations in milk

2.4.1 In addition to pigments that are present in milk, numerous genera and species of microorganisms may bring about changes in colour of milk. The well known colour fermentations in milk are described by Humpriss (1953). Blue and yellow milk result from the growth and development of Pseudomonas group of negative bacilli and red milk due to the multiplication in milk of Serratia aerobic gram negative bacilli.

2.4.2 Yellow and blue fermentation

Wrede and Strack (1929) elucidated the structure of pyocyanine the characteristic blue pigment of
Pseudomonas *syncyanea*, which is responsible for blue fermentation in milk, to be oxymethyl phenazine while Kogl and Postowsky (1930), established the structure of green pigment chlororaphin, produced by *Pseudomonas chlororaphin* as the amide of phenazine -1- carboxylic acid. The yellow fermentation of *Pseudomonas synxantha* was reported to be due to elaboration of yellow pigment phenazine -1- carboxylic acid (Haynes *et al.*, 1956).

1. Pyocyanine

2. Phenazine -1- carboxylic acid

3. Chlororaphin
2.4.3 Red fermentation in milk

The red pigment of Serratia marcescens "Prodigiosin", is the only known naturally occurring tri pyrrole pigment, whose structure was first elucidated by Wrede and Rothhaas (1934) to be tripyrryl methene. Hubbard and Rimington (1950), demonstrated the existence of two readily interconvertible forms of prodigiosin depending upon the pH of the solvent. In acid solution, the pigment was red with a sharp band at 535-540 nm. In alkaline solution, the pigment was orange yellow due to a broader less intense band at 470 nm. The comparison of the spectral properties of prodigiosin with synthetic tripyrryl methene supported the structure assigned to prodigiosin by Wrede and Rothhas (1934). It was found that prodigiosin was not a single component but could be separated into at least four components, one blue and three red. Williams et al. (1956), established that these pigments were very similar though not identical. It has been shown by Wasserman et al. (1960) and Rapoport and Holden (1960), that Prodigiosin is a pyrryl dipyrryl methene and not tripyrryl methene as was earlier suggested by Wrede and Rothhas (1934).
2.5 *Colour development in Dairy products*

2.5.1 Several species of bacteria occurring in milk produce coloured colonies in dairy products. A type of discolouration of butter has been noticed by Brown (1924). It was found that in certain districts of New South Wales (Australia), in some seasons of the year, a greenish colour developed which was ascribed to presence of aphides on the trefoil pastures. Ramsay et al. (1927) noted that a green colour developed in butter due to the decomposition of proteins with the formation of melanins. The green colour developed was not due to bacterial flora. Amongst the chromogenic micro-organisms, *pseudomonas* group of gram negative bacilli produce blue black to violet pigments throughout butter (White, 1940). Greenish discolouration of rendered butter in cold stores, particularly at 18°C was studied by Godel (1956), using spectrophotometer. It was found that the agent responsible was present in the liquid fraction separated by freezing butterfat at -18°C and some indication was given that the pigment was carotene.

2.5.2 Moulds growing on the surface of butter produce a range of colours like dark bluish black (*Cladosporium herbarum*), black, brown, pink, reddish orange, yellow and green. The greens are mostly confined to spores and colour produced is that of the mycelium itself. Luck (1966) found development of greenish colour in butter when stored at -10°C for over 18 months. The discoulouration was
accompanied by a change in carotene spectra. More recently, Fisker and Hedemann (1970), noticed that a paler butter was produced when the working temperature was reduced from 13° to 15° for 75 min to 5° to 11° for 35 min. Colour defects brought about by psychrotropic organisms in butter have been reviewed by Thomas and Druce (1971). However, such colour development is generally confined to surface unlike typical colour fermentations described that extends throughout the whole mass of milk. From soft cheese, Panetsos et al. (1971), extracted a pigment which was identified as water soluble chlorophyll type III.

2.6 Colour development due to processing of milk

Milk acquires a brown colour of varying intensity when subjected to high temperature as in the manufacture of sterilized milk and condensed milk and when condensed and dried products are stored under adverse conditions. This discoloration is due mainly to the interaction of lysine, and to a lesser extent of arginine and histidine, from casein with lactose, generally known as the Millard reaction (Patton, 1955; Ellis 1959). The reaction leading to the formation of the brown pigment melanoidin is autocatalytic and accelerated by high temperature and humidity. In addition to the Millard reaction, there is also direct caramalization of lactose though it is only of minor significance (Dutra et al., 1958). The Millard reaction is activated by phosphates, metals and oxygen, as well as, by
the decomposition products of lactose, like furfural and others. On the other hand, this reaction is inhibited by sulphydryl compounds produced when milk is subjected to high temperatures (Lea et al., 1943).

2.7 Greenish yellow pigment in Buffalo milk

Buffalo butterfat isolated from sour milk is known to contain a greenish yellow pigment. A reference to this colour development in desi buffalo ghee was made by workers from Anand (Inst.Agri., Anand, 1944). It was observed that buffalo ghee obtained directly from cream was almost white, creamery ghee had a shade of greenish-yellow colour and desi ghee had a marked greenish-yellow colour. Similarly, Srinivasan and Banerjee (1946) observed that the colour of desi ghee from milk stored for 5 h was lighter than that of samples made from 12, 24 and 48 h dahi (fermented sour milk). A more detailed study of desi ghee was made by Lalitha and Dastur (1956). It was found that the colour giving property differed in milk of individual buffaloes, the variation noticed being from 0.4 to 6.6 Lovibond units (1 cm transmission), and was characteristic of the animal. The colour development was more in butterfat from animals in early and late lactation, but was not connected with the quantity of green forage ingested. Colour development occurred in milk fat from milk allowed to sour either spontaneously or after addition of a starter culture. The development of colour proceeded rapidly when milk was allowed to stand for over 4 h after production at room temperature. Raising the storage temperature to 42°C and addition of starter culture stimulated
the development of colour in the initial stages. Colour reached a maximum, but blending the extracted fat with fresh skim milk and then storing further, enhanced the colour. No particular species of bacteria could be identified with the development of colour from amongst the limited organisms tried. Butterfat in boiled milk took longer time to reach the same intensity of colour, but butterfat in sterilized milk showed only a fourth of the colour intensity of ghee from raw milk of the same batch. It was also observed by these authors that the colour development had no relation with the acidity of milk. Addition of pure lactic acid and hydrochloric acid to milk did not help to increase the colour intensity in butterfat. The colour intensity was highest in fat from the bottom layer of sour milk than from the top layer. Colourless butterfat absorbed the greenish-yellow pigment when blended with raw skim milk and allowed to sour but not when sterilized separated milk was used. That microorganisms may play a role in the development of colour was shown by the fact that butterfat in aseptically collected milk did not develop any colour even on storage for several days, nor was the colour development noticed when preservatives were added to milk.
The broad conclusion drawn by these workers were that the colour development in milk fat is probably brought about by the action of microorganisms on some unidentified constituent of milk. It was also observed that the colour was not a carotenoid.

2.7.2 Recently buffalo milk was shown to contain a dormant blue-green pigment, non-carotene in nature, that is associated with casein moiety (Daniel & Dastur, 1975). During storage and souring of milk, the blue-green pigment underwent conversion to a yellow pigment with an absorption maximum at 450 nm. This yellow pigment was shown to be identical with the yellow pigment of buffalo butterfat, obtained from sour milk.

2.7.3 Besides the formation of pigments, milk constituents undergo several changes during the fermentation with starter cultures during the formation of dahi and other fermented products. The most important change is a decrease in the lactose content by about 40 percent in the first 24 h, rising to nearly 86 percent when dahi was stored for 7 days. Lactose in buffalo milk was observed to breakdown faster than in cow milk. For example, in raw milk the disappearance of lactose was 37.4 per cent in cow milk and 53.1 percent in buffalo milk. During the storage of dahi there was a decrease in solids-not-fat content and protein percent of original milk. This was accompanied by an increase in lactic acid, volatile acids, alcohol, dialysable calcium
and phosphorous, non-protein-nitrogen and ammonia nitrogen (Khambatta & Dastur, 1950, 1951). Some of the vitamins of B-group also decreased significantly (Venkateswara Rao & Basu, 1952). On the other hand, Reddy and Shahani (1972) have observed synthesis of folic acid by lactic acid cultures in milk. A number of carbonyl compounds and volatile acids are formed during lactic acid fermentation which impart special flavour to the resulting products, like dahi. Amongst these the most important is the production of diacetyl. Other substances that occur normally are acetoin, acetone, acetaldehyde and butyric, propionic, acetic and formic acids (Merth, 1974). Micro-organisms commonly associated with milk produce antibiotics, amongst which the most noteworthy is nisin by special strains of Streptococcus Lactis (Overby, 1954). Recently, Reddy and Shahani (1971) showed that during souring of milk and ripening of cream, Lactobacillus bulgaricus also produces antibiotic material. This illustrates the large variety of biochemical changes brought about during the fermentation of milk and their importance to the dairy industry. The published literature does not throw direct light on the biochemical changes involved in the conversion of blue-green to yellow pigment in buffalo milk.

2.7.4 In the course of the present investigation, the dormant blue-green pigment of buffalo milk was identified as bile pigment biliverdin and on souring of milk it was reduced to yellow pigment bilirubin. The literature pertaining to bile pigments is outlined in the subsequent paragraphs.
2.8 Bile pigments

2.8.1 As a complement to the green pigment chlorophyll of the vegetable kingdom, haemoglobin that gives red colour to the blood is of special importance as the oxygen carrying pigment in animals. The catabolism of haemoglobin and resulting rupture of protoporphyrin IX, the prosthetic group of haem, gives rise to iron free open chain tetra pyrrolic compounds, the most immediate one being the green pigment biliverdin. Biliverdin($H_{34}^4$) on reduction gives rise to yellow pigment Bilirubin ($H_{36}^6$). The free bile pigments bilirubin and biliverdin, formed mainly in the reticuloendothelial system of the liver, are water insoluble and they are converted to water soluble conjugates by esterification with glucuronic acid. In the human system, both biliverdin and bilirubin are waste products of metabolism and through a series of reduction and oxidations are excreted by the system as stercobilin or urobilin as per the broad scheme outlined.
Biliverdin (H$_{34}$)

$\xrightarrow{+H_2}$ Bilirubin (H$_{36}$)

Dihydrobilirubin (H$_{28}$)

$\xrightarrow{+H_2}$ +2H$_2$ $\rightarrow$ Urobilinogen (H$_{42}$) +2H$_2$

$\xrightarrow{+H_2}$ Mesobilirubin (H$_{40}$)

$\xrightarrow{+H_2}$ Mesobilirubinogen (H$_{44}$)

Urobilinogen-d (H$_{42}$) $\xleftarrow{-H_2}$ +2H$_2$

Urobilin-i (H$_{42}$) $\xrightarrow{-H_2}$ Autoxidation

Stercobilin-i (H$_{46}$) $\xrightarrow{-2H_2}$ Autoxidation

Urobilin-i (H$_{42}$)

**Metabolism of biliverdin in mammals**
Garner (1955) showed that the fate of bile pigments in cattle was similar to that in man. There are two current theories on the mechanism by which haemoglobin and its derivates are catabolised to the first detectable bile pigment, biliverdin. One holds that the process is enzymic while according to another, non-enzymic coupled oxidation of haem compounds followed by oxidation and hydrolysis are the major steps involved. According to Lamberg (1956), the methine bridge of protoporphyrin is first oxidised in situ by addition of hydrogen peroxide. During this process, the protoporphyrin remains linked to globin. By this oxidation choleglobin (Green intermediate) is formed. During biliverdin formation, the iron and the protein is split off from choleglobin. Biliverdin is then converted to bilirubin. On the other hand, Nakajima and collaborators (1958, 1963) found that the principal route of haemoglobin breakdown in vivo was initiated by formation of the haemoglobin-haptoglobin complex which is broken down by the enzyme haem-a-methylenyl oxygenase resulting in the formation of a substance absorbing at 656 nm. This 656 nm substance has a prosthetic group which is opened at a-bridge, contains a formyl group there, and is still bound to haptoglobin and iron. Formyl biliverdin iron complex is converted to biliverdin by the enzyme haem-a-methenyl-formylase according to the following scheme:
2.8.2 Biliverdin has four isomers $a$, $B$, $r$ and $d$ depending on which methine bridge of protoporphyrin IX is opened. It is now accepted that the naturally occurring tetrapyrrole bile pigments have a IX$_{a}$ structure, that is they are derived from biliverdin which is produced biologically by the rupture of $a$-methine bridge of protoporphyrin (Gray et al., 1958). The only natural bile pigment so far to be shown not to have the IX$_{a}$ substituent orientation, occurs in the integumental cells of the caterpillar of the cabbage white butterfly. (Rudiger and Abolins, 1966).
2.9 Bile pigments in blood

2.9.1 Biliverdin normally occurs in the bile of herbivorous animals. Although it is the primary breakdown product of haemoglobin, it is never present in normal sera. It is conceivable that at least traces of biliverdin can be found in icteric sera (Larson et al., 1947). There are no reports about the occurrence of biliverdin in the milk of the buffalo, the cow and the goat. On the other hand, bilirubin normally occurs in blood serum of animals. In human beings, the bilirubin value varies widely from day to day, with the time of sample collection (being high after fasting) and also depends on muscular activity. It has not been established definitely if the values are governed by hereditary factors. The value for bilirubin in blood serum runs parallel with the serum iron content, values below 1 mg/100 ml serum generally being taken as normal. Rudra (1946) was not able to detect bilirubin in normal and vitamin A deficient calf sera. A high bilirubin content (from 1.0 to 7.4 mg/100 ml) was found in horses to coincide with high vitamin A and carotene reserves. Soliman and Amrousi (1965) studied bilirubin content in blood of 348 Egyptian buffaloes of different age groups. The average values found were:
For a normal cattle, a range of $0.14-0.34$ (avg. 0.26) mg/100 ml was noted for 9 animals by Agarwala et al. (1962) while for 3 sheep, the average value was $0.34$ mg/100 ml of blood plasma. The values rose to 7.70 (range 4.22 to 10.76) in cattle with induced jaundice and to 5.22 (range 0.64 to 8.17) in sheep under similar circumstances.

2.9.2 Bilirubin in blood is bound to a protein. Bennhold (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. In highly icteric serum, he could detect a small bilirubin fraction which was localised before the albumin. Coolidge (1942) found that practically the entire bilirubin of icteric sera can be precipitated with ammonium sulphate. He found that precipitation occurs together with the albumin fraction and only insignificant amounts with globulins. Ostrow and Schmid (1963) using $^{14}$C labelled bilirubin of high specificity found that in all instances, at the pigments concentrations ranging from physiological levels to the limit of bilirubin binding capacity of serum $^{14}$C bilirubin migrated only with albumin.
Bile pigments though first identified in higher vertebrates are widespread in nature. Various members of bilichrome series are encountered in red and blue green algae. Phytochrome, a photomorphogenetic pigment of wide distribution in the plant world, is a biliprotein with a bile pigment prosthetic group (Siegelman et al., 1966). Bile pigments are also widely distributed among invertebrates. Biliverdin is found in the integumental cells and haemolymphs of insects such as praying mantis, locust and grasshopper (Siegelman et al., 1966), also in blue coral heliopora coerulea (Rudiger et al., 1968) and in the bones, fins and skin of certain fish (Fox and Milloft, 1954). Bile pigment biliverdin, occurs in the root nodules of certain plants (Viratanen and Miettan, 1949). Lemberg (1932, 1934) and Tixier (1945) identified biliverdin in the egg shells of gulls and other birds. Aplysiosviolbin, the prosthetic group of a chromoprotein component of purple secretion of Aplysia limacina, the sea hare is also a bile pigment (Rudiger, 1967). A bilidiene containing biliprotein has been reported in the ripe ova of key hole limpet (Bannister et al., 1968). The importance of bile pigments in nature where they play dormant and active roles is evidenced by the literature, certain phases of which have been discussed and reviewed by Lemberg and Legge (1949), Gray (1953), With (1968) and, Hudson and Smith (1975).
2.11 Scope of present studies

2.11.1 From the above review of literature, it is seen that a number of pigments are associated with milk and blood serum. Besides the differences exhibited by species, individuals of the species exhibit marked variations due to stage of lactation, feed, season etc. Alongwith major pigments like carotene and riboflavin, a number of species of bacteria which occur as contaminants often impart colouration to milk and milk products. However, a major biological pigment of buffalo milk has attracted very little attention. No attempts have been made regarding its quantitative estimation, elucidation of its chemical nature and its biological role. Perhaps the difficulty of extraction and assessment might have been deterrent factors. It is also probable, compared to the intense colour of carotenoid pigments of cow butter-fat, the greenish yellow colour of buffalo milk fat, was apparently mistaken to be of low quantitative significance (instead of a major significant biological pigment as revealed by the study in depth reported in the present thesis).

2.11.2 In course of the present study, biochemical studies on the greenish-yellow pigment formation in
buffalo milk have been carried out and the following aspects are dealt with:

1. Extraction, purification, estimation and characterisation of the pigments.
2. Role of microorganisms in pigment formation and biochemical changes involved.
3. Association of pigment with milk proteins.
4. Effect of heat processing of buffalo milk on the pigment.
5. Source of the pigment.