3. PIGMENTS IN MILK AND MILK PRODUCTS

3.1. Natural pigments:

It is known that pigmentation in milk varies from species to species. Cow's milk is rich in natural colour but comparatively goat and sheep milk have low concentration of pigment, while buffalo milk fat is entirely devoid of pigment.

3.1.1. Fat-soluble pigments:

The yellow colour of cow's milk is due to the presence of fat-soluble carotenes, among which β-carotene is the main component. Carotenes are derived from food and diet rich in green feeds. The intensity of colour in fat varies not only with the nature of feed but with the breed of the animal and the season. McGillivray (1957a) showed that the bulk of the carotene secreted in cow's milk was derived mainly from carotene of immediate dietary origin and not from the plasma. The amount of carotene transmitted from pasture to milk was also dependent on the nature of pasture lipids (MacDowell & McGillivray, 1963). Studying the differences in the high carotene values obtained by solvent extraction method compared to the boiling-off process, it was suggested that substantial amounts of both carotene and vitamin A in milk were protein bound, as in blood, the protein probably being globulin (McGillivray, 1957b).
3.1.2. The yellow pigment of cow's milk fat is 90% carotenes \((C_{40}H_{56})\) and the rest xanthophyll \((C_{40}H_{56}O_2)\). Along with β-carotene, milk contains a number of other pigments. β-Carotene makes up to 50% of the total vitamin A potency of cow's milk, the exact amount depending upon the type and amount of green feed ingested by the animals. With certain feeds, carotenoids appearing in milk are completely inactive as pro-vitamin A due to the absence of the β-ionone system. For example, α-carotene appears to the extent of 25% of the total carotenoids when animals are fed carrots. Yellow turnip increased the concentration of lycopene \((C_{40}H_{26})\), lutein \((C_{40}H_{56}O_2)\), xanthophyll \([C_{40}H_{56}(OH)_2]\), and zeaxanthin \((C_{40}H_{25}OH)\), neo-β-carotenes and stereoisomers of β-carotene containing cis-bonds (Gillam & Heilbron, 1935; Zschelle et al., 1944).

3.1.3. Though buffalo milk is rich in vitamin A (on account of its high fat%), there is no definite evidence for the presence of carotene. Carotene has not been detected even in buffalo colostrum, unlike in the case of goat, where in some cases presence of carotene has been shown in colostrum and early lactation milk, but not in normal milk. In reporting presence of carotene in buffalo milk fat, workers concerned have used colorimetric or tintometric methods to match the observed colour, assuming that the yellow tint was carotene on the analogy of cow's milk fat (Narayanan & Anantakrishnan, 1959; Singh et al., 1962). The only spectrophotometric study has been by Roy (1966). It was
found that in diethyl ether extract of buffalo milk, the 
characteristic maxima at 322, 335, 425 and 480 nm observed for 
cow's milk were absent. Buffalo milk fat showed maxima at 333 
and 345 nm.

3.1.4. Like buffalo milk, milk of goat is also white in colour. 
It has, however, been shown spectrophotometrically, after 
chromatographic purification of the extracted pigments, that 
goat colostrum fat contains β-carotene and traces of lutein 
(Chanda & Owen, 1952). It is interesting to note that in their 
studies on carotene metabolism in goats after giving massive doses 
of carotene in various forms, Goodwin & Gregory (1948) failed to 
detect carotene in the blood, though vitamin A increased in lymph. 
These workers found a non-carotenoid yellow pigment showing 
maximum inflexion at 433-434 nm and a very small one at 590 nm. 
The pigment was destroyed by treatment with alkali. Later, 
Dikshit & Ranganathan (1955) also observed that when massive 
doses of carotene were administered to goats by oral, intra-
muscular and intravenous routes, goat colostrum showed strong 
yellow colour due to the presence of a non-carotenoid pigment.

3.1.5. **Water-soluble pigments:**

Milk also contains water-soluble pigment riboflavin which 
gives a greenish-yellow colour to whey. Riboflavin occurs in milk 
mostly in free form, and in the bound form as flavin-mono-
nucleotide and flavin-adenine-dinucleotide, wide variations having
been observed in free and bound forms (Modi & Owen, 1956; Manson & Modi, 1957). Work in Egypt and India has demonstrated cow's milk to be richer in riboflavin content than buffalo milk (El-Rafey, 1962; Venkateshwara Rao & Basu, 1951; Boman, 1953).

3.1.6. "Red Protein" in milk:

Milk has also been shown to contain coloured proteins in trace quantities that chelate iron (red proteins). One of the proteins, transferrin, has been found to be identical in the blood and milk of the same animal, while lactoferrin is specific to milk (Groves, 1960, 1971). 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 & Heremann, 1971). An interesting property of the red proteins is that they are responsible for the bacteriostatic properties of milk (Oram & Reiter, 1966, 1968; Nutr. Rev., 1972). Though there are no data on lactoferrin and transferrin in buffalo milk it was observed by Natarajan & Dudani (1961), Anclair (1968) and Patel (1969) that buffalo milk has better bacteriostatic property than cow's milk.

3.2. Developed Pigments:

In addition to the natural pigments present, milk also develops pigments under varying conditions. A number of genera and species of microorganisms occurring in milk produce coloured
colonies. Among chromogenic microorganisms, *Pseudomonas* group of gram negative bacilli produce "blue milk" and also discolour butter (White, 1940). *Pseudomonas* species has been shown to produce a yellow filament on dairy equipment (Maxcy, 1973). *Pseudomonas fluorescens* produce a water-soluble greenish-blue pigment which shows fluorescence at low temperature. *Serratia*, aerobic gram negative bacilli, produce a characteristic red pigment; while *Sarcina* gram positive cocci, produce a yellow pigment (Humphriss, 1953). A number of organisms also synthesise carotenoid pigments (Liaaen-Jensen, 1965; Liaaen-Jensen & Andrews, 1972). Pigments mentioned above are not normal constituents of bovine milk but are contaminants.

3.2.1. Milk acquires a brown colour of varying intensity when subjected to high temperature as in the manufacture of sterilised milk and condensed milk, and when condensed and dried products are stored under adverse conditions. This discolouration is due mainly to the interaction of lysine, and to a lesser extent of arginine and histidine from casein with lactose to exhibit the Maillard 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 Maillard reaction, there may be direct caramalisation of lactose, though this is only of minor significance (Dutra *et al.*, 1959). The Maillard reaction is activated by phosphates, metals and oxygen, as well as, by the decomposed
products of lactose like furfural, etc. On the other hand, this reaction is inhibited by sulphydryl compounds produced when milk is subjected to high temperatures (Lea et al., 1943). Fluorescent substances are also formed during the decomposition of lactose (Simonson & Tarassuk, 1952).

3.2.2. Ramsey et al. (1927) noted that a green colour developed in butter during storage due to the decomposition of proteins with the formation of melanines. The colour development was not due to microbial action. A different type of discolouration of butter was noticed by Brown (1924) who found that in certain districts of New South Wales (Australia), in some seasons of the year, greenish colour developed, which was ascribed to the presence of aphides in the trefoil pastures. Lück (1966) observed development of greenish colour in dry butterfat, and other fats containing carotene, when stored below −10°C for over 18 months. The discolouration was accompanied by a change in carotene spectra. The colour change in butterfat was arrested by the addition of antioxidants, or by heating the butterfat with its solids-not-fat at 110°C. The results indicate a reaction of carotene with some products of fat oxidation. More recently, Fisker & Hedemann (1970) noticed that a paler butter was produced when the working temperature was reduced from 13°-15°C for 75 min to 5°-11°C, for 35 min.

3.2.3. Panetsos et al. (1971) extracted a pigment from soft cheese with 75% ethyl alcohol and diethyl ether (2:1). On further
purification, it was identified as water-soluble chlorophyll type III.

3.3. **Pigment in desi buffalo ghee:**

3.3.1. The paucity of literature on the development of a fat-soluble pigment in buffalo milk is due to the fact, that, the production of buffalo milk is limited to a few countries of the world. The use of buffalo milk being restricted, has failed to invite extensive investigations on the colour development. A reference to the colour development in desi buffalo ghee was made by workers from Anand (Inst. Agric.–Anand, 1946). It was stated that buffalo ghee obtained directly from cream was almost white, creamery ghee had a shade of greenish-yellow pigment, and the desi ghee had a marked greenish-yellow colour. Similarly, Srinivasan & 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 & 48 h dahi. A more detailed study of the pigment in desi ghee was made by Lalitha & Dastur (1956). It was found that the colouring property differed in milk of individual buffaloes, the variation noticed being from 0.4 to 6.6 Lovibond units (1 cm transmission), and was a 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 when milk was allowed to sour either spontaneously or after addition of a starter culture. Aseptically collected milk, or
milk subjected to sterilisation temperature, did not develop pigment. Similar was the case when milk was preserved with preservatives. The colour development took place over a wide range of temperatures from about 4°C to 56°C and the pigment intensity reached a maximum in about 24/48 h. Further enhancement of pigment intensity was noticed when the butterfat was emulsified with fresh lot of skim milk and stored. The pigment in butterfat was shown to be a non-carotenoid. From these studies the authors have concluded that the pigment development took place in milk by the action of microorganisms on an yet unidentified constituent of milk.

3.4. Other characteristics of desi ghee:

3.4.1. Apart from the pigmentation of butterfat during the process of souring of milk, certain other differences have also been noted in several studies. Doctor et al. (1940) found that the curd-process ghee had a firmer texture than the cream-process ghee, both being from the same bulk milk. The Reichert value of curd-process ghee was lower, though the Polenske and saponification values of the samples by the two different processes did not vary. Buffalo butterfat obtained by the indigenous method contained 0.398 per cent unsaponifiable matter, whilst cow butterfat showed an average value of 0.450. Cholesterol accounted for 77.6 per cent of the unsaponifiable matter in the case of cow and 70.0 per cent in the case of buffalo butterfat (Bindal & Jain, 1973a).
In a further study by the same authors, it was shown that desi buffalo ghee contained 10.2 per cent less cholesterol than the creamery ghee, whilst cow ghee samples showed a difference of 8.1 per cent (Sindal & Jain, 1973b). The average values of 10 trials for buffalo butterfat as such was 294 mg/100 g, and when extracted by the creamery process 276 mg and by the desi process 264 mg/100 g. Differences have been noted in the phospholipid content also. In ghee prepared by the creamery process, the average phospholipid content was 0.232 and 0.206 per cent in buffalo and cow ghee, respectively. The corresponding values in fat isolated by the indigenous method from the same lot of milk were 0.155 and 0.178 per cent, respectively, that is a decrease of 33.2 and 13.6 per cent (Rama Murthy & Narayanan, 1966).

3.4.2. With the above background it was of interest to study the influence of the pigments developed on the keeping quality of ghee. Almost one-third of the fatty acids of buffalo butterfat are unsaturated (Anantakrishna et al., 1947) and thus susceptible to oxidation and rancidity development under the influence of factors such as air, heat, light and metals like copper and iron. Development of rancidity gives rise not only to off flavours, but also results in the destruction of vitamin A content. The extensive literature on rancidity in edible fats has been reviewed by Lea (1939). Subsequently, Farmer (1946) postulated a new dynamic theory of autoxidation. Though the actual initiation of the reaction is yet imperfectly understood, it
has been assumed that initially oxygen attaches itself in a loose linkage to a double bond. This results in the activation of the adjacent -OH₂ group, from which a hydrogen atom or proton gets loosened. It draws the oxygen atom towards it to form not a peroxide but a hydroperoxide, creating in the process, a new double bond at a different place that has a trans configuration as opposed to the configuration of natural unsaturated fatty acids. Heaton & Uri (1961) believe that the presence of metal catalysts in traces is essential for autoxidation to be initiated. These react with molecular oxygen and give it a negative charge, making possible its further reaction with unsaturated fats. A new concept for the initiation of autoxidation was proposed by Khan (1965) according to which the electronic structure of oxygen is favourable for formation of transition cyclic pi-complexes with a group of carbon atoms in the vicinity of the double bond. Such complex formation allows free movement by reducing the electron density and explains better than the free radical concept, the shift from a cis to a trans double configuration, which occurs during autoxidation.

3.4.3. Butterfat contains a number of unsaturated fatty acids like oleic, linoleic, linolenic, and others of higher molecular weight. Besides, milk has as its natural constituents, hemoproteins, which bring about spontaneous non-enzymic oxidation, studied by Aurand & Woods (1959), with xanthine oxidase. Eriksson et al. (1970) and Eriksson (1970) have shown similar effect of catalase and lactoperoxidase. Both native and denatured
lactoperoxidase accelerated the development of aldehydes from linoleic acid. Catalase also increased oxidation of linoleic acid. Though the presence of cytochrome b5, a membrane heme protein and susceptibility of milk samples to lipid peroxidation could not be correlated, Gregory et al. (1976) have listed it as a suspect in its catalytic involvement. Buffalo milk has been found to contain higher amount of lactoperoxidase, though its xanthine oxidase activity is lower than in cow's milk. Catalase and cytochrome c reductase are present in almost similar amounts in the milk of both species (Rifaat et al., 1968, 1971).

3.4.4. Ghee contains natural antioxidants like tocopherols, in common with other animal fats. Other forms of tocopherols, all with antioxidant properties occur in vegetable oils, usually in much larger amounts than in ghee. A few vegetable oils also contain specific materials with antioxidant activity. Sesame oil carries powerful natural antioxidants sesamin and sesamolin; cotton seed oil contains a yellow pigment, gossypol, with marked antioxidant properties. Likewise many natural pigments are weak antioxidants. The influence of antioxidants is enhanced by the simultaneous presence of synergists which act as metal scavengers that tie up trace metals, inhibit their pro-oxidant activity and allow the primary antioxidant scope to function. Phospholipids are natural synergists which occur in fats, including ghee. Citric acid, phosphoric acid and ascorbic acid are well established synergists which are often added along with primary antioxidants.
to fats. Phospholipids constitute about 0.2 to 1 per cent of milk fat. Narayanan et al. (1966) found the phospholipid content of ghee to vary with the temperature and time of heating, the values rising with both the factors. In ghee prepared at 120°C, the phospholipid content in six samples varied from 0.8-10.7 mg/100 g fat. When the holding time was increased to 10 and 20 min, the values were 42.0-66.6 and 95.3-125.9 mg/100 g ghee, respectively. Samples with higher phospholipid content showed higher keeping quality supporting the earlier observation of Persai & Barnicoat (1949-50). In this connection El-Rafey et al. (1944) had drawn attention that the influence of sulphhydrils formed by subjecting the protein residue to high temperatures should be considered. The increase in keeping quality of ghee has been attributed to the liberation of lecithin and other phospholipids from the proteins at high temperature. Pruthi et al. (1971) showed that only cephalin fraction of the butterfat phospholipids has antioxidant effect when added at 0.05 per cent level. Heat treatments of butter and cream lead to release of phospholipids from lipoprotein combinations and their passage into the fat phase. As severe heat treatments can cause injury to the flavour and colour of fat, the almost equally effective procedure of heating to 90°C in the presence of secondary phosphate was suggested by Dobers (1969) as a promising alternative. Recently Cornell et al. (1971) made an interesting observation that added antioxidants were bound both to the protein and fat phases of milk.
3.4.5. The combined influence of natural antioxidants and synergists in ghee is not sufficient to withstand the severe conditions encountered in its manufacture and sale in packed tins or loose. A number of synthetic antioxidants have, therefore, been recommended and used from time to time amongst which the most important ones are: nordihydroguaiaretic acid (NDGA), hydroquinone, gallates (ethyl, octyl and dodecyl), tocopherol, wheat germ oil, cereal flours, flavones (dihydroquercetin, rutin, quercitrin), butylated hydroxyanisole, butylated hydroxytoluene, lecithin in combination with citric, phosphoric and ascorbic acids. Under the food laws in India, the antioxidants permitted in ghee are BHA and BHT, either singly or in combination, in a concentration not exceeding 0.02 per cent. It is not known if the pigment developed in desi ghee has any antioxidant properties to effect the storage life of ghee. This aspect was, therefore, examined in course of the present studies.

3.4.6. Objectives of the study and plan of work:

The above review of the literature has shown special properties of buffalo milk amongst which the one most noticeable is the apparent lack of pigment in fresh milk, and its development in butterfat prepared from fermented milk. In course of the present study, an attempt was made to investigate the yet unidentified constituent of milk which acts as the precursor for the pigment developed by desi ghee, as well to study the mechanism of this conversion in milk during souring. It was also of interest
to examine the milk of other species like cow and goat, to see whether similar pigment systems were present. The variations in the concentration of the pigment with factors like the stage of lactation, season and feed were studied to get an idea about the source of the pigment in milk. Finally the effect of the pigment on the shelf-life of desi ghee was studied to see whether it imparts any antioxidant properties to the fat.