10 SUMMARY

10.1 Buffalo milk was shown to contain green bile pigment biliverdin. It was found to be a normal constituent of milk and did not represent any abnormal condition of the animal. The concentration of biliverdin in Buffalo milk was found to be greater than the concentration of B-carotene that is normally present in cow milk.

10.2 During fermentation of milk, the central methine group of biliverdin was reduced with the resultant formation of a yellow pigment, bilirubin. Bilirubin so formed was extracted by the milkfat, and this accounts for the colour developed in buffalo butter fat during souring of milk.

10.3 Natural reducing systems of milk did not reduce the central methine group of biliverdin in milk. This was demonstrated using aseptically drawn milk.

Biliverdin did not undergo reduction in milk when incubated with preservatives. Hydrogen peroxide, formaldehyde and mercuric chloride completely inhibited the formation of bilirubin. The added preservatives, however, did not impair the precursor biliverdin in milk.
The reduction of biliverdin in milk was caused by the metabolic activities of the bacteria. All the 14 pure bacterial cultures studied were shown to reduce biliverdin to bilirubin in 24 hours. The reduction proceeded normally at 10°C also, provided organisms capable of growing comparatively rapidly at this temperature were present. The possible mechanism has been discussed.

All acids added, viz., citric, succinic, fumaric, lactic and acetic acids at concentration of 0.15% stimulated the accumulation of bilirubin in milk fat. Even mineral acid (H₂SO₄) when added to such an extent so as to bring the pH of milk around 5.6 enhanced the yellow colour in milk fat. Though the stimulation was little, it was very consistent. A reasonable explanation for this finding has been given.

10.4 Based on the above findings, the state of occurrence of biliverdin and bilirubin in milk has been discussed.

10.5 Biliverdin had no effect on the reduction of either methylene blue or resazurin added to milk.

10.6 Heat treatment of milk had an adverse effect on bilirubin accumulation in skim milk. The cause of this effect has been established. It was not due to destruction of bacterial activity in milk but, due to non-availability
of precursor 'biliverdin' in heat treated milk. The amount of biliverdin in heat treated samples showed the following gradation, raw milk = pasteurised milk > boiled milk > milk subjected to temperature of about 100°C for 1 hour > milk subjected to temperature of 120°C under pressure. Autoclaved milk samples almost showed no biliverdin absorption. Biliverdin was also not found in milk exposed to 65°C over long intervals.

Authentic biliverdin (Sigma) added to autoclaved milk underwent conversion to colourless compound during incubation of milk with or without starter. The colourless compound was identified as urobilinogen based on the colour reaction with Para dimethyl amino benzaldehyde reagent. It was thus demonstrated that the potent reducing systems that are formed during the heat treatment of milk, rapidly act on biliverdin to reduce it to a colourless urobilinogen. The mechanism of reduction in heat treated milk has been discussed.

10.7 Biliverdin obtained by solvent extraction (acetone) of buffalo milk was found to be associated with proteins. The spectra of the green protein in 80% aqueous acetone revealed only a single absorption maximum at 660 nm, unlike free biliverdin which showed two absorption maxima at 380 nm and 660 nm. The usual absorption maximum of biliverdin at 380 nm was completely quenched when bound to protein.

Polyacrylamide gel electrophoresis of the protein revealed it to be heterogeneous. The protein was purified
by gel filtration on Sephadex G-200. Biliverdin was shown to be associated with a minor component of the protein. The fractionated protein sedimented as a homogeneous component with a sedimentation coefficient of 13.99 S in 0.05 M tris-HCl buffer containing 0.1M sodium chloride at pH 9.0.

The purified protein fraction showed 0.437% phosphorous, 0.136% iron. Carbohydrates were found to be absent.

A similar protein (brown coloured) was isolated from cow milk by solvent extraction of casein. However, the concentration of the protein was less as compared to the concentration in buffalo milk.

10.8 A rapid and simple method of biliverdin estimation in milk with good reproducibility and recovery has been described. The biliverdin content of the milk of same animal showed wide variations from day to day.

The level of bilirubin in the blood plasma of buffaloes exerted no influence on the biliverdin content of buffalo milk. This perhaps excludes the possibility of direct uptake of plasma bilirubin by the mammary gland.

Normal cow blood plasma showed average bilirubin content of $127.2\pm21.15$ mg/100 ml approaching the value of bilirubin in buffalo blood plasma ($156.1\pm42.11$ ugs/100 ml). Hence, the wide difference in the biliverdin content of milk of these two species, could not be ascribed to the different levels of bilirubin in blood of these animals.