STUDIES ON FOLIC ACID CONTENT AND FOLATE BINDING CAPACITY OF MILK

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

IN

DAIRYING

(HUMAN NUTRITION AND DIETETICS)

TO THE KURUKSHETRA UNIVERSITY

KURUKSHETRA

BY

VEENA SINGH

DIVISION OF HUMAN NUTRITION AND DIETETICS

NATIONAL DAIRY RESEARCH INSTITUTE

(I. C. A. R.)

KARNAL (HARYANA) INDIA

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Registration No. 80-DK-50

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DEDICATED TO MY
LATE GRANDMOTHER
MRS. MATHURA DEVI

Dr. M.K. Sharma,
Scientist S-1

DIVISION OF DAIRY CHEMISTRY
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Karnal (Haryana)

Dated: 22nd February,1983

I certify that the thesis entitled "STUDIES ON FOLIC ACID CONTENT AND FOLATE BINDING CAPACITY OF MILK", submitted in partial fulfilment of requirements for the degree of MASTER OF SCIENCE (DAIRYING) IN HUMAN NUTRITION AND DIETETICS to the University of Kurukshetra, embodies the result of a bonafide research carried out by MISS VEENA SINGH under my guidance and supervision and no part of this has been submitted for any other degree or diploma.

I further certify that such help or source of information as has been availed of in this connection, is duly acknowledged by her.

MKSharma

(M.K. SHARMA)

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I thank all my friends - Nagesh, Dinakar, Shashi, Manja, Dolly, Reba, Amita, Sujatha and Sasikala for helping me out as and when required.

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Lastly, but not the least, I would like to thank Bhagwant, my husband, for his constant incitement.

Veena Singh

(VEENA SINGH)

KARNAL
February 22, 1983.

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CHAPTER I

INTRODUCTION

INTRODUCTION

Milk is sole natural food of human infants for the first few months of life. The nourishing of babies on processed milk is widely practiced in developed countries. However, in under-developed countries the choice remains to use milk available from dairy animals as food for infants largely due to higher prices of milk formulations and their low availability. According to Ford (1974) the breast feeding had certain advantages over bottle feeding. In that the former practice eliminated the chances of infection and made possible the relatively higher bioavailability of the nutrients present in the milk. The protecting agents against infection were recognized (Chandra, 1976) as metalloproteins like lactoferrin and transferrin in milk which act in combination with specific antibodies to check the growth of pathogenic organisms.

Like iron, certain other metals like zinc and copper are also found in complex form in the milk (Evans, 1980; Hurley and Lönnerdal, 1982). Similarly, some vitamins though micro-nutrient but of vital importance, like folic acid and cyanocobalamin have been found to be present in bound form. These vitamin binding factors were identified as minor whey proteins with a capacity to bind added folic acid

(Ford *et al*., 1969). It was further reported that these binding proteins influences the growth and composition of gut microflora (Ford, 1974). These folate-binding proteins were, therefore, thought to perform mainly two functions: first, that they may act in mammary gland as trapping mechanism to accumulate vitamins from blood and second, that subsequently in gut of infant they may facilitate the absorption of vitamins.

The vitamin-binding capacity was reported to vary among milks from different mammalian species (Ford and Scott, 1975). The human milk contained about 0.3 ng of vitamin B_{12} per ml and had the capacity to bind 80 ng additional of vitamin B_{12} per ml. Cow's mature milk contained about 3 ng vitamin B_{12} per ml and had little or no capacity to bind added vitamin B_{12}. The milk from goat was found to contain 6 ng of folic acid per ml as against 50 ng/ml in human and cow milk. The mature human and cow milk could bind about 60 ng folic acid per ml (Ford *et al*., 1972). The report is not available for buffalo milk in this regard. It appeared that a functional relationship exists in vitamin-binding proteins and vitamin content of milk.

Little information is available about the effect of pH on vitamin-binding activity of milk (Ghitis *et al*., 1969; Givas and Gutcho, 1975). The heat treatment was also found to inflict some loss in total folate content (Ghitis, 1966).

Jenness (1974) found a wide variation in the composition of milks in different species. The stage of lactation was found to have added effects on composition of milk even from one species (Jones, 1980). It was observed that there is increase in lactose and fat content in human milk with increased lactation period. In contrast, there was a decrease in protein nitrogen, zinc and copper in milk as the lactation advanced. Ford *et al*. (1972) reported a sharp fall in concentration in goat milk during early days of lactation.

In view of the above points, an interest was developed to have comparative study of milks from cow, buffalo and goat with following objectives:

i) estimation of free and bound folic acid in milk at different stages of lactation;

ii) to find out the total folate-binding capacity of milk at these stages of lactation;

iii) to study the effect of pH and heat treatment on folate-binding capacity of milk.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.0 HISTORY OF FOLIC ACID

McCarrison (1921) noted that monkeys maintained on a diet deficient in 'vitamin B' developed diarrhoea and anaemia. Lucy Wills (1934) recognized that there was a factor in yeast and crude liver extracts but not in the refined types which would relieve tropical macrocytic anaemia. She reported a similar clinical picture in monkeys on a polished rice, margarine, fruit diet and showed that same crude extracts of liver and yeast would eliminate the pathology. This factor was called the "Wills Factor". Day et al. (1938) induced a syndrome of anaemia, leukopenia, diarrhoea and stomatitis in monkeys. Later, they called this vitamin M deficiency and recognized that it was similar to sprue.

Stockstad and Manning (1938) described a growth factor for chicks which they named vitamin U. Hogan and Parott (1939) reported observations on a factor which they called vitamin B_c an anti-anaemic substance for chicks. Snell and Peterson (1940) described a new growth factor for lacto-bacilli, the Lacobacilli casei factor. Mitchell et al. (1941) obtained the substance in concentrated form from deep green leaves. This source suggested the name Folic Acid.

Pfiffner (1943) related folic acid to the chick factor, vitamin B_c. Folic acid was obtained from liver and was synthesized by Angier et al. (1945). This report brought to successful conclusion years of searching for a factor necessary for the growth of many species of animals and micro-organisms, and for the protection of man, monkeys, rats, chicks and ducks against leukopenia and anaemia. Day et al. (1945) successfully treated vitamin M deficiency in monkeys with folic acid. Soon it became apparent that all these factors were one and the same substance.

2.1 OCCURRENCE OF FOLIC ACID

Folic acid is a yellow crystalline compound that occurs widely distributed in nature, especially in the green foliage, hence the name. The richest food sources are found in chicken livers and vegetables such as asparagus, broccoli, endive, leaf lettuce and spinach. Liver, legumes and other green-leafy vegetables are good contributors of the vitamin.

Milk has a very low folic acid content although Welch and Wright (1944) failed to produce folic acid deficiency in rats when a milk diet was used in conjunction with a poorly absorbed sulphonamide such as succinyl sulphathiazole. They suggested that milk contained a substance itself micro-biologically inactive, which served as growth factor for rats.

Cooperman et al. (1946) observed that milk was a good source of monkey anaemia factor. Most of this was present in the skim milk and hardly any in the cream; raw whey was also a good source of the factor. Milk, as well as liver and certain grains, were good sources of the Streptococcus lactis R factor, but leafy materials were, in general, poor sources of both. Good correlation on anaemic monkeys and their effect on Streptococcus faecalis R, but correlation was less satisfactory with purified materials. Hodson (1949) confirmed the presence of a conjugated form of folic acid, assaying milk micro-biologically after digestion with chick pancreas conjugase.

The folate content in the sow and buffalo milk was apparently much lower than that in human milk and cow's milk, which contained about 50 ng/ml (Ford et al., 1969).

2.2 VITAMIN BINDERS

a) Folate Binder

i) In Milk

Metz and Herbert (1967) observed that milk had a maximal binding capacity for pteryl-glutamic acid and that the binding of fo lates in milk was stronger than in serum. Metz et al. (1968) further said that dextrancoated charcoal removed pterylqutamic acid bound to serum more rapidly than that bound to skim milk or human milk, thus, confirming that folate was much more tightly bound in milk than in serum. Ghitis et al. (1969) found out that the binding of folic acid in milk was quantitative, rapid and irreversible. They said that teropteroic tetrahydrofolic acids and 5-methyl-tetrahydrofolic acid competed with folic acid for binding sites. Ford et al. (1969) purified folate-binding protein from cow's milk. Its identity as a distinct minor whey protein was confirmed by comparative starch gel electrophoresis at various pH values. Svendsen et al. (1979) isolated and characterized the folate-binding protein from cow's milk. They observed that molecular weight was 30,000 ± 2000 and it contained six disulphide bridges and three per cent carbohydrate in the form of glucosamine residues.

ii) In Serum

Metz and Herbert (1967) developed a coated charcoal radioisotope dilution assay for folic acid using dextran-10-coated charcoal. The binding of folate in serum was weaker than that in milk. Metz et al. (1968) further studied the binding of pterylglutamic acid by skim milk and blood serum using (^{3}H) pterylglutamic acid. The binding was stronger in skim milk and human milk than that in the serum. They observed that the binder in milk was in the phosphate buffer eluate which was not present in the blood serum. Waxman and Schreiber

(1973) reported that the serum proteins were of two types: the high capacity, low affinity type mainly comprising albumin, and the low capacity, high affinity, specific binders. The latter were a heterogenous group of beta globulins.

Zamierowski and Wagner (1977) found three separate binding proteins in cytosol, one of which was identified as the 25,000 MW dihydrofolate reductase, and a fourth folate-binding fraction from mito-chondria. Franscisco et al. (1979) observed that the binding sites for methylfolate of the folic acid binding protein in serum were fully saturated in physiological state. Colman and Herbert (1980) reported that about 65% of the specific binders were saturated. Wittwer and Wagner (1980) concentrated their efforts on the mitochondrial fraction and obtained a 90,000 MW protein.

Holm et al. (1980) isolated a cofactor from whey of cows' milk which was important in binding of the folate. A similar cofactor was also demonstrated in sera from men, pregnant women and unblical cords. But they could not identify this cofactor which might act as an overall modulator of high affinity folate-binding.

b) Vitamin B_{12} Binders

Finkler et al. (1967) compared the protein in human milk which bound cyanocobalamin (vitamin B_{12}) with the two known cyanocobalamin - binding proteins in blood serum. They observed that *in vitro* binding protein was also the main binder of endogenous cyanocobalamin.

Samson *et al*. (1980) reported that pepsin digestion had no effect on vitamin B_{12} binding capacity, the ability of *Escherichia coli* to take up the vitamin or the growth inhibitory effect on a vitamin B_{12} dependent strain. But pepsin helped in releasing iron from milk and abolished its bacteriostatic effect. Trypsin digestion on the other hand did not affect iron-binding or bacteriostatic effects attributable to lactoferrin. It slightly reduced the molecular size of the vitamin B_{12} - binding protein without releasing free vitamin B_{12}. Vitamin B_{12} - binding protein had no bacteriostatic role as lactoferrin in the breast-fed neonate's intestine.

Sandberg *et al*. (1981) measured the cobalamin content of human milk, which averaged to be 0.87 ng/ml. They observed that there was no correlation between the cobalamin content and its supplementation. The milk was seen to have two types of binders, that is, R type binder and transcobalamin II. The human milk was found to contain adequate amounts of cobalamin which could be made available only if sufficient proteolytic enzymes were present to release it from binding to R binders.

2.3 ROLE OF VITAMINS AND THEIR BINDERS

The vitamins ushered a new era in nutrition. It was one in which small things in diet assumed great importance. The percentage of body weight attributable to vitamins was minute. Nevertheless, the amount, even though small, was indispensable for normal functioning (Wilson et al., 1968). The binding of vitamin B_{12} and folate in milk may be direct nutritional advantage to the young animal, but the influence of the large excess of unsaturated binder proteins on the intestinal micro-organisms might be more important. Uptake of cyanocobalamin by pure cultures of intestinal bacteria was inhibited by the presence of sow's milk (Ford, 1974).

Eichner et al. (1978) could not find out the role of folate-binding proteins but they reported that the proteins might be intracelluler which were released into the serum as an index of activity of liver cells, granulocytes and perhaps certain cancer cells.

Rubiniff et al. (1981) suggested that folic acid binding proteins might play a role in enterohepatic circulation of folates by directing non-methylated folates to the liver.

a) Biochemical Aspects

Nichols and Welch (1959) showed the inter-relationship of folic acid with certain other vitamins, for example, vitamin B_{12} and ascorbic acid. They reported that in the absence of vitamin B_{12}, redox potential might not be suitable for the conversion of folic acid to tetrahydrofolic acid. Ascorbic acid deficiency might interfere with folic acid metabolism in similar manner. Ponders et al. (1966) demonstrated that the antibiotics usually taken did not affect the folic acid activity in the rumen. Davidson et al. (1972) reported that tetrahydrofolic acid, which is the active form of the vitamin, had an essential role in many synthetic processes in the body. It acted as a carrier for one-carbon radicles. Tetrahydrofolic acid (THF)- CH=N.H, THF-CH_2OH and THF-CHO are formed from the metabolism of histidine, serine and formate respectively and each of these substances could be converted into THF-CH_3. Each of the one carbon radicles, when attached to THF, can be readily detached and incorporated into other molecules. Thus, THF-CH_3 and homocysteins yielded tetrahydrofolic acid and methionine. Methionine has an essential role in all transmethylation in the body. Tetrahydrofolic acid is also concerned in nucleic acid and protein synthesis.

Coats et al. (1979) observed that pyrimidine acted as an analogue of folic acid as they noticed that inhibition caused by pyrimidines to certain microorganisms could be reversed by folic acid. Colman and Herbert (1980) compared the uptake of free and bound folate by isolating the intestinal mucosal cells. They observed that the extraction was more when the folate was bound to milk folic acid binding proteins. Rubiniff et al. (1981) suggested that folic acid binding protein might play a role in enterohepatic circulation folates by directing non-methylated folates to the liver.

b) Physiological Aspects

Hertz (1948) demonstrated the importance of folic acid for the activity of certain hormones, for instance, estrogens had no effect on the oviducts of young folic acid-defficient chicks until folic acid was given.

Areekul et al. (1971) observed that folic acid and folic acid binding protein concentrations were significantly lower in pregnant women than those of non-pregnant women. They observed that there was a direct relationship between the serum folic acid binding proteins level and the gestation period.

Tamuta *et al*. (1980) conferred that breast-feeding was better as they observed that folate levels in breast fed infants were significantly higher.

Eichner *et al*. (1981) could not find out the role of folate-binding proteins but they reported that the proteins could be intracellular which were released into the serum as as index of activity of liver cells, granulocytes and perhaps certain cancer cells.

c) Clinical Aspects

Vitamin B_{12} deficiency might induce a secondary defficiency in the folic acid co-enzyme either by reducing the amount of folic acid or folinic conjugates converted to folic or folinic acids, or the amount of folic acid co-enzymes formed (Swendseid *et al*., 1947; Williams *et al*., 1950; and Will *et al*., 1959). DeGrazia *et al*. (1972) reported that there was a marked decrease in rate and amount of oxidation of 3-C^{14} serine to $^{14}CO_2$ in human folate defficiency. Pincus *et al*. (1972) studied clinical symptoms of severe folate deficiency which resembled classical pernicious anaemia with neurological involvement; megaloblastio anaemia, impairment of posterior column functions, absent reflexes and bilateral babinski signs. These symptoms or abnormalities were resolved with folic acid therapy. De Waard (1974) said that methotrexate induced folic acid deficiency which could not be prevented by giving adenine,thymine or both in the diet. Chandrashekhar *et al.* (1980) observed that Fe^{2+} and folic acid in serum and haemoglobin value increased in women given the tables with iron 200 and folic acid 500 ng.

Sneed *et al.* (1981) reported that concentrations of folate content increased significantly on supplementation. Lewis *et al.* (1982) correlated folic acid deficiency with other vitamin-deficiencies like vitamin C. They observed that although ascorbic acid did not seem to be needed for normal folate metabolism, the lower ascorbic acid values were associated with folate deficiency.

2.4 ASSAY METHODS

The methods suitable to estimate folic acid may be grouped into biochemical, microbiological and chemical procedures. O'Dell and Hogan (1943) presented the first technique to determine folic acid. According to them, the chicks were placed on the basal diet until they became anaemic; then supplements of folic acid and test material were administered. Later, Campbell *et al.* (1944) developed a prophylactic method based on growth and anaemic prevention for four weeks.

Microbiological methods require less preparation than chicks assays and are particularly well adapted to simultaneous assays of several samples. Microbiological methods are based on the observation that certain micro-organisms require specific vitamins for growth using a basal medium complete in all respects except for the vitamin under test, growth responses of the organism are compared quantitatively in standard and unknown solutions. In their original method Mitchell *et al.* (1943) used *Streptococcus faecalis* R which was also used by Elvehjem *et al.* (1944, 1945) who modified the medium in order to obtain greater acid production and, thus, increase the titre. Bird *et al.* (1945) described a method of hydrolysing vitamin B_c conjugate by means of vitaminB_c conjugate prepared from hog kidney or almonds; the total folic acid was then estimated microbiologically with *Lactobacillus helveticus.* Teply and Elvehjem (1945) found that the two organisms that gave the best results and reproducibility and so mostly used were *Lactobacillus casei* and *Streptococcus faecalis*.

Jukes and Stockstad (1948) reported that *Lactobacillus casei* had the advantage of requiring much lower levels of folic acid for maximum growth and, therefore, more sensitive. Baker and Frank (1967) modified the microbiological assay for folic acid.

2.5 FACTORS AFFECTING THE LEVEL OF MAJOR NUTRIENTS AND VITAMINS IN MILK AND SERUM

i) Genetic and Climatic

Basu *et al.* (1962) reported that the average composition of the milk of different species varied. Sheep milk was the richest in many aspects, followed by the milk of buffalo, goat and cow. The high-protein and total - solids contents of sheep milk and the high vitamin C contents of sheep and goat milks indicated their superiority over other milks but their yield was very poor. Variations were observed in individual cows and buffaloes, sheep and goats. Rangappa and Achaya (1974) observed a marked effect on the yield and composition of milk due to changes in temperature, humidity and precipitation and consequent differences in the animal's fodder from season to season.

ii) Nutritional

Rook (1961) noted effect of plane of nutrition on solids-not-fat content. Over feeding by 25-35% above a standard might increase solids-not-fat by about 0.2% whereas under feeding by 25% decreases it by as much as 0.4 - 0.5%. The effect was primarily on protein content with the greatest change was observed in the percentage of casein. Rook and Line (1961) observed some decreases in lactose percentage with under feeding. Garton (1963) reported that the amount of fat in the ration had little effect on the fat content of milk although fatty acid composition of the dietary fat did influence that of the milk fat. Dong and Oace (1975) found that the feeds given to the dairy herds also affected the concentration and the pattern of folates in milk. High folate concentrations were measured in milk from herds which grazed on green pastures than those fed with dry feeds. Sneed et al. (1981) noticed that in milk the concentration of vitamin B_6, vitamin B_{12} and folic acid increased on supplementation while that of ascorbic acid was not affected.

Areekul et al. (1971) reported that there was a direct relationship between the serum folic acid binding protein levels and the gestation period. Ford et al. (1972) showed that the folate content in goat milk had fallen from 210 ng/ml to 5 ng/ml and the folate binding capacity from 600 ng/ml to 130 ng/ml by the thirtieth day of lactation. But they could not throw any light on the concept that folate binders acted as a trapping mechanism that accumulated free folate from the plasma into the milk, and less lactose than later milk. Its fat content might be higher or lower than normal milk. The total solids content of colostrum might be as high as 25%. The content of proteins synthesized by the gland (caseins, β-lactoglobulin α- lactoalbumin) might be doubled at about the mid lactation level, but the immunoglobulins were present in even higher levels (upto 10%). Calcium, magnesium, phosphorus, and chloride were present in higher concentrations in colostrum than in milk but the potassium concentration was lower.

Freier and Eidelman (1980) observed significant variations in the first few days of lactation in human milk. The high protein concentration in colostrum was mainly due to accumulation of milk specific proteins (lactoferrin, alpha lactoalbumin, Ig^A) during late pregnancy. The lactose content increased from 5.9 ± 0.5 g % by day 5 to 7.3 ± 0.4 g % by the end of six months ; the fat content increased by 1.2 ± 0.7 gm % by day 5 to 3.5 ± 1.2 gm % by six months. The total hitrogen and so also the protein nitrogen decreased 3.0 ± 0.6 mg/ml to 1.6 ± 0.2 mg/ml and 2.5 ± 0.51 mg/ml to 0.22 ± 0.13 mg/ml respectively.

Tamura <u>et al</u>. (1980) reported that folate levels in breast-fed infants were significantly higher than in mothers. They observed a correlation between folate levels in breast milk and in the infants plasma. The folate activities of serum and red blood cells decreased from 42.2 ng/ml and 633.0 ng/ml at birth to 22.4 ng/ml and 45.8

ng/ml after six weeks in infants. It was seen that breast-fed infants had lower folate contents than bottle-fed infants (Smith _et al._, 1981).

iv) Others

Lumb _et al._ (1981) reported that nitrous oxide induced loss of folate from certain tissues like liver. This gas affected polyglutamates to a greater extent than monoglutamates. In contrast to the tissues, there was a marked rise in plasma folate on exposure to nitrous oxide.

2.6 FACTORS INFLUENCING THE VITAMIN binding activity in milk and serum

a) Effect of pH

Ghitis (1969) found that binding of folic acid was quantitative, rapid, irreversible and not dependent on temperature or pH between 5.5 and 8.0 and was calculated to be 5 ng folic acid per ml of milk.

Ford (1974) reported that uptake of free folate by _Lactobacillus bifidus_ was strongly influenced by pH and was at a maximum at about pH 6.0, where as uptake of the folate added with goat's milk was greatest at pH 5.6 and declined to a low level at pH 5.0 and above. This higher availability of the milk bound folate at pH 4.5 probably reflected dissociation of the folate-protein complex.

Ford et al. (1969) filtered a preparation of folate-protein in Sephadex gel G25, eluting with buffer solutions of different pH values. At pH 6.0 and above, the folate was eluted in the void volume together with the protein. At pH 5.9, only 61% of the folate emerged with the protein and 39% was recovered as free folate. At pH 3.6, only free folate was present in the eluate. The dissociation at pH 3.6 was reversed on adjustment of the pH value to 7.1

Givas and Gutcho (1975) compared the binding of folic acid and N-5-methyl-tetrahydrofolic acid to milk binder in the pH range 7.4 to 10.1. At pH 7.4, the relative affinities were quite disperate, with folic acid showing the greater affinity for milk binder. As the pH was increased from 7.4 to 9.3, the difference in affinities became smaller, and at pH 9.3, the affinities were nearly the same. As the pH was increased from 9.3 to 10.1 the relative affinities again began to differ, with N-5-methyleterahydrofolic acid displaying the greater affinity.

b) Heat Treatment

Ghitis and Canosa (1965) found that autoclaving of milk affected the folate activity of milk when they observed that the folate activity decreased from 36.0 - 43.0 ug/l to 14.0 to 22.0 ug/l.

Later, Ghitis (1966) studied the effect of pasteurization and drying of milk. He found that the losses of pterylglutamic acid were considerable in pasteurized and dried milk than in fresh milk when boiled for five minutes.

Ghitis (1967) reported that the free folate on boiling or autoclaving could be protected by addition of ascorbic acid from adsorbing to charcoal. Additional pterylglutamic acid could bind to fresh milk but not to autoclaved milk. Freshm milk, in turn, was unable to rebind natural folates released on autoclaving but could rebind pterylglutamic acid released by it.

Areekul et al. (1978) suggested that the infants should preferably be breast fed rather than bottle-fed as he observed that folic acid binding activity of human milk was 10.3 ng/ml compared to 17.9, 1.23, 0.07 and 5.6 ng/ml in raw cow's milk, pasteurized milk, evaporated milk and condensed milk respectively. Cows' milk was usually not given in raw form. On pasteurization and sterilization free folic acid content reduced by 27% and 69% respectively.

c) Others

Holm et al. (1980) found methotrexate to be a weak inhibitor of folate binding in milk as well as in serum. Another impurity, N^{10}-methylfolate was also an inhibitor of folate binding in milk and serum. Hansen et al. (1981) saw the biological significance of reactivation of purified folate-binding protein. The reactivation by number of surfactants (phospholipids and synthetic detergents) caused a lower affinity of the protein for folate.

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CHAPTER III

MATERIALS AND METHODS

MATERIALS AND METHODS

3.0 ANIMALS

The animals were selected from three species maintained at National Dairy Research Institute, Karnal given as follows:

i) Cows (Karan Swiss) numbering 40001, 47, 42, 290, 3102 and 236.

ii) Buffaloes (Murrah) numbering 1828, 1382, 1656, 368, 377 and 1097.

iii) Goats (Beetal) numbering 617 and372.

3.1 COLLECTION OF MILK SAMPLES

The milk samples from cows and buffaloes were collected at 5.00 am while those of goat at 11.00 am from the individual animal.

The sample (250 ml) from each animal was collected in plastic bottles containing 80 mg ascorbic acid in the form of Tris-ascorbate buffer (pH 7.2) and stored at -20^{o}C until used for assay.

3.2 CHEMICALS

Folic acid was purchased from Sigma Chemical Company, U.S.A. The folic acid assay media, Folic Acid Inoculum

Medium (Dehydrated) and Folic Acid Culture Agar (Dehydrated) were obtained from Hindustan Dehydrated Media, Bombay. Pepsin (1:3000) was obtained from S.D's Lab. Chem-Industry and Trypsin (2000 units/g) from E. Merck. Tris (Hydroxymethyl) methylamine was purchased from BDH Chemicals, England. All other chemicals used were of analytical grade.

3.3 PREPARATION OF MEDIA

i) Folic acid Inoculum Media

The composition of the medium was as follows:

Compounds	Quantity
Difco peptone	5 g
Difco Yeast Extract	1 g
Anhydrous Glucose	10 g
Anhydrous sodium Acetate	10 g
Salt solution A	5 ml
Salt solution B	5 ml

The above ingredients were dissolved in 200 ml of water. Then pH was adjusted to 6.8 with 1 N NaOH and was then diluted to 500 ml.

ii) FOLIC ACID CULTURE AGAR (FOR STOCK CULTURES)

The composition of this medium was as follows:

Compounds	Quantity
Anhydrous Glucose	2 g
Peptone	1 g
Cystein	100 mg
Salt solution A	1 ml
Salt solution B	1 ml
Agar	3.5 g

All the ingredients except agar were dissolved in approximately 150 ml of water. The pH was adjusted to 6.8 and the volume was made upto 200 ml. Agar was added and the mixture was steamed until agar was dissolved.

Salt Solution A: - 25 g of $K_2H\ PO_4$ and 25 g of $KH_2\ PO_4$ dissolved in 500 ml of water.

Salt Solution B:- The following amounts of salts were dissolved in 500 ml of water.

Magnesium sulphate ($Mg\ SO_4 7H_2O$)	10.0 g
Sodium chloride (NaCl)	0.5 g
Ferrous sulphate ($FeSO_4.7H_2O$)	0.5 g
Manganese sulphate ($Mn\ SO_4.7H_2O$)	0.5 g

iii) Folic Acid Assay Medium (Basal Medium)

This media is devoid of folic acid but contains all essential nutrients, amino acids and vitamins for the growth of Streptococcus faecalis ATCC 8043. The composition of media is as follows:

Compounds	Quantity
Acid-Hydrolysed casein solution	25 ml
Adenine-Guanine-Uracil solution	2.5 ml
Asparagine solution	15.0 ml
Manganese sulphate solution	5.0 ml
Polysorbate 80 solution	0.25 ml
Salt solution	5.0 ml
Tryptophan solution	25.0 ml
Vitamin solution	50.0 ml
Xanthine solution	5.0 ml
L-cysteine $HCl.H_2O$	0.19 g
Glucose, anhydrous	10.0 g
Glutathione	0.0013 g
Potassium phosphate (dibasic) anhydrous	1.6 g
Barium Citrate	13.0 g

All the above ingredients were mixed in water and diluted to a volume of 250 ml. The dehydrated media manufactured by the Hindustan Dehydrated Media, Bombay, was used throughout the present investigation.

3.4 ORGANISM AND ITS MAINTENANCE

The micro-organisms, Streptococcus faecalis ATCC 8043 was used for the assay. The pure culture was obtained in two tubes from the Division of Dairy Bacteriology. This culture was kept in the refrigerator under aseptic conditions till further use. After a week, this culture was transferred to the enriched culture medium (broth), incubated at 37°C (± 0.5°C) for 18 hrs. and then transferred to the Enriched Agar Medium for stock culture. During transferring of the cultures the following precautions were observed strictly.

i) One stab culture was reserved unopened for use in the preparation of subsequent stock culture stabs. The others were used in the preparation of inoculum. They were not more than one week old and sterile technique was observed.

ii) Whenever the stock culture had not been used for several weeks or months, new culture was obtained or revived the old one by making daily successive stab transfers for at least three days before preparing the inoculum.

3.5 ASSAY PROCEDURE FOR FOLIC ACID

The stock cultures were prepared as explained earlier.

The assay procedure for folic acid was used as described by Freed (1966).

<u>Preparation of Inoculum:</u> - The cells from the stock culture were transferred to a sterile tube of enriched culture medium (also known as broth) and incubated for 18 hrs. at 37°C. The clear solution turned turbid indicated the growth of the organism. This was then transferred to a sterilised centrifuge tube plugged with cotton. It was centrifuged at 3,000 rpm for 15 min.

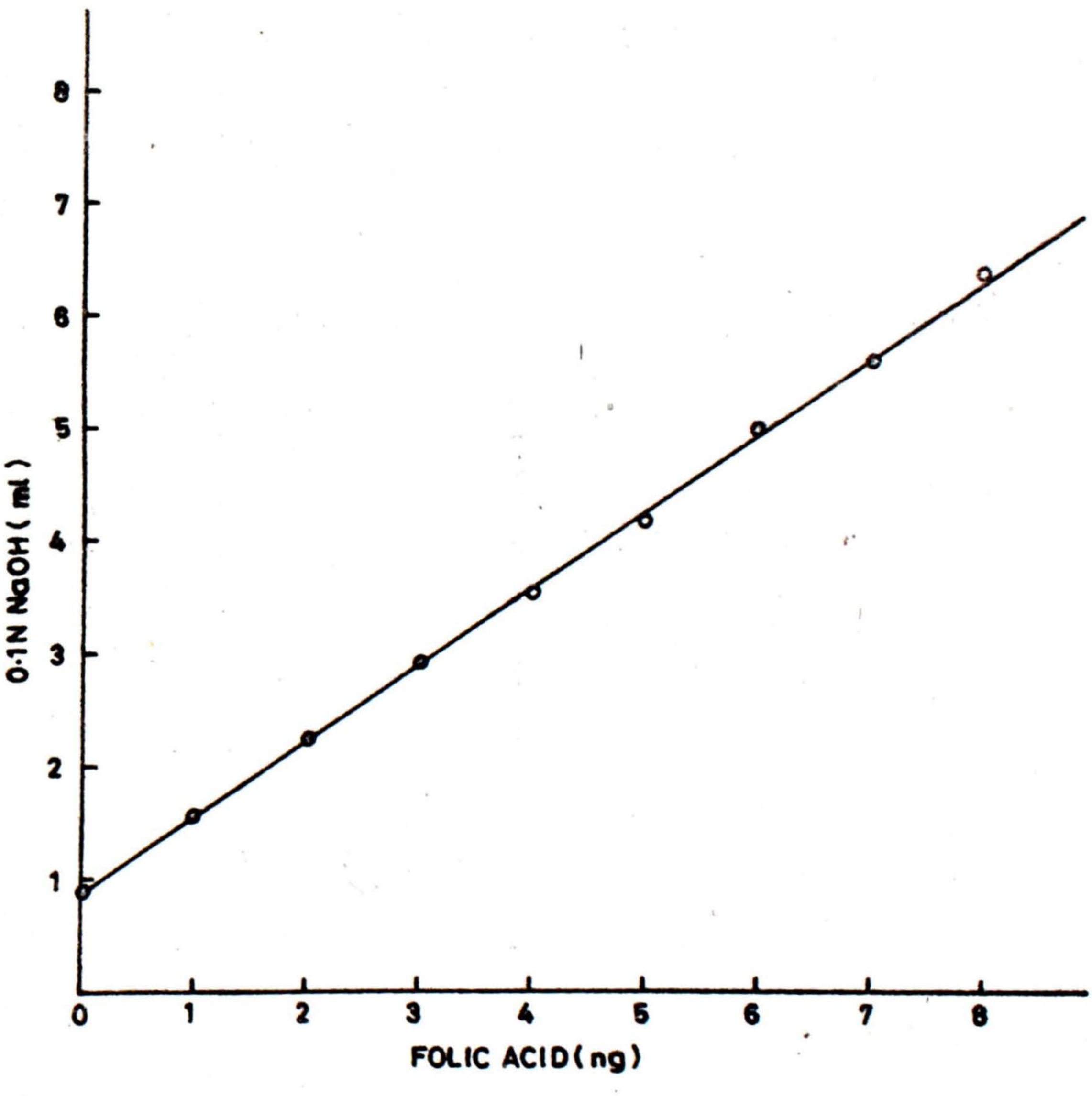
The supernatant was decanted and the cells were resuspended in 10 ml of sterile isotonic sodium chloride (0.9%) under aseptic conditions. This suspension was again centrifuged for 15 min. The cells were washed in a similar manner for 3-4 times so as to remove the traces of media completely. Lastly, the cells were resuspended in 10 ml of saline solution. The suspended cells were used for further inoculations to assay folic acid.

3.6 PREPARATION OF STANDARD CURVE

Stock solution of Folic Acid: Dissolved 100 mg of folic acid in 0.01 N NaOH in 20% ethanol and made upto one litre. The solution was covered with few drops of tolune and stored in dark glass bottle in refrigerator.

FIG. I

Standard curve for folic acid
(using *Streptococcus faecalis*)



0·1N NaOH (ml)
1
2
3
4
5
6
7
8
0
1
2
3
4
5
6
7
8
FOLIC ACID(ng)

<u>Working solution of Folic Acid:</u> One millilitre of folic acid stock solution was diluted to 1 litre in a volumetric flask. Further 10 ml of this solution was made to 500 ml. The final solution contained 2 ng(millimicrograms) of folic acid per ml.

a) The different volumes of working standard folic acid i.e., 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 3.5, 4.0, 4.5 and 5.0 ml were taken in duplicate test tubes.

b) Sufficient water was added tobring the volume in each tube to 5.0 ml.

c) Then to each tube, 5.0 ml of folic acid assay medium (basal medium) was added.

d) All the tubes were plugged with cotton and sterilized in an autoclave at 15 lb. pressure for 15 min.

e) The tubes were brought to room temperature and they were inoculated with the help of sterilised pipettes under aseptic conditions. They were then put for incubation at 37^{o}C for 72 ho-urs.

f) The content of each tube was transferred to a Erlenmeyer flask. The tubes were again rinsed with 10 ml water and content was put in flask. After adding 0.2 ml of 0.1% bromothymol blue the content was titrated with 0.1 N NaOH to determine the corresponding amount of lactic acid produced.

3.7 ASSAY OF FOLIC ACID IN MILK SAMPLES

a) Defattening of milk samples

The milk was defatted as described by Rose and Gottleieb (1959). An aliquot of whole milk (15 ml) was taken in graduated measuring cylinder. Absolute alcohol (15 ml) was added to the milk and shaken vigorously. It was followed by addition of 20 ml diethyl ether and again shaken thoroughly. The content was kept for 15-20 min. The upper layer so separated containing ether and fat was removed. The defattening of milk sample was done by adding ether repeatedly so as to make the sample free from fat.

b) Digestion of milk with pepsin and trypsin

The defatted milk was subjected to digestion to liberate folic acid enzymatically by the action of pepsin and trypsin as described by Ford (1974).

To 5 ml of defatted milk sample, were added 10 ml of 0.1 M and 1 M HCl to bring the pH to 2.0 of the sample. Ten milligrams of crystallized porcine pepsin were then added and content was stirred. The mixture was incubated for 90 min at 37° with occassional swirling. The digest was then brought to pH 6.8 by addition of 1 M NaOH and diluted to 40 ml. A portion of this pepsin digest was further digested with trypsin as follows:

To 20 ml of the digest were added 50 mg $CaCl_2$ and 1 M NaOH to pH 8.2 and 16 mg of a preparation of crystallized bovine trypsin. The mixture was incubated for 15 minutes at 37°C and its pH was against adjusted to 8.2 by addition of 0.1 M NaOH. Incubation was continued for a further 105 minutes with occasional swirling.

c) Assay of Folic Acid

i) The desired aliquots of digested milk samples ranging from 0.02 ml to 2 ml were taken in different tubes. The content of these tubes was brought to 5 ml with distilled water. Five millilitres of folic acid assay medium was then added to these tubes making total volume to 10 ml.

ii) Sterilization

a) The contents of each tube was mixed thoroughly. The tubes were plugged with cotton and were autoclaved at 15 lb. pressure for 10-15 min.

iii) Inoculation and incubation

a) All the tubes were cooled at room temperature

b) Each tube was inoculated with 0.2 ml of the inoculum aseptically.

iv) Titration

a) The contents of each tube was transferred to a Erlenmeyer flask. The tube was rinsed with 10 ml of distilled into flask. About 0.2 ml of 0.1% bromothymol blue was added and the content was titrated with 0.1 N NaOH to the end point with is Sea-green colour. A flask was kept for a reference colour for about 10 titrations and then substituted a new flask.

Calculations

a) A standard curve was drawn by plotting 0.1 N NaOH used in titrating the standard tubes against ng of folic acid per tube in the standard series.

b) The folic acid content of the tubes in the unknown series was determined by interpolation of the titre values on the standard curve.

c) The folic acid content of the test sample was calculated from the average of the values for each ml of test samples, which was obtained from three sets of the tubes.

3.8 MEASUREMENT OF FOLATE BINDING CAPACITY OF MILK

To measure the folic acid binding capacity of the milk sample, folic acid was added to the defatted milk in excess and then dialysed to remove the excess folic acid as described by Ford et al. (1972).

To 1 ml of sample added 2 ml of buffer solution (pH 7.2) containing 0.15 M Nacl, 0.02 M-sodium phosphate and 0.001 M ascorbic acid and graded amounts (500-1800 ng) of folic acid dissolved in 1 ml of buffer. The content was incubated for 2 hr under cold conditions. This mixture was then transferred to dialysis sac. and dialysed for 48 hours under cold conditions against eight successive portions of buffer. The residual folate activity in the sacs was then assayed in the similar manner as described.

3.9 EFFECT OF SOME FACTORS ON FOLATE BINDING ACTIVITY OF MILK

i) pH :- In order to study the effect of pH on binding of folic acid with milk, the buffers of different pH were prepared as follows:

a) 0.05 M citrate buffer (pH 3.5 - 5.0);

b) 0.02 M phosphate buffer (pH 7.2) ;

c) 0.05 M Tris-HCl buffer (pH 8.0 - 9.0);

d) 0.05 M Carbonate/Bicarbonate buffer (pH 10.0).

The milk sample (5 ml) was brought to desired pH by adding HCl and NaOH. The content was dialysed overnight against buffer of pH as maintained for the sample. After dialysis the pH of milk sample was brought to 7.2 and assayed for folic acid content.

ii) Heat treatment

To study the effect of heat treatment on folate binding activity of milk, the milk samples from three species were treated as follows:

a) The milk sample (50 ml) was kept at 75°C for 15-20 sec. which is known as High Temperature Short Time (HTST) treatment.

b) In another experiment the milk sample 50 ml was boiled for 5 min and then cooled.

After giving above treatments the folic acid content in these samples was determined as described.

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CHAPTER IV

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.0 FOLATE CONTENT OF MILK IN RELATION TO STAGE OF LACTATION

The folate content was estimated in the milk collected from cows, buffaloes and goats at various stages of lactation.

a) Cow

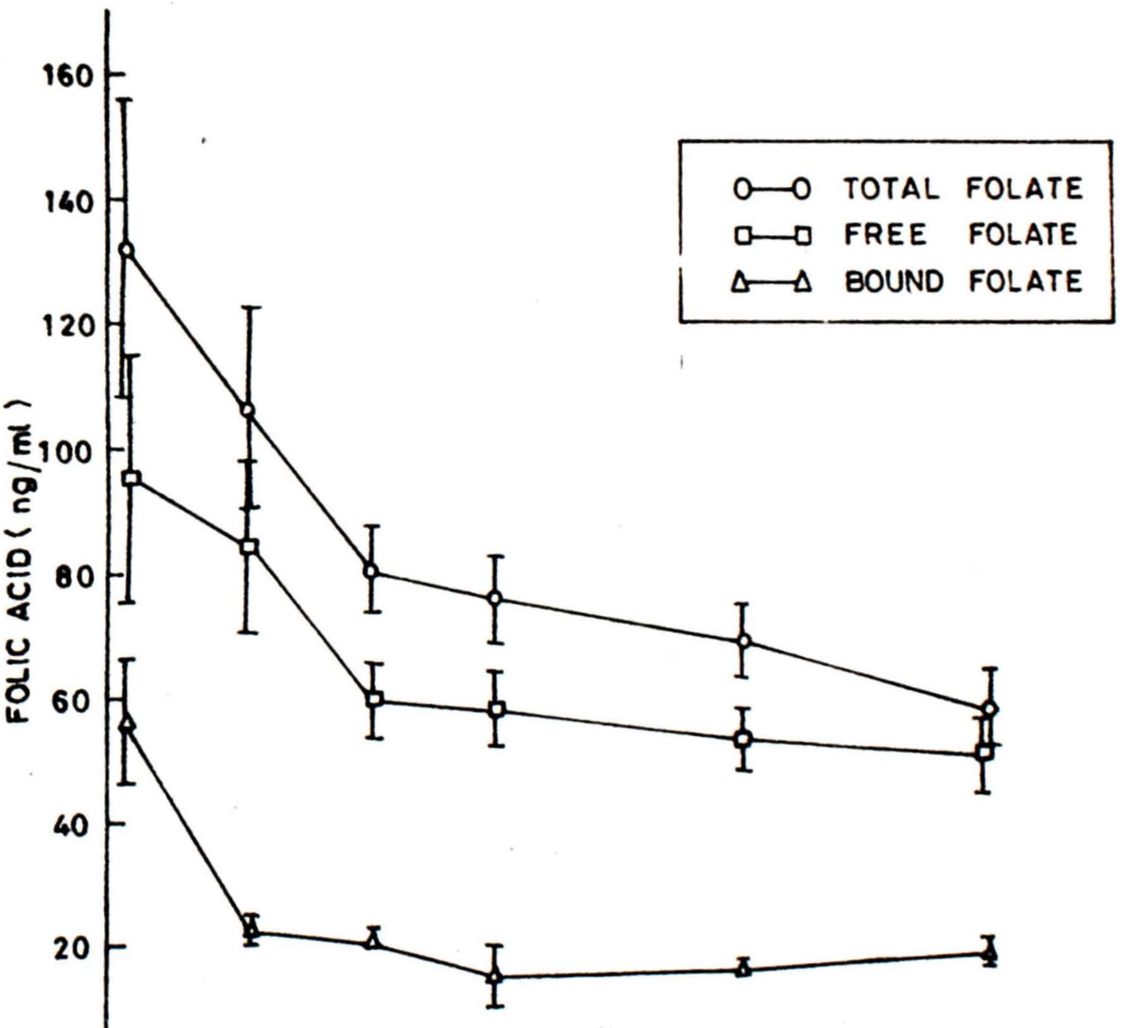
The results are shown in Table I and Fig. 2. The highest concentration of folic acid was obtained on the first day of parturition. The total content ranged from 60 - 226 ng/ml. There was a gradual decrease in total folate content with increase in lactation period. On day 55 after parturition, the values for folic acid ranged from 43.6 - 82.0 ng/ml. The bound and free forms of folic acid were also determined at these stages of lactation. On an average, the total free form was found to be 95 ng/ml ranging from 40.0 - 181.3 ng/ml. The bound form of folic acid was obtained in the range from 20.00 - 56.00 ng/ml on first day of lactation. The similar pattern, as observed regarding the gradual decrease in total folate, was also found with regards to free and bound form of folic acid. Out of the total folate content at various stages of lactation about 28 - 30% was in bound form and 70- 72% was present as free form. There was a significant

Table I. Folic acid content in cow milk at various stages of lactation (ng/ml)

Anim. No.	1st day Free	1st day Bound	1st day Total	8th day Free	8th day Bound	8th day Total	16th day Free	16th day Bound	16th day Total	24th day Free	24th day Bound	24th day Total	40th day Free	40th day Bound	40th day Total	55th day Free	55th day Bound	55th day To[tal]
4001	181.33	43.33	226.66	129.60	28.40	158.00	75.60	15.75	91.35	71.30	18.90	90.20	69.20	16.40	85.60	69.40	12.60	82
47	40.00	20.00	60.00	44.30	15.70	60.00	30.70	20.20	50.90	30.76	15.34	46.10	29.50	15.50	45.00	29.00	14.60	43.
290	48.00	32.00	80.00	51.60	20.91	72.51	49.50	18.50	68.00	47.30	13.70	61.00	46.50	13.70	60.20	44.40	15.93	60.
3102	96.00	56.00	152.00	82.00	24.00	106.00	72.00	20.50	92.50	66.00	14.90	80.90	59.00	11.33	70.33	40.60	25.15	65.
42	101.33	42.67	144.00	125.90	23.60	149.50	68.50	21.83	90.33	69.40	21.90	91.30	59.90	20.43	80.13	60.33	19.67	80.6
236	103.20	55.80	129.00	72.31	21.69	94.00	61.60	24.53	86.13	60.90	20.00	80.90	52.80	17.11	69.91	50.91	16.69	67.6
Total	569.86	221.80	791.66	505.71	134.30	640.01	357.90	121.31	479.21	345.66	82.84	450.44	316.90	94.47	411.17	294.16	104.64	399.2
Av.	94.976	36.966	131.943	84.285	22.383	106.668	59.650	20.218	79.868	57.610	13.807	75.066	52.816	15.745	68.528	49.106	17.440	56.54

Fig.II

Folate content in cow milk at various stages of lactation (ng/ml)



160
140
120
100
80
60
40
20
FOLIC ACID (ng/ml)
0
8
16
24
32
40
48
55
DAYS AFTER PARTURITION
TOTAL FOLATE
FREE FOLATE
BOUND FOLATE

fall in all the forms of folic acid during first week of lactation. There appeared a relationship between folic acid content and age of the animal which was evident by obtaining highest value in the milk from youngest animal. This was simply an observation and can authentically be claimed when more studies are done in this regard.

b) Buffalo

The results for folic acid content in milk from buffalo have been shown in Table II and Fig. 3. The total content varied from 32 - 80 ng/ml on day 1. There was again a gradual decrease till day 55, after parturition where the values were recorded in the range from 25.5 - 50.6 ng/ml. Similarly, there was a decrease in free and bound form of folic acid as the days advanced after parturition. In case of buffalo milk folate, there was 40.7% bound folate and about 59% free folate, on first day of lactation. Approximately, similar ratio between bound and free form was maintained on day 55 of parturition. From day 1 to day 55, the total content of folic acid declined from 54.6 ± 6.6 to 37.7 ± 4.5 ng/ml

c) Goat

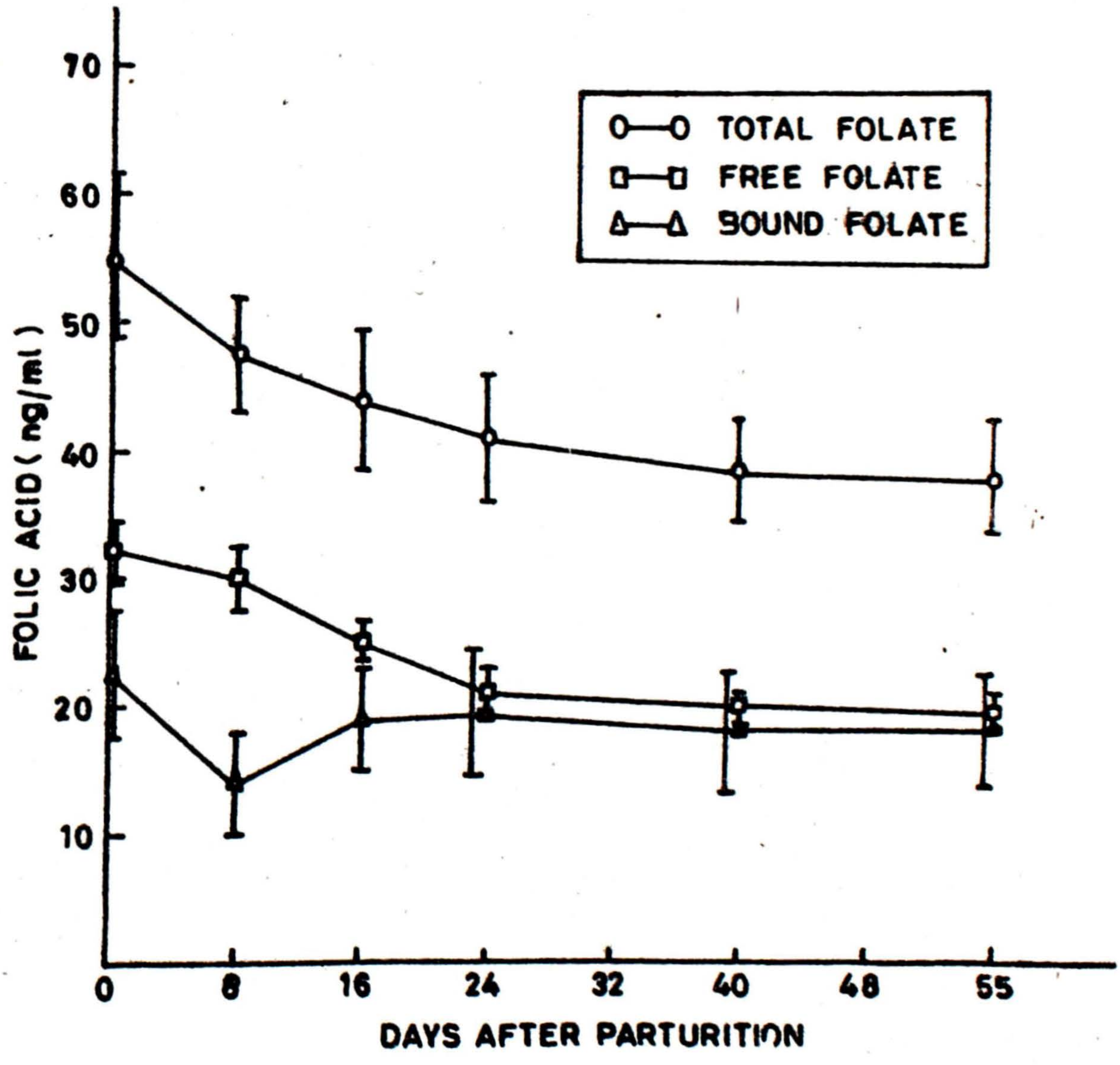
The folic acid content in goat milk have been given in Table III and Fig. 4. The total folic acid content on

Table II. Folic acid content in buffalo milk at various stages of lactation (ng/ml)

Anim.	1st day			8th day			16th day			24th day			40th day			55th day		
No.	Free	Bound	Total	Free	Bound	Total	Free	Bound	Total	Free	Bround	Total	Free	Bound	Total	Free	Bound	Total
1656	25.20	30.80	56.00	24.60	19.73	44.33	22.50	18.41	40.91	20.60	15.00	35.60	20.20	14.80	35.00	18.50	14.83	33.33
1097	29.65	14.49	44.14	27.50	5.50	33.00	20.90	9.79	30.69	20.83	9.87	30.10	19.60	7.30	26.90	19.50	7.11	26.61
368	38.12	21.88	60.00	39.50	19.30	58.80	31.00	19.50	50.50	30.66	16.24	46.90	23.00	21.30	44.30	23.10	18.49	41.59
377	24.40	7.60	32.00	22.70	8.10	30.80	21.30	4.90	26.20	20.05	6.18	26.23	21.00	5.00	26.00	20.80	4.90	25.50
1383	38.65	17.28	55.93	35.50	3.40	38.90	28.66	28.00	56.66	19.60	35.50	55.10	20.00	29.19	49.19	18.93	29.98	48.91
1828	36.00	44.00	80.00	30.65	29.70	60.35	25.90	34.43	60.33	17.00	35.66	52.66	15.60	34.73	50.33	16.90	33.66	60.56
Total	192.02	136.046	328.066	180.45	85.73	268.18	150.26	115.03	265.29	128.74	118.45	246.59	119.40	112.32	231.72	117.53	108.97	226.50
Av.	32.003	22.674	54.678	30.075	13.288	47.696	25.045	19.170	44.215	21.456	19.742	41.098	19.900	18.720	38.680	19.588	18.162	37.75

Fig. III

Folate content in buffalo milk at
various stages of lactation (ng/ml)



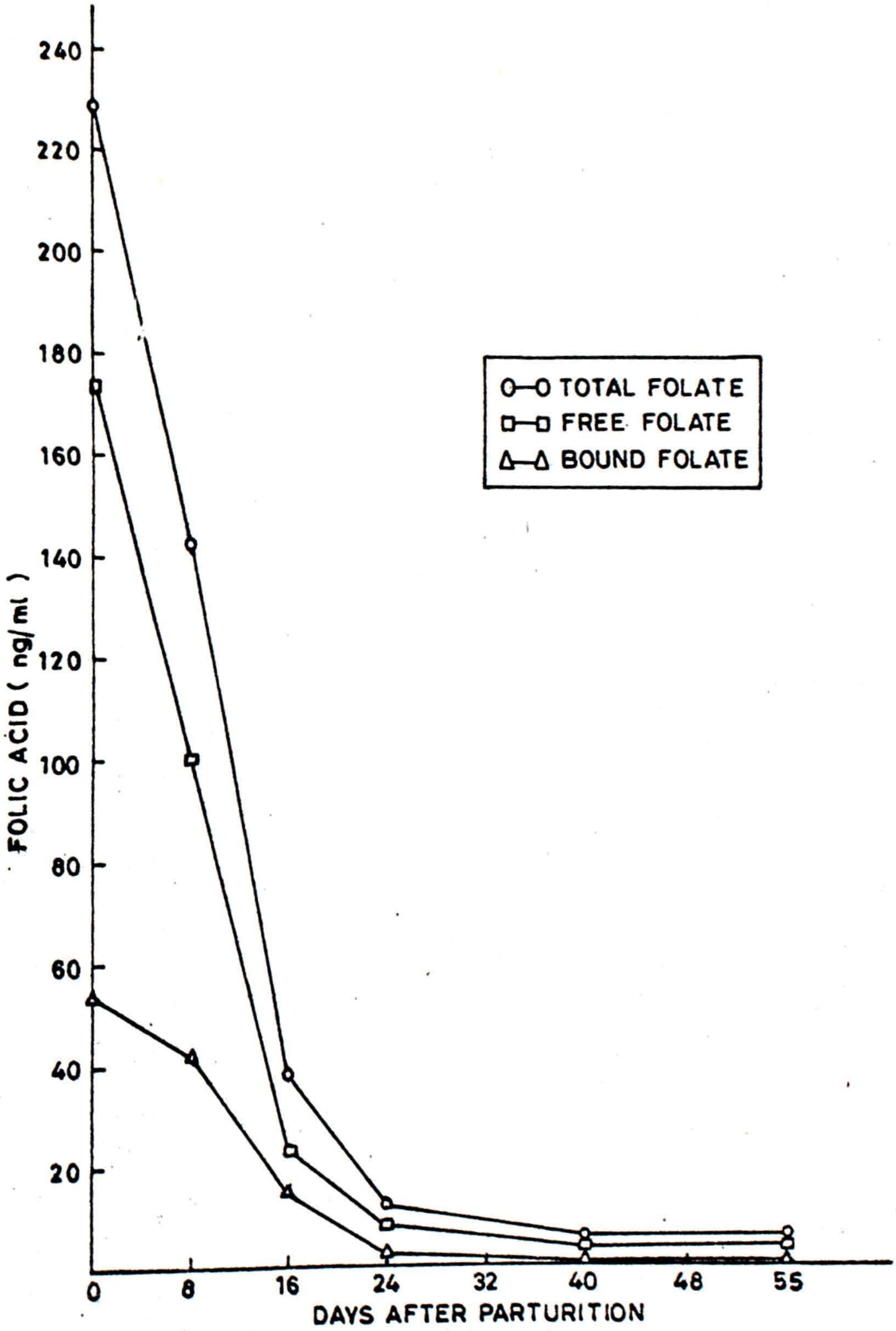
TOTAL FOLATE
FREE FOLATE
BOUND FOLATE
FOLIC ACID (ng/ml)
70
60
50
40
30
20
10
0
8
16
24
32
40
48
55
DAYS AFTER PARTURITION

Table III. Folic acid content in goat milk at various stages of lactation(ng/ml)

Anim.	1st day			8th day			16th day			24th day			40th day			55th		
No.	Free	Bound	Total	Free	Bound	Total	Free	Bound	Total	Free	Bound	Total	Free	Bound	Total	Free	Bound	Total
617	205.31	55.67	260.98	123.60	59.98	183.58	26.80	18.59	45.39	16.50	2.35	18.85	4.25	2.13	6.38	4.16		6.[illegible]
372	243.50	53.36	296.86	76.91	24.18	191.09	19.89	11.76	31.65	6.86	2.77	9.63	3.75	1.90	5.65	3.02		4.[illegible]
Total	448.81	109.03	557.84	200.51	84.16	374.67	46.69	30.35	77.04	16.36	5.12	22.48	8.00	4.03	12.03	7.18		11.[illegible]
Av.	224.405	54.55	278.920	100.255	42.080	187.335	23.345	15.175	38.520	8.180	3.100	11.240	4.000	2.015	6.015	3.590	2.005	.595

Fig. IV

Folate content in goat milk at various stages of lactation (ng/ml)



240
220
200
180
160
140
120
100
80
60
40
20
FOLIC ACID (ng/ml)
O—O TOTAL FOLATE
□—□ FREE FOLATE
Δ—Δ BOUND FOLATE
0
8
16
24
32
40
48
55
DAYS AFTER PARTURITION

first day of parturition was found to be 278.92 ng/ml. Later on, there was a drastic fall in total folate for which the value was obtained as 187.34 ng/ml on day 8 after parturition. On day55, the average value for total folate remained only 5.60 ng/ml. Like the patterns of fall in folic acid content obtained for cow and buffalo milk, the goat milk samples also exhibited similar pattern. In contrast to the extent of bound folate shown by cow and buffalo colostrum, the goat colostrum contained only about 19% bound form of folic acid. Unlike cow and buffalo milk, the bound form of folic acid appeared to be increased randomly with the increased period of lactation. On day 8, day 16, day 24, day 40 and day 55, the values for bound folic acid were 22.45, 38.96, 27.27, 33.00 and 35.71% respectively.

It is quite evident from Table IV and Fig. V that all three species have shown a similar trend for the fall of folic acid content from day 1 upto day 55 after parturition. All the species also produced highest concentration of folic acid in colostrum and lowest in the milk secreted on day 55. However, the extent of fall in folic acid content with increased lactation period is quite different for all the three species during early period of lactation. There was a slow decrease in folic acid content with increased lactation period incase of buffalo milk samples. Comparatively,

Table IV. Comparative values of folic acid content in milk from cow, buffalo and goat at various stages of lactation (ng/ml)

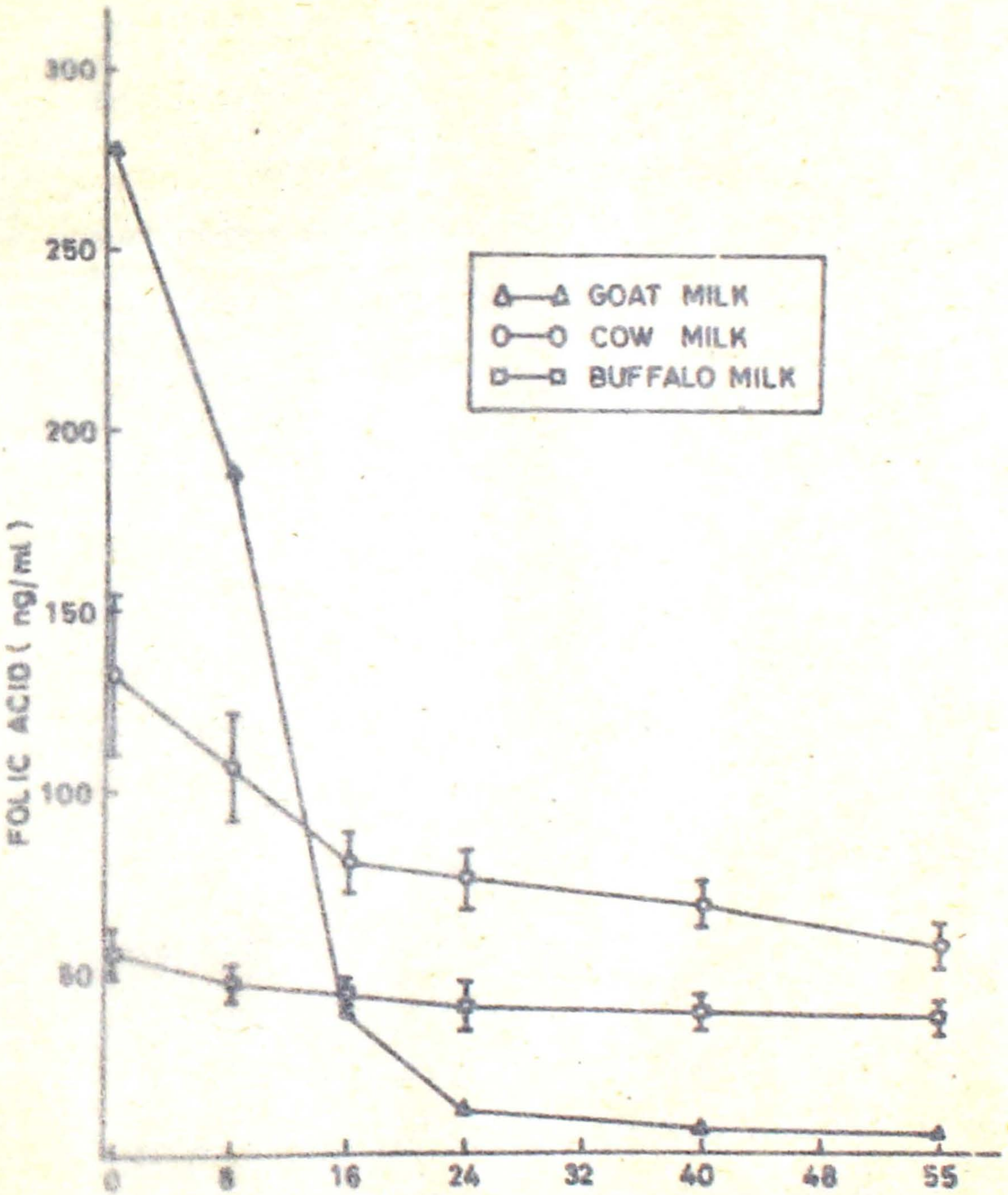
Species	1st day	8th day	16th day	24th day	40th day	55th day
Cow milk *	131.943 ± 24.0842	106.668 ± 16.3107	79.868 ± 6.8801	75.066 ± 7.2973	68.528 ± 5.9277	56.546 ± 6.0085
Buffalo milk*	54.678 ± 6.5793	47.696 ± 4.5782	44.215 ± 5.6934	41.098 ± 4.9519	38.680 ± 4.4376	37.750 ± 4.4613
Goat milk**	278.920	187.335	38.520	11.240	6.015	5.595

* Average of six observations ± S.E

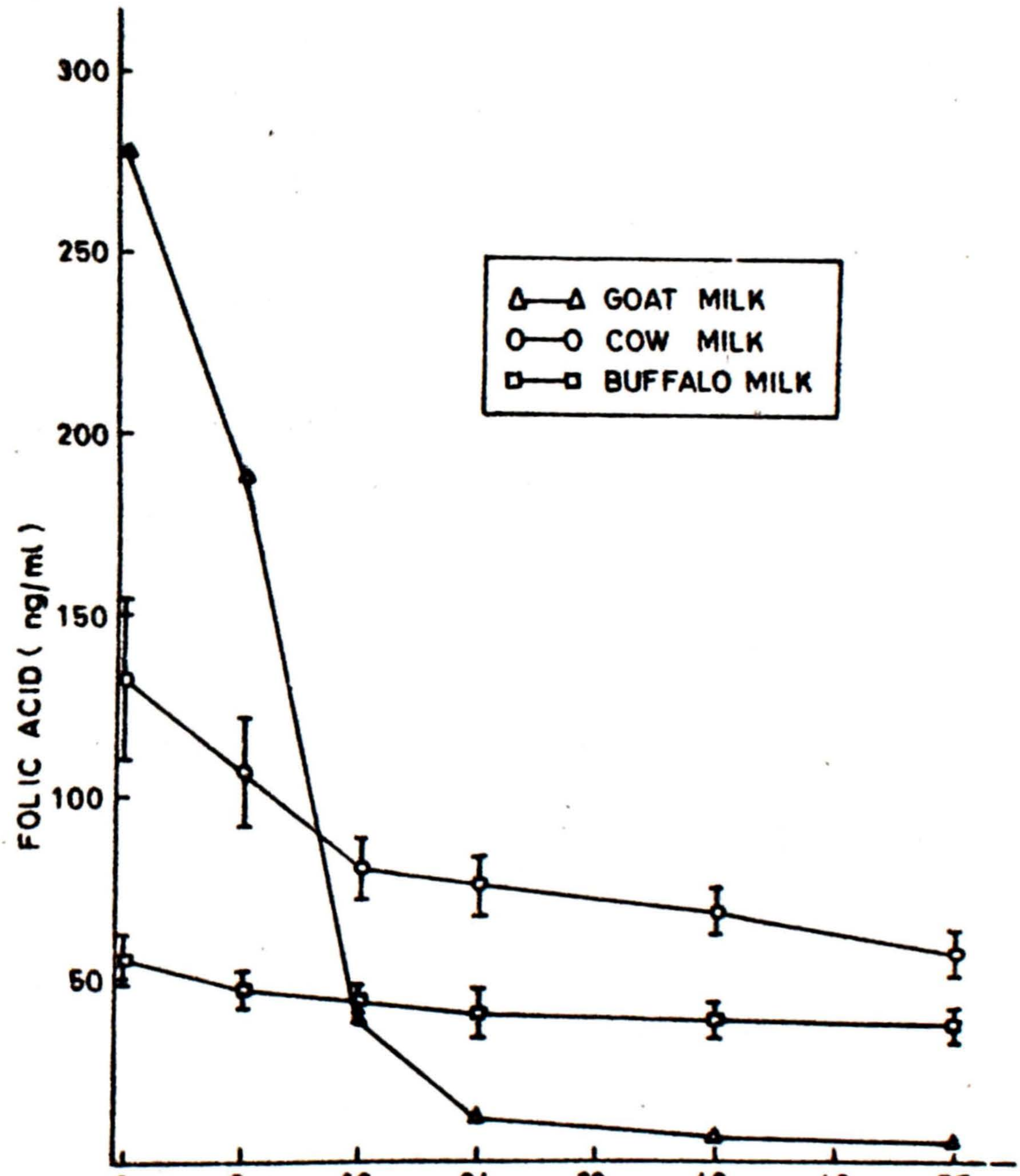
** Average of two observations.

Fig.V

Folate content in milk from buffalo cow and goat at various stages of lactation (ng/ml)



GOAT MILK
COW MILK
BUFFALO MILK
300
250
200
150
100
50
FOLIC ACID (ng/ml)
0
8
16
24
32
40
48
55
DAYS AFTER PARTURITION



GOAT MILK
COW MILK
BUFFALO MILK
FOLIC ACID (ng/ml)
300
250
200
150
100
50
0
8
16
24
32
40
48
55
DAYS AFTER PARTURITION

there was a significant fall in cow's milk folate during early lactation. At the same time, there was a drastic fall in folic acid content of goat milk on day 8. After day 24 onwards, all the three species showed a similar decline in folic acid content which was almost a steady rate in fall.

Ford et al. (1972) reported a comparatively high concentration of folate in goat colostrum which ranged upto about 300 ng/ml. It fell sharply during early days of lactation and by day 14 averaged only 9.5 ng/ml. They also reported a more extreme contrast between the folate concentration in colostrum and mature milk. In human milk, the pattern was normally different; the folate concentration was low in colostrum and immature milk and increased to a sustained high level around 150 ng/ml in mature milk.

Among all these species, the highest concentration of folic acid was found in goat colostrum (278.92 ng/ml) which was followed by cow (132.0 ± 34.00 ng/ml) and buffalo (54.68 ± 6.60 ng/ml). Swaminathan (1974) reported that cows mature milk contained 85 n /ml total folate, out of which 29 ng was in bound form; buffalo mature milk contained 56 ng/ml, out of which 23 ng was in bound form. Since the animals were maintained on routine feeding schedule, the drastic difference in folic acid content of colostrum from three species may not be due to feed. It appears to be a physiological event for accumulation of folic acid by all the three species where goat has highest capacity. It may be that the marked difference in folic acid content is due to genetic variability among species.

Different theories have been put forward regarding the fall in folic acid content of milk with increased lactation period. Ford et al. (1972) proposed that the rapid fall in milk folate concentration during the few days after parturition might be largely attributable to dilution of the initial folate content. After parturition, the milk was secreted at a faster rate and its folate content might be limited by the availability of free folate in the plasma, or by the rate at which plasma folate taken up into the milk was replenished from the body stores. Another possible explanation was that there was a corresponding fall in the content of folate binding protein in milk, and therefore in the capacity of milk to accumulate the folate from blood plasma against a concentration gradient.

On comparing the values of bound folic acid in milks from cow, buffalo and goat, it can be observed (Table V) that goat colostrum (54.5 ng/ml) has bound folate more than cow and buffalo colostrum. Buffalo colostrum contained lowest bound folate (22.67 ± 5.30 ng/ml). On day 55, cow milk

Table V. Comparative values of bound folic acid in milk from cow, buffalo and goat at various stages of lactation (ng/ml)

Species	1st day	8th day	16th day	24th day	40th day	55th day
Cow*	36.966 ± 10.284	22.383 ± 1.709	20.218 ± 1.214	13.807 ± 4.960	15.745 ± 1.265	17.440 ± 1.814
Buffalo**	22.674 ± 5.307	14.288 ± 4.189	19.170 ± 4.484	19.742 ± 5.221	18.720 ± 4.853	18.162 ± 4.793
Goat**	54.515	42.080	15.175	3.100	2.015	2.005

* Average of six observations ± SE

** Average of two observations.

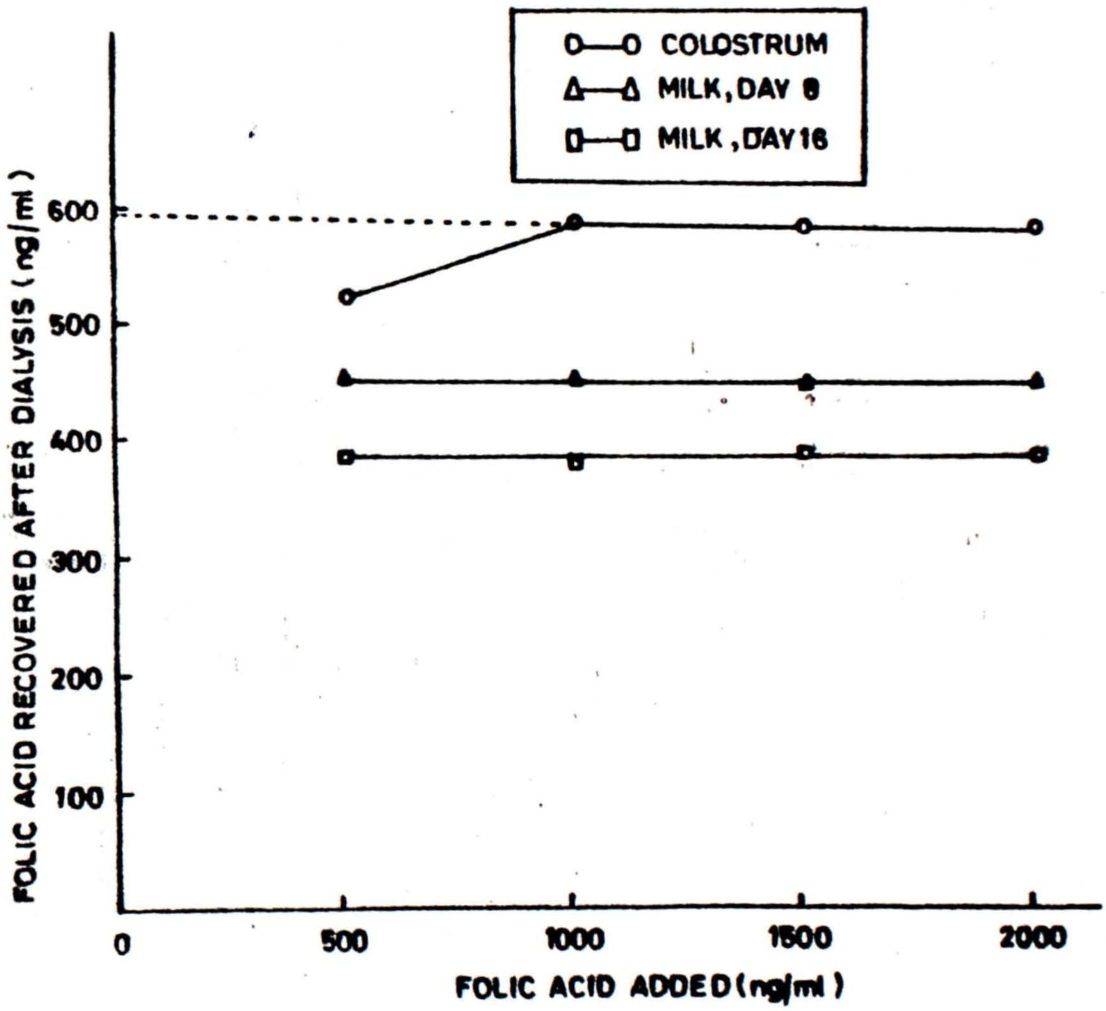
retained 17.44 $\pm$ 1.81 ng/ml which was 50% of the bound folate present in colostrum. There was negligible difference in bound form of folate in buffalo colostrum and its mature milk on day 55. Unlike cow and buffalo, goat mature milk on day 55 contained only 2 ng of bound folate per ml against 54.5 ng/ml on day 1. The differences in bound form of folate may again be attributable to genetic differences. So far it has been observed that there is a large variation in total and bound folate content in milk from cow, buffalo and goat. There is also a difference in extent of fall in folate with increased period of lactation. Goat has shown a very rapid change in folate profile throughout the period under study whereas it is least in case of buffalo. It may justbe possible that in goat, being a small animal comparatively, the high rate of metabolism is responsible for such a drastic change in folate profile which may also be possible due to short span of lactation. Buffalo being a slow animal (metabolically) was not able to produce a significant change in folate profile during lactation period under study. However, there has been a considerable change regarding folic acid content in relation to stage of laćtation in case of cow, being more active than buffalo.

4.1 FOLATE BINDING CAPACITY OF MILK

The folate binding capacity of milk was studied by taking two different colostrum and milk sample from each species. It has been reported that the milk has capacity

Fig. VI

Binding colostrum of folic acid added to goat's colostrum and milk (ng/ml)



COLOSTRUM
MILK, DAY 8
MILK, DAY 16
FOLIC ACID RECOVERED AFTER DIALYSIS (ng/ml)
600
500
400
300
200
100
0
500
1000
1500
2000
FOLIC ACID ADDED (ng/ml)

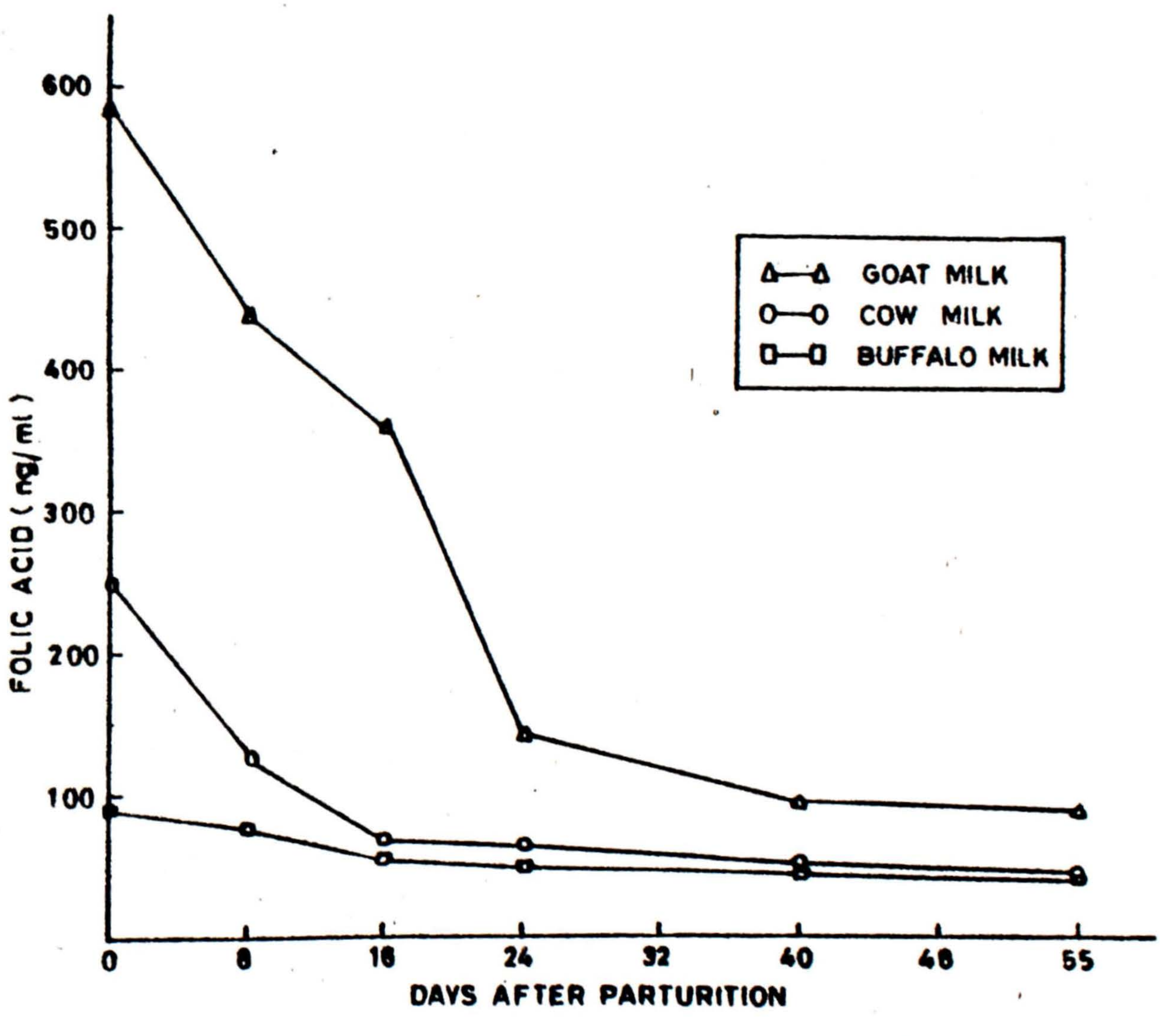
to bind extra amount of folate in addition to the natural one present. In order to know the maximum exogenous folate which could be bound by milk of any species, the graded concentrations of folic acid ranging from 500 - 2000 ng were added in different milk samples and dialysed as described. Since goat colostrum contained highest amount of folic acid, it was taken for standardising the condition. It can be seen fromFig. 6 that there was increased binding of folicacid till the concentration of added folic acid was 1000 ng. Afterwards, no additional binding was obtained at higher concentration of folic acid added and the curve was flattened. This indicated that 1000 ng of folic acid is enough to saturate the folate binding protein in rest of the samples. The addition of relatively higher concentration of folate did not appear to produce any advantageous results. Hence, 1000 ng of folic acid was added in all the samples to find out the total binding capacity of milk at different stages of lactation. The values for total bound folic acid have been given in Table VI and Fig. 7. As shown inTTable VII, goat colostrum had highest binding capacity which was followed by cow and buffalo respectively. This trend was maintained until day 24 after parturition. On day 40 and day55 cow and buffalo did not show any significant difference in total binding capacity of milk. On the other hand, goat milk at these stages of lactation still

Table VI. Total folic acid bound in milk from different species at various stages of lactation (ng/ml)

Species Animal No.	1st day	8th day	16th day	24th day	40th day	55th day
Cow						
4001	207.98	136.32	78.75	74.60	62.32	45.36
3102	291.26	117.68	57.42	54.05	48.72	42.58
Buffalo						
1097	82.10	68.65	40.13	38.49	32.62	31.99
368	98.46	84.92	70.36	60.46	58.38	55.17
Goat						
617	594.05	453.83	368.85	146.95	102.55	98.68
372	579.47	421.65	352.22	138.36	90.60	85.33

Fig.7

Total folate bound in milk from cow, buffalo and goat at various stages of lactation (ng/ml)



600
500
400
300
200
100
FOLIC ACID (ng/ml)
GOAT MILK
COW MILK
BUFFALO MILK
0
8
16
24
32
40
48
55
DAYS AFTER PARTURITION

Table VII. Comparative average value of total folate bound in milk from cow, buffalo and goat at various stages of lactation (ng/ml)

Species	1st day	8th day	16th day	24th day	40th day	55th day
Cow	249.620	127.000	68.085	70.825	55.520	43.970
Buffalo	90.280	76.785	55.245	49.475	45.500	43.580
Goat	586.760	437.740	360.535	142.655	96.575	92.005

maintained higher binding capacity. There was a gradual fall in total binding capacity of milk from all the three species. The values fell from 249.62 to 43.97 ng/ml for cow, 90.28 to 43.58 ng/ml for buffalo and 586.76 to 92.00 ng/ml in case of goat.

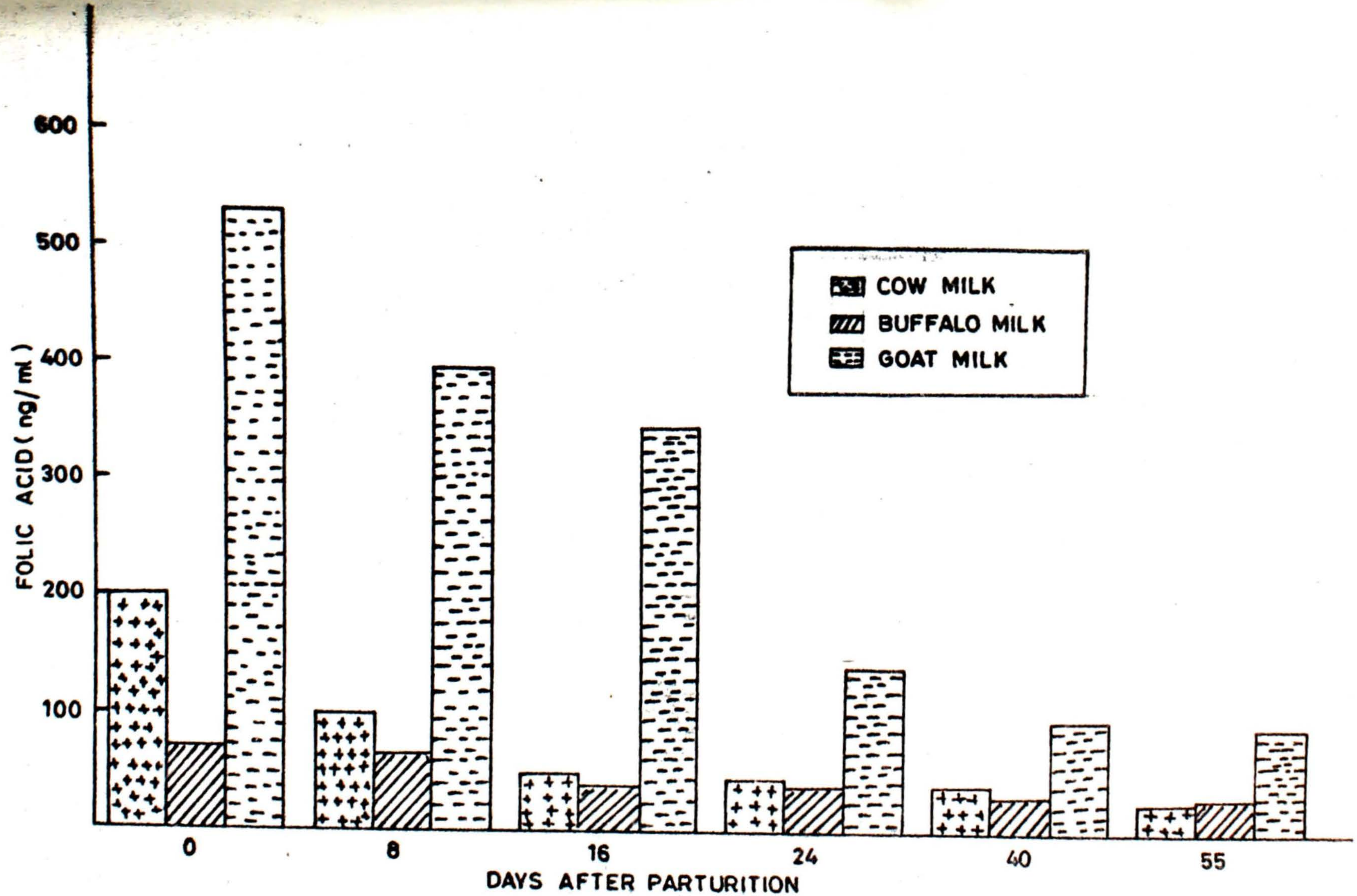
It was further calculated that how much additional amount of folate could be bound by each sample. The results have been shown in Table VIII, Table IX and Fig. 8. There were individual variations in additional folate bound by different milk from each species. The colostrum from all the species could bind highest amount of additional folate. On an average, the values for cow, buffalo and goat were 199.95, 72.1 and 532.24 ng/ml at this stage. As the period of lactation advanced, a declining pattern was obtained for the additional bound folate. On day 55, the values remained as 25.1, 30.7 and 90.00 ng/ml for cow, buffalo and goat respectively. It is also obvious from these results that cow colostrum bound the extra folate about more than five times the natural bound form present. Buffalo colostrum could only bind little more than three times the natural bound folate present. Strikingly, the goat colostrum showed a capacity to bind the extra folate about ten times the natural bound folate present. On day 55, the milk samples from cow and buffalo could hardly bind folate less than double the amount present in bound form. However,

Table VIII. Additional folate bound in milk from different species at various stages of lactation (ng/ml)

Species Animal No.	1st day	8th day	16th day	24th day	40th day	55th day
Cow						
4001	164.65	107.92	63.00	55.70	45.92	32.76
3102	235.26	93.68	36.92	39.15	37.39	17.43
Buffalo						
1097	67.61	63.15	30.34	28.62	25.32	24.88
368	76.58	65.62	50.86	44.22	37.08	36.68
Goat						
617	538.38	393.85	350.26	144.60	100.42	96.63
372	526.11	397.47	340.46	135.59	88.70	83.38

Fig. VIII

Additional folate bound in milk from cow, buffalo and goat at various stages of lactation (ng/ml)



600
500
400
300
200
100
FOLIC ACID (ng/ml)
COW MILK
BUFFALO MILK
GOAT MILK
0
8
16
24
40
55
DAYS AFTER PARTURITION

Table IX. Comparative average values of additional folate bound in milk from cow, buffalo and goat milk at various stages of lactation (ng/ml)

Species	1st day	8th day	16th day	24th day	40th day	55th day
Cow	199.955	100.800	49.960	47.425	41.655	25.095
Buffalo	72.095	64.385	40.600	36.420	31.200	30.780
Goat	532.245	395.660	345.360	140.095	94.560	90.005

the goat milk on this day surprisingly bound 45 times the natural bound form present. It can be concluded that the additional amount of bound folate by all the species at different stages of lactation showed a variability similar to what was obtained for either total content or bound form of folate. Here, again, goat milk maintained the highest capacity throughout the lactation period under study regarding the binding capacity as compared to cow and buffalo.

These results indicate that there is a direct functional relationship between folic acid an folate binding factor present in the milk. High binding capacity shown by colostrum means that the folate binding proteins are high unsaturated. The loss in binding capacity of milk at later stages of lactation may be attributable to the possibility that the folate binding protein are being synthesized with a reduced rate as the lactation advances. This can only be proved if the total concentration of these folate binding proteins is determined at respective stages of lactation. It may also be possible that some unknown factor regulating the binding activity of milk is either inactive or absent. It is worth studying the folate binding mechanism to explain these possibilities. Ford et al. (1972) reported that goat colostrum had the capacity to bind about 600 ng folate per ml and folic acid added in excess of this threshhold concentration was not retained against dialysis. They further observed that by day 30, the natural folate concentration fell from 210 to 5 ng/ml and folate binding capacity from 600 to 130 ng/ml. However, the mature milk still contained more folate binder. From these findings it seems that the milk folate concentration is primarily determined by the rate of secretion in relation to availability of free folate in blood plasma and that the milk's content of folate binder does not limit the milk folate concentration. This can be supported by the fact that colostrum contained highest folate which is secreted at a slow rate whereas mature milk contained reduced amount of folate since it is secreted relatively faster rate.

Ford and Scott (1975) reported that human and cow's milk can bind 60 ng of extra folic acid per ml. Cow's milk also bound around 60 ng of extra folic acid per ml. However, it had higher affinity for cyanocobalamin than for folic acid.

4.2 FACTORS INFLUENCING THE FOLATE BINDING CAPACITY OF MILK

a) pH

The effect of pH was studied on folate binding capacity by treating the milk at different pH as described in Table X and XI. The highest amount of folate was retained at pH 7.2

Table X. Effect of pH on folate binding capacity of milk (ng/ml)

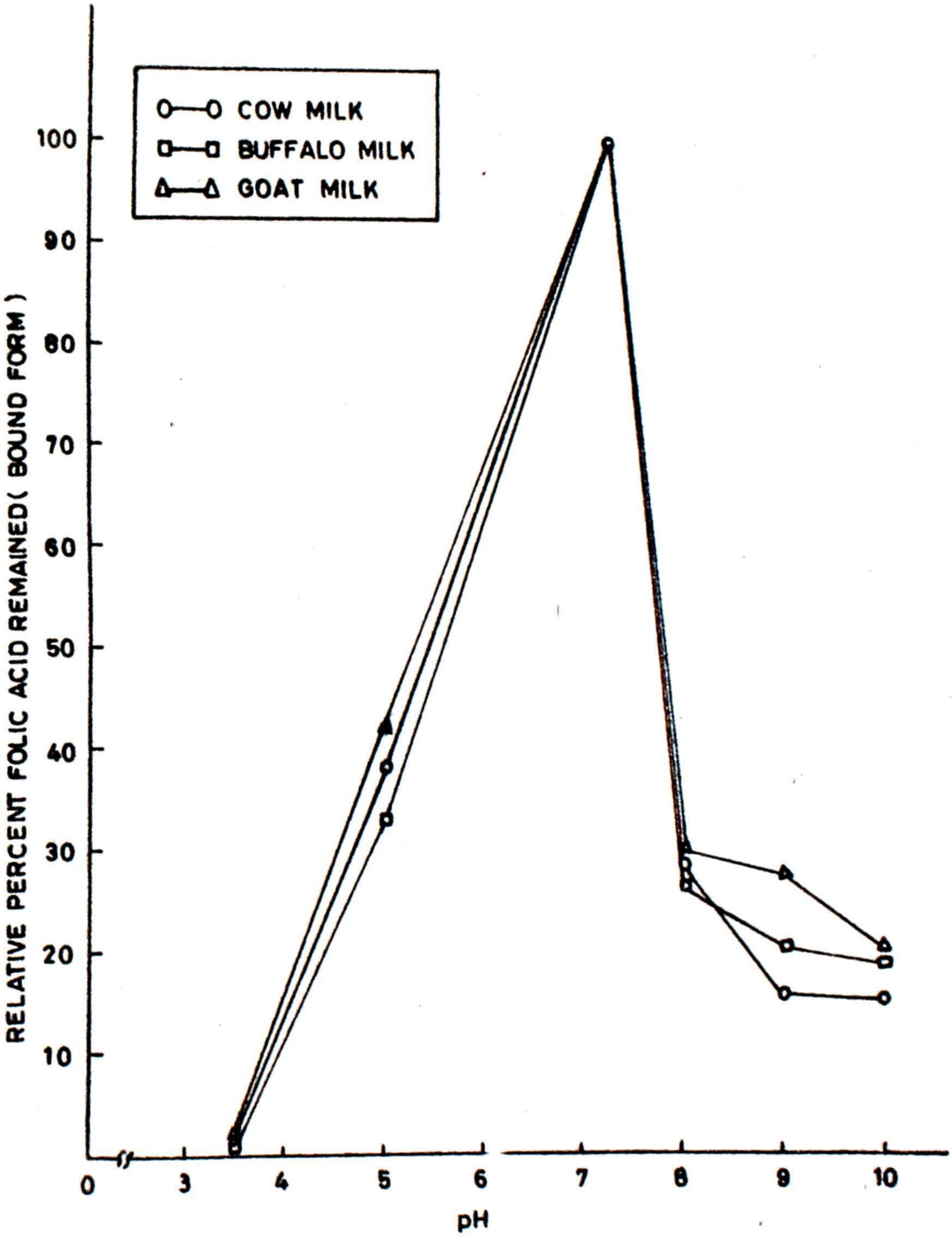
Species	3.5	5.0	7.2	8.0	9.0	10.0
Cow	0.58	10.83	28.50	8.36	4.56	4.45
Buffalo	0.25	5.48	16.54	4.46	3.85	3.19
Goat	0.12	2.32	5.48	1.65	1.52	1.13

Table XI. Relative per cent folic acid remained (Bound Form)

Species	3.5	5.0	7.2	8.0	9.0	10.0
Cow	2.03	38.00	100	29.33	16.00	15.61
Buffalo	1.51	33.13	100	26.96	20.44	19.29
Goat	2.19	42.33	100	30.11	27.74	20.62

Fig. IX

Relative percent folic acid remained (Bound form)


COW MILK
BUFFALO MILK
GOAT MILK
RELATIVE PERCENT FOLIC ACID REMAINED (BOUND FORM)
100
90
80
70
60
50
40
30
20
10
0
3
4
5
6
7
8
9
10
pH

by milks from all the three species. There was a drastic dissociation of bound folate as pH was reduced. This dissociation was also observed at pH higher than the pH 7.2 in alkaline range. In case of cow milk, out of 28.5 ng/ml folate retained at pH 7.2, only 10.83 and 0.58 ng/ml could be retained at pH 5.0 and 3.5 respectively. On the other hand, 8.36, 4.56 and 4.45 ng/ml folate was retained at pH 8.0, 9.0 and 10.0 respectively. The pattern regarding the extent of dissociation was almost similar in other species also.

On comparing the extent of dissociation caused by pH on bound folate, it was quite clear (Table XI) that goat milk was fairly stable against the pH either in alkaline or acidic range. This was followed by cow and buffalo respectively. In alkaline range, buffalo milk retained folate higher than cow's which was not true in acidic pH. The values for folic acid retained by milk from different species ranged from 1.51 - 2.19% at pH 3.5. However, at pH 10.0, the folate was retained in the range from 15.6 - 20.6% of the total present at pH 7.2 The milk samples from all the species were fairly stable against alkaline pH as compared to their stability against dissociation in acidic pH.

Ford *et al*.(1969) and Ford (1974) reported that uptake of free folate was strongly influenced by pH and was at a maximum at about pH 6.0, whereas uptake of folate added with goat's milk was greatest at pH 4.5 and declined to a low level at pH 5.0 and above. This higher availability of the milk bound folate at pH 4.5 probably reflected dissociation of the folate protein in Sephadex gel G25, eluting with buffer solutions of different pH values. At pH 6.0 and above, the folate was eluted in the void volume together with the protein. At pH 5.0, only 61% of the folate emerged with the proteins and 39% was recovered as free folate. At pH 3.6, only free folate was present in the eluate. The dissociation at pH 3.6 was reversed on adjustment of the pH value to 7.1. Givas and Gutcho (1975) compared the binding of folic acid and N-5-methyl-tetrahydrofolic acid to milk binder in the pH range 7.4 - 10.1. At pH 7.8, the relative affinities were quite disparate, with folic acid showing the greater affinity for milk binder. As the pH was increased from 7.4 to 9.3, the difference in affinities became smaller, and at pH 9.3, the affinities were nearly the same. As the pH was increased from 9.3 to 10.1, the relative affinities again began to differ, with N-5-methyltetrahydrofolic acid displaying the greater affinity.

b) Heat Treatment

The effect of heat treatment was investigated by pasteurizing and boiling the milk as described. On pasteurisation and boiling, there was a little loss in total folate present in

Table XII. Effect of heat treatment on the folate binding activity of milk (ng/ml)

Species	Normal			Pasteurised			Boiled		
	Free	Bound	Total	Free	Bound	Total	Free	Bound	Total
Cow	66.00	14.90	80.90	65.94	10.32	76.26	61.93	8.75	70.68
Buffalo	20.60	15.00	35.60	22.57	10.08	32.65	21.77	7.98	29.75
Goat	6.86	2.77	9.63	6.66	2.40	9.06	5.52	1.76	7.28

Table XIII. Relative percentage of folic acid remained after heat treatment

Species	Normal (Raw milk)		Pasteurised milk		Boiled milk	
	Free	Bound	Free	Bound	Free	Bound
Cow	81.58	18.42	81.50	12.76	76.55	10.82
Buffalo	57.86	42.13	63.39	28.31	61.15	22.41
Goat	71.24	28.74	69.16	24.92	57.32	18.28

the milk from all the species. Out of a total of 80.9 ng/ml folate, 76.26 and 70.68 ng/ml remained after pasteurisation and boiling in cow's milk. Similar patterns were also observed in case of buffaloes and goats. In addition to little loss in total content observed, it was found that there was a dissociation of bound folate on pasteurisation and boiling which caused an increase in free form of folate. This dissociation of bound folate was higher when milk was boiled than that obtained for pasteurised milk.

From a total of 18.42 per cent bound folate in cow's milk, 12.75 and 10.82 per cent could be retained after pasteurisation and boiling respectively. In case of goat milk, buffalo milk only 28.31 and 22.41 per cent bound folate was retained on pasteurisation and boiling respectively against 42.13 per cent bound folate present in normal milk. In case of goat milk, 22.42 and 18.28 per cent bound folate could be retained against 28.74 per cent in normal milk. Overall, it appears that buffalo milk is relatively highly resistant to heat treatment as compared to goat and cow. Further, goat milk is more stable than cow milk in this regard. Probably, heat treatment affected the bound form of folate since some kind of denaturation of the protein was caused by such treatment. These results indicated that such treatments usually practised during consumption of raw milk still offer some bound folate which could be useful from nutrition point of view.

Ghitis and Canosa (1965) reported that autoclaving of milk affected the folate activity of milk when they observed that the folate activity decreased from 36.0 - 43.0 ug/l to 14.0 - 22.0 ng/l. Later on, Areekul _et al_. (1978) suggested that the infants should preferably be breast fed rather than bottle fed as cow's milk was usually not given in raw form. They reported that binding activity of human milk was 10.3 ng/ml while that of pasteurised cow's milk was 1.23 ng/ml.

The three species, namely, cow, buffalo and goat have shown similar profile for folic acid content in milk at various stages of lactation. They have also shown similar decline in pattern for total folate binding capacity in milk with increased lactation period. The information can further be enriched in this regard if such studies are conducted in milk from other ruminants like sheep, in particular. It is still not clear whether the synthesis of folate binding proteins is declined or there is some other factor regulating this activity becomes inactive or absent. This can only be confirmed if the vitamin binders are isolated from milk at different stages of lactation and some work is initiated on the vitamin-binding mechanism. Further studies are also required to know the nature of vitamin binders present in blood plasma and milk and milk of the same animal and to explore the possibility of establishing some structural relationship between the vitamin-binders from two sources.

CHAPTER V

SUMMARY

SUMMARY

The folic acid content in the milks from cow, buffalo and goat was estimated on day 1, day 8, day 16, day 24, day 40 and day 55 after parturition. The highest folate content was found in colostrum from all the species which was (mean ± SE) 131.94 ± 24.08, 54.68 ± 6.56 and 278.92 ng/ml on day 1 in cow, buffalo and goat milk respectively. There was gradual decrease in milk folate with increased lactation period. On day 55, the values were 56.55 ± 6.00, 37.75 ± 4.46 and 5.60 ng/ml in milks from three species in similar order as given for day 1. The total folate binding capacity in milk was determined at all these stages of lactation under study . The total folate binding capacity of colostrum from cow, buffalo and goat was 249.62, 90.28 and 582.76 ng/ml respectively. A further decline was obtained on the pattern similar to total folate content. The goat colostrum and mature milk showed highest capacity to bind added folate as compared to buffalo and cow milk at all the stages of lactation. The additional amount of folate bound by cow, buffalo and goat milk was 199.95, 72.1 and 532.24 ng/ml on day 1 and 25.1, 30.7 and 90.0 ng/ml on day 55 after parturition.

Goat milk was fairly stable with regards to its folate binding capacity either at alkaline pH or acidic pH. This was

followed by cow and buffalo respectively. The amount of bound folic acid retained by milk from different species ranged from1.51 - 2.19% at pH 3.5 and from 15.6 - 20.6% at pH 10.0 out of the total bound folate present at pH 7.2 There was 6 - 10% loss in total content of folate after pasteurisation and this loss was increased on boiling which ranged from 13 - 24 %. By both these types of treatments, there was significant dissociation of bound folate where buffalo milk was fairly stable as compared to milk from cow and goat in this regard.

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