Br. J. Nutr. (1974), 31, 243

Some observations on the possible nutritional significance of vitamin B_{12}- and folate-binding proteins in milk

By J. E. FORD

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT

(*Received* 12 *June* 1973 – *Accepted* 26 *September* 1973)

1. The folate and vitamin B_{12} in milk are strongly bound to minor whey proteins. These binder proteins are present in excess, and so milk has capacity – which varies between milks of different mammalian species – to sequester added cyanocobalamin and folic acid.

2. A study was made of the influence of different milks on uptake of [^{3}H]cyanocobalamin and [^{3}H]folic acid in selected bacteria, mostly of types that are commonly found in the intestine.

3. None of the test cultures required exogenous vitamin B_{12}. Nevertheless, when free cyanocobalamin was added, in seven of nine cultures it was rapidly taken up into the cells, though there were large differences between the different bacterial species in their absorptive capacity. In presence of sow's milk, little or none of the added cyanocobalamin was taken up, even after incubation of the test mixture for 1 h at 37°. The avidity of sow's milk for cyanocobalamin, as judged from its retention of the vitamin against competition by bacterial cells, was greater than that of a preparation of porcine Intrinsic Factor of similar binding capacity.

4. Of ten cultures of bacteria representing seven species, only the five cultures that required exogenous folate took up added folic acid into the cells. Uptake was severely depressed by the simultaneous addition of goat's colostrum, even after incubation of the test mixture for 1 h at 37°.

5. The physiological role of the binders is discussed. It is postulated that they may act in the mammary gland as trapping mechanisms to accumulate the vitamins from blood plasma into milk and in the gut to facilitate their absorption, both directly, and indirectly by preventing their uptake by intestinal micro-organisms. It is concluded that the binders might well influence the vitamin economy in the neonatal period, and the ecology of the gut microflora.

The rearing of babies on processed cow's milk is so widely practised as to have become accepted as the 'normal' procedure, in Britain as in several other developed countries. There appears to be no decisive nutritional advantage in breast-feeding: the bottle-fed baby generally thrives, despite suspicions that he may be more susceptible to gastro-intestinal and respiratory infections, and perhaps also to a variety of less common maladies (for a review see Davies, 1971). With farm animals – piglets and calves – the possible hazards of early weaning onto milk replacement formulas are more apparent, and it is recognized that the suckling animal is comparatively much more resistant to gastroenteritis in the neonatal period. Part of the explanation lies in the passive immunity conferred by antibodies present in the colostrum and milk, but there is evidence (cf. Bullen, Rogers & Leigh, 1972) that the iron-binding proteins lactoferrin and transferrin are an important component of a protective mechanism in human milk, and act in combination with specific antibody to check the growth of *Escherichia coli* in the intestine.

The vitamin B_{12} and folate in milk, like the Fe, are strongly and specifically bound to minor whey proteins which are present in excess, so that the milk has capacity to bind added vitamin. This prompts the speculation that these vitamin-binding proteins

might similarly influence the growth and composition of the gut microflora. The vitamin B_{12} in sow's milk is not accessible to vitamin B_{12}-requiring micro-organisms (Gregory, Ford & Kon, 1952; Gregory & Holdsworth, 1955), and milk folate might prove equally unavailable to folate-requiring species. Further, it could well be that it is not only the vitamin-dependent microbial species that are inhibited since, even in species that synthesize vitamins, leakage from the cells into the surrounding medium constitutes a burden on synthesis during the early phase of growth (Ford & Goulden, 1959). The presence of external vitamin binders might increase this drain and so reduce growth rate; and under the conditions in the gut small changes in relative growth rates could have marked effects on the composition of the flora.

The vitamin binders in milk may have a complex physiological role. They may act in the mammary gland as trapping mechanisms to accumulate the vitamins from blood plasma into milk and in the gut to facilitate their absorption, both directly, and indirectly by preventing their uptake by intestinal micro-organisms. The present paper describes a study of the influence of the binders on the uptake of vitamin B_{12} and folic acid in selected micro-organisms.

MATERIALS AND METHODS

Milk samples. The sow's milk was a bulked sample from three Large White sows in their 2nd–4th week of lactation. It was collected as described by Braude, Coates, Henry, Kon, Rowland, Thompson & Walker (1947). The sample of goat's colostrum was taken from a British Saanen goat within a few hours of kidding and the sample of mature milk from another animal in the 11th month of lactation. The cow's milk was bulked morning milk from the herd of British Friesians maintained at the National Institute for Research in Dairying (NIRD). The human milk was from one donor, in the 4th month of lactation. The milks were centrifuged at about 2°, for 30 min at 5000 *g*, and the aqueous phase was decanted and filtered through Kleenex tissue. The fat was discarded.

Sow's milk was chosen as being particularly rich in vitamin B_{12}-binder. Gregory & Holdsworth (1955) compared the binding activity in milk of several mammalian species. The amount of cyanocobalamin bound by milk of the following species was (ng/ml): mature sow 245; rat 126; human 80; goat 2·7 and cow 0·5. Goat's colostrum was selected as being a rich source of the folate binder; 1 ml binds about 600 ng folic acid (Ford, Knaggs, Salter & Scott, 1972).

Digestion of sow's milk and goat's colostrum with pepsin and trypsin. To 10 ml samples of the colostrum and milk were added 10 ml 0·1 M-HCl, and 1 M-HCl to pH 2·0. Crystallized porcine pepsin (10 mg) (Koch–Light Laboratories, Colnbrook, Bucks.) was then added and the mixture incubated for 90 min at 37°, with occasional 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 (Koch–Light Laboratories) containing

< 50% $MgSO_4$. The mixture was incubated for 15 min at 37° and its pH was again adjusted to 8·2 by addition of 0·1 M-NaOH. Incubation was continued for a further 105 min, with occasional swirling.

Test micro-organisms. Escherichia coli 0101K was the causal organism of neonatal diarrhoea in a calf; it was kindly supplied by my colleague Dr J. Brock. *Streptococcus faecalis* B 65 (group D, serological type 6; Sharpe, 1964) and *Lactobacillus fermenti* ATCC 9338 were isolates from infants' faeces, and the cultures were kindly supplied by Dr M. E. Sharpe. The following cultures were obtained from the National Collection of Dairy Organisms at NIRD: *Lactobacillus acidophilus*, NCDO 4 (isolated from human vagina); *Lactobacillus bifidus*, NCDO 1452 (from faeces of a breast-fed infant); *Enterobacter aerogenes*, NCDO 1487 (from a mastitis-infected bovine udder); *Streptococcus faecalis*, NCDO 581; *Streptococcus zymogenes*, NCDO 592; *Proteus mirabilis*, NCDO 1880; *Staphylococcus aureus*, NDCO 949. This small selection is not of course representative of enteric organisms; the cultures were chosen largely for their ease of cultivation in defined media. The object in this preliminary study was to test the concept that vitamin B_{12} and folate binders in milk might inhibit the uptake of the vitamins into bacteria, and so influence the ecology of the gut microflora and the nutrition of the host animal.

Culture media. The stock cultures of *E. coli*, *Ent. aerogenes* and *P. mirabilis* were stored at 4° on nutrient agar slopes. *Staph. aureus*, *Strep. faecalis* and *Strep. zymogenes* were stored in stab culture in yeast–dextrose agar, and *L. bifidus*, *L. acidophilus* and *L. fermenti* in stab culture in MRS agar (De Man, Rogosa & Sharpe, 1960).

For the experiments on vitamin uptake, *E. coli* was grown in the chemically-defined, vitamin-free medium of Burkholder (1951). *L. bifidus* was grown in the medium of Skeggs, Nepple, Valentik, Huff & Wright (1950), modified in that *p*-aminobenzoic acid was omitted and the content of folic acid reduced to 0·5 μg/l. *L. fermenti* and *L. acidophilus* were grown in a medium based on that of Ford & Rogosa (1961). Sodium acetate trihydrate was included at 10 g/l final strength medium, and the content of both KH_2PO_4 and K_2HPO_4 was reduced to 0·5 g/l. Cyanocobalamin and *p*-aminobenzoic acid were omitted, the content of folic acid was reduced to 0·5 μg/l, and 10 mg thymidine were added/l. *Ent. aerogenes*, *Staph. aureus*, *Strep. faecalis* and *Strep. zymogenes* were grown in the medium of Ford (1962), modified in that cyanocobalamin and *p*-aminobenzoic acid were omitted, and the content of folic acid was reduced to 0·5 μg/l. All the test cultures were grown at 37° for 18 h, in 250 ml Pyrex conical flasks each containing 150 ml basal medium. After incubation, 2 ml portions of culture were withdrawn and diluted with 2 ml preserving fluid (Fernell & Rosen, 1956), for subsequent microscopic cell counting and measurement of absorbance at 580 nm. The remainder of the culture was adjusted to pH 6·8 by the addition of 1 M-NaOH, and cooled in a bath of iced water.

Radioactive vitamins. [G-^{3}H]Cyanocobalamin (1·2 mCi/mg) and [G-^{3}H]folic acid (potassium salt; 11·3 mCi/mg) were purchased from the Radiochemical Centre, Amersham, Bucks. They were diluted with non-radioactive cyanocobalamin and folic acid to convenient concentrations of the labelled compounds.

Porcine Intrinsic Factor (IF) concentrate. IF concentrate, batch number 934C-186-1,

was supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

Measurement of binding activity. The folic acid-binding capacity of the goat's colostrum was measured as described by Ford *et al.* (1972). The capacity of the sow's milk to bind added cyanocobalamin was measured as follows. The milk was diluted fourfold with water. To 1 ml portions were added graded amounts of cyanocobalamin (10–800 ng) in 1 ml water. A 1 ml sample of each mixture was then transferred to a sac of 8 mm cellulose dialysis tubing and dialysed for 48 h at 4° against eight successive 100 ml portions of buffer solution of pH 7·0 containing 0·15 M-NaCl and 0·02 M-sodium phosphate. The residual cyanocobalamin in the sacs was then assayed microbiologically as described by Gregory (1954). The cyanocobalamin-binding activity of the IF concentrate was measured similarly, using 1 ml portions of aqueous solution containing 1 mg IF/ml.

Measurement of bacterial uptake. Giannella, Broitman & Zamcheck (1971, 1972) studied the uptake of cyanocobalamin by intestinal bacteria, and the competitive binding of the vitamin by IF and by bacterial cells. They demonstrated that bacterial uptake is a two-stage process. The primary uptake stage was rapid, independent of temperature, and did not require viable organisms. This phase was thought to represent attachment of the vitamin to receptor sites on the cell wall. The second stage was slower, required actively metabolizing bacteria, and was presumed to reflect transport of the vitamin into the bacterial cell. Only the primary uptake stage was influenced by IF, and in competition with IF all the bacteria studied bound 10–27% of the available cyanocobalamin, except *P. mirabilis* and *Klebsiella pneumoniae* which bound > 50%. In the present study, first-stage uptake was assessed by addition of chilled bacterial culture to 15 ml conical centrifuge tubes containing the test preparations of radioactive vitamin, and immediately centrifuging for 5 min at 3500 *g*. The supernatant fluid was decanted and the pellet of bacterial cells dispersed in 5 ml of the appropriate culture medium by stirring with a glass rod. The suspension was centrifuged again and the supernatant fluid decanted. The pellet was dissolved in 1 ml hyamine hydroxide and the solution transferred to a scintillation vial, to which were then added 10 ml of scintillator solution containing, per l toluene, 4 g 2,5-diphenyloxazole and 0·3 g 1,4-bis-(5-phenyloxazol-2-yl) benzene. The radioactivity was measured in a Philips type PW 4510/01 liquid scintillation analyser, in which the efficiency of counting is automatically determined by use of an external standard.

In further experiments the test mixtures were incubated at 37° for 1 h before the initial centrifugation.

RESULTS

Influence of sow's milk and IF concentrate on the uptake of cyanocobalamin

All the test cultures grew freely in media that contained no measurable vitamin B_{12} activity. It was presumed, therefore, that none was dependent on exogenous vitamin B_{12}. Nevertheless, all but two of the nine test cultures took up the free vitamin avidly, though there were large differences between the different species in their absorptive capacity (Table 1). Thus, *E. coli* rapidly took up 1·84 ng cyanocobalamin/10^9 cells,

Table 1. *Influence of sow's milk on uptake of cyanocobalamin by different bacteria*

[^{3}H]Cyanocobalamin (10 ng) was added, (1) alone and (2) bound with 0·1 ml sow's milk, to 3 ml portions of test culture. The bacterial cells were harvested by centrifugation, immediately ('rapid uptake') and after incubation for 1 h at 37° ('1 h uptake')

Organism	No. of bacteria added per test	Recovery of [^{3}H]cyanocobalamin in bacterial cells (%)			
		'Rapid uptake'		'1 h uptake'	
		(1)	(2)	(1)	(2)
Enterobacter aerogenes	$4{\cdot}2 \times 10^{9}$	64·3	6·1	59·4	4·3
Lactobacillus bifidus	$6{\cdot}0 \times 10^{9}$	36·6	3·5	58·0	6·3
Escherichia coli	$2{\cdot}4 \times 10^{9}$	44·1	2·9	63·6	9·8
Staphylococcus aureus	$11{\cdot}7 \times 10^{9}$	14·8	1·4	47·0	3·1
Streptococcus faecalis	$11{\cdot}1 \times 10^{9}$	1·8	0·4	11·6	1·3
Strep. zymogenes	$9{\cdot}9 \times 10^{9}$	0·5	0·2	9·4	1·5

Table 2. *Influence of sow's milk and Intrinsic Factor (IF) concentrate on 1 h uptake of cyanocobalamin by different bacteria*

[^{3}H]Cyanocobalamin (10 ng) was added, (1) alone, (2) bound with 0·15 mg IF and (3) bound with 0·15 ml sow's milk, to 5 ml portions of test culture. The bacteria cells were harvested by centrifugation after incubation for 1 h at 37°

Organism	No. of bacteria added per test	Recovery of [^{3}H]cyanocobalamin in bacterial cells (%)		
		(1)	(2)	(3)
Enterobacter aerogenes	$10{\cdot}5 \times 10^{9}$	73·7	20·1	0·94
Lactobacillus bifidus	$5{\cdot}0 \times 10^{9}$	50·1	1·98	0·14
Escherichia coli	$4{\cdot}9 \times 10^{9}$	77·3	16·3	1·39
Staphylococcus aureus	$18{\cdot}0 \times 10^{9}$	20·7	0·71	0·22
Streptococcus faecalis, NCDO 581	$16{\cdot}0 \times 10^{9}$	13·0	1·55	0·32
Lactobacillus fermenti	$7{\cdot}5 \times 10^{9}$	0·16	0·30	0·28
Proteus mirabilis	$3{\cdot}5 \times 10^{9}$	46·0	14·3	1·16
Streptococcus faecalis, B 65	$6{\cdot}6 \times 10^{9}$	5·7	0·57	0·35
Lactobacillus acidophilus, A 4	$3{\cdot}5 \times 10^{9}$	0·47	0·35	0·17

whereas only 0·005 ng/10^{9} cells were taken up by *Strep. zymogenes*. In the presence of sow's milk the uptake of added cyanocobalamin by the test bacteria was sharply reduced.

Before the effects of sow's milk and IF concentrate on uptake were compared, the capacity of these two substances to retain added cyanocobalamin against prolonged dialysis was measured, as described on page 246. The sow's milk bound 140 ng added cyanocobalamin/ml, and a solution containing 1 mg IF/ml bound 120 ng/ml.

The effects of sow's milk and IF on 1 h uptake are compared in Table 2. Both binders were added in excess of the amounts needed to bind the test dose of 10 ng cyanocobalamin: IF had the capacity to bind 18 ng, and the sow's milk 21 ng. In seven of the nine bacterial species tested, uptake of cyanocobalamin was clearly depressed by the binders, much more by the milk than by the IF. However, since part of the binding capacity of gastric juice is not due to IF (cf. Gottlieb, Lau, Wasserman & Herbert, 1965), and since the dried preparation of IF used in the present

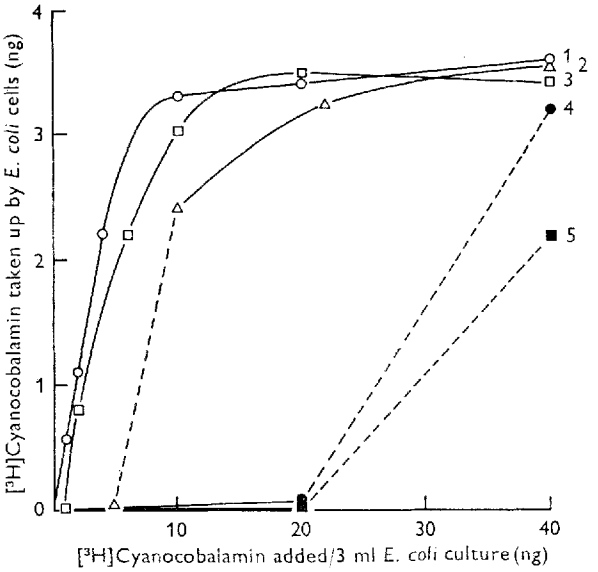

Fig. 1. Influence of milk on the uptake of [³H]cyanocobalamin in *Escherichia coli*. To 3 ml portions of chilled culture were added (1) graded amounts of cyanocobalamin, with (2) 2 ml goat's colostrum, or (3) 5 ml cow's milk, or (4) 0·2 ml sow's milk, or (5) 0·5 ml human milk. The mixtures were centrifuged immediately, and the pellet of bacterial cells was washed before being dissolved in hyamine hydroxide for scintillation counting.

experiments might have been partly denatured during manufacture, it cannot be inferred that the binders in porcine IF and sow's milk are necessarily dissimilar.

In one of the test cultures, *L. fermenti*, the apparent uptake of cyanocobalamin during the incubation period was very small (0·002 ng/10^9 cells as against 1·58 ng/10^9 cells in *E. coli*) and might well have been largely attributable to insufficient washing of the cells. The same is true for the apparent small uptake of sow's milk-bound cyanocobalamin in *L. bifidus*, *Staph. aureus*, *Strep. faecalis* and *L. acidophilus*.

Influence of milk on the uptake of cyanocobalamin in E. coli. Fig. 1 shows the influence of milk from different mammalian species on first-stage uptake of cyanocobalamin, added in graded amounts to portions of *E. coli* culture. Uptake of cyanocobalamin added alone (1) was proportional to the amount added to the culture up to about 3·2 ng, which was presumably a measure of saturation of first-stage uptake. In the presence of 0·2 ml sow's milk (4) there was little uptake of cyanocobalamin until the amount added exceeded 20 ng; thus, the milk had the property of sequestering > 100 ng cyanocobalamin/ml and making it unavailable to *E. coli*. With human milk (5) there was no uptake of cyanocobalamin added at 20 ng/0·5 ml milk; the milk therefore bound > 40 ng/ml. The corresponding value for goat's colostrum was > 2·5 ng/ml, and for cow's milk > 0·2 ng/ml.

Effects of digestion with pepsin and trypsin. In the first of two experiments, digestion of the sow's milk with pepsin caused no apparent change in its capacity to inhibit uptake of added cyanocobalamin in *E. coli*; the pattern of uptake was similar to that for undigested sow's milk shown in Fig. 1. However, this comparison would not have revealed any small change resulting from the pepsin digestion. It showed only

Table 3. *Influence of pH on uptake of cyanocobalamin in* Lactobacillus bifidus

([^{3}H]Cyanocobalamin (10 ng) was added (1) alone and (2) bound with 0·1 ml sow's milk, to 3 ml portions of test culture. The bacterial cells were immediately harvested by centrifugation, washed, and dissolved in hyamine hydroxide for scintillation counting)

	Recovery of [^{3}H]cyanocobalamin in bacterial cells (%)	
pH	(1)	(2)
4·5	35·8	0·79
5·0	45·0	0·34
5·5	42·8	0·25
6·0	45·3	0·17
6·5	51·7	0·33
7·0	46·5	0·30
7·5	41·5	0·32
8·0	41·3	0·28
8·5	44·1	0·20
9·0	45·3	0·30

that the peptic digest, like the raw milk, had the capacity to bind 100–200 ng added cyanocobalamin/ml. In a second experiment, carried out a few days later with a further sample from the same batch of sow's milk and under apparently similar conditions, pepsin digestion reduced the binding capacity to 50–100 ng/ml. It appears therefore that the vitamin B_{12}-binder largely survived peptic digestion for 90 min at pH 2·0 and retained much of its capacity to bind added cyanocobalamin.

On further digestion with trypsin there was a considerable loss of binding activity, but about 20% of the activity of the raw milk survived digestion successively with pepsin and trypsin.

Influence of pH on uptake of [^{3}*H*]*cyanocobalamin in* L. bifidus. The predominance of *L. bifidus* in the intestine of breast-fed infants is associated with a low pH in the contents of the large intestine, which may act to depress growth of *E. coli* (Bullen & Willis, 1971). *L. bifidus* grows over a wide pH range, and the present experiment was set up to test the influence of pH on the uptake of [^{3}H]cyanocobalamin, free and bound to sow's milk.

Ten 10 ml portions of an 18 h culture of *L. bifidus* were adjusted to different pH values in the range 4·5–9·0, in increments of 0·5 pH units. Two 3 ml samples were taken from each and cooled in iced water. To one was added 0·1 ml of aqueous solution containing 10 ng cyanocobalamin, and to the other a mixture containing 0·1 ml of the cyanocobalamin solution and 0·1 ml sow's milk. The bacterial cells were immediately harvested by centrifugation, washed and dissolved in 1 ml hyamine hydroxide for scintillation counting. The results are shown in Table 3. They show that the efficiency of first-stage uptake was affected very little, if at all, by variation of pH within the range 5·0–9·0. Similarly, pH variation within this range had little influence on the low availability of sow's milk-bound cyanocobalamin. At pH 4·5 the efficiency of uptake of free cyanocobalamin was perhaps marginally lower, and the availability of the bound cyanocobalamin slightly higher, but the significance of these small differences is uncertain and requires further investigation.

Table 4. *Influence of goat's colostrum on uptake of folic acid by different bacteria*

([^{3}H]Folic acid (100 ng) was added (1) alone and (2) bound with 1·5 ml goat's colostrum, to 5 ml portions of test culture. The bacterial cells were harvested by centrifugation, immediately ('rapid uptake') and after incubation for 1 h at 37° ('1 h uptake'))

Organism	No. of bacteria added per test	Recovery of [^{3}H]folic acid in bacterial cells (%)			
		'Rapid uptake'		'1 h uptake'	
		(1)	(2)	(1)	(2)
Lactobacillus bifidus	$5{\cdot}0 \times 10^9$	21·0	0·22	76·0	0·31
Streptococcus faecalis, NCDO 581	$22{\cdot}0 \times 10^9$	9·2	0·14	37·5	1·40
Strep. faecalis, B 65	$9{\cdot}1 \times 10^9$	5·8	0·23	37·9	0·19
Strep. zymogenes	$31{\cdot}0 \times 10^9$	6·3	0·16	48·5	0·76
Lactobacillus acidophilus	$3{\cdot}6 \times 10^9$	38·0	0·19	49·9	0·46
Staphylococcus aureus	$17{\cdot}0 \times 10^9$	0·07	0·14	0·09	0·16
Enterobacter aerogenes	$7{\cdot}5 \times 10^9$	0·10	0·17	0·38	0·38
Proteus mirabilis	$12{\cdot}0 \times 10^9$	0·12	0·12	0·21	0·15
Escherichia coli	$6{\cdot}3 \times 10^9$	0·10	0·10	0·16	0·46
Lactobacillus fermenti	$1{\cdot}4 \times 10^9$	0·03	0·10	0·17	0·24

Influence of goat's colostrum on the uptake of folic acid

Table 4 shows the influence of goat's colostrum on the uptake of folic acid in ten cultures of bacteria representing seven species. The colostrum had unsaturated capacity to bind 560 ng folic acid/ml, and so the 100 ng folic acid added with 1·5 ml colostrum was entirely bound.

The cultures could be classified into two groups on the basis of their capacity to take up folic acid. Five of the cultures (*L. bifidus*, *L. acidophilus* and the three faecal streptococci) rapidly absorbed 5·8 – 38 % of the added vitamin, and at 1 h had taken up 38 – 76 %. In all of these five cultures the capacity to absorb folic acid was associated with a requirement for exogenous folate, which was readily demonstrable by serial cultivation in culture medium with and without added folic acid. In the absence of folic acid, growth failed by the second or third transfer.

The remaining five cultures (*Staph. aureus*, *Ent. aerogenes*, *P. mirabilis*, *E. coli* and *L. fermenti*) took up very little, if any, of the added folic acid, and at 1 h had absorbed at most 0·38 %. None required exogenous folate.

In the five cultures that took up added folic acid, uptake was severely depressed by the simultaneous addition of goat's colostrum, and even after 1 h incubation with the test cultures little of this bound folate was taken up by the bacterial cells.

Influence of goat's colostrum on uptake of folic acid in L. bifidus. Fig. 2 shows the patterns of uptake of folic acid added in graded amounts of 10–60 ng to portions of *L. bifidus* culture, with and without goat's colostrum. Free folate was rapidly taken up, with efficiency ranging from 67 % of the 10 ng dose to 36 % of the 60 ng dose. In the presence of 0·05 ml goat's colostrum no folic acid was absorbed until the test dose exceeded 20 ng. By extrapolation of the line of best fit describing the uptake of the 30, 40, 50 and 60 ng doses, it appears that 0·05 ml colostrum had the capacity to sequester 29 ng added folic acid. This measure of the folate-binding capacity (580 ng/

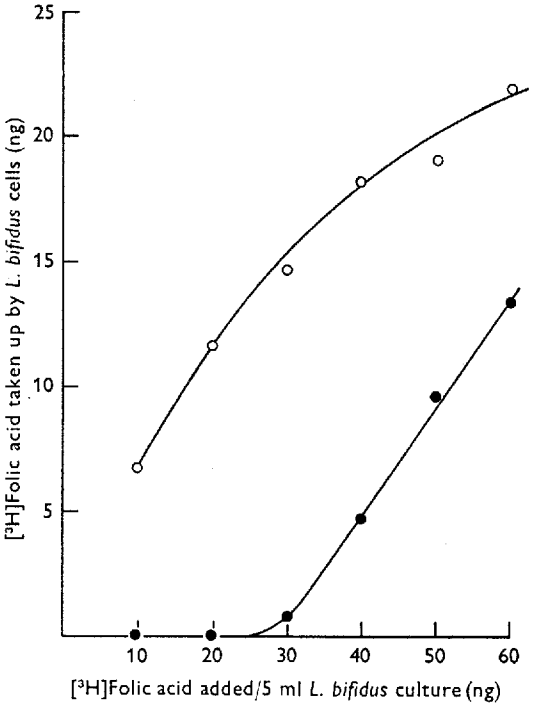

Fig. 2. Influence of goat's colostrum on the uptake of [³H]folic acid in *Lactobacillus bifidus*. To 5 ml portions of chilled culture were added graded amounts of [³H]folic acid, alone (○—○) and with 0·05 ml goat's colostrum (●—●). The bacterial cells were immediately harvested by centrifugation, washed, and dissolved in hyamine hydroxide for scintillation counting.

ml) agreed closely with the value 560 ng/ml obtained by the dialysis procedure (see page 246).

Effects of digestion with pepsin and trypsin. Ford, Salter & Scott (1969) isolated folate binder from a preparation of cow's milk by starch-gel electrophoresis at pH 2·0 in the presence of 5 M-urea. They showed that this purified preparation, after dialysis against buffer of pH 7·0, had retained capacity to bind added folic acid despite the drastic procedures to which it had been subjected. In the present experiment the influence of peptic digestion at pH 2·0 was examined, and the further effects of subsequent digestion with trypsin. Folate-binding activity was assessed by measuring the capacity of the test preparations to inhibit uptake of added folic acid in *L. bifidus*. The results are shown in Fig. 3.

As in Fig. 2, free folate (1) was rapidly taken up, whereas in the presence of 0·1 ml goat's colostrum (2) no folic acid was absorbed until the test dose exceeded 56 ng. Digestion of the colostrum with pepsin (3) reduced its binding activity to about 24 ng/0·1 ml, and it seemed that this bound folate was perhaps not quite so completely inaccessible to the bacteria as was the folate added to raw colostrum. On further digestion with trypsin (4) the colostrum no longer hindered the uptake of added folate.

Influence of pH on the uptake of [³H]folic acid in L. bifidus. Ten 10 ml portions of

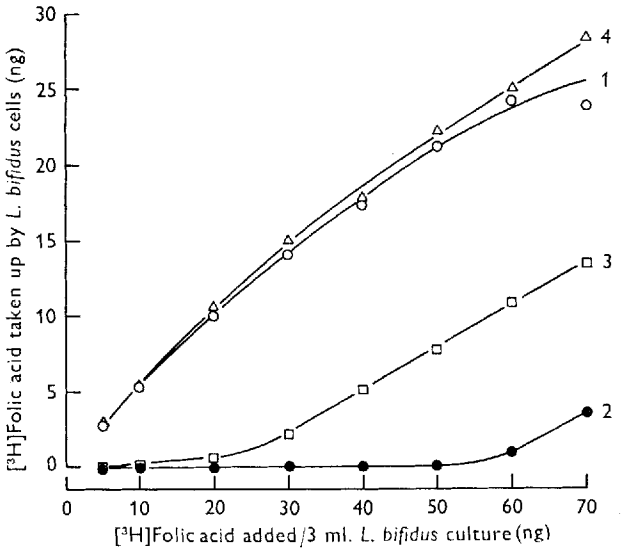

Fig. 3. Effects of digesting goat's colostrum with pepsin and trypsin on its capacity to bind added [³H]folic acid. To 3 ml portions of chilled culture were added (1) graded amounts of [³H]folic acid, with (2) 0·1 ml colostrum, or (3) pepsin digest equivalent to 0·1 ml colostrum, or (4) pepsin–trypsin digest equivalent to 0·1 ml colostrum. The mixtures were centrifuged immediately, and the bacterial cells were washed before being dissolved in hyamine hydroxide for scintillation counting.

an 18 h culture of *L. bifidus* were taken and adjusted to different pH values, in the range 4·5–9·0, in increments of 0·5 pH units. Two 3 ml samples were taken from each. To one was added 1 ml of an aqueous solution containing 20 ng [³H]folic acid, and to the other 1 ml of a solution containing 20 ng [³H]folic acid and 0·5 ml mature goat's milk. The small changes in pH that occurred on adding the milk to the cultures at the two extremes of the pH range were ignored. The bacterial cells were immediately harvested by centrifugation, washed and dissolved in hyamine hydroxide for scintillation counting. The results are shown in Fig. 4.

Uptake of free folate was strongly influenced by pH and was at a maximum at about pH 6·0, whereas uptake of the 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 reflects dissociation of the folate–protein complex. Ford *et al.* (1969) filtered a preparation of folate–protein in Sephadex gel G 25, 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 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.

Influence of heating on the vitamin binders. A study is now being made of the effects of heating and other treatments on the vitamin binders, and the findings will be reported in due course. For the purposes of the following discussion, it is convenient to record here that the binders are heat-labile. Thus, heating at 115° for 5 min

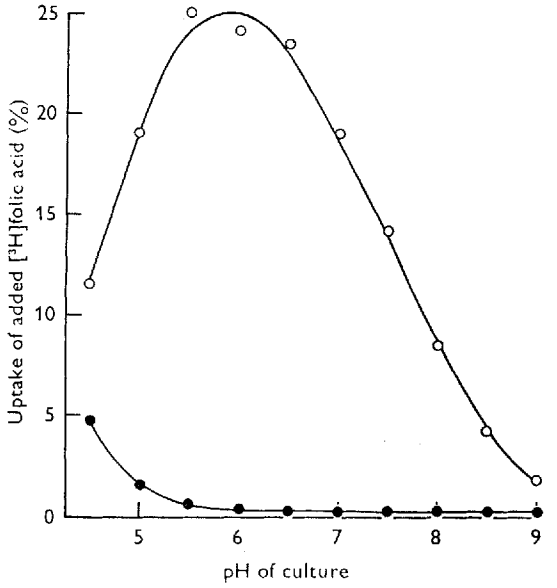

Fig. 4. Influence of pH and of goat's milk on the uptake of folic acid in *Lactobacillus bifidus*. Portions of a culture of *L. bifidus* were adjusted to different pH values in the range 4·5–9·0. Two 3 ml samples were taken from each. To one was added 20 ng [^{3}H]folic acid (○), and to the other 20 ng [^{3}H]folic acid in 0·5 ml goat's milk (●). The bacterial cells were immediately harvested by centrifugation, washed and dissolved in hyamine hydroxide for scintillation counting.

destroyed the capacity of sow's milk to bind added cyanocobalamin, and of goat's milk and cow's milk to bind added folic acid.

DISCUSSION

As long ago as 1949, Ternberg & Eakin (1949) showed that vitamin B_{12} combines with a non-dialysable component of normal gastric juice, and that the bound vitamin was not available to *Lactobacillus leichmannii*. Hoff-Jorgensen (1952) further showed that several wild strains of *E. coli*, none of which required exogenous vitamin B_{12}, absorbed large amounts of the vitamin from the culture medium, and that extracts of pig's stomach inhibited this absorption. He suggested that 'this finding may offer an explanation of the effect of IF, as it is known that in patients suffering from pernicious anaemia large numbers of *E. coli* are found in the upper intestine and even in the stomach'. Giannella *et al.* (1971) concluded that competition by enteric microorganisms may explain the malabsorption of vitamin B_{12} that frequently accompanies the clinical syndromes of bacterial overgrowth in the intestine, and Giannella *et al.* (1972) suggested that IF may have a dual function, namely to promote the absorption of vitamin B_{12} across the ileal mucosa, and to protect the vitamin from uptake and utilization by the normal flora in the terminal small bowel. An interesting question then arises, namely whether in the newborn mammal the vitamin B_{12}-binder in the milk similarly acts in these dual roles to facilitate uptake and protect the vitamin from the competition of intestinal bacteria. In the newborn rat, vitamin B_{12} is absorbed

with high efficiency by a process that is independent of gastric IF. Uptake by this process falls off rapidly and by 14 d has largely disappeared. Meanwhile, the content of IF in the stomach wall increases, from a very low value at birth to the adult level at weaning (Boass & Wilson, 1963; Williams & Spray, 1968). Transmission of passive immunity in the rat is mostly by way of the colostrum and milk and continues for most of the lactation period. There is very little uptake of vitamin B_{12} by passive diffusion, and attachment of the vitamin to binder protein clearly offers a plausible explanation for the high efficiency of IF-independent uptake during the early post-natal period. In the newborn baby very small amounts of intact proteins may be transmitted from the gut to the circulation, but the weight of evidence is that little or no antibody is absorbed from the colostrum and milk, though colostral antibodies may afford protection against infections of the alimentary tract (Brambell, 1970). In the piglet, transmission of passive immunity declines very rapidly after birth and effectively ceases at the end of the 1st day. Thus, in these two species, unless uptake of the protein-bound vitamin continues long after transmission of immunity has ceased, we must suppose that the bound vitamin B_{12} is absorbed in a less direct manner. One possibility is that the binder acts by relaying the vitamin to receptor sites at the mucosal surface, as has been postulated for IF (Glass, 1963).

Vitamin B_{12}-binding is only one facet of IF activity; the essential characteristic of IF is its ability to promote the uptake of the vitamin, and there is need for unequivocal evidence that the milk binders do not share this property. If they do not, then we must envisage the transfer to IF of the milk-bound vitamin B_{12}, though the evidence now presented (Table 2) might suggest that the transfer would be predominantly in the reverse direction. The avidity of the sow's milk binder for cyanocobalamin, as judged from its capacity to retain the vitamin against competition by the bacterial cells, was distinctly greater than that of the preparation of porcine IF of equivalent binding capacity. In the presence of sow's milk, little or none of the added cyanocobalamin was taken up by the bacterial cells, even after incubation of the test mixture for 1 h at 37°.

Seven of the nine test cultures rapidly took up free cyanocobalamin. The extent of uptake varied, and ranged from 0·08 ng/10^9 cells in *Strep. faecalis* to 1·58 ng/10^9 cells in *E. coli*. Uptake might have been greater in some of the test cultures if they had been centrifuged and the cells resuspended in fresh medium. Thus, in cultures of *Ochromonas malhamensis* the property of binding cyanocobalamin is not confined to the cells. It appears also in the culture fluid during growth, and in proportion to the vitamin B_{12} requirement of the organism this extracellular binding capacity is very large. Cyanocobalamin linked to the extracellular binder is much less readily absorbed than the free vitamin (Ford, 1958). It is possible that similar extracellular binders could have depressed the uptake of added cyanocobalamin in the present experiments.

Fig. 1 shows that milks of several different mammalian species were all potent inhibitors of cyanocobalamin uptake by *E. coli*, though their capacity to bind added cyanocobalamin varied widely. The high capacity of human milk and sow's milk to bind added cyanocobalamin prompts the speculation that the binder may act during the neonatal period in the reabsorption of biliary vitamin B_{12}. The bile is the main

excretory vehicle for vitamin B_{12}, though in the adult animal most of this vitamin B_{12} is reabsorbed through the agency of IF. The flux of vitamin B_{12} in this enterohepatic circulation may be considerable: Gräsbeck, Nyberg & Reizenstein (1958) estimated that, in the human adult, about 43 μg vitamin B_{12} were secreted each day in the bile and about 31 μg were reabsorbed. These values may be too high (Ardeman, Chanarin & Berry, 1965), but it is apparent that, failing an efficient recovery mechanism, losses of vitamin B_{12} in the bile would be considerable and probably greater than the supposed dietary requirement. It seems reasonable to postulate that the milk binder constitutes just such a recovery mechanism in the neonate, and operates until its function is taken over by the developing IF system.

The hypothesis that the vitamin B_{12} binder in milk promotes the absorption of the vitamin from the gut and protects it against the intestinal microflora could apply equally to the folate binder. The large amount of bound folate in goat's colostrum passes intact into the kid's blood circulation, together with much unsaturated binder protein (Ford *et al.* 1972). The period of time during which the intact folate–protein complex is absorbed is brief and probably corresponds to the period during which passive immunity is transmitted from the colostrum. Thereafter, as with vitamin B_{12}, the bound folate must be transmitted in a less direct way, since unsaturated binder no longer passes from the milk into the kid's blood plasma.

Like vitamin B_{12}, folate passes into the gut with the bile. Baker, Kumar & Swaminathan (1965) showed that in man the daily biliary excretion may average 60–90 μg, which is much the same as the minimal nutritional requirement in the adult male (Herbert, 1962). The unsaturated folate-binder in milk might facilitate recovery of this biliary folate and so be important in the folate nutrition of the nursling.

Izak, Galewski, Rachmilewitz & Grossowicz (1972) compared the uptake of free and goat's milk-bound folic acid from intestinal segments from weaned 100 g rats. They reported that the bound folate was readily absorbed, predominantly from the ileum, where uptake was three times as great as that of free folic acid. The conditions of their experiment were of course unphysiological and their finding begs the question whether, in the intact animal, milk-bound folate survives into the ileum. The same question arises from the above speculations concerning the possible role of the binder in the recovery of biliary folate and in protecting the milk folate from intestinal micro-organisms. Under the conditions of in vitro digestion described on page 244 the folate-binding activity was destroyed by trypsin, and it may be that, with the rapid decline in the content of trypsin inhibitor in milk with advancing lactation, the binder is similarly destroyed in the intestine.

Of the eight species of bacteria examined in the present study, only those that could be shown to require exogenous folate took up the free vitamin. *L. acidophilus* rapidly took up 10·6 ng [^{3}H]folic acid/10^9 cells and *L. bifidus* 4·2 ng/10^9; the three faecal streptococci took up 0·20–0·63 ng/10^9. The corresponding '1 h uptake' values were 13·9, 15·2 and 1·6–4·2 ng/10^9 cells. The remaining five species showed no significant rapid uptake, and '1 h uptake' ranged from 0·005 to 0·12 ng/10^9 cells. In the presence of goat's colostrum, uptake of folic acid in the folate-requiring strains was considerably reduced; the 1 h uptake values were only 0·4–3·7 % of those for free folate.

It is well known that the nature of the diet may greatly influence the composition of the intestinal flora. In mice given a folate-free diet there was a marked increase in the incidence of folate-synthesizing coliforms and other Gram-negative organisms (Klipstein & Lipton, 1970). The notion that the vitamin B_{12} and folate binders in milk might similarly influence the ecology of the gut microflora by withholding the vitamins from dependent bacteria raises questions to which we have at present few answers. We need to know much more about the metabolism of the binders within the intestine, and about the numbers and varieties of bacteria in the absorptive region of the small intestine and their quantitative requirements for vitamin B_{12} and folate.

To suggest that the folate and vitamin B_{12} binders in milk facilitate the absorption of the vitamins is to imply that destruction of the binders, as might occur in the manufacture and subsequent preparation for use of milk foods for babies, could be positively detrimental. Matoth, Pinkas & Sroka (1965) found that blood folate concentrations were about 50% higher in breast-fed infants than in artificially fed children of the same age. They showed that the difference could probably be accounted for by loss of folate during heat-treatment of the milk, but suggested that 'quantitative differences in folate intake may not necessarily be the sole factor responsible for the higher blood folate concentrations in breast-fed infants. Other factors, such as folate absorption, may be involved. Furthermore, the role played by the intestinal flora as a producer or a consumer of folates in either breast-fed or artificially-fed infants is not known'.

My conclusion is that the binders may well exert a considerable influence on the vitamin economy in the neonatal period, and on the composition of the gut microflora.

I thank Dr M. E. Coates and Dr J. W. G. Porter for their helpful interest in this study. I am indebted to Dr G. F. Spray for the gift of IF concentrate, to Dr K. G. Mitchell for organizing the collection of sows' milk, and to Sara Staker and John Siriwardawa for technical help.

REFERENCES

Ardeman, S., Chanarin, I. & Berry, V. (1965). *Br. J. Haemat.* **11**, 11.
Baker, S. J., Kumar, S. & Swaminathan, S. P. (1965). *Lancet* i, 685.
Boass, A. & Wilson, T. H. (1963). *Am. J. Physiol.* **204**, 101.
Brambell, F. W. R. (1970). *The Transmission of Passive Immunity from Mother to Young* p. 269. Amsterdam: North-Holland Publishing Co.
Braude, R., Coates, M. E., Henry, K. M., Kon, S. K., Rowland, S. J., Thompson, S. Y. & Walker, D. M. (1947). *Br. J. Nutr.* **1**, 64.
Bullen, J. J., Rogers, H. J. & Leigh, L. (1972). *Br. med. J.* i, 69.
Bullen, C. L. & Willis, A. T. (1971). *Br. med. J.* iii, 338.
Burkholder, P. R. (1951). *Science, N.Y.* **114**, 459.
Davies, P. A. (1971). *Br. med. J.* iv, 351–354.
De Man, J. C., Rogosa, M. & Sharpe, M. E. (1960). *J. appl. Bact.* **23**, 130.
Fernell, W. R. & Rosen, G. D. (1956). *Br. J. Nutr.* **10**, 143.
Ford, J. E. (1958). *J. gen. Microbiol.* **19**, 161.
Ford, J. E. (1962). *Br. J. Nutr.* **16**, 409.
Ford, J. E. & Goulden, J. D. S. (1959). *J. gen. Microbiol.* **20**, 267.
Ford, J. E., Knaggs, G. S., Salter, D. N. & Scott, K. J. (1972). *Br. J. Nutr.* **27**, 571.
Ford, J. E. & Rogosa, M. (1961). *J. gen. Microbiol.* **25**, 249.
Ford, J. E., Salter, D. N. & Scott, K. J. (1969). *J. Dairy Res.* **36**, 435.
Giannella, R. A., Broitman, S. A. & Zamcheck, N. (1971). *J. clin. Invest.* **50**, 1100.
Giannella, R. A., Broitman, S. A. & Zamcheck, N. (1972). *Gastroenterology* **62**, 255.

Glass, G. B. J. (1963). *Physiol. Rev.* **43**, 529.
Gottlieb, C., Lau, K., Wasserman, L. R. & Herbert, V. (1965). *Blood* **25**, 875.
Gräsbeck, R., Nyberg, W. & Reizenstein, P. (1958). *Proc. Soc. exp. Biol. Med.* **97**, 780.
Gregory, M. E. (1954). *Br. J. Nutr.* **8**, 340.
Gregory, M. E., Ford, J. E. & Kon, S. K. (1952). *Biochem. J.* **51**, proc. xxix.
Gregory, M. E. & Holdsworth, E. S. (1955). *Biochem. J.* **59**, 329.
Herbert, V. (1962). *Archs intern. Med.* **110**, 649.
Hoff-Jorgensen, E. (1952). *Archs Biochem.* **36**, 235.
Izak, G., Galewski, K., Rachmilewitz, M. & Grossowicz, N. (1972). *Proc. Soc. exp. Biol. Med.* **140**, 248.
Klipstein, F. A. & Lipton, S. D. (1970). *Am. J. clin. Nutr.* **23**, 132.
Matoth, Y., Pinkas, A. & Sroka, C. (1965). *Am. J. clin. Nutr.* **16**, 356.
Sharpe, M. E. (1964). *J. gen. Microbiol.* **36**, 151.
Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W. & Wright, L. D. (1950). *J. biol. Chem.* **184**, 211.
Ternberg, J. L. & Eakin, R. E. (1949). *J. Am. chem. Soc.* **71**, 3858.
Williams, D. L. & Spray, G. H. (1968). *Br. J. Nutr.* **22**, 297.

Printed in Great Britain