Vitamin B_{12} absorption in the neonatal piglet

2. Resistance of the vitamin B_{12}-binding protein in sows' milk to proteolysis in vivo

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1. It has been postulated that the vitamin B_{12}-binding protein in the sows' milk may facilitate the intestinal absorption of vitamin B_{12} in the piglet. This implies that the binder is not rapidly and completely degraded by gut proteases, and the present experiments were devised to test this assumption.

2. Piglets aged 7 and 28 d were given a test feed of sows' milk whey both with and without cyano[^{3}H]cobalamin to saturate the binder, and with ^{14}C-labelled polyethylene glycol (PEG) as a marker. Control piglets were given a milk substitute containing no vitamin B_{12}-binder. At 80 min after the test meal the piglets were killed and the contents of the stomach and small intestine were removed for analysis.

3. Recovery of [^{14}C]PEG, mainly from the middle and distal portions of the small intestine, ranged from 49 to 78%. Apparent loss of vitamin B_{12}-binder from the intestine was calculated from the ratio, PEG:binder. In both 7- and 28-d-old piglets, the content of saturated binder in the middle and lower small intestine was about 20% depleted relative to that in the test feed. With the unsaturated binder the loss was more variable and generally greater, at about 50%.

4. At slaughter the stomach was nearly empty and the high unsaturated binding capacity of the contents was attributed to endogenous binders. However, in the middle and lower small intestine, the binding capacity was derived mainly from sows' milk given with the test meal. No free cyano[^{3}H]cobalamin was found in the intestinal contents of piglets given sows' milk to which enough cyano[^{3}H]cobalamin had been added to saturate the binder.

5. There was no change in the molecular weight of the vitamin B_{12}-binder during its passage down the intestine, as judged by its behaviour on filtration in Sephadex gel G-150.

6. The results indicate that a high proportion of saturated binder, and a smaller proportion of unsaturated binder, survived unchanged in the intestine of piglets at both ages.

Ford (1974) and Ford *et al.* (1975) postulated that the vitamin B_{12}-binding protein in milk may strongly influence the vitamin B_{12} economy of the sucking animal and the ecology of its gut microflora, by facilitating the intestinal absorption of the vitamin and preventing its uptake by intestinal micro-organisms. This implies that the binder persists in the intestine and is not rapidly degraded under the influence of gut proteases, and Ford *et al.* (1975) offered evidence that this is indeed the case. However, studies in vitro showed that, although digestion of sows' milk with pepsin (*EC* 3.4.23.1) caused no apparent change in the capacity of the vitamin B_{12}-binder to inhibit uptake of vitamin B_{12} by *Escherichia coli*, treatment with trypsin (*EC* 3.4.21.4) greatly reduced this inhibitory effect (Ford, 1974). Samson *et al.* (1980) found with human milk that its capacity to prevent the growth of a vitamin B_{12}-dependent strain of *E. coli* was lost when the milk was digested with trypsin although treatment with pepsin had no such effect. The situation in vivo may be quite different, however. In the piglet there are marked changes in the quantities of proteases produced in the stomach, pancreas and intestine during the first few weeks after birth. Levels are low during the first 3–4 weeks, but thereafter they increase rapidly (Manners, 1976; Corring *et al.* 1982). It is reasonable to suppose, therefore, that much of the vitamin B_{12}-binder in the sows' milk might survive digestion during these early weeks of life. The present

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experiments were designed to determine the extent to which vitamin B_{12}-binder, present in a test meal containing sows' milk whey, survived digestion in the gastrointestinal tract of 7- and 28-d-old piglets. A preliminary report of this work has been published (Trugo & Newport, 1983).

EXPERIMENTAL

Preparation of the test feeds

Sows' milk (2 litres) was taken from a bulk supply representing mature milk from several sows. It had been stored at −20°. After thawing, it was centrifuged at 30000 *g* for 30 min at 4°. The aqueous phase was carefully decanted from the residue of micellar casein and other particulate matter and the supernatant layer of hardened fat, and filtered through a Whatman no. 4 filter paper. The unsaturated vitamin B_{12}-binding capacity (B_{12}-UBC) of the filtrate (whey) was then determined by the charcoal adsorption method (see p. 259).

A 50 mg (0·5 μCi)/ml solution of ^{14}C-labelled polyethylene glycol (PEG) was prepared by diluting the specific radioactivity of [^{14}C]PEG (20 μCi/mg) with non-radioactive PEG (PEG-4000; BDH Chemicals, Poole, Dorset) and added to 1·5 litres whey to give a concentration of 1 mg PEG/ml. To half this mixture was added an aqueous solution of cyano[G-^{3}H]cobalamin to give a concentration of 89 ng (0·29 μCi) cyano[G-^{3}H]cobalamin/ml. This added vitamin was precisely sufficient to saturate the vitamin B_{12}-binder in the whey. The test feeds, one containing binder saturated with cyano[G-^{3}H]cobalamin and the other containing unsaturated binder, were stored as 50 or 100 ml portions at −20°, and thawed just before being given to the piglets.

Cyano[G-^{3}H]cobalamin (specific activity 3·25 mCi/mg) and [^{14}C]PEG (specific activity 20 μCi/mg) were obtained from Amersham International plc (Amersham, Bucks).

Procedure with piglets

The piglets used in this experiment were from the Institute's herd of Large White × Landrace pigs. Two litters, each of nine piglets, were weaned at 5- and 26-d-old. They were caged individually and given a milk substitute (diet A), prepared as described by Newport (1980). This had very low (0·1 ng/ml) B_{12}-UBC. After 2 d the piglets were starved overnight and assigned to three treatment groups: (1) three piglets at 7- and 28-d-old given sows' milk whey containing cyano[G-^{3}H]cobalamin-saturated binder; (2) four piglets at 7- and 28-d-old given whey containing no added cyano[G-^{3}H]cobalamin (unsaturated binder); (3) two piglets at 7- and 28-d-old given the milk substitute. The purpose of this control treatment (group 3) was to provide a measure of the B_{12}-UBC in the gut contributed by the endogenous secretion of cobalophilins and intrinsic factor. Each piglet was given a single test feed of 50 ml (7-d-old) or 100 ml (28-d-old) and killed by intracardiac injection of sodium pentobarbitone 80 min after receiving the test feed.

Removal and preparation of gut contents

Immediately after death, the stomach and small intestine were ligated and removed. The small intestine was cut into three approximately equal lengths (upper, middle and lower segments). The contents of the stomach and the three intestinal segments were washed out with 0·02 M-sodium phosphate buffer, pH 7·0, containing 0·15 M-sodium chloride. Approximately 50 and 100 ml buffer were used for the 7- and 28-d-old piglets respectively. A second washing was then carried out with a larger volume of buffer, until the effluent was colourless. Volumes were recorded and the washings kept separately. The washings were centrifuged at 28000 *g* for 20 min at 4° and the supernatant volumes measured. The sediments (solid phase) were combined, twice resuspended in 10 ml buffer and centrifuged, and finally resuspended in 5 ml buffer.

Analysis of gut contents

^{14}C and ^{3}H. Radioactivity was measured in duplicate 1 ml portions of liquid phase and resuspended sediment, and also in 1 ml portions of the test feeds. Samples were digested with 1 ml hyamine hydroxide (BDH Chemicals) and neutralized with 0·5 ml citric acid solution (12 g/l) before addition of 10 ml Instagel scintillant (Packard Instruments, Reading), and counted in a Packard B2450 Tricarb liquid-scintillation spectrometer, with external standardization of counting efficiency. ^{14}C and ^{3}H were counted simultaneously as described by Neame & Homewood (1974). The concentrations of [^{14}C]PEG and cyano[G-^{3}H]cobalamin were determined by the use of standards.

B_{12}-UBC. This was measured by the charcoal adsorption method of Gottlieb *et al.* (1965), modified in that polyvinylpyrrolidone (PVP-360; Sigma Chemical Co., Poole, Dorset) was used as the coating agent instead of albumin. Duplicate 1 ml samples of the test feeds and of the liquid phase from the contents of stomachs and intestinal segments were mixed with 50 μl of a solution containing 1 μg cyano[G-^{3}H]cobalamin (3·25 μCi)/ml, and with 50 μl 0·02 M-sodium phosphate buffer, pH 7·0, containing 0·15 M-NaCl. After 20 min, 2 ml of the charcoal suspension was added and the mixtures left standing for a further 30 min, with occasional shaking. They were then centrifuged at 3000 ***g*** for 20 min and a portion of the supernatant taken for counting as described previously. Controls containing 1 ml buffer instead of sample were also analysed.

As the samples from piglets in group 1 already contained cyano[G-^{3}H]cobalamin from the test feed, a further control was needed and was set up as follows. Samples (1 ml) were mixed with the charcoal suspension and the radioactivity of the supernatant fraction obtained after centrifugation was measured. This radioactivity was subtracted from that present in supernatant fractions obtained from samples to which additional cyano[G-^{3}H]cobalamin had been added before mixing with the charcoal suspension. The difference corresponded to the B_{12}-UBC of any excess of vitamin B_{12}-binders.

The contribution of gastric intrinsic factor to the total B_{12}-UBC in the gut contents was determined by use of a modification of the antibody-blocking technique of Gottlieb *et al.* (1967). Rabbit antiserum to cobalophilin isolated from sows' milk (see p. 260) was used to block the binding of added cyano[G-^{3}H]cobalamin to cobalophilins present in the gut contents, derived from milk or from the endogenous secretion. Antiserum (50 μl) was added to duplicate 1 ml portions of the samples and the mixtures incubated at 37° for 30 min before addition of cyano[G-^{3}H]cobalamin for the determination of the B_{12}-UBC by the charcoal method. The difference in B_{12}-UBC as measured before (total B_{12}-UBC) and after adding the antiserum (B_{12}-UBC due to intrinsic factor) represented the contribution of cobalophilins, irrespective of their origin, to the total B_{12}-UBC present in the sample.

Cyano[G-^{3}H]cobalamin-saturated binder. The concentration of cyano[G-^{3}H]cobalamin was determined in the liquid phase of the gut contents of piglets from group 1, which had received cyano[G-^{3}H]cobalamin-saturated binder in the test feed. After the charcoal treatment and centrifugation, radioactivity was counted in the supernatant fractions. The concentration of free cyano[G-^{3}H]cobalamin was determined by subtracting the concentration of bound radioactive vitamin B_{12} from that of total radioactive vitamin B_{12} present in the same samples before charcoal treatment.

Chromatography on Sephadex G-150. A column (25 mm × 600 mm) of Sephadex G-150 (Pharmacia Fine Chemicals, Hounslow, Middx) was employed for the chromatography of the whey used in the test feeds and of the liquid phase of the gut contents. Because of the large number of samples, only the liquid phase from the first of the two washings from one piglet at each age in groups 1 and 2 was analysed. The column was calibrated by determining the elution volumes of cyano[G-^{3}H]cobalamin and of proteins of known molecular weight:

haemocyanin 400000, human γ-globulin 155000, bovine serum albumin 67000, myoglobin 17500.

The samples (5–15 ml), except those obtained from piglets in group 1, were mixed with cyano [G-^{3}H]cobalamin in excess of their B_{12}-UBC before they were applied to the column. The column was eluted with 0·02 M-sodium phosphate buffer, pH 7·0, containing 0·15 M-NaCl and 5-ml fractions were collected and monitored for protein content by measurement of absorbance at 280 nm. Portions (0·1–1 ml) of each fraction were analysed for cyano[G-^{3}H]cobalamin. The chromatography was conducted in a cold room at 4°.

Preparation of antiserum to purified vitamin B_{12}-binding protein isolated from sows' milk

Isolation of the vitamin B_{12}-binder. Purified vitamin B_{12}-binder was isolated from sows' milk whey by affinity chromatography in a column of vitamin B_{12}–Sepharose, essentially as described by Burger & Allen (1974) for the isolation of vitamin B_{12}-binder from human milk. Sepharose 6B was purchased from Pharmacia Fine Chemicals. Purification of the binder by this technique was 3920-fold, and the yield 53·8%. Its homogeneity was checked by the use of sodium dodecylsulphate–polyacrylamide gel electrophoresis. A full description of the procedure and the characterization of the binder thus obtained is given elsewhere (Trugo, 1984).

Preparation of the antiserum. A male New Zealand White rabbit was injected intramuscularly with 0·5 ml portions (2 ml total) of an emulsion containing equal parts of the purified vitamin B_{12}-binder solution (0·5 mg/ml) in 0·15 M-NaCl and Freund's complete adjuvant (Miles Laboratories, Slough). Blood samples were taken from the marginal ear vein at intervals after immunization, and the titre of specific antibodies to the vitamin B_{12}-binder was determined in the serum by the indirect enzyme-linked immunosorbent assay (Voller *et al.* 1976) as described elsewhere (Trugo, 1984). Antiserum obtained at day 60 after injection was stored at −20° in 2 ml portions until required for use. The assessment of the antiserum specificity was performed by the double-immunodiffusion analysis of Ouchterlony (1958) and by immunoelectrophoresis (Hudson & Hay, 1976). The anti-(sows' milk vitamin B_{12}-binder) serum cross-reacted with porcine gastric cobalophilin (non-intrinsic factor from porcine gastric mucosa; Sigma Chemical Co.) but not with intrinsic factor (from porcine gastric mucosa; Sigma Chemical Co.).

Statistical analysis

In Tables 2 and 3, data for each site in the intestine were considered separately and subjected to analysis of variance after logarithmic transformation.

RESULTS

Total recovery of [^{14}C]PEG varied from 49 to 78% (Table 1). The marker was recovered mostly from the middle segment of the small intestine in the 7-d-old piglets and from the lower segment in the 28-d-old piglets. Thus, by 80 min after ingestion of the test meal, some of the digesta which contained the marker had already passed to the large intestine, but half, or more, was still present in the small intestine. No [^{14}C]PEG was present in the solids phase obtained after centrifugation of the gut contents.

The determination of B_{12}-UBC due to cobalophilin in the gut contents of piglets given different test feeds is shown in Table 2. Comparison of results for the control piglets, which had received little or no vitamin B_{12}-binder in their feed, with those for piglets given the unsaturated binder in sows' milk shows that most of the cobalophilin in the stomach was of endogenous origin, whereas most of that in the middle and lower small intestine was

Table 1. *Recovery (%) of ^{14}C-labelled polyethylene glycol (PEG) in the gut contents (liquid phase) of piglets*

(Mean values)

Vitamin B_{12}-binder in test feed*	Age (d)	No. of piglets	[^{14}C]PEG recovered (%)				
				Small intestine			
			Stomach	Upper	Middle	Lower	Total
Saturated	7	3	3·0	5·1	47	23	78
	28	3	4·4	2·8	15	46	68
Unsaturated	7	4	2·4	4·7	24	18	49
	28	4	2·0	3·2	16	50	71
SD (8 df)	—	—	1·66	2·16	6·82	6·67	8·80

* For details of test feeds, see p. 258.

derived from the milk. The B_{12}-UBC values for the gut contents of piglets given milk with saturated binder were similar to those found for the controls.

The cyano[^{3}H]cobalamin in the liquid phase of the gut contents from piglets given milk in which the binder was saturated with cyano[G-^{3}H]cobalamin was recovered in bound form. No free cyano[G-^{3}H]cobalamin was detected in the liquid phase of the gut content and neither bound nor free cyano[^{3}H]cobalamin was present in the solids phase, which probably consisted of intestinal bacteria, mucosal debris and food residues. The ratio, bound cyano[G-^{3}H]cobalamin (ng):[^{14}C]PEG (mg) (Table 3), used to express the ratio saturated vitamin B_{12}-binder:PEG, changed little during passage of the digesta down the intestine and remained broadly similar to that in the test feed. In contrast, with the milk containing unsaturated binder, the ratio, B_{12}-UBC:PEG was much higher in the stomach but lower in the middle and lower segments of the small intestine. The ratio, B_{12}-UBC:PEG in the gut contents was then corrected for endogenous cobalophilin (Table 3) by subtraction of the values obtained from the control group.

The apparent extent of survival of the milk vitamin B_{12}-binder is shown in Table 4. The values were calculated by dividing the ratio, concentration of bound cyano[G-^{3}H]cobalamin, or the B_{12}-UBC attributable to milk: content of marker [^{14}C]PEG at each site in the gut (Table 3) by the corresponding value in the test feeds. These results show that saturated vitamin B_{12}-binder largely survived digestion in the gut, assuming that the vitamin remained bound to the milk cobalophilin, rather than transferring to endogenous cobalophilin. However, the survival of unsaturated binder was poorer.

Fig. 1 shows the Sephadex G-150 elution profile for a sample of sows' milk whey, to which had been added an amount of cyano[G-^{3}H]cobalamin equivalent to twice its B_{12}-UBC. Nearly half (48%) of the added cyano[G-^{3}H]cobalamin was eluted in a bound form, as a single peak, in the region corresponding to a molecular weight of approximately 150000 (115 ml effluent volume). The remainder was eluted as free vitamin (260 ml effluent volume). Dilution of the sows' milk whey, or addition of cyano[G-^{3}H]cobalamin to only 5% saturation of the vitamin B_{12}-binding capacity did not alter the elution volume of the binder.

Fig. 2 shows the Sephadex G-150 elution profile for the liquid phase from contents of the stomach and upper, middle and lower segments of the small intestine of a 28-d-old piglet that had been given sows' milk whey containing unsaturated B_{12}-binder. Excess of cyano[G-^{3}H]cobalamin was added to the test samples before they were applied to the

Table 2. *Unsaturated vitamin B_{12}-binding capacities (B_{12}-UBC) due to cobalophilins in gut contents (liquid phase) of piglets*
(Means of $\log_{10}$ transformed values with antilogs in parentheses)

Vitamin B_{12}-binder in test feed†	Age (d)	No. of piglets	B_{12}-UBC (μg) due to cobalophilins				
				Small intestine			
			Stomach	Upper	Middle	Lower	Total
Saturated	7	3	−0·365 (0·43)	−1·119** (0·08)	−1·062*** (0·09)	−1·741*** (0·02)	−0·200*** (0·63)
	28	3	−0·169 (0·68)	−0·762* (0·17)	−0·588*** (0·26)	−0·173*** (0·67)	0·253*** (1·79)
Unsaturated	7	4	−0·462 (0·35)	−0·520 (0·30)	−0·169 (0·68)	−0·602 (0·25)	0·214 (1·64)
	28	4	−0·223 (0·60)	−0·408 (0·39)	−0·006 (0·99)	0·596 (3·94)	0·776 (5·97)
Absent (control)	7	2	−0·372 (0·42)	−0·759 (0·17)	−0·828*** (0·15)	−1·412** (0·04)	−0·103*** (0·79)
	28	2	−0·173 (0·67)	−0·722 (0·19)	−0·631*** (0·23)	−0·246*** (0·57)	0·222*** (1·67)
SD (12 df)	—	—	0·0732	0·1891	0·1298	0·2128	0·0617

Mean values were significantly lower than those from piglets at the same age given the unsaturated binder: * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† For details of test feeds, see p. 258.

Table 3. *Ratio, vitamin B_{12}-binder from the milk: ^{14}C-labelled polyethylene glycol (PEG) in the gut contents (liquid phase) of piglets*

(Means of $\log_{10}$ transformed values with antilogs in parentheses)

Vitamin B_{12}-binder in test feed†	Age (d)	No. of piglets	Vitamin B_{12}-binder from milk:[^{14}C]PEG			
				Small intestine		
			Stomach	Upper	Middle	Lower
Saturated‡	7	3	1·861 (73)	1·937 (87)	1·783 (61)	1·763*** (58)
	28	3	1·877 (75)	1·880 (76)	1·926* (84)	1·898 (79)
Unsaturated§	7	4	2·459*** (288)	2·146*** (140)	1·741 (55)	1·457 (29)
	28	4	2·487*** (307)	2·140*** (138)	1·789 (62)	1·903 (80)
Unsaturated‖	7	4	—	—	45	24
	28	4	—	—	49	69
SD (10 df)	—	—	0·0768	0·0547	0·0638	0·0799

B_{12}-UBC, unsaturated vitamin B_{12}-binding capacity.

Statistically significant differences at each site in the intestine between feeds given to piglets of the same age: * $P < 0{\cdot}05$, *** $P < 0{\cdot}001$.

† For details of test feeds, see p. 258.

‡ Expressed as bound cyano[G-^{3}H]cobalamin (ng):[^{14}C]PEG (mg).

§ Expressed as B_{12}-UBC due to cobalophilins (endogenous + milk) (ng):[^{14}C]PEG (mg).

‖ Expressed as B_{12}-UBC due to milk cobalophilin (ng): [^{14}C]PEG (mg), and was calculated by subtracting the mean value of B_{12}-UBC due to endogenous cobalophilin (control group, Table 2) from that due to (endogenous + milk) cobalophilin (unsaturated group, Table 2). As the stomach and upper small intestine contained no B_{12}-UBC attributable to milk cobalophilin, there are no values for these two sites. For this reason this diet group was excluded from the statistical analysis.

Table 4. *Apparent extent of survival of saturated and unsaturated vitamin B_{12}-binder from sows' milk whey in gut contents (liquid phase)*

Vitamin B_{12}-binder in test feed*	Age (d)	No. of piglets	Apparent extent of survival			
				Small intestine		
			Stomach	Upper	Middle	Lower
Saturated†	7	3	0·82	0·98	0·69	0·65
	28	3	0·84	0·85	0·94	0·89
Unsaturated‡	7	4	—§	—§	0·51	0·27
	28	4	—	—	0·55	0·78

B_{12}-UBC, unsaturated vitamin B_{12}-binding capacity.

* For details of test feeds, see p. 258.

† Ratio, bound cyano[G-^{3}H]cobalamin (ng):^{14}C-labelled polyethylene glycol (PEG) (mg) in each gut segment (see Table 3) divided by the corresponding value in the test meal (89 ng bound cyano[G-^{3}H]cobalamin/mg PEG).

‡ Ratio, B_{12}-UBC due to milk cobalophilin (ng):[^{14}C]PEG (mg) in each gut segment (see Table 3) divided by the corresponding value in the test meal (89 ng B_{12}-UBC/mg PEG).

§ In the stomach and upper small intestine there was no B_{12}-UBC attributable to the milk cobalophilin (see Table 2).

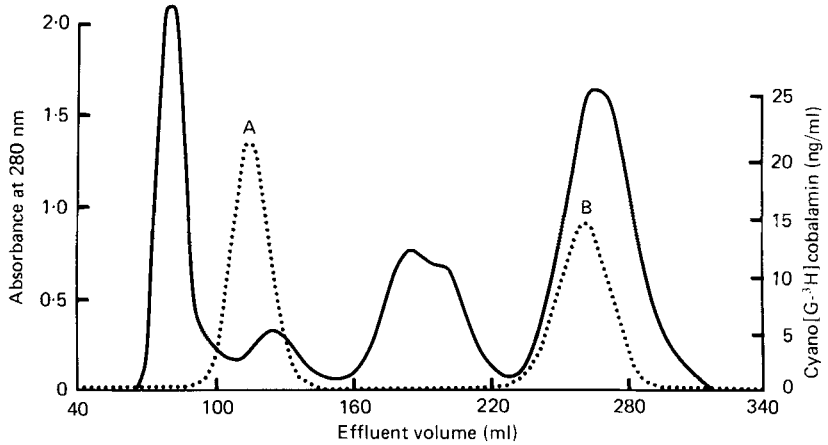

Fig. 1. Chromatography of sows' milk whey in which the vitamin B_{12}-binder was saturated with cyano[G-^{3}H]cobalamin, on a column of Sephadex G-150. Sows' milk whey (5 ml) mixed with cyano[G-^{3}H]cobalamin in excess of its vitamin B_{12}-binding capacity was applied to a Sephadex G-150 column (25 × 600 mm) and eluted with buffer, pH 7·0, containing 0·02 M-sodium phosphate and 0·15 M-sodium chloride. The effluent was collected in 5-ml fractions. Peak A, protein-bound vitamin B_{12}; peak B, free vitamin B_{12}. (——), Absorbance at 280 nm; (.....), cyano[G-^{3}H]cobalamin (ng/ml).

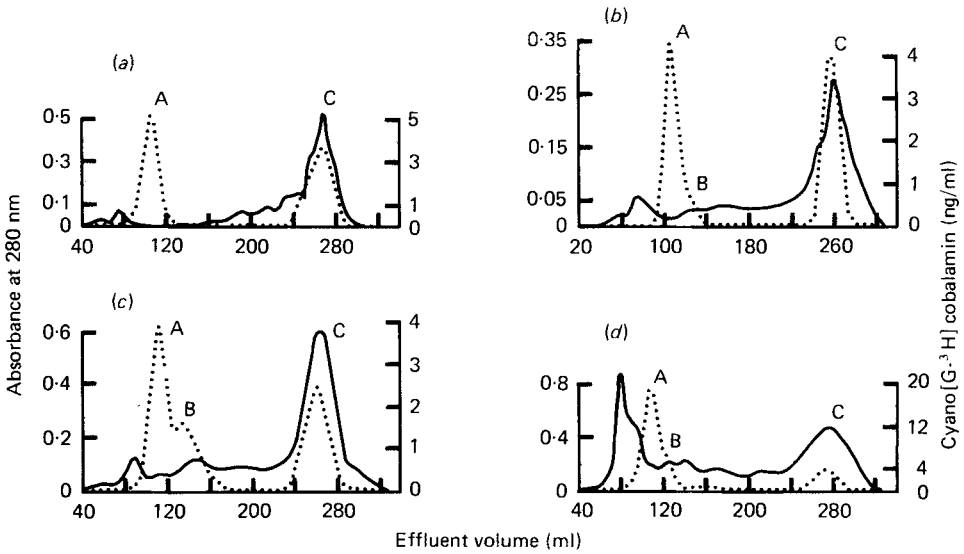

Fig. 2. Chromatography of the contents of the stomach and small intestine of a 28-d-old piglet given a test feed of sows' milk whey. A portion of each sample was mixed with cyano[G-^{3}H]cobalamin in excess of the unsaturated binding capacity for vitamin B_{12}, applied to a Sephadex G-150 column (25 × 600 mm) and eluted with buffer, pH 7·0, containing 0·02 M-sodium phosphate and 0·15 M-sodium chloride. The effluent was collected in 5-ml fractions. The samples used were of the liquid phase (see p. 259) from (*a*) stomach, (*b*) upper small intestine, (*c*) middle small intestine, (*d*) lower small intestine. Peak A, cobalophilin; peak B, protein-bound vitamin B_{12} (see p. 258); peak C, free cyano[G-^{3}H]cobalamin. (——), Absorbance at 280 nm; (.....) cyano[G-^{3}H]cobalamin (ng/ml).

Sephadex column, to saturate their B_{12}-UBC fully. With the stomach contents (Fig. 2(*a*)), bound vitamin B_{12} was eluted as a single symmetrical peak (A) representing the milk binder together with cobalophilins of endogenous origin. Besides peak A, the contents of the small intestine (Fig. 2(*b*,*c*,*d*)) gave a second, smaller peak (B), representing a binder of somewhat smaller apparent molecular weight. It is probable that this corresponds to intrinsic factor, which contributed 6, 14, 34 and 17% respectively, towards the binding capacity of the contents in the stomach and upper, middle and lower segments of the small intestine. Alternatively, it is conceivable that the peak represents a derivative of cobalophilin formed by proteolysis in the intestine. There were no other significant peaks containing bound vitamin B_{12}. A generally similar picture (not shown here) was obtained for the corresponding samples from a 7-d-old piglet.

Results of Sephadex G-150 chromatography of samples obtained from one 7-d-old and one 28-d-old piglet, which had been given sows' milk whey in which the binder had been precisely saturated with the cyano[G-^{3}H]cobalamin, are not presented here but they showed that all the bound vitamin was eluted as a single peak, corresponding to the elution volume of sows' milk cobalophilin. There was no cyano[G-^{3}H]cobalamin eluted in the position corresponding to that of the free vitamin.

The elution volumes (mean and SD, *n* 17) determined as the peak maximum for cobalophilin (113 (SD 5) ml) and for free cyano[G-^{3}H]cobalamin (267 (SD 6) ml) were highly reproducible. Total recovery of radioactive vitamin added to the column in each chromatographic run was 94 (SD 9)%. The binding capacity of the samples, calculated as the amount of bound cyano[^{3}H]cobalamin eluted from the column, varied between 82 and 109% (mean 93 (SD 8) %) of the values obtained by the charcoal adsorption method.

DISCUSSION

In postulating a role in the neonate for the vitamin B_{12}-binder present in milk, it is assumed that the binder is not readily degraded by gut proteases. The present findings indicate that a considerable proportion of the cobalophilin from sows' milk does indeed survive in the gut, and is resistant to proteolysis in vivo. Saturation of milk cobalophilin increased its resistance to proteolysis. This protective effect of saturation is also seen with other vitamin B_{12}-binders. Saturated cobalophilins from gastric juice and saliva are more resistant to proteolysis in vitro than the unsaturated form (Carmel *et al.* 1983), as is saturated intrinsic factor (Allen *et al.* 1978; Andersen & Von der Lippe, 1979; Nicolas *et al.* 1981).

Recovery of the saturated vitamin B_{12}-binder might be overestimated, since it was not possible to determine whether there was any transfer of the cyano[G-^{3}H]cobalamin from the milk binder to the endogenous binders. However, the affinity of the milk cobalophilin for cyanocobalamin is similar to that of endogenous cobalophilin and even greater than that of intrinsic factor (McGuigan, 1967; Hippe & Olesen, 1971; Allen & Mehlman, 1973) and so it is reasonable to suppose that any such transfer would be negligible. Further evidence for the preferential binding of vitamin B_{12} by cobalophilin is that the affinity of the binder from sows' milk for vitamin B_{12} was greater than that of porcine intrinsic factor, as judged from the retention of the vitamin by the binders against competition from bacterial cells (Ford, 1974). A similar prevention of vitamin B_{12} uptake by bacterial cells is indicated in the present findings; neither free nor bound cyano[G-^{3}H]cobalamin was detected in the centrifuged residue of the gut contents, indicating that the bound vitamin was unavailable to the large numbers of micro-organisms present throughout the piglets' small intestine.

A difficult problem in the present investigation has been to assess the contribution of the endogenous secretion of cobalophilin to the B_{12}-UBC in the gut. Endogenous cobalophilins

derived from saliva, gastric secretion and bile are immunologically indistinguishable from, and have similar molecular weights to, those present in milk (Allen, 1975; Stenman, 1976). The antiserum used in measuring the relative contributions of intrinsic factor and cobalophilin to the binding capacities did not distinguish between cobalophilins from different sources. However, from comparison of the values found for the B_{12}-UBC in the gut of piglets given sows' milk containing unsaturated binder with those for the control animals that had received no exogenous binder (see Table 2), it was evident that most of the B_{12}-UBC recovered in the gut contents of piglets given sows' milk could be attributed to the milk cobalophilin. The contribution of the milk binder to the B_{12}-UBC in the stomach was negligible but it contributed a high proportion in the middle and lower segments of the small intestine. The recovery of saturated binder also showed the same pattern, which reflects the progress of the digesta along the intestine as indicated by the recovery of the non-digestible and non-absorbable [^{14}C]PEG marker (Table 1).

The use of serial slaughter, one of the most commonly used methods for studying the progress of digestion of nutrients, in the present study would have required a very large number of piglets and was impracticable. Instead, a time for slaughtering the experimental piglets after feeding was arbitrarily chosen. The choice was based on studies reported in the literature (Newport, 1968; Braude *et al.* 1970; Wilson & Leibholz, 1981) on the transit time of a test meal in the gut of piglets of similar age to those used in the present work. The time interval between feeding and slaughter had to be such that most of the digesta would have left the stomach but would still be present throughout the small intestine. The choice of 80 min proved to be satisfactory in that most of the digesta was present in the middle and lower segments of the small intestine, where most of the digestive processes are completed.

The digestive system in piglets undergoes dramatic changes in development between birth and 3 weeks of age, with the greatest changes occurring between 2 and 3 weeks (Efird *et al.* 1982), and the protease activities in the gut increase markedly after 3–4 weeks (Manners, 1976; Corring *et al.* 1982). In the present experiments, however, the vitamin B_{12}-binder was no less resistant to digestion in the 28-d-old piglets than in the 7-d-old animals. Indeed, the milk binders appeared more resistant to digestion in the older piglets, despite the higher secretion of gastric and pancreatic proteases.

Concentration-dependent aggregation and a tendency to aggregate in the presence of excess folate have been reported for the folate-binder in goats' and cows' milk (Ford *et al.* 1969; Salter & Mowlem, 1983). This behaviour was not observed with the vitamin B_{12}-binder; neither the extent of dilution nor the degree of saturation with vitamin B_{12} affected the apparent molecular weight, as judged by chromatography in Sephadex G-150. Similarly, there was no change in the molecular weight of vitamin B_{12}-binder during its passage down the intestine. Comparative studies of intrinsic factor and gastric cobalophilin recovered from different parts of the gastrointestinal tract of adult pigs also showed that there is no modification of these proteins during their passage down the intestine (Marcoullis *et al.* 1978). However, studies in vitro showed that digestion with pancreatic proteases slightly reduces the molecular weight of cobalophilins from human milk, saliva and gastric secretions (Allen *et al.* 1978; Samson *et al.* 1980; Carmel *et al.* 1983), whereas pepsin has no effect.

In conclusion, it seems from the results of the present experiment that a high proportion of the saturated binder, and a smaller proportion of unsaturated binder survived unchanged in the intestine of piglets at both ages. It seems that the evidence of experiments in vitro (Ford, 1974; Samson *et al.* 1980; Carmel *et al.* 1983) showing that the binders were highly susceptible to tryptic digestion may have little relevance to the physiological situation.

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