Vitamin B_{12} absorption in the neonatal piglet

3. Influence of vitamin B_{12}-binding protein from sows' milk on uptake of vitamin B_{12} by microvillus membrane vesicles prepared from small intestine of the piglet

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1. The influence of the vitamin B_{12}-binding protein isolated from sows' milk on the uptake of vitamin B_{12} was investigated using microvillus membrane vesicles prepared from the small intestine of 7- and 28-d-old piglets. Uptake of radioiodinated purified binder was also examined.

2. The binder strongly promoted uptake of vitamin B_{12} at both ages; in the absence of the binder there was little uptake. The uptake mechanism was specific and operative in vesicles prepared from all regions of the small intestine. Uptake was a rapid process, exhibiting saturation kinetics, with a pH optimum at about 7·0, and dependent on the presence of magnesium or calcium ions for maximum activity. Affinity constants of the binding sites for the milk binder were determined.

3. Competition experiments using complexes of the binder with vitamin B_{12} and with non-cobalamin analogues (cobinamide and Co-α-[2-methyladenyl]cobamide) showed that the bound analogues competed with bound vitamin B_{12} for uptake but with lower efficiency.

4. Intrinsic factor also promoted vitamin B_{12} uptake by the vesicles but it did not compete with the milk binder for the same binding sites. It promoted uptake only in microvilli isolated from the lower third of the small intestine, and was more effective with preparations from 28-d-old piglets, whereas the milk binder was more effective with the 7-d-old piglets. Porcine gastric cobalophilin competed with the milk cobalophilin, but with lower efficiency.

5. It was concluded that a specific transport mechanism for absorption of vitamin B_{12}, mediated by the vitamin B_{12}-binder in milk, exists at the intestinal brush border of neonatal piglets and strongly reinforces the developing intrinsic factor-mediated mechanism during the early days or weeks of life.

6. It is suggested that the binder in the milk has a wider physiological significance and acts also as a 'host protective' factor and as a scavenger of adventitious vitamin B_{12}.

Intrinsic factor promotes the intestinal absorption of vitamin B_{12} in all the mammalian species that have so far been studied (Chanarin, 1979). However, the absorption process has been investigated mainly in adult animals. The situation in the neonatal period may be different, in that absorption of vitamin B_{12} may occur in the absence of intrinsic factor. Thus, in the rat, a specific mechanism of vitamin B_{12} uptake operates in the small intestine until about the third week of life and is independent of intrinsic factor (Boass & Wilson, 1963; Gallagher & Foley, 1971). The presence of vitamin B_{12}-binding protein (cobalophilin) in milk, raises questions concerning its possible influence on absorption of the vitamin during the neonatal period. Ford (1974) suggested that the binder in milk 'bridges the gap' until the production of intrinsic factor in the gut becomes fully established. In the piglet this occurs by about 2–3 weeks of age (Ford *et al.* 1975).

Experiments in vivo with sucking and early-weaned piglets showed that absorption and retention of cyano[^{58}Co]cobalamin was greater in the sucking piglets (Trugo *et al.* 1985), but it was not clearly established that this greater uptake was specifically related to the presence of vitamin B_{12}-binding protein in the sows' milk. There were several uncontrollable variables in the experimental design, which admitted of alternative explanations.

Brush-border membrane vesicles from intestinal epithelial cells have been much used in

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studies on intestinal absorption (Beesley & Faust, 1979; Selhub & Rosenberg, 1981; Ling & Faust, 1982), and microvillus membrane vesicles prepared from the small intestine of hamsters (Donaldson *et al.* 1967; Mackenzie & Donaldson, 1972; Beesley & Bacheller, 1980) and guinea-pigs (Mathan *et al.* 1974) have been used to examine the influence of intrinsic factor on the uptake of vitamin B_{12}. The present investigation was undertaken to complement the experiments in vivo described in a previous paper (Trugo *et al.* 1985). Its main objective was to determine the effect of vitamin B_{12}-binding protein from sows' milk on the uptake of vitamin B_{12} by microvillus membrane vesicles prepared from the small intestine of piglets aged 7 and 28 d. The kinetics of uptake of the binder, and of vitamin B_{12} in the presence and absence of the binder, were also determined.

MATERIALS AND METHODS

Preparation of microvillus membrane vesicles

Four sucking male piglets were taken from a Large White sow, two at 7 d and two at 28 d post partum. They were fasted for 16 h and then killed by an intracardiac injection of sodium pentobarbitone. The entire small intestine was quickly excised and divided into three approximately equal segments (upper, middle and lower). Each segment was flushed with ice-cold 0·15 M-sodium chloride solution and then divided into smaller pieces which were placed on a polyethylene sheet lying on a tray of crushed ice. Each piece was opened to expose the mucosal surface, which was quickly and gently scraped off with a glass slide. Scrapings representing the upper segment from the two 7-d-old piglets were pooled, as also were those from the middle and the lower segments. The same procedure was followed with scrapings from the 28-d-old piglets. This pooling of replicates was necessary to provide a sufficient yield of membrane vesicles, so that all the subsequent experiments on uptake could be carried out on the same test preparations. The scrapings were frozen on solid carbon dioxide and stored at $-20°$.

The isolation of the microvillus membrane vesicles from the frozen scrapings of intestinal mucosa was carried out by an adaptation of the methods described by Booth & Kenny (1974) and Schmitz *et al.* (1973). Homogenates of mucosal scrapings (3 g/100 ml) were made in 50 mM-mannitol–2 mM-Tris, pH 7·1, at 4°, using a Braun homogenizer at full speed for 2 min. Subsequent operations were also performed at 4°. Each homogenate was filtered through no. 40 nylon mesh and portions (5 ml) were taken for protein and enzyme assays. Solid calcium chloride was then added to the remainder, to give a concentration of 10 mM. Subsequent steps were carried out as described by Booth & Kenny (1974). The final preparations of microvillus membrane vesicles were suspended in 50 mM-mannitol–2 mM-Tris, pH 7·1, and samples taken for protein and enzyme assays. The remainder was divided into 0·2 ml portions, to each of which was added 0·2 ml glycerol. Storage at $-20°$ for up to 6 months caused no change in the properties of these preparations. Immediately before use the preparations were centrifuged at 38000 ***g*** for 40 min, the supernatant liquid was decanted, and the pellets containing the vesicles were resuspended in an incubation buffer to give a concentration of 1 mg microvillus protein/ml. Protein assays were performed by the method of Lowry *et al.* (1951).

Purification of the microvillus preparation was demonstrated by the 15–35-fold increase, as compared with the original homogenates, in the specific activity of the enzymes aminopeptidase M (*EC* 3.4.11.2) and alkaline phosphatase (*EC* 3.1.3.1), used as markers for the microvillus membrane and assayed as described by George & Kenny (1973) and Murer *et al.* (1976) respectively. Acid phosphatase (*EC* 3.1.3.2) assayed according to Scalera *et al.* (1980), as a marker for lysosomes, and [Na+, K+]ATPase (*EC* 3.6.1.3), assayed as described by Colas & Maxoux (1980), as a marker for the basolateral membrane

and mitochondria, showed no enrichment in any of the preparations (relative specific activity < 1). The integrity of the final preparations was also examined by electron microscopy, using ruthenium red to assess the permeability of the membrane (Luft, 1971).

Experiments on uptake of vitamin B_{12}

The uptake of radioactively labelled cyanocobalamin by microvillus membrane vesicles was examined in the absence and presence of purified vitamin B_{12}-binder isolated from sows' milk (Trugo & Newport, 1985). The effects of pH, period of incubation, Ca^{2+} and Mg^{2+} ions, concentration of cyanocobalamin and vitamin B_{12}-binder, and osmolality of the incubation medium were investigated, as were the effects on vitamin B_{12} uptake of other milk proteins: α-lactalbumin and β-lactoglobulin from bovine milk (Sigma Chemical Co., Poole, Dorset); intrinsic factor from porcine gastric mucosa (11000 units/μg protein; Sigma Chemical Co.); and complexes of non-radioactive corrinoids and vitamin B_{12}-binder. The corrinoids used were the non-cobalamin analogues of vitamin B_{12}, Co-α-[2-methyladenyl]cobamide (factor A) and cobinamide (factor B). Incubations were performed at 25° in disposable polycarbonate tubes, and each experiment was done in triplicate.

Incubation mixtures contained 50 μl (50 μg protein) of microvillus preparation and cyano[^{57}Co]cobalamin or cyano[G-^{3}H]cobalamin, with and without purified vitamin B_{12}-binding protein, and incubation buffer to 0·5 ml. The incubation buffer contained 50 mM-HEPES, 50 mM-2-[*N*-morpholino]ethanesulphonic acid (MES), 50 mM-NaCl and 2 mM magnesium sulphate (final concentrations, unless otherwise stated), and the pH value was adjusted when necessary with hydrochloric acid or potassium hydroxide. In experiments carried out in the presence of the vitamin B_{12}-binder, the mixture of labelled cyanocobalamin and binder was pre-incubated for 20 min in the incubation buffer before addition of the microvillus preparation to tubes containing the other test components, and stopped by dilution with 5 ml ice-cold washing solution (10 mM-HEPES–150 mM-NaCl, pH 5·0) followed immediately by filtration through microporous filters (25 mm diameter, 0·45 μm pore size; Amicon Ltd, Stonehouse) using a Millipore vacuum aspiration apparatus, which allowed filtration in 5–10 s. The incubation tubes were rinsed with a further 5 ml washing solution, which was also passed through the filter to ensure quantitative transfer of the incubation mixtures. The filters with the retained vesicles were then washed twice with 5-ml portions of the washing solution and transferred to scintillation vials for measurement of radioactivity.

Duplicate control incubations were performed using the same conditions as were employed in the experiment except that the microvilli were added to the tubes containing the mixtures only after the addition of the washing buffer, followed by immediate filtration. For each experiment the amount of radioactivity retained by the filters in the control incubations was subtracted from that obtained for the experimental incubations.

Before use, each microporous filter was washed with 10 ml of the washing solution containing 1 g bovine serum albumin (fraction V; Miles Laboratories, Slough)/l. The specific radioactivity of labelled cyanocobalamin used in each experiment was reduced by dilution (5- to 100-fold) with non-radioactive cyanocobalamin (Koch-Light Laboratories Ltd, Colnbrook) to give convenient levels for radioactivity measurements. Cyano[G-^{3}H]cobalamin (4·4 Ci/mmol) and cyano[^{57}Co]cobalamin (20 μCi/mmol) were obtained from Amersham International plc (Amersham, Bucks). HEPES and MES were purchased from Sigma Chemical Co.

Uptake of radioiodinated vitamin B_{12}-binding protein

Purified vitamin B_{12}-binding protein (vitamin B_{12}-binding capacity 21 μg/mg protein) isolated from sows' milk was radioiodinated by the lactoperoxidase (*EC* 1 . 11 . 1 . 7) method

of Frantz & Turkington (1972). Lactoperoxidase (from bovine milk, activity 80–100 units/mg protein) and $Na^{125}I$ (specific radioactivity 15·1 mCi $^{125}I/\mu g$ iodine) were obtained from Sigma Chemical Co. and Amersham International plc respectively. The specific radioactivity of the ^{125}I-labelled binder preparation obtained was 53·5 $\mu Ci/\mu g$ protein, and was reduced by 20- to 200-fold dilution with non-radioactive binder before use in the uptake experiments.

The general procedure described for experiments on the uptake of vitamin B_{12} was also followed in experiments on the uptake of ^{125}I-labelled binder, which was tested both unsaturated and saturated with non-radioactive cyanocobalamin.

The specificity of binding sites on the microvillus membrane for ^{125}I-labelled binder was investigated by incubating the microvilli with the ^{125}I-labelled binder in the absence and in the presence of other proteins. The test proteins used were non-radioactive binder from sows' milk, α-lactalbumin and β-lactoglobulin from cows' milk, porcine intrinsic factor and porcine gastric cobalophilin (non-intrinsic factor, from porcine gastric mucosa, 17500 units/mg protein; Sigma Chemical Co.).

Measurement of radioactivity

When cyano[^{57}Co]cobalamin or ^{125}I-labelled binder were used in the incubation mixtures, the radioactivity retained on the microporous filters was measured in an Intertechnique CG 4000 gamma counter. When cyano[G-^{3}H]cobalamin was used, the radioactivity was measured in a Packard B 2450 Tricarb liquid-scintillation spectrometer, after the addition of 10 ml Insta-gel (Packard Instruments Co., Reading) to the vials. Standard solutions of the radioactively labelled substances were applied to unused microporous filters and included together with each batch of samples for counting. Background counts were always at least ten times lower than those for the samples.

RESULTS

Uptake of vitamin B_{12} in the presence of vitamin B_{12}-binder from sows' milk

Influence of pH. Fig. 1 shows the effect of pH on the uptake of cyano[G-^{3}H]cobalamin by microvillus membrane vesicles in the presence of purified vitamin B_{12}-binder from sows' milk. Variation of pH had a marked influence on uptake, which was at a maximum at pH 7·0. The same was found for vesicles obtained from the upper, middle and lower segments of the small intestine, and for 7- and 28-d-old piglets. In the absence of the binder, uptake of cyano[G-^{3}H]cobalamin was much lower and no effect of pH on uptake was observed.

Influence of Ca^{2+} and Mg^{2+}. Fig. 2 shows the uptake of vitamin B_{12} as a function of Ca^{2+} and Mg^{2+} concentrations. Only vesicles prepared from the lower small intestine were used in this experiment. Increase in the concentration of either cation had no effect on uptake of vitamin B_{12} in the absence of the binder. Addition of the binder to the incubation mixture greatly enhanced uptake of the vitamin. With increasing concentrations of Ca^{2+} or Mg^{2+}, uptake of vitamin B_{12} increased and was nearly twice as great in the presence of 2 mM-Mg^{2+} as in the absence of Mg^{2+}. Mg^{2+} had a greater effect than Ca^{2+} and was included in the incubation buffer used in all the experiments. The effects of Ca^{2+} and Mg^{2+} on vitamin B_{12} uptake were similar for microvilli obtained from both 7- and 28-d-old piglets.

Influence of time of incubation. Fig. 3 shows the time-course of uptake of vitamin B_{12} by microvilli prepared from the lower small intestine of 7-d-old (L7) and 28-d-old (L28) piglets in the absence and in the presence of the binder. At 1 min, uptake was at least ten times greater in the presence of the binder. Uptake was also much more rapid in the presence of the binder and was apparently linear up to 1 min, and reached a maximum corresponding to the equilibrium in about 20 min. Time dependency curves similar to those shown in Fig. 3

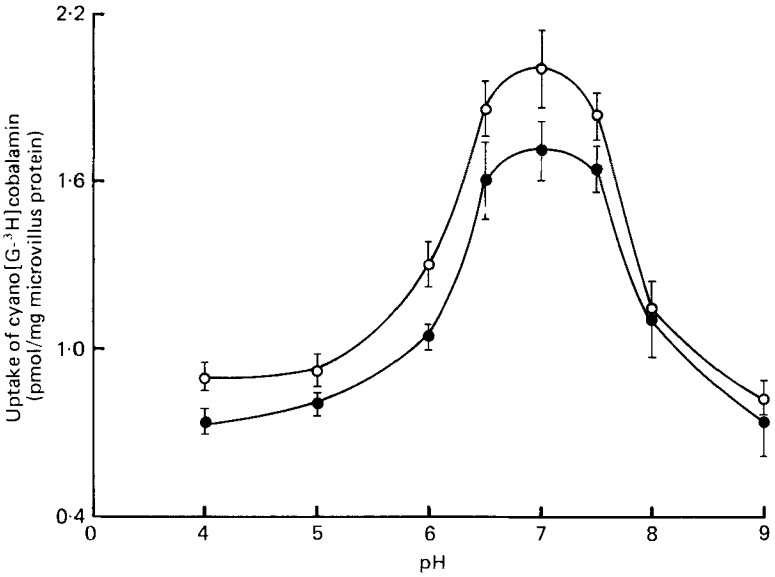

Fig. 1. Influence on pH on uptake of cyano[G-^{3}H]cobalamin by microvillus membrane vesicles. Membrane vesicles (0·05 mg protein) were incubated for 30 min in HEPES–MES buffer solution (p. 271) at varied pH values, with cyano[G-^{3}H]cobalamin bound to purified vitamin B_{12}-binder from sows' milk (0·37 nmol (1·6 μCi) bound vitamin/mg microvillus protein). The vesicles were prepared from the lower small intestine of 7-d-old (○), and 28-d-old (●) piglets. Values are means with their standard errors, represented by vertical bars, of triplicate determinations.

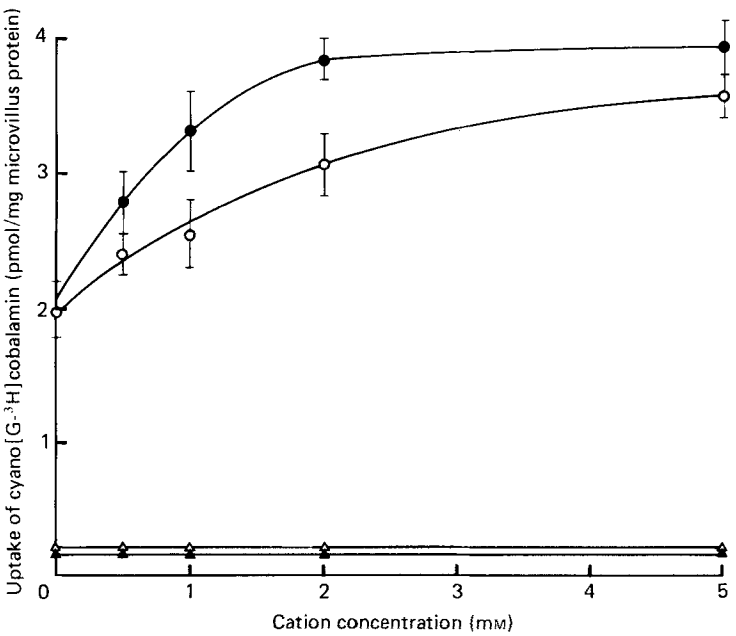

Fig. 2. Influence of calcium and magnesium ions on uptake of cyano[G-^{3}H]cobalamin by microvillus membrane vesicles. Microvillus membrane vesicles (0·05 mg protein) were incubated for 30 min with cyano[G-^{3}H]cobalamin (0·75 nmol/mg microvillus protein), free (△, ▲) and bound to purified vitamin B_{12}-binder from sows' milk (○, ●), in HEPES–MES buffer solution (p. 271) at pH 7·0 containing graded concentrations (0–5 mM) of calcium chloride (○, △) or magnesium chloride (●, ▲). The vesicles were prepared from the lower small intestine of 7-d-old piglets. Values are means with their standard errors, represented by vertical bars, of triplicate determinations.

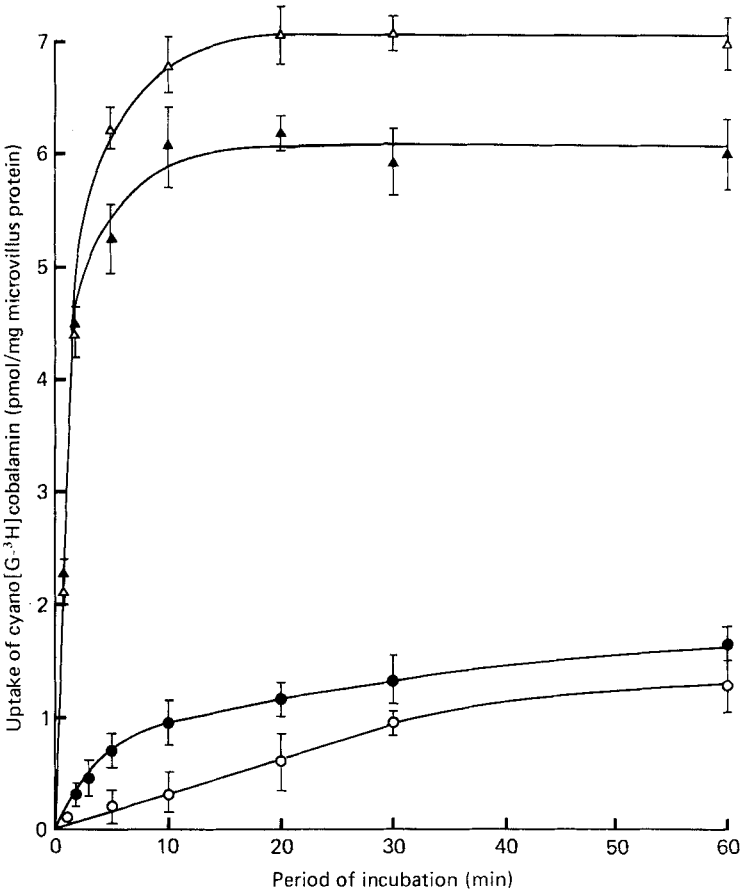

Fig. 3. Time-course of uptake of cyano[G-^{3}H] cobalamin by microvillus membrane vesicles. Membrane vesicles (0·05 mg protein) were incubated in HEPES–MES buffer solution (p. 271) at pH 7·0. The incubation mixtures contained 3 nmol cyano[G-^{3}H]cobalamin/mg microvillus protein, without (○, ●) and with (△, ▲) an equivalent amount of vitamin B_{12}-binder. Vesicles were prepared from the lower small intestine of 7-d-old (○, △) and 28-d-old (●, ▲) piglets. Values are means with their standards errors, represented by vertical bars, of triplicate determinations.

were obtained when 0·15 or 30 nmol vitamin B_{12}, with and without the binder, were added per mg microvillus protein in the incubation mixtures. At all time-points, increase in the concentration of vitamin B_{12} resulted in greater uptake. It was observed that, in the presence of the binder, uptake of vitamin B_{12} with preparation L7 was higher than with L28; the same was also found in experiments in which the effects of pH and cations were examined.

Influence of other proteins. Table 1 summarizes the effects of the purified sows'-milk vitamin B_{12}-binder, bovine α-lactalbumin and β-lactoglobulin and porcine intrinsic factor, on the uptake of cyano[^{57}Co]cobalamin by microvillus vesicles isolated from different regions of the small intestine.

Ca^{2+} (as 1 mM-$CaCl_2$) was added to the incubation buffer to optimize intrinsic factor-mediated uptake of vitamin B_{12} (Beesley & Bacheller, 1980). Uptake of free vitamin B_{12} was low and much the same for all three intestinal segments; analysis of variance showed that it was not influenced by the presence of α-lactalbumin or β-lactoglobulin in the incubation mixture. Purified vitamin B_{12}-binder sharply increased uptake ($P < 0·001$).

Table 1. *Uptake of vitamin B_{12} by microvillus membrane vesicles in the presence of purified sows' milk vitamin B_{12}-binding protein, porcine intrinsic factor, α-lactalbumin and β-lactoglobulin*

(Mean values based on triplicate determinations. The tests were carried out in HEPES–MES buffer at pH 7·0 (p. 271). Incubation mixtures contained 0·15 nmol cynao[^{57}CO]cobalamin/mg microvillus protein, with and without 0·15 nmol of the test protein, and were incubated with the vesicles for 30 min)

Small intestine segment	Test proteins	Uptake of cyano[^{57}Co]cobalamin (pmol/mg microvillus protein)	
		L7*	L28*
Upper	None	0·08	0·14
	Vitamin B_{12}-binder (from sows' milk)	0·72	0·64
	Intrinsic factor	0·15	0·24
	α-Lactalbumin	0·10	0·14
	β-Lactoglobulin	0·09	0·13
Middle	None	0·07	0·14
	Vitamin B_{12}-binder	0·70	0·65
	Intrinsic factor	0·16	0·26
	α-Lactalbumin	0·05	0·14
	β-Lactoglobulin	0·06	0·12
Lower	None	0·07	0·13
	Vitamin B_{12}-binder	0·90	0·80
	Intrinsic factor	0·60	1·21
	α-Lactalbumin	0·06	0·15
	β-Lactoglobulin	0·07	0·13
SED (60 df)		0·031	

* L7, L28, vesicles obtained from the lower small intestine of 7-d-old and 28-d-old piglets respectively.

Again, the effect was consistently greater with microvilli from 7-d-old than 28-d-old piglets ($P < 0·001$). Intrinsic factor had a comparatively small effect on vitamin B_{12} uptake in vesicles prepared from the upper and middle segments ($P < 0·001$), but it greatly enhanced uptake in preparations from the lower segment. It caused a slightly higher increase in uptake with preparation L28 (9·3-fold) than with L7 (8·6-fold). Thus, with vesicles obtained from the 7-d-old animals the sows' milk binder was the more active promoter of vitamin B_{12} uptake, whereas with the 28-d-old animals it was the intrinsic factor.

Distinction between transport and binding. To distinguish between binding of vitamin B_{12} to the vesicle membrane and transport into the intravesicular space, uptake of vitamin B_{12} in the presence of the binder was measured as a function of the osmolality of the incubation medium (Selhub & Rosenberg, 1981). Increase or decrease in the osmotic pressure, and the resultant change in the volume of the intravesicular space, should have a significant effect on transport, while causing little or no change in binding (Beesley & Faust, 1979; Halestrap & McGivan, 1979). As shown in Fig. 4, uptake of vitamin B_{12} was strongly dependent on the osmolality of the incubation medium and showed an inverse linear relationship. Extrapolation to zero at infinite osmolality is indicative of intravesicular transport rather than binding of vitamin B_{12} to the microvillus membrane. Similar results were obtained when the incubation time was extended to 30 min.

Influence of the binder concentration. The molar ratio, binder (as vitamin B_{12}-binding capacity):vitamin B_{12} in the incubation medium has an important influence on the uptake

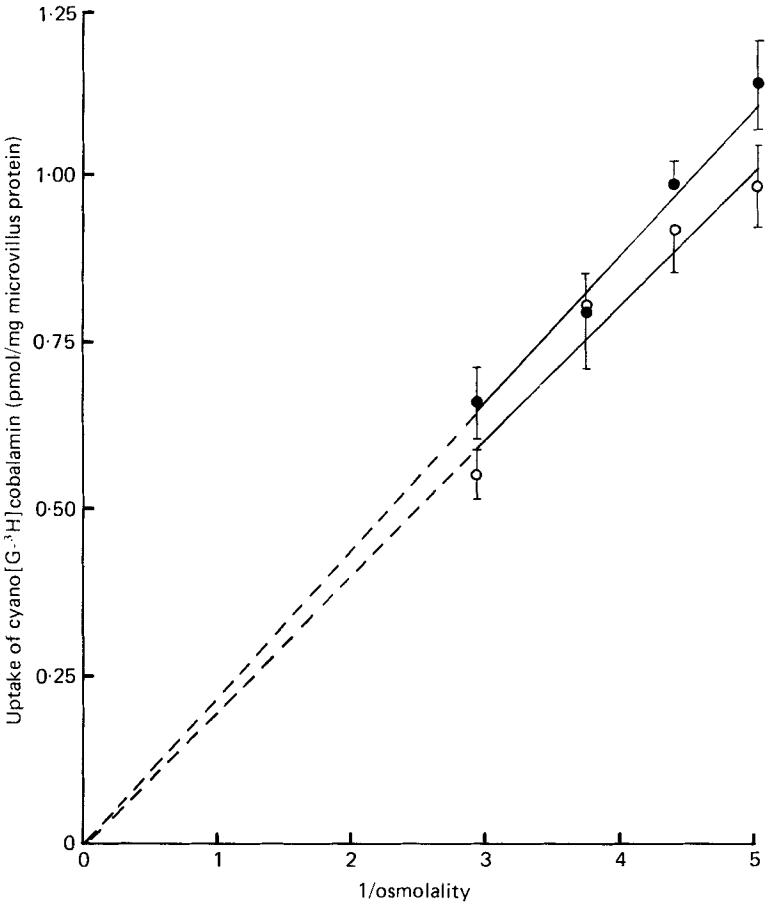

Fig. 4. Effect of osmolality of the incubation medium on uptake of cyanocobalamin by microvillus membrane vesicles. The vesicles were first incubated for 30 min in HEPES–MES buffer at pH 7·0, with graded concentrations of cellobiose (0–50 g/l). They were then incubated for 1 min with cyano[G-^{3}H]cobalamin (0·3 nmol/mg microvillus protein) that had been previously bound to an equivalent amount of vitamin B_{12}-binder. The vesicles were prepared from the lower small intestine of 7-d-old (●) and 28-d-old (○) piglets. The osmolality of the incubation buffer was calculated from values given in the *Handbook of Chemistry and Physics* (Weast & Astle, 1981). Values are means with their standard errors, represented by vertical bars, of triplicate determinations.

of the vitamin. Uptake was examined using graded concentrations of cyano[^{57}Co]cobalamin, varying the ratio, binder:vitamin B_{12} for each of these concentrations. Uptake of the vitamin increased with increase in the binder:vitamin value (from 0·2:1 to 1:1), reaching a maximum when the molar ratio was 1:1. When the concentration of binder exceeded that of vitamin B_{12} (at ratios of 2:1 and 4:1), uptake decreased. Assuming that for the molar ratios 0·2:1–1:1 practically all the binder molecules were saturated with cyano[^{57}Co]cobalamin, the decrease in uptake at higher values indicates that excess of unsaturated binder competes with the binder–vitamin complex for the binding sites, and so causes inhibition of vitamin B_{12} uptake.

Determination of dissociation constant (K_d) values. Fig. 5 shows the uptake of cyano[^{57}Co]cobalamin by the vesicles during 30 min incubation, in relation to the concentration of the binder–cyano[^{57}Co]cobalamin complex (molar ratio 1:1). Uptake of the

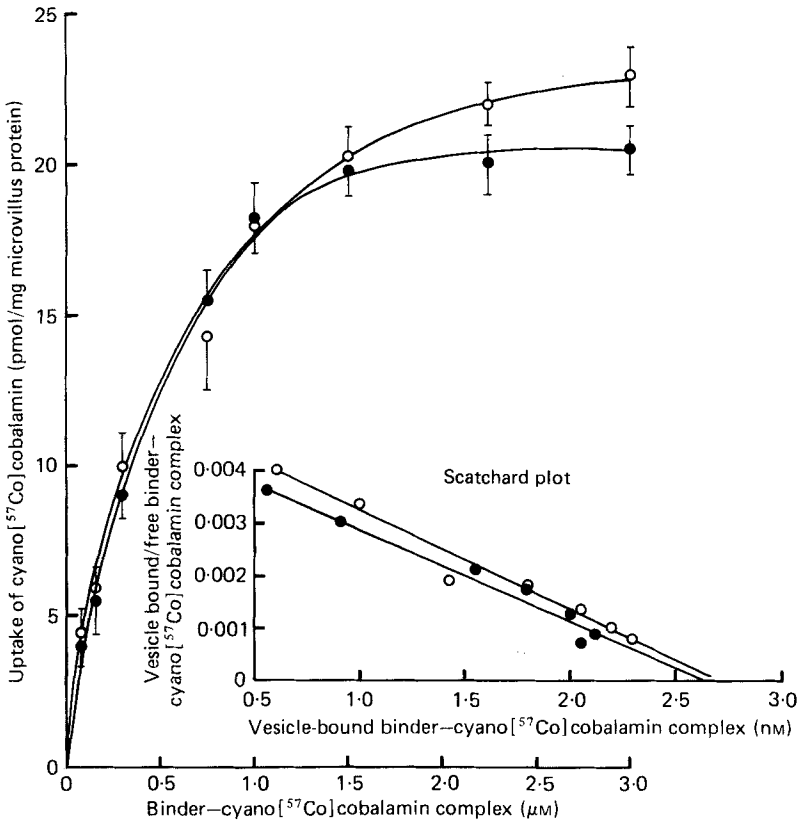

Fig. 5. Effect of concentration of binder–cyanocobalamin complex on uptake of cyanocobalamin by microvillus membrane vesicles. Graded concentrations of cyano[^{57}Co]cobalamin (0·15–3·0 μM) were mixed with equivalent concentrations of vitamin B_{12}-binder and incubated with the vesicles (0·05 mg protein) for 30 min in 0·5 ml HEPES–MES buffer at pH 7·0. The vesicles were prepared from the lower small intestine of 7-d-old (○) and 28-d-old (●) piglets.

The Scatchard plot (inset) gave a straight line of slope $-k_a$ (or $-1/K_d$). The abscissa intercept gives the concentration of potential binding sites. The concentration of the complex remaining free in solution was calculated by subtracting the concentration of bound complex from the total concentration of the complex originally present in the incubation mixture. The data points were fitted by linear regression analysis.

vitamin as a function of the complex concentration showed a tendency towards saturation at concentrations of substrate higher than 1·5 μM. As the time-course experiment (Fig. 3) had demonstrated that incubation for 30 min was sufficient to reach equilibrium uptake, the values shown in Fig. 5 were analysed using the Scatchard plot (Dahlqvist, 1978), from which the K_d was then calculated. The Scatchard plot (inset in Fig. 5) gave a straight line, indicating a single class of binding sites on the microvillus membrane at the concentrations of substrate and vesicles used in the experiment. The K_d for L7 was 0·53 μM (correlation coefficient −0·98) and for L28 it was 0·57 μM (correlation coefficient −0·97). The maximum capacity of the binding sites for the complex was 27 pmol/mg microvillus protein for L7 and 26·5 pmol/mg for L28.

Influence of other corrinoid–binder complexes on uptake of bound cyano[^{3}H]cobalamin. Fig. 6 shows the effects of complexes of the binder with non-radioactive cyanocobalamin, factor A and factor B on uptake of binder–cyano[^{3}H]cobalamin complex. With increase in

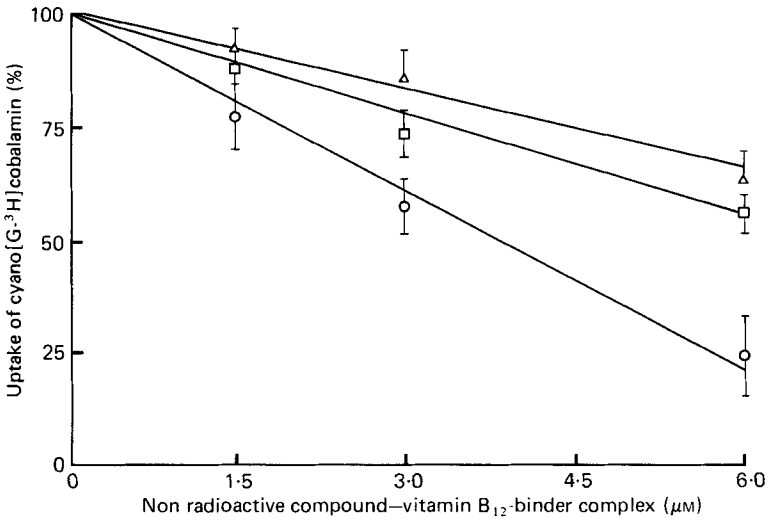

Fig. 6. Effects of concentration of cyanocobalamin, cobinamide (factor B) and Co-α[2-methyladenyl]cobamide (factor A) bound to vitamin B_{12}-binder, on uptake of bound cyano[G-^{3}H]cobalamin by microvillus membrane vesicles. Membrane vesicles (0·05 mg protein) were incubated for 30 min in HEPES–MES buffer at pH 7·0 with 0·15 μM-cyano[G-^{3}H]cobalamin–vitamin B_{12}-binder complex, alone and with graded concentrations of bound cyanocobalamin (○), factor A (□) or factor B (△). Uptake of cyano[G-^{3}H]cobalamin in the absence of other bound ligands was taken as 100%. Membrane vesicles were prepared from the lower small intestine of 7-d-old piglets. Values are means with their standard errors, represented by vertical bars of triplicate determinations.

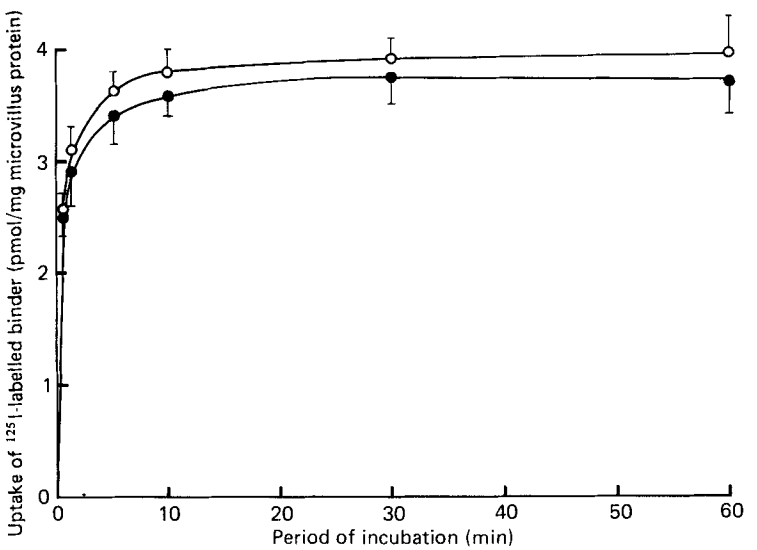

Fig. 7. Time-course of uptake of radioiodinated vitamin B_{12}-binder by microvillus membrane vesicles. ^{125}I-labelled vitamin B_{12}-binding protein (30 pmol/mg microvillus protein) was incubated with membrane vesicles (0·05 mg protein) from the lower small intestine of 7-d-old (○) and 28-d-old (●) piglets, in HEPES–MES buffer at pH 7·0. Values are means with their standard errors, represented by vertical bars of triplicate determinations.

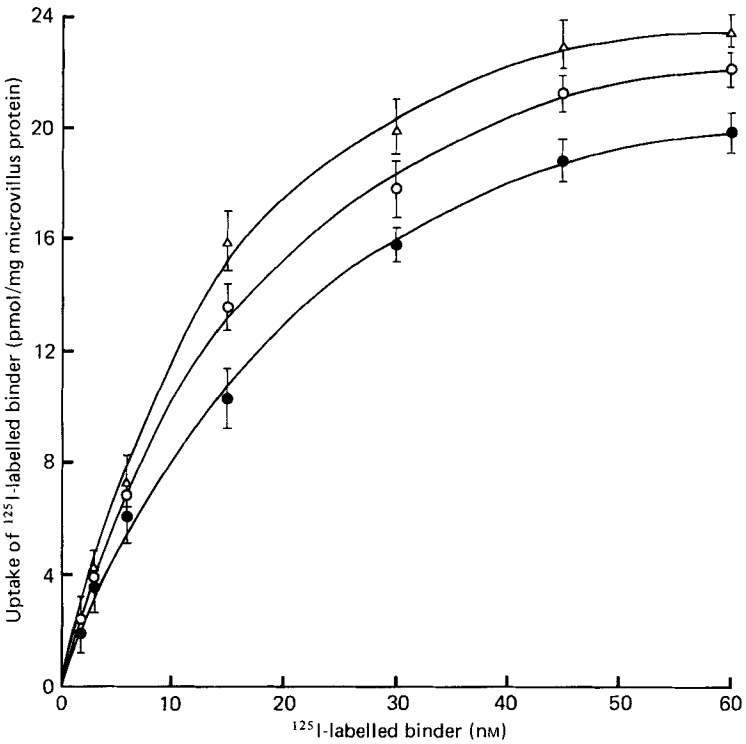

Fig. 8. Effect of substrate concentration on uptake of radioiodinated vitamin B_{12}-binder by microvillus membrane vesicles. Microvillus membrane vesicles (0·05 mg protein in 0·5 ml incubation mixture) were incubated for 30 min in HEPES–MES buffer at pH 7·0, with graded concentrations of ^{125}I-labelled binder, unsaturated (○, ●) and saturated (△) with cyanocobalamin. The vesicles were prepared from the lower small intestine of 7-d-old (○, △) and 28-d-old (●) piglets. Values are means with their standard errors, represented by vertical bars, of triplicate determinations.

concentration of the binder–cyanocobalamin complex there was a progressive decrease in the amount of cyano[G-^{3}H]cobalamin taken up by the vesicles: a 40-fold excess of binder–cyanocobalamin complex caused 75% inhibition of uptake. Uptake of cyano[G-^{3}H]cobalamin was also inhibited by the binder–factor A and binder–factor B complexes, although a 40-fold excess of these caused smaller inhibition of uptake (35 and 45% respectively) than did the corresponding excess of binder–cyanocobalamin complex. Similar results were obtained with vesicle preparations L7 and L28.

Uptake of radioiodinated vitamin B_{12}-binding protein

Influence of period of incubation. Fig. 7 shows the time-course of uptake of radioiodinated vitamin B_{12}-binder by the microvillus vesicle preparations L7 and L28. Uptake of the ^{125}I-labelled binder approached equilibrium after about 10 min and was very rapid, more so than that of radioactively labelled vitamin B_{12} (Fig. 3).

Determination of K_d values. Fig. 8. shows the relation between equilibrium uptake and concentration of ^{125}I-labelled binder in the incubation medium. Scatchard plots (^{125}I-labelled binder bound by the vesicles divided by ^{125}I-labelled binder free in solution in the incubation mixture *v.* ^{125}I-labelled binder bound) of values presented in Fig. 8 gave straight lines (correlation coefficients −0·99 and −0·96 for unsaturated and saturated binder respectively

Table 2. *Effects of vitamin B_{12}-binders and of α-lactalbumin and β-lactoglobulin, on uptake of radioiodinated vitamin B_{12}-binder from sows' milk by microvillus membrane vesicles*

(Mean values based on triplicate estimations. The tests were carried out in HEPES–MES buffer at pH 7·0 (p. 271). Incubation mixtures contained 30 pmol ^{125}I-labelled binder/mg microvillus protein in the absence and in the presence of other proteins, and were incubated with the vesicles for 30 min)

Other proteins	Ratio, ^{125}I-labelled binder: other proteins	Uptake of ^{125}I-labelled binder (pmol/mg microvillus protein) L7*	L28*
None	1:0	4·1	3·7
Unlabelled binder	1:10	2·4	2·1
	1:100	1·6	1·4
Gastric cobalophilin	1:100	3·2	2·8
Intrinsic factor	1:50	4·0	3·8
α-Lactalbumin	1:100	4·3	4·1
β-Lactoglobulin	1:100	4·2	4·0
SED (28 DF)		0·25	

* L7, L28, vesicles obtained from the lower small intestine of 7-d-old and 28-d-old piglets.

with L7, and 0·98 for the unsaturated binder with L28) suggesting that a single class of binding site was involved. With L7 the K_d was 16 nM for the unsaturated binder and 14 nM for the saturated binder; with L28 it was 17 nM for the unsaturated binder. With L7 the maximum capacities of the binding sites for unsaturated and saturated binder were 28 and 30 pmol/mg microvillus protein respectively. For preparation L28 the maximum capacity for unsaturated binder was 25 pmol/mg microvillus protein. Similar results were obtained for the binder– cyano[^{57}Co]cobalamin complex.

Specificity of binding sites. The specificity of the uptake of vitamin B_{12}-binder by the microvillus vesicles was assessed by determining the ability of selected unlabelled proteins to compete for the binding sites. The results are summarized in Table 2. Uptake of ^{125}I-labelled binder in the presence of a 100-fold excess of unlabelled binder or of porcine gastric cobalophilin was reduced to about 40 ($P < 0·001$) and 77% ($P < 0·01$) respectively of the original value, indicating competition for the same microvillus membrane binding sites. Intrinsic factor did not reduce uptake of the ^{125}I-labelled binder, indicating that the binding sites for intrinsic factor and ^{125}I-labelled binder are different. α-Lactalbumin and β-lactoglobulin similarly had no effect on uptake of ^{125}I-labelled binder.

DISCUSSION

The present paper provides evidence that uptake of vitamin B_{12} by microvillus membrane vesicles was strongly promoted by the vitamin B_{12}-binding protein isolated from sows' milk. Uptake of bound vitamin B_{12} was saturable and dependent on pH, with maximum uptake at around pH 7·0. Mg^{2+} or Ca^{2+} were required for maximum uptake. No source of energy was supplied in the incubation medium. Uptake of vitamin B_{12} in the absence of the binder was very small and much less dependent on pH, and independent of Ca and Mg.

Kinetic analyses, using the Scatchard plot, of equilibrium binding data revealed a single class of binding site and a maximum binding capacity of 25–30 pmol/mg microvillus protein. The dissociation constant, K_d, determined under equilibrium conditions, was 0·53

and 0·57 μM for the bound vitamin, for vesicles isolated respectively from 7- and 28-d-old piglets. However, when K_d was calculated for radioiodinated binder a value of about 76 nM was found, which is approximately thirty times lower than that based on the uptake of the radiolabelled vitamin B_{12} bound to the milk binder. This difference may be explained in terms of binding to the membrane surface and transport into the vesicles. Uptake of ^{125}I-labelled binder was most probably a measure of binding to the membrane, whereas uptake of bound radiolabelled vitamin B_{12} reflected transport of the vitamin itself into the vesicles. The higher value of K_d for the binder–vitamin B_{12} complex, measured as uptake of radiolabelled vitamin B_{12}, reflects the sequential processes of binding, transmembrane transport and release into the intravesicular space. Experiments in which uptake of the labelled vitamin was measured both by incubating the vesicles with the binder–vitamin B_{12} complex, and by attaching the unsaturated binder to the vesicles before incubating with the vitamin, gave similar results. This indicates that unsaturated binder, after attachment to the vesicle membrane, retained its capacity to bind and to promote the transport of the vitamin.

The effect of the purified vitamin B_{12}-binder on vitamin B_{12} uptake by microvilli obtained from 7- and 28-d-old piglets was compared with that of intrinsic factor. The reason for comparing uptake in piglets at both ages was to determine whether maturation of the intrinsic factor-mediated process of uptake influences the milk-binder-mediated process. Intrinsic factor was found to promote uptake of vitamin B_{12}, but its effect was only pronounced in vesicles prepared from the lower third of the small intestine. The milk binder promoted uptake in vesicles obtained from all three segments of the small intestine. Intrinsic-factor-mediated uptake of vitamin B_{12} is restricted to the ileum in the pig as in most animal species so far studied (Seetharam & Alpers, 1982). Comparing the effects of intrinsic factor and the milk binder on vitamin B_{12} absorption by microvilli isolated from the lower third of the small intestine, the milk binder was the more effective in promoting uptake with 7-d-old piglets and intrinsic factor with 28-d-old piglets. The milk binder must constitute only a transitory mechanism for vitamin B_{12} transport; its role might be continued by endogenous cobalophilins, but it is clear that the intrinsic-factor mechanism becomes the predominant mode of uptake in older animals. A similar substitution or complementation of one mechanism of absorption with another has been reported for the neonatal rat (Boass & Wilson, 1963; Gallagher & Foley, 1971), where a transitory specific mechanism of vitamin B_{12} uptake operates until it is replaced by the intrinsic-factor-mediated mechanism at about the third week of life. Also in the rat, the intestinal transport of immunoglobulin through specific receptors declines with age and is no longer demonstrable by about 20 d of age (Borthistle *et al.* 1977; Mackenzie *et al.* 1983).

The binding sites for the uptake of vitamin B_{12} mediated by the milk binder were found to be specific for this binder, whether it was unsaturated or saturated with vitamin B_{12}; the equilibrium K_d was practically the same for both forms. Intrinsic factor did not compete for these binding sites. The intrinsic-factor-mediated and the milk-binder-mediated mechanisms for vitamin B_{12} uptake were both present in the 7- and 28-d-old piglets, and were similar in their pH optimum and Ca dependence (Donaldson *et al.* 1967; Mackenzie & Donaldson, 1972; Beesley & Bacheller, 1980), but they appeared to act at different sites in the brush-border membrane.

Cobalophilin from porcine gastric mucosa belongs to the same family of binders as that from milk and has common antigenic and chemical properties. It inhibited uptake of radioiodinated milk-binder when added in a 100-fold excess, but not to the same extent as did the unlabelled milk binder. Thus, although gastric cobalophilin binds to the same binding sites as the milk binder, the affinity of the commercial preparation used in these experiments was lower. No experiments on the influence of endogenous cobalophilin on

the absorption of vitamin B_{12} were carried out in the present study. In the adult pig, as in man and in the guinea-pig, cobalophilins from milk, saliva, gastric juice and blood plasma had no effect on vitamin B_{12} uptake by ileal mucosal homogenates (Hooper *et al.* 1973).

The affinity of the binding sites for complexes of the binder with the non-cobalamin analogues, factor A and factor B, was lower than for the complex with cyanocobalamin. Also, the affinity of the binder for these analogues was less than half of that for cobalamins (Trugo, 1984). These properties imply the preferential uptake of cobalamins in vivo. No such effect was found by Trugo *et al.* (1985), for the likely reason that the selectivity would have been obscured by the large excess of the analogues used in these experiments.

The present experiments show that a vitamin B_{12} transport mechanism mediated by the milk binder exists at the intestinal brush border of young piglets, but its physiological importance for the vitamin B_{12} nutrition of the piglet remains to be further elucidated. It is in the early days or weeks of life that the function of this binder in facilitating vitamin B_{12} absorption would seem to be especially appropriate, not only in 'bridging the gap' before the intrinsic factor-mediated mechanism becomes predominant in the regulation of uptake, but also in strongly reinforcing the developing endogenous systems. The milk binder may ensure the efficient uptake of the small content of vitamin B_{12} in the sows' milk, and the large excess of unsaturated binder may further benefit the piglet by enabling it to exploit richer adventitious sources of vitamin B_{12} such as bacterial synthesis in the gut and ingested soil, faeces-contaminated litter and other forage. The binder may be presumed to intervene between vitamin B_{12}-producing and vitamin B_{12}-requiring intestinal bacteria, diverting vitamin B_{12} to the benefit of the host animal and, in so doing, depriving the vitamin B_{12}-requiring bacteria of an essential nutrient. In this way, as has been suggested by Ford (1974) and Ford *et al.* (1975), the unsaturated binder may strongly influence the ecology of the intestinal microflora and fulfil a 'protective' function by preventing the stimulation and consequent overgrowth of vitamin B_{12}-dependent bacteria. The interactions of the binder with the microflora need to be investigated further, in relation to the similar 'protective' functions that have been postulated for other binders such as unsaturated folate-binding protein and unsaturated lactoferrin, both of which are present at significant concentrations in the milk of several mammalian species.

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