The effect of streptomycin and erythromycin on vitamin B_{12} nutrition in rats in which coprophagy was prevented

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1. Vitamin B_{12} nutrition was studied in normal, coprophagy-prevented and antibiotic-treated rats on vitamin B_{12}-deficient diets with and without a vitamin B_{12} supplement; the indices used were excretion of total urinary ether-soluble acid (TUESA) and methylmalonic acid, and vitamin B_{12} assays on the liver and intestinal tract.

2. A significant positive correlation ($r = 0{\cdot}61$) was found between TUESA excretion and weight of rats, and a significant negative correlation ($r = -0{\cdot}89$) between TUESA excretion and liver vitamin B_{12} contents.

3. Although prevention of coprophagy reduced the contents of vitamin B_{12} in the stomach and small intestine, no effect on vitamin B_{12} nutrition, as assessed by TUESA excretion and liver vitamin B_{12} contents, was found. Rats in which coprophagy was permitted became vitamin B_{12}-deficient, when given a diet in which vitamin B_{12} was low.

4. The amounts of TUESA and methylmalonic acid excreted indicated that streptomycin and erythromycin administered orally prevented vitamin B_{12} deficiency in rats on a diet deficient in vitamin B_{12}. Liver vitamin B_{12} contents were, however, very low in these rats. This anomaly was thought to be due to the non-specificity of the *Euglena gracilis* assay for vitamin B_{12}.

5. It was concluded that, under the conditions of these experiments, coprophagy was not necessary to the vitamin B_{12}-sparing action of antibiotics.

Various antibiotics have been shown, by growth assay to spare vitamin B_{12} in the rat (Tappan, Lewis, Register & Elvehjem, 1950; Cuthbertson, 1952*a*; Barnes, Bourdeau, Kwong & Fiala, 1960). The mechanism of this action is controversial, but it is almost certainly through an effect on the intestinal flora, since parenteral antibiotics are ineffective (Whitehill, Oleson & Hutchings, 1950) and no effect is seen in germ-free animals (Mickelsen, 1962). It has been suggested that antibiotics may increase the availability of vitamin B_{12} synthesized by intestinal micro-organisms (Stokstad & Jukes, 1951) either by destroying vitamin B_{12}-utilizing organisms or by favouring vitamin B_{12}-synthesizing forms (Cuthbertson, 1952*b*).

The use of single antibiotics certainly does not necessarily sterilize the intestine (Johansson, Peterson & Dick, 1953) and increased amounts of vitamin B_{12}-active substances have been demonstrated in the intestinal contents of aureomycin-fed animals (Davis & Chow, 1951). Johansson *et al.* (1953), however, failed to find increased amounts of vitamin B_{12} in the intestinal contents of aureomycin-fed rats and aureomycin supplements given to pigs did not increase the tissue vitamin B_{12} content (Kelly, Bray & Phillips, 1954), although growth was stimulated. Thus growth stimulation alone, since it is relatively non-specific, cannot be taken as conclusive evidence for an increased availability of vitamin B_{12}.

If antibiotics do increase the availability of vitamin B_{12} from intestinal micro-organisms in rats, this could be dependent on coprophagy, since some vitamin B_{12} is normally obtained by these means (Morgan, Gregory, Kon & Porter, 1964). Barnes & Fiala (1958) showed that the depression of growth of vitamin B_{12}-deficient rats below that of controls given vitamin B_{12} supplements was greater when coprophagy was prevented than when it was permitted, this suggests that the prevented rats were deprived of a source of vitamin B_{12}. Barnes & Fiala (1958) also showed that succinyl-sulphathiazole further depressed the growth of the vitamin B_{12}-deficient rats in which coprophagy was prevented, suggesting that a further deprivation of vitamin B_{12} had occurred. This may have been due to a decrease in vitamin B_{12} absorbed direct from the site of synthesis in the intestine. However, when coprophagy was prevented, the sparing effects of penicillin, aureomycin and bacitracin for vitamin B_{12}, as assessed by growth assay, were not observed (Barnes *et al.* 1960).

To elucidate further the relationship of the intestinal flora to vitamin B_{12} nutrition in the rat, experiments are here described using a method of coprophagy prevention (Armstrong & Softly, 1966), administration of streptomycin and erythromycin and estimation of the state of vitamin B_{12} nutrition from the amounts of urinary ether soluble acid (TUESA) and methylmalonic acid excreted (Armstrong, 1967). The vitamin B_{12} contents of the intestinal tract and liver were also measured at the end of the experiment.

EXPERIMENTAL

Expt 1. *Investigation of the effect of simultaneous administration of streptomycin and erythromycin and the prevention of coprophagy on TUESA and methylmalonic acid excretion and liver and intestinal levels of vitamin* B_{12} *in the rat*

Weanling male and female Wistar albino rats were placed on the vitamin B_{12}-deficient diet previously described (Armstrong, 1967). After 3 weeks they were divided into experimental groups of five rats, and each was offered 10 g of the diet per day. The groups were treated as follows:

Group 1A vitamin B_{12}-supplemented, coprophagy permitted
Group 1B vitamin B_{12}-deficient, coprophagy permitted
Group 2A vitamin B_{12}-supplemented, coprophagy prevented
Group 2B vitamin B_{12}-deficient, coprophagy prevented
Group 3A Vitamin B_{12}-supplemented, treated with antibiotics, coprophagy prevented
Group 3B vitamin B_{12}-deficient, treated with antibiotics, coprophagy prevented

Each rat was housed in an individual, $\frac{1}{2}$ in. mesh, screen-floor cage and received $CoCl_2.6H_2O$ (1 mg/100 ml) in the drinking water. Coprophagy was prevented by the use of a jacket previously described (Armstrong & Softly, 1966).

Vitamin B_{12}-supplemented rats received 1 μg hydroxocobalamin/100 ml drinking water. Antibiotic-treated rats were given 10 mg streptomycin and 2 mg erythromycin in 0·3 ml water, by stomach tube, on the 1st day of the experiment and then 1·5 mg streptomycin and 0·025 mg erythromycin/ml drinking water thereafter. This treatment was the same as that used by Kasuya (1964) in mice and was intended to produce

a relatively sterile intestine. Drinking water was renewed and fresh vitamin B_{12} and antibiotics were added from refrigerated stock each day.

TUESA and methylmalonic acid excretion was estimated on three or four occasions during the 6-week experimental period by the method previously described (Armstrong, 1967). The rats were weighed at regular intervals.

At the end of the experimental period the rats were killed with diethyl ether and the liver, stomach, small bowel and large bowel removed, intact with contents, and deep-frozen. Each organ, with contents, was weighed and homogenized with an equal weight of water, and 2 g of the homogenate (1 g for the stomach) were taken for vitamin B_{12} assay.

Homogenates were prepared for assay by dilution to 20 ml with water and digestion for 1 h at 58° with 50 mg papain. The digest was steamed for 10 min and then filtered. The filtrate was diluted appropriately with sterile distilled water and assayed for vitamin B_{12} activity by the method of Nicholas & Pitney (1958) using *Euglena gracilis* as the test organism. These assays were performed routinely with serum samples in the Haematology Department, Royal Perth Hospital. Tissues from antibiotic-treated rats were assayed both in the light and in the dark and a sample of the antibiotic solution was assayed in the dark.

Expt 2. An investigation of the changes in liver vitamin B_{12} levels and TUESA and methylmalonic acid excretion in rats fed close to their daily requirement of vitamin B_{12}: an attempt to resolve an anomaly in the results of Expt 1 (*see p.* 533)

Male and female Wistar albino rats were derived from two litters which had been on the vitamin B_{12}-deficient diet since weaning. They were housed in individual, screen-floor cages and divided as follows:

Group A six rats, each given 10 g of the vitamin B_{12}-deficient diet/day

Group B five rats, each given 10 g of the vitamin B_{12}-deficient diet/day; 0·5 μg vitamin B_{12} was added to the diet each day

The rats were weighed and TUESA and methylmalonic acid excretion were measured before, and at intervals during, the experiment until the methylmalonic acid excretion by the vitamin B_{12}-supplemented group was reduced to 'trace' or zero levels. They were then killed with ether; the livers were removed and deep-frozen and the vitamin B_{12} content was determined as previously described.

Estimates of statistical significance of means of results were made by means of Student's *t* test.

RESULTS

The results of Expt 1 are given in detail in Fig. 1 and Table 1. The mean values are given with their standard deviations.

The mean TUESA excretion in vitamin B_{12}-deficient rats not given antibiotics (group 1 B, 0·91 ± 0·24 m-equiv./100 g 24 h; group 2 B, 0·91 ± 0·35 m-equiv./100 g 24 h) was significantly greater than that of their vitamin B_{12}-supplemented controls (group 1 A, 0·42 ± 0·14 m-equiv./100 g 24 h; group 2 A, 0·30 ± 0·13 m-equiv./100 g 24 h).

In the rats given antibiotics the mean TUESA excretion in those on the vitamin B_{12}-deficient diet (group 3 B, 0·44 ± 0·11 m-equiv./100 g 24 h) was not significantly greater than that in the supplemented controls (group 3 A, 0·39 ± 0·09 m-equiv./100 g 24 h). There was no difference between the mean TUESA excretion in rats in which coprophagy was prevented (group 2 B) or permitted (group 1 B) but that in the antibiotic-treated, vitamin B_{12}-deficient rats (group 3 B) was significantly less than either ($P < 0·005$).

Weights of the vitamin B_{12}-deficient rats (group 1 B) were compared with the TUESA excretion (m-equiv./24 h) on the day of weighing and the correlation coefficient between them was calculated. The resulting positive correlation coefficient ($r = 0·61$) was highly significant ($P < 0·005$).

The distribution of methylmalonic acid excretion over six levels of estimation in each of the six groups of rats is shown in Fig. 1. The scoring system represents a semi-quantitative estimate of the 24 h excretion of methylmalonic acid made from the size and density of the methylmalonic acid spot seen on thin-layer chromatography (Armstrong, 1967).

Table 1. *Expt* 1. *Vitamin* B_{12} *content* (*μg*/*g*) *of organs of rats, at the end of the* 6*-week period* (*group means and standard deviations*)

Group	Treatment and diet	Liver	Stomach	Small bowel	Large bowel
1	Coprophagy permitted.				
	(A) Vitamin B_{12}-supplemented	0·100 ± 0·031	0·099 ± 0·032	0·061 ± 0·010	1·31 ± 0·28
	(B) Vitamin B_{12}-deficient	0·039 ± 0·008	0·067 ± 0·041	0·065 ± 0·027	1·03 ± 0·24
2	Coprophagy prevented				
	(A) Vitamin B_{12}-supplemented	0·111 ± 0·004	0·080 ± 0·061	0·102 ± 0·036	0·58 ± 0·57
	(B) Vitamin B_{12}-deficient	0·039 ± 0·023	0·033 ± 0·021	0·019 ± 0·012	0·71 ± 0·45
3	Coprophagy prevented, antibiotic-treated				
	(A) Vitamin B_{12}-supplemented	0·145 ± 0·041	0·080 ± 0·044	0·063 ± 0·018	0·17 ± 0·13
	(B) Vitamin B_{12}-deficient	0·020 ± 0·004	0·024 ± 0·015	0·017 ± 0·007	0·23 ± 0·26

Results of vitamin B_{12} assays on livers and intestines of rats in groups 1–3 are shown in Table 1. There was no difference between assays performed in the light and in the dark on antibiotic-treated rats. The differences between mean contents of vitamin B_{12} in the livers of supplemented and deficient rats of groups 1–3 were highly significant ($P < 0·005$). The differences were not significant between the vitamin B_{12}-deficient groups 1 B, 2 B and 3 B.

The difference between the concentration of vitamin B_{12} in the stomach and stomach contents of vitamin B_{12}-supplemented and vitamin B_{12}-deficient rats were significant only between groups 3 A and 3 B ($P < 0·05$). The mean concentration in group 3 B was significantly lower than that in group 1 B ($P < 0·05$), but the difference was not significant between groups 2 B and 1 B or between groups 2 B and 3 B. However, the mean concentration in groups 2 B and 3 B combined was significantly less ($P < 0·05$) than that in group 1 B.

The mean vitamin B_{12} concentration of the small bowel and small bowel contents of deficient rats in each of the coprophagy-prevented groups 2 B and 3 B was significantly less ($P < 0·005$) than that of their supplemented controls (group 2 A and 3 A). This

difference was not significant between groups 1 A and 1 B ($P > 0{\cdot}30$). Mean levels for groups 2 B and 3 B were not significantly different but were significantly lower than that for group 1 B ($P < 0{\cdot}05$).

In no group was there a significant difference between mean vitamin B_{12} levels in the large bowel and large bowel contents in deficient rats and their supplemented controls. However, the mean level for the antibiotic-treated rats (group 3) was significantly lower ($P < 0{\cdot}05$) than that for the non-treated rats (groups 1 and 2). The differences between the mean levels for groups 1 and 2 were not significant ($P < 0{\cdot}10$).

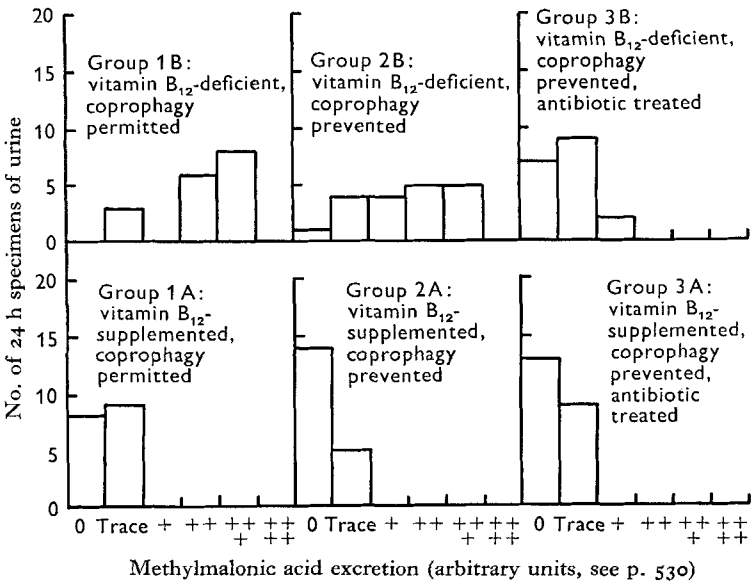

Fig. 1. Expt 1. Histogram showing number of 24 h specimens of urine giving each of the six levels of methylmalonic acid excretion in each group of rats.

Liver vitamin B_{12} content and TUESA excretion in the vitamin B_{12}-deficient rats in groups 1 B and 2 B were compared at the end of the experiment and a highly significant ($P < 0{\cdot}005$) negative correlation ($r = -0{\cdot}91$) was found between them. A regression line for liver vitamin B_{12} (Y) in μg/g, on TUESA excretion (X) in m-equiv./100 g 24 h was also derived giving $Y = 0{\cdot}088 - 0{\cdot}051X$

Assay, in the dark, of the antibiotic stock solution suggested that each rat received $< 0{\cdot}0005$ μg vitamin B_{12} daily from this source assuming a daily water intake of 25 ml/rat.

The results of Expt 2 are given in Table 2. There was no significant difference between mean TUESA excretion in groups A and B at the beginning of the experiment. The mean TUESA excretion in non-supplemented rats (group A) showed no significant fall over 5 weeks, whereas the fall in the rats of group B was highly significant ($P < 0{\cdot}005$). The difference in mean TUESA excretion in the two groups at the end of the period was significant ($P < 0{\cdot}05$). The mean liver vitamin B_{12} level of the rats in group B at the end of the experiment was significantly greater ($P < 0{\cdot}025$) than

that of the rats in group A. A correlation coefficient of −0·89, calculated between mean TUESA excretion and liver vitamin B_{12} levels for all the rats at the end of the experiment, was highly significant ($P < 0{\cdot}005$).

Table 2. *Expt 2. Total urinary ether-soluble acids (m-equiv./100 g 24 h) and methylmalonic acid excretion by individual rats before and after the 5-week experimental period and liver vitamin B_{12} content at the end of the 5-week period*

	At beginning of period*		At end of period†		
	TUESA	MMA‡	TUESA	MMA‡	Liver vitamin B_{12} (μg/g)
			Group A: no vitamin B_{12} supplement		
	0·47	+ +	0·52	+ +	0·024
	0·74	+ + +	0·82	+ + +	0·016
	0·47	+ +	0·80	+ + +	0·018
	0·32	+	0·29	Trace	0·044
	0·60	+ +	0·40	+	0·031
	0·66	+ + +	0·30	Trace	0·027
Mean and SD	0·54		0·52		0·027
	±0·14		±0·22		±0·009
			Group B; 0·05 μg vitamin B_{12}/day		
	0·43	+	0·28	0	0·052
	0·51	+	0·32	Trace	0·041
	0·47	+ +	0·34	Trace	0·043
	0·38	+	0·23	0	0·047
	0·52	+	0·34	0	0·045
Mean and SD	0·46		0·30		0·046
	±0·05		±0·04		±0·004

* Mean of three measurements.
† Mean of two measurements.
‡ Armstrong (1967); for semi-quantitative scoring system, see p. 530.

DISCUSSION

The success of the jacket in preventing access to faecal vitamin B_{12} can be judged from the results of the vitamin B_{12} assays on the stomach, small bowel and their contents. The significant reduction in the vitamin B_{12} content of these organs when the jacket was used indicates at least partial success. These levels were somewhat higher than the levels for small-bowel contents alone found by Morgan *et al.* (1964) using *Lactobacillus leichmanii* assays and the faecal cup method of coprophagy prevention (Barnes, Fiala, McGehee & Brown, 1957). However, they were lower than the levels found by Morgan *et al.* (1964) for the walls of the gastro-intestinal tract, and since these, at least in the stomach, would have made a considerable contribution to the assays reported here it may be concluded that the jackets were about as effective as the faecal cups.

The excretion of TUESA and methylmalonic acid, and liver vitamin B_{12} content gave no indication that the prevention of coprophagy exerted an effect on vitamin B_{12} nutrition. TUESA excretion and liver vitamin B_{12} contents in vitamin B_{12}-deficient rats were the same whether coprophagy was prevented or permitted. The work of Morgan *et al.* (1964) suggests a possible effect of coprophagy on vitamin B_{12} nutrition

which is confirmed by the growth difference found by Barnes & Fiala (1958). It may be that TUESA excretion and liver vitamin B_{12} contents are insufficiently sensitive to show small differences, but it is certain that coprophagy by the rat does not prevent the development of vitamin B_{12} deficiency.

Although bacteriological examinations of faeces and bowel contents were not made, it is evident that the antibiotics used did not eliminate the intestinal flora. The antibiotics reduced the vitamin B_{12} activity of the contents of the large bowel (Table 1), but this was still much greater than that of the small bowel; it may be inferred that there was some bacterial action in the intestine.

The results of determinations of TUESA and methylmalonic acid excretion (Fig. 1) of rats fed on a vitamin B_{12}-deficient diet suggest that coprophagy prevention and the administration of an antibiotic mixture (group 3B) did not produce vitamin B_{12} deficiency. This is consistent with a sparing action of the antibiotics for vitamin B_{12}. Such an action for streptomycin has previously been reported in chicks (Stokstad & Jukes, 1950). However, the very low liver vitamin B_{12} content in group 3B (Table 2) clearly contradicts this conclusion. Further, in Expt 2 there was a significant elevation of liver vitamin B_{12} above that in unsupplemented controls in rats supplemented with an amount of vitamin B_{12} (0·05 μg) close to their daily requirement (Armstrong, 1967). There was a significant negative correlation between liver vitamin B_{12} contents and TUESA excretion. Assuming that rats in group 3B were receiving amounts of vitamin B_{12} close to their daily requirements, which is reasonable in view of the trend of methylmalonic acid excretion (Fig. 1), it may be estimated from the regression line of liver vitamin B_{12} on TUESA excretion in the vitamin B_{12}-deficient rats in Expt 1 that the mean liver vitamin B_{12} of group 3B rats would be expected to be 0·066 μg/g; this is considerably higher than the observed 0·020 μg/g.

This anomaly is unlikely to have been due to enhanced absorption of vitamin B_{12} in the presence of antibiotics since, although this would reconcile the moderate fall in vitamin B_{12} content of the large bowel with increased availability to the animal, it would not explain the low liver vitamin B_{12} level.

It is possible that an increase in synthesis of mammalian-active vitamin B_{12} may not always be paralleled by an increase in vitamin B_{12} activity for *Euglena*, since *Euglena* is sensitive to a number of vitamin B_{12} analogues which are not active for mammals (Ford, 1953). It has recently been shown than many bacteria isolated from the rat large bowel synthesize more of these vitamin B_{12} analogues than of vitamin B_{12} active for *Ochromonas malhamensis* (Raibaud, Valencia, Dickinson & Cong, 1965). Further, in a normal rat these analogues constitute the greater part of the vitamin B_{12} activity of faeces and of the content of the large bowel (Morgan *et al.* 1964; Valencia, Sacquet, Raibaud, Cong & Charlier, 1965). Thus a fall in the *Euglena* vitamin B_{12} activity of the large bowel contents might be observed, with a net increase in mammalian-active vitamin B_{12}, if organisms synthesizing mainly mammalian-active vitamin B_{12} were favoured. Since also the livers of normal and vitamin B_{12}-deficient rats usually contain some four to five times as much of the vitamin B_{12} analogues as of mammalian-active vitamin B_{12} (Valencia *et al.* 1965), it is possible that an increase in the content of mammalian-active vitamin B_{12} in the livers of rats in group 3B may have been completely masked by

a fall in the content of analogues. It has been shown that streptomycin reduces the number of coliforms, which absorb and utilize vitamin B_{12} (Davis & Mingioli, 1950; Oginsky, 1952), and favours some bacteria, in particular *Bacillus megatherium* (Smith & Robinson, 1945) which synthesize vitamin B_{12} (Garibaldi, Kjichi, Snell & Lewis, 1950). Thus it appears probable that an increased availability of mammalian-active vitamin B_{12}, synthesized by the intestinal flora, is the explanation for the low TUESA and methylmalonic acid excretion by rats in group 3B.

The vitamin B_{12}-sparing action of antibiotics observed in rats in which coprophagy was prevented suggests that vitamin B_{12} or the factor responsible for the sparing action is absorbed from the site of synthesis by the intestinal flora. This is in contradiction with the findings of Barnes *et al.* (1960). These results do not show whether this absorption occurs in the small or large bowel. However, since there was no apparent effect of the antibiotics on the vitamin B_{12} content of the small bowel, the absorption may have occurred in the large bowel. Tracer studies have shown this to be possible in the rat (Merzbach & Grossowicz, 1965). Nor do these results show whether, under normal circumstances, vitamin B_{12} is obtained from this source. However, since Merzbach & Grossowicz (1965) observed no effect of sulphaguanidine on vitamin B_{12} absorption from the large bowel of the rat, it may be concluded that, in the absence of antibiotics, the absorptive mechanism of the large bowel is active and at least some vitamin B_{12} is obtained from it.

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