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Coprophagy and vitamin B_{12} in the rat

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The importance of coprophagy in nutritional studies has long been recognized; already in the early days of vitamins numerous workers demonstrated the presence of B vitamins in the excreta of birds and mammals (cf. Kon, 1945; Elvehjem, 1948; Mickelsen, 1956) and began keeping experimental rats on screens to prevent their consuming their droppings. Rats kept in this way have been used for assays of members of the vitamin B complex and have shown characteristic signs of deficiency in the absence of any one of them. However, additional measures have usually been necessary to cause rats to exhibit deficiency of biotin, folic acid or vitamin B_{12}. These vitamins are needed in only small amounts by the rat and are synthesized in appreciable quantity by many micro-organisms. It has been generally assumed that sufficient biotin and folic acid were synthesized and absorbed from the caecum to supply the animals' requirements, for the appropriate deficiency could be induced when synthesis was depressed by the inclusion in the diet of sulphonamide drugs (cf. Mickelsen, 1956). The picture with vitamin B_{12} was less clear; the absorptive mechanism for this vitamin is more complicated and it seemed unlikely that absorption occurred from the caecum. However, it was considered that the difficulty in inducing deficiency was due to storage of the vitamin in the tissues, and it was found that the onset of deficiency could be hastened on increasing the metabolic rate by inclusion of desiccated thyroid in the diet (Ershoff, 1947). It now seems probable that coprophagy was the main reason for these difficulties in inducing certain vitamin deficiencies, and it is noteworthy that du Vigneaud, Chandler, Moyer & Keppel (1939) pointed out already 25 years ago that rats can practise coprophagy even when kept on screens. The full extent of this practice was not really appreciated until Barnes, Fiala, McGehee & Brown (1957) designed an effective cup for collecting faeces (described in the rest of this paper simply as 'cup') which they used to show that rats kept on screens normally ingest about half their daily output of faeces. Later, using the same technique, they were able to induce deficiencies of biotin (Barnes, Kwong & Fiala, 1959), folic acid (Barnes, Fiala & Kwong, 1963) and vitamin B_{12} (Barnes & Fiala, 1958) in rats given appropriate deficient diets and without recourse to other expedients. Subsequently, Morgan (1960*a*, *b*) showed that the condition of refection

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in rats did not occur, as was previously thought, through direct absorption of vitamins from the gut, but that it could be explained by the rats eating their faeces.

Barnes & Fiala (1958) showed that rats given a diet without added vitamin B_{12} and fitted with cups grew less well than rats fed similarly and not fitted with cups, but that the growth of the latter was improved when vitamin B_{12} was added to the diet. They did not, however, measure the amount of vitamin B_{12} normally available to the rat through coprophagy.

The object of the investigation now described was to study whether rats in the course of depletion on a vitamin B_{12}-deficient diet excrete such quantities of vitamin B_{12} that they could, by eating their faeces, obtain sufficient of the vitamin to defeat the attempt to produce deficiency. The amount of vitamin B_{12}-active compounds excreted in the faeces of rats receiving a vitamin B_{12}-deficient diet has been determined. Further tests showed that the vitamin B_{12} activity of the contents of stomach and small intestine of rats killed shortly after a meal of the vitamin B_{12}-deficient diet alone or mixed with faeces or with cyanocobalamin accorded closely with the nature of the meal. Thus it was evident that coprophagy was the cause of the higher content of vitamin B_{12} found in the stomachs of rats given the deficient diet and kept on screens in the usual way than in those of rats fitted with the cup described by Barnes *et al.* (1957).

A brief preliminary account of part of this work was given by Morgan, Gregory, Kon & Porter (1961).

EXPERIMENTAL

Animals

Male albino rats were reared on a stock diet until about 140 g in weight. Then for 10–14 days before each experiment they were housed individually in cages with raised $\frac{5}{16}$ in. wire-mesh screens and were given *ad lib.* a diet containing 80% α-protein (an isolated soya-bean protein; Glidden Co., Chicago), 15% arachis oil (semi-hardened), 5% salts (de Loureiro, 1931) and a supplement of all vitamins except vitamin B_{12} (Cuthbertson & Thornton, 1952).

Vitamin B_{12} activity in faeces

Cups, similar to those described by Barnes *et al.* (1957), were fitted to six rats. The collected faeces were removed from the cups daily on each of the next 3 days. The faeces from each rat were separately weighed, and the vitamin B_{12} activity of each sample of faeces was measured microbiologically with *Lactobacillus leichmannii* and with *Ochromonas malhamensis*, as described below.

Vitamin B_{12} activity in the walls and contents of the stomach and small intestine of rats fitted with cups and given a meal of the vitamin B_{12}-deficient diet or of the diet supplemented with rat faeces or with cyanocobalamin

Sixteen rats were fitted with cups and starved overnight. The next morning six of the rats were offered weighed amounts of the vitamin B_{12}-deficient diet, five were offered the diet mixed with bulked faeces collected overnight (1·5 parts diet + 1 part

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wet faeces) and the remaining five rats were offered the diet supplemented with cyanocobalamin (0·1 μg/g diet). The rats were allowed to eat for 20 min, the amount of food eaten was recorded, and the animals were immediately killed with diethyl ether. The abdomen was opened, cannulas were inserted into the small intestine at the pylorus and just anterior to the caecum, and the intestinal contents were washed into a beaker by passing 150 ml isotonic saline through the intestine. The small intestine and the stomach (with contents) were then removed. The vitamin B_{12} activities of the diet and of the walls and contents of the stomach and small intestine were measured microbiologically with *Lb. leichmannii* and with *O. malhamensis*, as described below.

Vitamin B_{12} activity of the stomach contents of normal rats and of rats fitted with cups

Thirty-one rats were fitted with cups; the collected faeces from sixteen of them were removed from the cups daily and discarded, whereas the faeces from the other fifteen were removed from the cups, mixed together and offered to these rats daily, ground up in their diet. The fifteen rats in the third group were kept in individual cages and were not prevented from eating their faeces. After 1 week on these treatments the rats were killed. The abdomen was opened, ligatures were tied round the lower end of the oesophagus and the pylorus, and the stomach was removed. The vitamin B_{12} activity of the stomach contents was measured microbiologically with *Lb. leichmannii*, as described below.

Treatment of material and preparation of extracts for assay

Faeces and diets. A weighed sample of faeces was suspended in about 50 ml water containing two drops of a 1% (w/v) aqueous solution of sodium cyanide. The pH was adjusted to 4·6 with 0·1 N-HCl and the mixture heated at 115° for 10 min in an autoclave. After cooling, the volume was made up to 100 ml and the extract filtered. The diets were treated similarly.

Stomach contents. The stomach wall was slit, and the contents were washed into a 100 ml Erlenmeyer flask with about 20 ml water. One drop of a 1% (w/v) aqueous solution of sodium cyanide was added, and the flask and contents were heated in flowing steam for 30 min. After cooling, the pH was adjusted to 4·6 with 0·1 N-HCl, and 1 ml of a solution containing 50 mg papain (British Drug Houses Ltd) and a trace of cyanide was added. After incubation for 2 h at 55°, the volume of the digested mixture was made up to 50 ml, and the digest was filtered.

Stomach walls. The stomach walls were first heated in flowing steam for 30 min in about 40 ml water containing one drop of a 1% (w/v) aqueous solution of sodium cyanide and then homogenized. The pH was adjusted to 4·6 with 0·1 N-HCl, 100 mg of papain were added, and the homogenate was incubated for 2 h at 55°. The flask containing the digested homogenate was transferred to an autoclave and heated at 115° for 10 min. After cooling, the volume was made up to 100 ml, and the extract was filtered.

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Small intestine. The washings containing the intestinal contents, and the intestinal walls, were treated in the same way as the stomach contents and walls.

Microbiological assay

The filtrates prepared from the faeces and stomach and intestinal contents and walls were each diluted to contain about 0·04 ng vitamin B_{12} activity/ml for assay with *Lb. leichmannii* and with *O. malhamensis*. The method used for *Lb. leichmannii* assay was based on that of Skeggs, Nepple, Valentik, Huff & Wright (1950) as described by Gregory (1954). The method used for *O. malhamensis* assay was that described by Ford (1953), except that the concentration of the vitamin B_{12} standard was reduced from 0·2 to 0·04 ng/ml and the period of incubation was increased to 7 days.

RESULTS

Vitamin B_{12} activity in faeces

The results in Table 1 show considerable variation both in the amount of faeces excreted by individual rats and in the concentration of vitamin B_{12} activity in the faeces. The mean weight of faeces excreted was 1·05 g, with a range from 0·37 to 2·06 g. The mean vitamin B_{12} activities were 3·84 μg/g (range 2·05–5·90) for *Lb. leichmannii* and 0·22 μg/g (range 0·04–0·71) for *O. malhamensis*. The results for individual samples show that faeces may contain markedly different ratios of activity for the two micro-organisms. Thus, to take extremes, one sample had activity for *Lb. leichmannii* of 4·61 μg/g and for *O. malhamensis* of 0·71 μg/g, whereas another had activities of 2·05 and 0·04 μg/g for these micro-organisms, i.e. ratios of 6·5:1 and 50:1.

Table 1. *Concentration of vitamin B_{12} activity in, and weight of, faeces excreted daily by individual rats fitted with cups for collecting faeces, measured with* (*A*) Lb. leichmannii *and* (*B*) O. malhamensis

	Day 1			Day 2			Day 3		
Rat no.	Wt of faeces (g)	Vitamin B_{12} activity (μg/g faeces) *A*	*B*	Wt of faeces (g)	Vitamin B_{12} activity (μg/g faeces) *A*	*B*	Wt of faeces (g)	Vitamin B_{12} activity (μg/g faeces) *A*	*B*
1	0·98	2·36	0·09	0·98	5·45	0·12	0·70	5·90	0·13
2	1·11	2·90	0·08	1·05	3·25	0·08	1·31	2·05	0·04
3	2·06	2·10	0·07	0·81	3·18	0·05	0·37	4·30	0·08
4	1·20	4·05	0·11	0·85	3·85	0·07	—*	—*	—*
5	2·02	2·90	0·41	1·09	3·96	0·58	0·71	4·60	0·71
6	1·34	4·38	0·36	0·81	5·05	0·47	0·64	5·11	0·42

* Cup pulled off by rat.

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Vitamin B_{12} activity of the stomach walls and contents and of the small intestine walls and contents of rats fitted with cups and given a meal of vitamin B_{12}-deficient diet or of the diet supplemented with rat faeces or with cyanocobalamin

The mean weights of diet consumed by rats offered the three different diets were: vitamin B_{12}-deficient diet, 1·8 g (range 1·4–2·0 g); vitamin B_{12}-deficient diet with rat faeces, 1·4 g (range 0·81–2·0 g); vitamin B_{12}-deficient diet with cyanocobalamin, 1·3 g (range 0·34–2·2 g).

The amounts of vitamin B_{12} activity for the two micro-organisms found in the contents and walls of the stomachs and intestines of the rats are shown in Table 2. Since each rat consumed a different amount of the diet, the total vitamin B_{12} activity of the contents of the stomach and the small intestine is expressed in the table as ng vitamin B_{12} activity/g diet consumed. It is evident from these results that the major part of the ingested vitamin B_{12} activity for *Lb. leichmannii* was recovered from the stomach and intestinal contents. Thus, for rats given the diet with faeces a total of 157 ng was recovered out of 188 ng consumed, and for the rats given the diet with cyanocobalamin the whole intake of 100 ng was recovered.

Table 2. *Mean values (with their standard errors) for vitamin B_{12} activity of the contents and walls of the stomach and small intestine of rats fed on a vitamin B_{12}-deficient diet and on the same diet containing added cyanocobalamin or rat faeces, measured with (A)* Lb. leichmannii *and (B)* O. malhamensis

		Vitamin B_{12} activity								
				Contents (ng/g diet eaten)			Walls (ng)			
		Diet (ng/g)		Stomach		Small intestine,	Stomach		Small intestine	
Diet	No. of rats	*A*	*B*	*A*	*B*	*A*	*A*	*B*	*A*	*B*
Vitamin B_{12}-deficient	6	0·3	NM	2·2 ± 2·1	1·3 ± 1·0	3·6 ± 2·2	36 ± 16	39 ± 13	36 ± 11	41 ± 12
Vitamin B_{12}-deficient + rat faeces	5	188	48	125 ± 16	29 ± 6	32 ± 25	32 ± 15	34 ± 14	43 ± 18	45 ± 25
Vitamin B_{12}-deficient + cyanocobalamin	5	100	100	84 ± 19	83 ± 19	16 ± 9	38 ± 22	42 ± 19	42 ± 8	49 ± 11

NM, not measurable.

Intestinal contents contained some substance that interfered with the *O. malhamensis* assay, so that this organism could only be used to measure the vitamin B_{12} activity of stomach contents, the results for which accorded well with the vitamin B_{12} activity of the diet.

The content of vitamin B_{12}-active substances, as measured by both micro-organisms, in the walls of the stomach and intestine was not increased after the meals containing faeces or cyanocobalamin. This indicates that little or no absorption of vitamin B_{12} activity had taken place during the short period between feeding and killing the rats.

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Vitamin B_{12} activity in the stomach contents of normal rats and of rats fitted with cups

Table 3 shows the mean values obtained for the total amounts of vitamin B_{12} activity present in the stomach contents of rats in the different groups. The normal rats and those fitted with cups but having faeces added to their diet had similar mean amounts of vitamin B_{12} activity in the stomach contents. A wide range of values was found within each group, since the rats had access to their food at any time, and therefore different amounts of food were present in the stomach at the time the animals were killed. The third group of rats, fitted with cups and deprived of faeces had markedly lower amounts of vitamin B_{12} in their stomach contents. These small amounts of vitamin B_{12} were similar to those found in the stomach contents of the rats offered the vitamin B_{12}-deficient diet in the previous experiment (see Table 2).

Table 3. *Mean vitamin B_{12} activity of the stomach contents of normal rats and of rats fitted with cups for collecting faeces, measured with* Lb. leichmannii

Treatment	No. of rats	Vitamin B_{12} activity (ng) Value	Range
None	15	15	1·2–40
Fitted with cups:			
Faeces returned	16	13	0·5–72
Faeces discarded	15	0·8	0·2–3·8

DISCUSSION

It is apparent from the results shown in Table 1 that rats given a vitamin B_{12}-deficient diet excreted appreciable, though variable, amounts of vitamin B_{12} activity in their faeces. The assays were carried out with two test organisms as *Lb. leichmannii* gives a measure of total vitamin B_{12} activity, for it responds to vitamin B_{12} and to several of its analogues that are inactive for higher animals, whereas *O. malhamensis* utilizes only the biologically active forms of vitamin B_{12}. Thus we have shown that not only did the activity measured in the faeces by *Lb. leichmannii* vary from rat to rat but that a much greater variation occurred in the proportion of this activity due to vitamin B_{12}. However, since the mean daily excretion of faeces was 1·05 g and their mean vitamin B_{12} content was 0·22 μg/g, it is evident that the mean daily faecal excretion of vitamin B_{12} by rats receiving this diet and of 150 g body-weight would be some 0·2 μg.

The assays of vitamin B_{12} activity of stomach and intestinal contents and walls after ingestion of the vitamin B_{12}-deficient diet alone or with known amounts of vitamin B_{12} activity showed that good recoveries could be obtained. Moreover, since the faeces, and hence the gut contents, would contain appreciably more activity for *Lb. leichmannii* than for *O. malhamensis*, it seemed likely, as was found, that the former organism would be the more sensitive indicator of the occurrence of coprophagy.

The results shown in Tables 2 and 3 make it clear that appreciable amounts of

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vitamin B_{12} activity were present in the stomach contents of rats given with their diet faeces or cyanocobalamin, or allowed to practise coprophagy, whereas much smaller amounts were present in the stomach contents of rats deprived of access to faeces. These findings confirm that rats kept on screens eat at least some of their faeces. However, since vitamin B_{12} and its analogues are rapidly absorbed from the intestine, it was not practicable to attempt to estimate the extent of coprophagy during 24 h periods by measurement of the amount of vitamin B_{12} activity in the stomach and intestinal contents.

The daily requirement for vitamin B_{12} of the non-coprophagous rat is not known, but Henry & Porter (1958) found that optimal growth of rats of body-weight 50–100 g occurred with a daily supplement to a vitamin B_{12}-deficient diet of 0·1–0·2 μg vitamin B_{12}. Coprophagous rats of this weight eating half their output of faeces would obtain 0·05–0·1 μg vitamin B_{12}/day—that is, a considerable proportion of their daily requirement. Thus it is evident that coprophagy during vitamin B_{12} assays with rats will markedly reduce the range of the dose–response curve and may allow rats whose intestinal flora synthesizes particularly large amounts of vitamin B_{12} to grow normally even in the absence of a vitamin B_{12} supplement.

SUMMARY

1. Rats of 150 g body-weight given a vitamin B_{12}-deficient diet, and fitted with cups for collecting faeces, excreted in their faeces daily about 4 μg of vitamin B_{12} compounds, of which about 0·2 μg was vitamin B_{12} itself, as measured with *Lactobacillus leichmannii* and *Ochromonas malhamensis*.

2. Vitamin B_{12} added to a vitamin B_{12}-deficient diet was almost quantitatively recovered from the contents and walls of the stomach and small intestine of rats killed shortly after a meal.

3. The practice of coprophagy by rats kept on screens was confirmed by the finding that the stomach contents of such rats contained appreciable amounts of vitamin B_{12} activity, whereas only small amounts were present in the stomach contents of rats fitted with cups.

4. The consequences of coprophagy by rats during vitamin B_{12} assays are discussed.

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